SnoN residue (1–366) attenuates hypertrophic scars through resistance to transforming growth factor- β 1-induced degradation

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Abstract

Hypertrophic scars (HSs) are characterized by fibroblast hyperproliferation and excessive matrix deposition. During wound healing, transforming growth factor (TGF)- β 1/Smad signaling acts as a key regulator. As a transcriptional corepressor of TGF- β 1/Smads, SnoN is expressed at low levels in many fibrotic diseases due to TGF- β 1/Smad-induced degradation. SnoN residue (1–366; SR) is resistant to TGF- β 1-induced degradation. However, the expression and role of SR in HSs are unknown. Here, we inhibited TGF- β 1/Smad signaling via overexpression of SR to block fibroblast transdifferentiation, proliferation, and collagen deposition during HS formation. Our results showed that SnoN was downregulated in HS fibroblasts (HSFs) owing to TGF- β 1/Smad-induced degradation. Overexpression of SR in normal human dermal fibroblasts (NHDFs) and HSFs successfully blocked phosphorylation of Smad2 and Smad3, thereby inhibiting NHDF transdifferentiation and HSF proliferation and reducing type I collagen (ColI) and type III collagen (ColIII) production and secretion. In addition, we applied overexpressed full-length SnoN (SF) and SR to wound granulation tissue in a rabbit model of HSs. SR reduced wound scarring, improved collagen deposition and arrangement of scar tissue, and decreased mRNA and protein expression of ColI, ColIII, and α -smooth muscle actin (α -SMA) more effectively than SF in vivo. These results suggest that SR could be a promising therapy for the prevention of HS.

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Introduction

Many patients suffer from hypertrophic scars (HSs) due to deep dermal injury after surgeries, burn, or trauma, leading to deformation of appearance and excessive contracture of neogenetic tissues, thus causing cosmetic, functional, and psychological problems [1]. HS is characterized by over-proliferation of fibroblasts and excessive deposition of extracellular matrix (ECM) [2]. Novel strategies for preventing HS are urgently needed.

When skin tissues are injured, fibroblasts near the injured site transdifferentiate into myofibroblasts expressing α -smooth muscle actin (α -SMA), which promotes wound healing. During this process, transforming growth factor (TGF)- β 1/Smad signaling acts as a key regulator [3, 4]. When TGF- β 1 binds to its receptor (T β RII), phosphorylation of Smad proteins is triggered, resulting in the transcription of downstream target genes, e.g., type I and III collagen (ColI and ColIII), and the transdifferentiation of fibroblasts [4–6]. During wound healing, redundant ECM is degraded through the concerted functions of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs)

[7], and myofibroblasts undergo apoptosis [3], creating a flat scar known as a physiological scar. However, continuous activation of TGF- β 1/Smad signaling after wound healing causes hyperproliferation of myofibroblasts and excessive production of ECM, leading to HS formation.

Researchers have attempted to inhibit wound scarring by blocking TGF- β 1/Smad signaling, e.g., using TGF- β 1 antagonists [8] or anti-TGF- β 1 antibodies [9]. However, TGF- β 1 is continuously produced by various cells involved in wound healing [10] and accelerates wound repair during the early stage [11], making appropriate inhibition difficult. Therefore, other targets have been explored, e.g., truncated T β RII [12], overexpression of dominant-negative mutant T β RII [13], and T β RI (also known as activating receptor-like kinase (ALK) siRNA [14]. ALK1 and ALK5, activated by binding of TGF- β 1 with T β RII, mediate the phosphorylation of Smad1, Smad, Smad3, and Smad5 [15, 16]. Moreover, Smad2 and Smad4 both mediate the transdifferentiation of fibroblasts to myofibroblasts and result in the upregulation of α -SMA. Smad2–4 are associated with increased production of all collagen types [17, 18]. Additionally, wound scarring is inhibited by Smad3 siRNA [19], knockout of Smad3 [20], or dominant-negative Smad3/4 and ectopic expression of Smad7 [21]. However, because of the independent functions of these proteins, modulating the functions of these proteins is challenging.

Human SnoN is a ubiquitously expressed nuclear protein of 684 amino acids [22] that shares 50% amino acid homology and 36% identity with Ski proteins [23]. SnoN contains several important structural domains mediating interactions with Smad proteins, including 89–92 residues required for binding to Smad2 or Smad3, a 100-amino acid Dachshund homology domain (DHD), which can bind directly to nuclear receptor corepressors (N-CoRs) to recruit the transcriptional corepressor complex of N-CoR, mammalian Sin3 (mSin3) and histone deacetylase complex (HDAC) and mediate the degradation of SnoN by being targeted by three E3 ubiquitin ligases, e.g., Smad ubiquitin regulatory factor [24], the anaphase promoting complex (APC/C) [25], and Arkadia [26], among other interactions [22, 23]. SnoN maintains the repressed state of the responsive genes of TGF- β 1 in the absence of ligand and participates in negative feedback upon stimulation of TGF- β 1. Immediately after TGF- β 1 stimulation, phosphorylated Smad3 and Smad2 form complexes with Smad4, resulting in rapid ubiquitination and degradation of SnoN; this permits TGF- β 1 signaling activation through binding with SnoN and recruitment of Smurf2, APC/C, and Arkadia. However, SnoN can interact with Smad2 or Smad3 via residues 89–92 to compete with Smad4 for binding to Smad2, disrupting the functional Smad heteromeric complexes. This mechanism also recruits the transcriptional corepressor complex of N-CoR, mSin3, and HDAC1 to the target promoters,

antagonizing TGF- β 1/Smad signaling. SnoN expression is altered under many pathological conditions, including renal fibrosis due to diabetic nephropathy and obstructive injury [27–29], paraquat-induced pulmonary fibrosis [30], liver fibrosis [31], and cardiac fibrosis [32]. MG132 attenuates diabetic nephropathy-induced renal fibrogenesis by inhibiting ubiquitination and degradation of SnoN [29]. However, the role of SnoN in HS formation has not been studied.

Therefore, in this study, we evaluated the role of SnoN residue (1–366; SR), which can interact with Smads but blocks SnoN degradation [25], in HS formation and ECM deposition. Our findings provide important insights into the use of SR as a potential therapy for the prevention of HS.

Results

Downregulation of SnoN protein expression in HS fibroblasts (HSFs) by ubiquitin-dependent degradation

Because SnoN is degraded in multiple organ fibrotic diseases by ubiquitin-dependent degradation through the TGF- β 1/Smad signaling pathway [27, 28, 30–34], we evaluated these pathways in normal human dermal fibroblasts (NGDFs) and HSFs in vitro. In both HSFs and NHDFs, *SnoN* mRNA expression was upregulated by TGF- β 1 treatment ($P < 0.001$; Fig. 1a), although there were no significant differences between the two cell lines. SnoN protein was degraded by incubation with a ubiquitin mixture (UM) in HSFs to a greater extent than that in NHDFs ($P < 0.05$, $P < 0.01$). After stimulation with TGF- β 1, SnoN was higher in NHDFs than HSFs ($P < 0.001$) but was degraded dramatically in both cell lines ($P < 0.001$; Fig. 1b), indicating that ubiquitin-dependent degradation of SnoN existed in HSs and was induced by TGF- β 1. Moreover, TGF- β 1 stimulation increased the phosphorylation of Smad2 and Smad3 in HSFs and NHDFs ($P < 0.05$), with that in the former remaining higher than that in the latter ($P < 0.001$; Fig. 1c), indicating that TGF- β 1 induced SnoN degradation by mediating Smad2 and Smad3 phosphorylation. Immunocytofluorescence analysis showed that SnoN was mainly localized in the cytoplasm of NHDFs but in the nucleus of HSFs. After TGF- β 1 stimulation, SnoN translocated into the nucleus in both cell lines. Moreover, SnoN expression nearly disappeared in HSFs (Fig. 1d) owing to nuclear degradation [33]. Thus, TGF- β 1-induced translocation contributed to the degradation of SnoN in HSs.

Lentivirus-mediated overexpression of SnoN

Next, to examine the effects of SnoN overexpression on HSs in vitro and in vivo, we created a lentivirus

Fig. 1 SnoN protein was downregulated in HSFs through TGF-β1-induced degradation. NHDFs and HSFs were treated with or without 5 ng/mL TGF-β1 for 24 h. NSDFs and HSFs, the paired samples from the same individual ($n = 3$). **a** *SnoN* mRNA was evaluated by RT-qPCR. **b, c** Cell lysates were incubated with a ubiquitin mixture, and western blot analysis was carried out for the detection of SnoN, phospho (p)-Smad2, and p-Smad3. **b** mRNA and protein levels of SnoN after incubation with the ubiquitin mixture with or without TGF-β1. mRNA and protein levels from NHDFs in the absence of TGF-β1 were set at 1 for normalization. Data are expressed as the fold change.

Values are means $\pm$ SEMs ($n = 5$; **a** $F = 34.732$, **b** $F = 48.302$, **c**: p-Smad2: $F = 10.01$, p-Smad3: $F = 6.19$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NHDFs with no TGF-β1; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. HSFs with no TGF-β1; $\Delta\Delta\Delta P < 0.001$ vs. HSFs incubated with the ubiquitin mixture; $\Delta P < 0.05$ vs. NHDFs with TGF-β1 treatment). **d** Immunocytofluorescence staining of SnoN (red) in TGF-β1-stimulated NHDFs and HSFs. DAPI was used for nuclear counterstaining (blue). Scale bar = 20 μ m. HSF hypertrophic scar fibroblast, TGF transforming growth factor, NHDF normal human dermal fibroblast

expression system in NHDFs and HSFs. After transfection for 3 days, more than 90% of the cells displayed green fluorescent particles (Supplementary Fig. s1). Transfection with LV-GFP-SF (encoding full-length SnoN [SF]) or LV-GFP-SR for 3 days showed that SF was mainly distributed in the cytoplasm in NHDFs but was partially

translocated into the nucleus and degraded following TGF-β1 treatment. In HSFs, SF was localized in both the cytoplasm and nucleus, and TGF-β1 treatment resulted in the degradation of SF, with low levels remaining in the cytoplasm. In the absence of TGF-β1 stimulation, SR showed trends similar to those of SF in NHDFs and HSFs;

Fig. 2 SnoN residue (SR) and full-length SnoN (SF) were successfully overexpressed using a lentivirus overexpression plasmid. **a** NHDFs and HSFs were separately transfected with 107 TU/mL LV-GFP-SF or LV-GFP-SR and treated with or without TGF- β 1 stimulation. SF and SR localizations were evaluated by immunocytofluorescence of SnoN (red). DAPI was used for nuclear counterstaining (blue). Scale bar = 20 μ m. **b–f** SnoN mRNA and protein levels in NHDFs and HSFs transfected with LV-GFP-SF (SF group), LV-GFP-SR (SR group), LV-GFP (Vec group), or nothing (Ctrl group), with or without 5 ng/mL TGF- β 1. **b** *SnoN* mRNA in NHDFs and HSFs. **c** Protein levels and western blot analysis of SnoN in NHDFs.

however, after TGF- β 1 treatment, SR was translocated into the nucleus and not degraded in both NHDFs and HSFs (Fig. 2a). Quantitative reverse transcription PCR (RT-qPCR) and western blotting showed that SnoN mRNA and protein levels were elevated in cells expressing SF and SR compared with those in control cells, with or without TGF- β 1 treatment ($P < 0.001$; Fig. 2b–d). TGF- β 1 treatment resulted in the upregulation of *SnoN* mRNA in cells expressing SR and SF ($P < 0.001$; Fig. 2b). In contrast, TGF- β 1 treatment downregulated SnoN protein in cells expressing SF ($P < 0.001$), whereas no differences were observed in cells expressing SR, regardless of TGF- β 1 treatment (Fig. 2c, d).

Notably, TGF- β 1 treatment decreased Smad2 and Smad3 phosphorylation levels in NHDFs expressing SR ($P < 0.05$, $P < 0.001$, respectively), whereas only Smad3

phosphorylation was decreased in NHDFs expressing SF ($P < 0.05$) compared with control cells. Phosphorylation of Smad2 and Smad3 was lower in cells expressing SR than in cells expressing SF ($P < 0.05$, $P < 0.01$, respectively). In HSFs, phosphorylation of Smad2 and Smad3 was obviously decreased compared with that in the control, regardless of TGF- β 1 treatment ($P < 0.05$, $P < 0.001$, respectively). Additionally, Smad2 and Smad3 phosphorylation was significantly lower in cells expressing SR than in cells expressing SF following TGF- β 1 treatment ($P < 0.01$; Fig. 2e, f).

In vivo, SnoN expression was increased in the SF and SR groups compared with the control and vector groups. However, α -SMA expression was gradually reduced, with the lowest expression observed in the SR group (Fig. 5c). RT-qPCR showed that *SnoN* mRNA levels were higher in

the SF and SR groups than the control group on days 0, 30, and 40 ($P < 0.001$; Fig. 5d).

Effects of SnoN on proliferation, transdifferentiation, and ECM production in NHDFs

Because TGF- β 1/Smad signaling promotes NHDF transdifferentiation and ECM production, resulting in HS formation [35], and SnoN suppresses TGF- β 1/Smad signaling [36], we hypothesized that overexpressing SnoN would inhibit NHDF transdifferentiation and ECM production. Cell counting kit (CCK)-8 assays showed that in the absence of TGF- β 1 stimulation, cell proliferation was accelerated by SF and SR on days 3 and 7 ($P < 0.05$). However, in the presence of TGF- β 1, cell proliferation was first promoted on day 3 and then suppressed dramatically on day 7 ($P < 0.05$, $P < 0.001$). On day 10, proliferation in cells expressing SR was significantly suppressed compared with the control. SR was more efficient than SF at all time points ($P < 0.05$; Fig. 3a).

Notably, following TGF- β 1 treatment, the mRNA and protein levels of ColI, ColIII, and α -SMA were significantly downregulated in cells expressing SF and SR ($P < 0.05$, $P < 0.001$), with SR showing superior effects ($P < 0.05$, $P < 0.01$; Fig. 3b, c). Enzyme-linked immunosorbent assays (ELISAs) showed that ColI and ColIII secretion was decreased by both SF and SR ($P < 0.01$, $P < 0.001$), with SR showing superior effects ($P < 0.05$; Fig. 3d).

Effects of SnoN on proliferation and ECM production in HSFs

Next, we evaluated the effects of SnoN on proliferation and ECM production in HSFs. The results showed that SR significantly inhibited proliferation, regardless of TGF- β 1 treatment, at all time points ($P < 0.05$, $P < 0.001$), whereas SF significantly inhibited HSF proliferation at all time points except day 10 ($P < 0.05$, $P < 0.01$). SF showed slightly lower effects than SR in the presence of TGF- β 1 ($P < 0.05$, $P < 0.01$; Fig. 4a). Similarly, both SR and SF significantly downregulated ColI, ColIII, and α -SMA protein and mRNA levels, regardless of TGF- β 1 treatment ($P < 0.05$, $P < 0.001$), with the exception of ColIII, which was not downregulated by SF in TGF- β 1-treated cells (Fig. 4b, c).

In HSFs, ELISAs showed that ColI and ColIII secretion was markedly decreased only by SR ($P < 0.01$; Fig. 4d). Thus, SnoN suppressed proliferation and ECM production in HSFs.

Effects of SnoN on hyperplasia and ECM deposition in rabbit ear scars

To verify the function of SnoN in HSs in vivo, we established an ear HS model in rabbits [14, 37]. SF and SR

treatment of rabbit ears on days 7 and 14 post surgery improved the appearance of the repaired wound on days 20 and 40 (Fig. 5a). Immunohistofluorescence staining of Ki67, a cell proliferation marker, on day 30 demonstrated the suppressive effects of SnoN on HSF proliferation in vivo (Fig. 5b). Repaired wounds were evaluated by the Vancouver scar scale (VSS). Total VSS scores were significantly lower in the SF and SR groups than in the control and vector groups at all time points after wounding ($P < 0.05$, $P < 0.001$), and SR had significantly greater effects on VSS than SF on days 30 and 40 ($P < 0.05$; Fig. 5e). All tissue sections were evaluated by the scar elevation index (SEI), the ratio of the scar area to the normal skin dermis, to quantify scar hypertrophy [14]. As with VSS, the SEIs in rabbit ear wounds treated with SF and SR were much lower than those in the control and vector groups at all time points ($P < 0.05$, $P < 0.001$), with SR showing a greater decrease in SEI than SF on days 30 and 40 ($P < 0.05$). The SEIs of all groups were higher on day 30 than on day 20 and decreased on day 40 (Fig. 5e).

Collagen deposition was lower in the SF and SR groups than in the control and vector groups at all time points (Fig. 5f). Furthermore, collagen fibers in the control and vector groups were arranged irregularly and compactly, whereas those in the SF and SR groups were arranged in a regular pattern (Fig. 5f). RT-qPCR and western blotting showed that SF and SR notably reduced the mRNA and protein expression of ColI, ColIII, and α -SMA on days 20 and 30 ($P < 0.01$, $P < 0.001$; Fig. 5g).

Taken together, these data showed that SnoN relieved hypertrophy, collagen deposition, and scar contracture (suggested by α -SMA expression), thus attenuating HS formation in vivo.

Discussion

Many studies have shown that blocking the pathways upstream of TGF- β 1 signaling using inhibitors or siRNAs is not satisfactory and is associated with off-target effects [14, 20, 38]. The dead-end inhibitor SnoN provides more specific inhibition [39] but has not been well studied. Downregulation of SnoN by ubiquitination and degradation has been observed in cardiac fibrosis, pulmonary fibrosis, liver fibrosis, and renal fibrosis [27, 30–32, 36, 40]. Consistent with these studies, we found that the protein expression of SnoN was lower in HSFs than in NHDFs, suggesting the occurrence of SnoN degradation. SnoN protein was further downregulated when UM was added to HSFs, indicating that SnoN was degraded via the ubiquitin system, and treatment with both UM and TGF- β 1 also downregulated SnoN in both HSFs and NHDFs. Phosphorylation of Smad2 and Smad3 mediates TGF- β 1-induced SnoN degradation [24, 25]. Indeed,

Fig. 3 SR significantly inhibited TGF-β1-induced NHDF transdifferentiation and collagen deposition. **a** Effects of SR and SF on NHDF proliferation in absence or presence of TGF-β1. **b** Effects of SR and SF on *Coll*, *ColIII*, and *α-SMA* mRNA levels with and without TGF-β1. **c** Effects of SR and SF on *Coll*, *ColIII*, and *α-SMA* protein levels with and without TGF-β1. **d** Effects of SR and SF on TGF-β1-induced *Coll* and *ColIII* secretion in NHDFs ($n = 5$, (a) D0: $F = 35.116$, D3: $F =$

12.781, D7: $F = 6.699$, D10: $F = 8.055$, (b) *Coll*: $F = 113.202$, *ColIII*: $F = 199.134$, *α-SMA*: $F = 773.195$, (c) *Coll*: $F = 9.074$, *ColIII*: $F = 12.776$, *α-SMA*: $F = 10.376$, (d) *Coll*: $F = 17.901$, *ColIII*: $F = 15.070$; * $P < 0.05$ vs. the Ctrl group; # $P < 0.05$, ### $P < 0.001$ vs. the Ctrl + Tβ1 group; ▲ $P < 0.05$, ▲▲ $P < 0.01$ vs. the SF + Tβ1 group). NHDF normal human dermal fibroblast, TGF transforming growth factor

our results showed that phosphorylation of Smad2 and Smad3 was increased in HSFs compared with NHDFs and was enhanced following treatment with TGF-β1 in both cell lines, providing support for the observed degradation of SnoN in HSFs in particular.

SnoN is predominantly located in cytoplasm in normal human tissues and becomes exclusively nuclear in cancer cells. In cancer cells, SnoN is associated with cell differentiation, during which SnoN exhibits altered localization [40–43]. Furthermore, this molecule is resistant to

Fig. 4 SR significantly inhibited HSF proliferation and collagen deposition in the presence of TGF-β1. **a** Effects of SR and SF on HSF proliferation in the presence and absence of TGF-β1. **b**, **c** Effects of SR and SF on the mRNA (**b**) and protein (**c**) expression of Coll, ColIII, and α-SMA with and without TGF-β1. **d** Effects of SR and SF on TGF-β1-induced Coll and ColIII secretion in HSFs ($n = 5$, (**a**) D0: $F = 0.17$, D3: $F = 8.537$, D7: $F = 14.772$, D10: $F = 9.059$, (**b**) Coll: F

$= 30.996$, ColIII: $F = 17.197$, α-SMA: $F = 121.673$, (**c**) Coll: $F = 31.512$, ColIII: $F = 28.285$, α-SMA: $F = 31.747$, (**d**) Coll: $F = 5.24$, ColIII: $F = 5.576$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the Ctrl group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. the Ctrl + Tβ1 group; ▲ $P < 0.05$, ▲▲ $P < 0.01$, ▲▲▲ $P < 0.001$ vs. the SF + Tβ1 group). HSF hypertrophic scar fibroblast, TGF transforming growth factor

TGF-β1-induced degradation in the cytoplasm [33]. We found that SnoN was mainly localized in the cytoplasm of NHDFs but was predominantly localized in the nucleus of HSFs. Following TGF-β1 stimulation, NHDFs transdifferentiate into myofibroblasts, showing a phenotype similar to that of HSFs [11, 17, 35, 43], and SnoN

translocated into the nucleus accordingly. Thus, our findings showed that SnoN protein was downregulated in HSFs through ubiquitination and degradation resulting from TGF-β1-induced and p-Smad3- and p-Smad2-mediated cell phenotype alterations causing nuclear translocation of SnoN.

◀ **Fig. 5** SR attenuated hypertrophy and collagen deposition in rabbit ear scars. **a** Representative photographs of rabbit ear scars in each group on days 20, 30, and 40 after wounding. **b** Immunofluorescence staining for Ki67 in paraffin sections of scar tissues. DAPI was used for nuclear counterstaining. Scale bar = 20 μ m. **c** Immunohistochemistry staining of SnoN (red) and α -SMA (green) in rabbit ear scar paraffin sections on day 40 post injury. DAPI was used for nuclear counterstaining (blue). Scale bar = 100 μ m. **d** SnoN mRNA expression in rabbit ear scar on day 20 (D20), day 30 (D30), and day 40 (D40) post injury. mRNA and protein levels in the Ctrl group were normalized to those at 1. Data are expressed as fold changes. Values are means $\pm$ SEMs ($n = 5$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. the Ctrl group on D20 in vivo; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the Ctrl group on D30 in vivo; $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$, $\blacktriangle\blacktriangle\blacktriangle P < 0.001$ vs. the Ctrl group on D40 in vivo). **e** Rabbit ear scars were evaluated by the Vancouver scar scale (VSS). The effects of SR and SF on the VSS of scars were evaluated. Values are means $\pm$ SEMs ($n = 24$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the Ctrl group breadthwise or vs. D20 lengthwise; $^{\#}P < 0.05$ compared with the SF group breadthwise). Hypertrophy of scars was evaluated using the scar elevation index (SEI), which was similar to the VSS. Values are means $\pm$ SEMs ($n = 24$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the Ctrl group breadthwise or D20 lengthwise; $^{\#}P < 0.05$ compared with the SF group breadthwise). **f** Masson staining of rabbit ear scar paraffin sections on D20, D30, and D40 post injury, illustrating the effects of SR and SF on collagen (green) content and arrangement. Scale bar = 20 μ m. **g** Effects of SR and SF on protein and mRNA expression of Coll, ColIII, and α -SMA on D20, D30, and D40 in rabbit ear scars. Values are means $\pm$ SEMs ($n = 6$; $**P < 0.01$, $***P < 0.001$ compared with the Ctrl group on D20; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the Ctrl group on D30. $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ compared with the SF group on D30)

SF has been reported to be degraded rapidly following TGF- β 1 stimulation. Three crucial lysine residues in SnoN (89–92) are required for combination with Smads or Smad3; residues 164–172, also called the destruction box (D box), are required for interaction with a ubiquitin ligase complex; and residues 440, 446, and 449 are necessary for ubiquitin transfer or attachment. In addition, lysines 30 and 31 are required for the nuclear localization of SnoN. SnoN mutants lacking lysines 30, 31, 440, 446, and 449 exhibit impaired ubiquitination, thereby stabilizing SnoN and enhancing its function [22, 25, 33, 44]. Our SR mutant lacked lysines 440, 446, and 449; indeed, treatment with TGF- β 1 did not result in substantial degradation of the SR protein, verifying previous studies. This finding could also explain the observed differences in p-Smad2 and p-Smad3 levels. Thus, our results showed that SR was more active and fully overexpressed, and hence, SR was superior to SF in blocking the TGF- β 1/Smad signal.

c-Ski shares regions of high homology with SnoN in its N-terminus, which contains several structural regions critical for protein function, explaining the high functional similarities of SnoN and c-Ski [23]. c-Ski promotes the proliferation of fibroblasts derived from normal rat skin and decreases Coll by inhibiting Smad3, thereby modulating wound healing and scar formation [18, 45]. This molecule also inhibits fibroblast-to-myofibroblast phenocconversion in cardiac fibrosis [40].

Indeed, our results showed that SnoN accelerated the proliferation of NHDFs in the absence of TGF- β 1 but repressed proliferation in the presence of TGF- β 1. We postulated that SnoN functioned similarly to c-Ski in the stimulation of normal skin fibroblast proliferation but suppressed the proliferation of myofibroblasts induced by TGF- β 1 through depression of TGF- β 1/Smad signaling depending on the ratio of NHDFs to myofibroblasts, i.e., SnoN may promote proliferation when NHDFs are the predominant cell type but inhibit proliferation when the myofibroblast phenotype is the predominant phenotype. This hypothesis could be indirectly proven by a previous study showing that c-Ski overexpression led to a reduction in the number of myofibroblasts [46]. Additionally, our findings that overexpression of SnoN suppressed HSF proliferation, downregulated α -SMA (a marker of myofibroblasts), and blocked the transdifferentiation of NHDFs also supported this hypothesis. In addition, SnoN decreased Coll and ColIII production and secretion. Thus, our results demonstrated that SR promoted NHDF proliferation but suppressed myofibroblast proliferation, blocked transdifferentiation of NHDFs into myofibroblasts, and decreased ECM deposition.

TGF- β 1/Smad signaling enhances proliferation, augments contractility by upregulating α -SMA [47, 48], and induces ECM deposition by HSFs, resulting in HS formation [35, 49–51]. Consistent with these results, we found that SnoN suppressed proliferation, contractility, and ECM deposition by HSFs. Notably, in the presence of TGF- β 1, SF did not have substantial effects because it was degraded; however, SR still blocked these processes. Thus, based on our results showing that SnoN improved the appearance of scars and prevented hyperplasia in vivo, SR could alleviate HS formation.

SnoN affected the mRNA and protein levels of Coll, ColIII, and α -SMA in HSFs, but not in NHDFs, in the absence of TGF- β 1. This finding could be explained by the high levels of Smad2 and Smad3 phosphorylation in HSFs and the absence of Smad2 and Smad3 phosphorylation in NHDFs. Additionally, in the presence of TGF- β 1, SF did not effectively block ColIII expression compared with its effects on Coll expression. There are three possible explanations for this observation: (1) SF was degraded by TGF- β 1 stimulation; (2) ColIII is expressed at higher levels than Coll during wound healing and scar maturation [52], and (3) c-Ski has been shown to have a preference for Coll [45, 46].

Additionally, retaining SnoN in the cytoplasm has been shown to repress TGF- β 1-induced transcription of target genes in Hep3B cells and growth arrest in Ba/F3 cells, similar to SR in our study [33]. Thus, further studies are required to determine whether sequestering SnoN in the cytoplasm may also potentially repress TGF- β 1/Smad signaling and affect fibrosis.

In conclusion, we demonstrated that overexpression of SR inhibited the transdifferentiation of fibroblasts into myofibroblasts, suppressed the proliferation of HSFs, and reduced collagen deposition and arrangement, thereby attenuating scar hypertrophy. The mechanism was thought to involve amplification of SnoN function in TGF- β 1/Smad signaling due to impairment of degradation. Thus, this primary study on SR revealed its potential therapeutic value for preventing HS formation. In the future, we may demonstrate more sufficiently the role of SnoN by knock-down analysis, which was absent in the study.

Materials and methods

Cell culture and treatment

HSs and surrounding normal skin samples were collected from five patients (two men and three women; age range: 18–46 years). All patients received no treatment before HS revision surgery and signed written consent to donate their tissue specimens. The sample collection protocol was approved by the Ethics Committee of Affiliated Putuo Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China. Samples were packaged in gauze saturated with physiological saline and placed into sterile gloves on ice. Immediately after receipt, samples were trimmed of excessive epidermis and adipose tissues, rinsed with phosphate buffer saline (PBS) three times, cut into pieces, and incubated with DMEM (Gibco, Grand Island, NY, USA) containing 0.1% collagenase type I (Sigma, St. Louis, MO, USA) at 37 °C for 4 h to dissociate fibroblasts. Isolated cells were then cultured with DMEM containing 10% fetal calf serum (Gibco), 1% penicillin, and 1% streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. Fibroblasts were used at passages 3–6.

Construction, identification, and transfection of the SnoN lentivirus overexpression plasmid

The overexpressed SnoN lentivirus plasmid was constructed and identified by GeneChem (Shanghai, China). The coding sequences of human SF and SR were amplified by RT-PCR using the following primers: SF (forward) 5'-GAGGATCC CCGGTACCGGTCGCCACCATGGAAAACCTCCAG ACAAATTTTC-3' and (reverse), 5'-TCCTTGTAGTCCAT ACCTTCTTTAGCAGTCTTTGATGATTTTC-3'; SnoN-M (forward), 5'-GAGGATCCCCGGGTACCGGTCGCCACC ATGGAAAACCTCCAGACAAATTTTC-3' and (reverse), 5'-TCCTTGTAGTCCATACCCTTGGATTGATTCTCT TTCC-3'. Using the restriction enzyme AgeI, the PCR products were embedded into the pGV-LV vector and then transformed into DH5 α competent cells. Positive

transformants containing the correct coding sequence were selected by PCR and cultured at 37 °C for 16 h. Green fluorescent protein (GFP) was fused to the N-terminus of SF and SR. Using 100 μ L Lipofectamine 2000, 293T cells were cotransfected with 20 μ g pGC-LV plasmid, 15 μ g pHelper 1.0 plasmid, and 10 μ g pHelper 2.0 plasmid to generate the recombinant lentiviruses LV-GFP-SF and LV-GFP-SR. The recombinant virus was harvested and quantified. An empty lentivirus plasmid carrying no specific gene fragment, e.g., LV-GFP, was used as the vehicle (Vec).

Cells reaching approximately 30% confluence were transfected with 10⁷ TU/mL LV-GFP-SF (the SF group), LV-GFP-SR (the SR group), or LV-GFP (the vector group) diluted with 50 μ g/mL polybrene (P.E.) in enhanced infection solution (Eni.s.) or 50 μ g/mL P.E. in Eni.s. only (control group). The medium was replaced by DMEM with or without TGF- β 1 (5 ng/mL) 12 h later. Forty-eight hours later, transfection was confirmed by visualizing GFP fluorescence.

RT-qPCR

Total RNA was purified and extracted using a MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan). RNA was then converted to cDNA with a Prime Script RT Master Kit (Perfect Real Time; TaKaRa). In brief, 2 μ L of 5 $\times$ Perfect Real Time, together with 500 ng total RNA, was combined with RNA-free double distilled water to 10 μ L. The reaction conditions were 37 °C for 15 min, 85 °C for 5 s, and 4 °C. The resulting cDNA was then amplified using a Vii ATM7 Real-Time PCR system (Bio-Rad, USA) with a SYBR Premix Ex Taq Kit (Tli RNaseH Plus; Takara) with the primers shown in Table S1 [14]. The *GAPDH* gene served as an internal control. The PCR protocol was as follows: initial denaturation at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative expression of target genes was determined by the cycle threshold (Ct) at which the specific fluorescence became detectable. The Ct value was used for kinetic analysis and was proportional to the initial number of target copies in the sample. The RT-qPCR data were exported and processed using the $\Delta\Delta$ CT method.

Western blotting

Western blotting was performed as previously described [53]. Briefly, the cells or sheared scar tissues were collected and lysed with 1 $\times$ RIPA lysis buffer on ice for 10–15 min, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. Protein quantification was performed using bicinchoninic acid (BCA) assays. Twenty micrograms of total protein was subjected to SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore, USA). After blocking with 5% nonfat milk, the membranes were

incubated with specific primary antibodies as shown in Table S2 at 4 °C overnight. Membranes were then washed three times with Tris-buffered saline with 0.1% Tween-20 and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 37 °C for 2 h. Immunoreactive bands were detected with a Chemiluminescent HRP Substrate ECL kit (Millipore). The density of each protein band on the membrane was scanned using an Alpha Imager scanner (Alpha Innotech, San Jose, CA, USA) and analyzed with AlphaEase FC image processing software (Alpha Innotech). GAPDH was used to ensure equal protein loading.

Degradation assays for SnoN

To investigate the degradation activity of SnoN, we conducted *in vitro* degradation assays for endogenous SnoN in NHDF and HSF lysates. Mixtures of cell lysates (15 µg) in 15 µL (50 mM Tris-HCl [pH 8.3], 5 mM MgCl₂, 2 mM dithiothreitol, 5 mM adenosine-50-triphosphate, 2 mg/mL ubiquitin) were incubated for 24 h at 37 °C. After incubation, each sample was subjected to SDS-PAGE, followed by immunoblot analysis with anti-SnoN antibodies⁽²⁷⁾ (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cellular immunofluorescence

Briefly, 300 µL NHDF and HSF suspensions (1000 cells/mL) were seeded in 35-mm glass-bottomed plates overnight, and cells were then treated with or without 5 ng/mL TGF-β1 for 24 h. Cells were fixed with 1 mL of 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and blocked by 1% Albumin from bovine serum (BSA) in PBS for 30 min. Rabbit polyclonal anti-SnoN antibodies (Abcam, USA) diluted at 20 µg/mL were added overnight, followed by incubation with Alexa Fluor 647 (Abcam) secondary antibodies (1:250 dilution) at 37 °C for 30 min. Finally, cells were mounted using antifade fluorescence mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (HelixGen, Guangzhou, China).

Fibroblast proliferation assays

Cell proliferation was quantified using CCK-8 assays (Dojindo, Japan). Briefly, cells from five donors were seeded at 3×10^3 cells/well in 96-well plates and transfected with LV-GFP-SF, LV-GFP-SR, empty lentivirus plasmid (vector), or nothing (control). After 48 h, the medium was replaced with dulbecco's modified eagle medium (DMEM) with or without 5 ng/mL TGF-β1 (R&D Systems, USA). The medium was refreshed every 2 days. On days 3, 7, and 10, 10% CCK-8 solution in 100 µL DMEM was added to each well. Two hours later, the absorbance was recorded at 450 nm.

ELISA

To determine ColI and ColIII production in the culture fluid of fibroblasts after transfection, the medium was collected from day 5 to day 7 during the experimental period. The samples were subjected to ELISAs using human collagen type I and III ELISA kits (BlueGene, Shanghai, China). Absorbance was recorded at 450 nm using a spectrophotometer (Bio-Rad), and concentrations were determined using a standard curve. All measurements were performed in triplicate, and data are expressed in µg/mL.

Rabbit ear scar model

To evaluate the outcomes of treatment *in vivo*, we used a chronic rabbit ear scarring model, as established by Morris et al., with minor modifications [37]. In brief, 36 adult New Zealand white rabbits weighing 2–2.5 kg each (50% male/50% female; Songlian Experimental Animal Institute, Songjiang District, Shanghai) were acclimated and housed under a 12-h light/12-h dark cycle with free access to water and food. All surgical approaches and procedures were approved by the Institutional Ethical Committee of Shanghai University of Traditional Chinese Medicine. Rabbits were anesthetized with xylazine (5 mg/kg) and prepared for wounding under sterile conditions. Four 1-cm full-thickness skin-defect wounds with removal of the perichondrium were created on the ventral side of each ear. The wounds were then covered with sterile gauze for 1 day. On day 7, the 288 wounds were randomly divided into four groups based on the ear, and each was injected with 20 µL of 2×10^8 TU/mL LV-GFP-SF (SF group), LV-GFP-SR (SR group), or LV-GFP (vector group) in normal saline. Wounds in the control group were not treated. The treatment was repeated 1 week later to enhance the transfection efficiency. The scars were evaluated by VSS [14] on days 20, 30, and 40 after surgery. The evaluation criteria for VSS included pliability, height, vascularity, and pigmentation (Supplementary Table s3). Animals were then sacrificed to collect scar specimens. Hypertrophy of the dermis was evaluated using the SEI [14]. Scar specimens were also prepared as 5-µm-thick paraffin sections for immunofluorescence staining.

Masson staining

Scar tissues were excised *in situ* from sacrificed rabbits on days 20, 30 and 40, fixed with 10% formalin, embedded in paraffin, sectioned at 5-µm thickness onto glass slides, and stained using a Masson Trichrome Staining kit (Showbio Biotech, China). Scars were examined for the expression and arrangement of collagen fibers under an FSX100 microscope (Olympus, Shinjuku-ku, Tokyo, Japan).

Immunohistofluorescence staining

Rabbit ear scars were excised on days 20, 30, and 40, embedded in paraffin, and cut into 4- μ m-thick sections for immunofluorescence staining. Sections were deparaffinized, dehydrated, and subjected to antigen retrieval. Sections were then incubated with anti-Ki67 (Abcam), anti- α -SMA (Sigma), or anti-SnoN (Santa Cruz Biotechnology) primary antibodies overnight incubated at 4 °C. Sections were then incubated with Alexa Fluor 488 goat anti-rabbit IgG H&L and Cy3-conjugated goat anti-rabbit IgG H&L secondary antibodies at 37 °C for 1 h. DAPI was used for nuclear staining.

Statistical analyses

All values are expressed as the mean $\pm$ standard error of the mean (SEM). Differences between groups were examined for statistical significance using analysis of variance. When a significant difference was found, further statistical analysis was performed using least significant difference *t* tests between the two groups. *P* values of less than 0.05 denoted statistically significant differences.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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