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The Role of SnoN in Transforming Growth Factor β1-induced Expression of Metalloprotease-Disintegrin ADAM12*[S]

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Emilia Solomon, Hui Li, Sara Duhachek Muggy, Emilia Syta, and Anna Zolkiewska[1]

From the Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506

Increased expression of metalloprotease-disintegrin ADAM12 is a hallmark of several pathological conditions, including cancer, cardiovascular disease, and certain inflammatory diseases of the central nervous system or the muscoskeletal system. We show that transforming growth factor β1 (TGFβ1) is a potent inducer of ADAM12 mRNA and protein in mouse fibroblasts and in mouse and human mammary epithelial cells. Induction of ADAM12 is detected within 2 h of treatment with TGFβ1, is Smad2/Smad3-dependent, and is a result of derepression of the *Adam12* gene. SnoN, a negative regulator of the TGFβ signaling pathway, is a master regulator of ADAM12 expression in response to TGFβ1 stimulation. Overexpression of SnoN in NIH3T3 cells reduces the magnitude of ADAM12 induction by TGFβ1 treatment. Down-regulation of SnoN expression by short hairpin RNA enhances TGFβ1-induced expression of ADAM12. In a panel of TGFβ1-responsive cancer cell lines with high expression of SnoN, induction of ADAM12 by TGFβ1 is significantly impaired, suggesting that the endogenous SnoN plays a role in regulating ADAM12 expression in response to TGFβ1. Identification of SnoN as a repressor of the *ADAM12* gene should contribute to advances in the studies on the role of ADAM12 in tumor progression and in the development of other pathologies.

ADAM12, a member of the metalloprotease-disintegrin family of proteins, has been implicated in the progression of cancer, cardiovascular disease, osteoarthritis, and neurological disorders (1). The *ADAM12* gene is frequently mutated in human breast cancers (2, 3), and cancer-associated mutations cause mislocalization of the ADAM12 protein in cells and alter its function (4). Missense single nuclear polymorphism in the *ADAM12* gene shows strong association with osteoarthritis (5, 6). In addition to changes in its amino acid sequence, expression levels of ADAM12 are significantly increased in many pathological states. For example, ADAM12 expression levels are 20–30-fold higher in human breast tumors than in normal mammary epithelium (7–12). ADAM12 expression is also markedly up-regulated in cancers of the liver, lung, stomach, colon, prostate, bladder, and in glioblastoma (13–18). Increased ADAM12 expression levels are found in the cardiac tissue of patients with hypertrophic obstructive cardiomyopathy (19) and in mice with angiotensin II-induced hypertension and cardiac hypertrophy (20, 21). During inflammatory responses and aseptic osteolysis associated with loosened hip replacement implants, ADAM12 is up-regulated in the interface tissue around loosening implants (22). In experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis, ADAM12 level is markedly increased in the T cells that infiltrate spinal cords (23).

The mechanisms regulating ADAM12 expression, in particular those that may be responsible for altered levels of ADAM12 in various pathological states, are poorly understood. Previous studies employing hepatic stellate cells, a mesenchymal cell type in hepatic parenchyma, have indicated that ADAM12 expression is induced by transforming growth factor β (TGFβ)[2] (13, 24). The TGFβ signaling pathway is initiated when one of the family members, *e.g.* TGFβ1, -β2, or -β3, binds to a complex of TGFβ type I and type II serine/threonine kinase receptors (TβRI and TβRII, respectively) and induces phosphorylation and activation of TβRI by TβRII. TβRI then phosphorylates receptor Smads (R-Smads), Smad2 and Smad3. Phosphorylated Smad2/3 associate with the common partner Smad4 and translocate to the nucleus, where they regulate transcription of target genes (25, 26). In addition, receptor activation in certain cell types leads to Smad-independent responses via the activation of mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase, and Rho family members (27, 28).

SnoN and the related Ski protein are negative regulators of TGFβ signaling. They bind to nuclear Smad complexes and repress their transcriptional activities (29–31). In response to TGFβ stimulation, SnoN (and to a lesser extent Ski) undergoes ubiquitination and rapid proteasomal degradation (32, 33). The ubiquitin ligases implicated in ubiquitination of SnoN, the anaphase promoting complex, Smurf2, and Arkadia, are recruited to SnoN via the phosphorylated R-Smads (34–38).

Previous study on the regulation of ADAM12 expression by TGFβ in hepatic stellate cells used rather long (24–72 h) stimulation times and showed that ADAM12 induction was par-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

[1] To whom correspondence should be addressed: Dept. of Biochemistry, Kansas State University, 141 Chalmers Hall, Manhattan, KS 66506. Tel.: 785-532-3082; Fax: 785-532-7278; E-mail: zolkiea@ksu.edu.

[2] The abbreviations used are: TGFβ, transforming growth factor β; TβRI and TβRII, TGFβ type I and type II serine/threonine kinase receptors, respectively; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT, reverse transcription; qRT-PCR, real time quantitative RT-PCR; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R-Smad, receptor Smad; shRNA, short hairpin RNA; DMSO, dimethyl sulfoxide.

tially blocked by inhibitors of MAPKs, phosphoinositide 3-kinase, or p70S6 kinase (13, 24). Based on these results, it was postulated that induction of ADAM12 expression by TGFβ might be Smad-independent, but a direct role of R-Smads in the regulation of ADAM12 expression has not been tested. In this report, we investigate short term (0–24 h) effects of TGFβ on ADAM12 mRNA and protein levels in mouse fibroblasts. We find that TGFβ causes derepression of the *Adam12* gene in a Smad2/3-dependent manner, and that the repressor responsible for the negative regulation of ADAM12 expression is SnoN. Our studies uncover a new mechanism of ADAM12 regulation by TGFβ that may contribute to aberrant expression of ADAM12 in various diseases.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 fibroblasts, HT1080 fibrosarcoma cell line, DU145 prostate cancer cell line (American Tissue Culture Collection), and retroviral packaging cell line Phoenix Eco (G. P. Nolan, Stanford University) were grown in DMEM supplemented with 10% FBS. *Smad2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) (E. Bottinger, Mount Sinai School of Medicine), *Smad3*$^{-/-}$ MEFs (K. Flanders, NCI), *Ski*$^{-/-}$ MEFs (C. Colmenares, Cleveland Clinic), *Adam9/12/15*$^{-/-}$ MEFs (C. P. Blobel, Hospital for Special Surgery), and wild-type MEFs were grown in DMEM containing 10% FBS and 1% penicillin/streptomycin. Normal mouse mammary gland epithelial cell line NMuMG and human MCF7 breast cancer cells were grown in DMEM with 10% FBS and 10 μg/ml of insulin. Normal human mammary epithelial cells MCF-10A were cultured in DMEM/F-12 supplemented with 5% horse serum, 0.5 μg/ml of hydrocortisone, 20 ng/ml of human epidermal growth factor, 10 μg/ml of bovine insulin, 100 ng/ml of cholera toxin, and 1% penicillin/streptomycin. MDA-MB-468 breast cancer cells were cultured in Liebovitz's L-15 medium supplemented with 10% FBS. MDA-MB-435S melanoma cells were maintained in Liebovitz's L-15 medium supplemented with 10% FBS and 10 μg/ml of bovine insulin. MDA-MB-231 breast cancer cells were cultured in DMEM/F-12 medium supplemented with 10% FBS. T47D breast cancer cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Cells were treated with 2 ng/ml of TGFβ1 (R&D Systems) for 24 h (unless indicated otherwise), 10 μM MG132 (EMD Biosciences) for 1 h prior to and during TGFβ1 treatment, 5 μg/ml of actinomycin D (Sigma) for 15 min prior to and during TGFβ1 treatment, 5 μg/ml of cycloheximide (Sigma) for 2 h prior to and during TGFβ1 treatment, or 10 μM SB-431542 (Sigma) 30 min prior to and during TGFβ1 treatment; control incubations with vehicle alone were included in each experiment.

Viral Transduction—Human SnoN cDNA was transferred from pCI-Neo HA-hSnoN plasmid vector into the retroviral pBMN-I-GFP vector (both from Addgene). Phoenix Eco cells were transfected with SnoN retroviral vector (15 μg of plasmid DNA/100-mm plate) using the calcium phosphate precipitation method. Viral supernatants were harvested 48 h later, supplemented with 5 μg/ml of Polybrene, and used without further dilution for infection of NIH3T3 cells. For SnoN knockdown, NIH3T3 cells were incubated with MISSION™ Lentiviral shSnoN Transduction Particles (Sigma, clone ID TRCN0000088306) or with MISSION™ Non-Target shRNA Control Transduction Particles (Sigma, SHC002V), according to the manufacturer's instructions. After 1 day, medium containing retroviral particles was replaced with fresh medium, and after an additional 24 h, stably transduced cells were selected with 2 μg/ml of puromycin for 7 days.

Immunoblotting—Immunoblotting was performed as described (4). For ADAM12 detection, cell extracts were enriched for glycoproteins using concanavalin A-agarose prior to SDS-PAGE and Western blotting (39). The following primary antibodies were used: rabbit anti-ADAM12 cytoplasmic peptide antibody (Ref. 39, 1:3,000), rabbit anti-ADAM9 (Ref. 39, 1:400), goat anti-ADAM15 (R&D Systems, 1:100), rabbit anti-SnoN (H-317, Santa Cruz Biotechnology, 1:1,000), mouse anti-Ski (G8, Santa Cruz Biotechnology, 1:100), and mouse anti-α-tubulin (Sigma, 1:100,000). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG antibodies. Signal detection was performed using WestPico or WestFemto (for anti-Ski blots) chemiluminescence detection kit (Pierce).

RNA Analysis—Total RNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System containing TRIzol (Invitrogen). Northern blot analysis was performed using the Northern Max kit (Ambion). Membranes were hybridized with ADAM12 cDNA probe (nucleotides 161–2202) or β-actin probe provided with the kit. Probes were labeled using DECAprime II Random Primed DNA Labeling kit (Ambion) and [α-^{32}P]dATP. For reverse transcription (RT)-PCR analysis, RNA (1 μg) was treated with deoxyribonuclease I (Invitrogen), followed by reverse transcription using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. Semi-quantitative PCR was performed in 50-μl reaction volumes using 1 μl of cDNA, 0.2 mM dNTPs, 2 units of BIO-X-ACT Short DNA Polymerase (Bioline), and 1 μM primers (supplemental Table S1). PCR conditions were: 94 °C, 30s; 55 °C, 30s; 72 °C, 45s; 29–32 cycles for mADAM12, and 24–26 cycles for mGAPDH. PCR products were resolved in 2% agarose/TAE gels, visualized after ethidium bromide staining and UV illumination, and quantified by densitometry. Real time quantitative RT-PCR (qRT-PCR) was performed in a total volume of 25 μl in a 96-well spectrofluorometric thermal cycler (iCycler, Bio-Rad). The final reaction mixture contained 10.5 μl of diluted cDNA (1:5 for human cDNA, 1:50 for mouse cDNA), 12.5 μl of iQSYBR Green Supermix, and 0.4 μM primers (supplemental Table S1). PCR conditions were: 95 °C, 30s; 55 °C, 30s; 72 °C, 40s. The relative expression of ADAM12 mRNA, normalized to mouse GAPDH or human β-actin, was calculated using the $2^{(-\Delta\Delta C_t)}$ method.

Gene Reporter Assays—Fragments of the genomic region located upstream of the ADAM12 translation initiation site (see Fig. 4*A*) were amplified from mouse genomic DNA using Pfu Turbo DNA polymerase and inserted into the multiple cloning site of pGL4.10luc2 vector (Promega) to generate pA12.Luc reporters. NIH3T3 cells grown in 6-well plates were transfected at 50% confluence with 0.5 μg of pA12.Luc reporters or empty pGL4.10luc2 vector, together with 0.05 μg of *Renilla* luciferase vector (pRL-TK), using FuGENE 6 transfection reagent. After 24 h, firefly and *Renilla* luciferase activities were measured

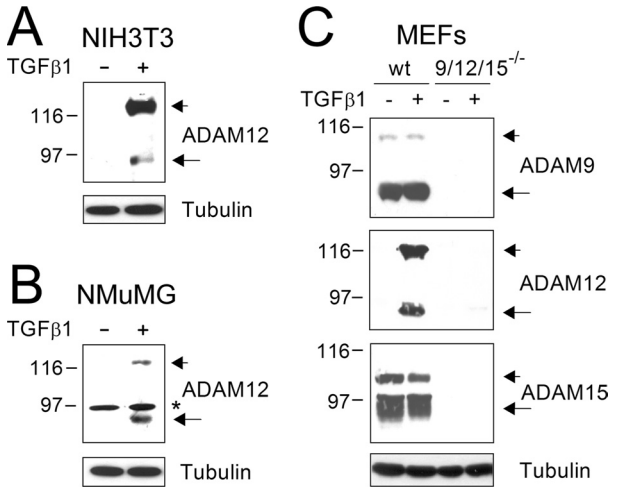

FIGURE 1. **Induction of ADAM12 expression by TGFβ1.** *A*, NIH3T3 fibroblasts; *B*, normal mouse mammary gland epithelial cells NMuMG; and *C*, MEFs (*wt*, wild-type; $9/12/15^{-/-}$, isolated from triple knock-out mice lacking ADAM9, -12, and -15) were incubated for 24 h in the presence or absence of 2 ng/ml of TGFβ1, as indicated. Cells were lysed and glycoprotein-enriched fractions were analyzed by Western blotting using anti-ADAM antibodies; tubulin is a gel loading control. The full-length ADAM proteins are indicated by *short arrows*, the mature forms lacking the pro-domains are indicated by *long arrows*, the *asterisk* denotes a nonspecific band.

using the Dual-Luciferase Reporter Assay System (Promega). To study the effect of TGFβ1 on the *Adam12* promoter, 24 h after transfection cells were stimulated with 2 ng/ml of TGFβ1 for an additional 24 h. The p3TP-Lux plasmid (Addgene) was used as positive control. The effect of TGFβ1 was also studied in NIH3T3 cells stably transfected with pA12.Luc1 vector. Stable transfectants were selected for 7 days in the presence of 2 μg/ml of puromycin; pooled populations of cells were used without isolation of individual clones. All assays were performed in duplicates.

Statistical Analysis—Paired *t* test was used to compare values of two groups. When fold-change in ADAM12 expression was calculated (stimulus- or inhibitor-treated cells *versus* vehicle control), data were analyzed by one sample *t* test (GraphPad Prism Software, San Diego, CA). Linear regression analysis was performed using the Linear Fit tool, with direct error weighting (Origin 8.0 software, OriginLab, Northampton, MA).

RESULTS

Previous reports have shown that treatment of human hepatic stellate cells with TGFβ1 induces expression of ADAM12 (13, 24). Here, we extended the analysis of TGFβ1 effects on ADAM12 expression to other cell types. We observed that treatment of mouse NIH3T3 fibroblasts or normal mouse mammary epithelial cell line NMuMG for 24 h with TGFβ1 led to dramatic increase in ADAM12 protein levels (Fig. 1, *A* and *B*). The levels of two other ADAMs, ADAM9 and ADAM15, were not changed after similar TGFβ1 treatment (Fig. 1*C*), demonstrating that among the three ADAMs tested, the effect of TGFβ1 was specific for ADAM12.

Induction of ADAM12 protein in cells that were starved in 0.5% serum for 24 h prior to adding TGFβ1 was comparable with the induction observed in the presence of 10% serum (Fig. 2*A*). Therefore, all subsequent experiments were performed using medium supplemented with 10% FBS, without starvation. It has to be stressed that when cells were incubated for prolonged times (~48 h) without adding fresh medium, the basal level of ADAM12 expression was significantly elevated, and it was efficiently reduced by adding SB-431542, an inhibitor of TβRI (Fig. 2*A*). This result suggests that the increase in the basal level of ADAM12 expression was most likely due to the autocrine/paracrine effect of the endogenous TGFβ1 produced in NIH3T3 cells that was accumulating over time in cell medium. The induction of ADAM12 protein in NIH3T3 cells by exogenously added TGFβ1 was dose-dependent and reached a maximum at 2 ng/ml of TGFβ1 (Fig. 2, *B* and *C*). This concentration of TGFβ1 was used in the remaining part of this study. The up-regulation of ADAM12 protein was evident after 8 h of stimulation of cells with TGFβ1 (Fig. 2, *D* and *F*). Semi-quantitative RT-PCR analysis further demonstrated that TGFβ1 treatment increased the level of ADAM12 mRNA, and the changes in ADAM12 mRNA preceded the changes in ADAM12 protein levels (Fig. 2, *E* and *F*).

Pre-treatment of cells with actinomycin D, an inhibitor of transcription, completely blocked the up-regulation of ADAM12 protein (Fig. 3*A*), indicating that induction of ADAM12 expression by TGFβ1 required new transcription. Consistently, qRT-PCR (Fig. 3*B*) or Northern blot analysis of NIH3T3 cells treated with TGFβ1 (Fig. 3*C*) showed that the induction of ADAM12 mRNA by TGFβ1 was also blocked by pre-treatment of cells with actinomycin D. As TGFβ1 did not have any effect on the stability of ADAM12 mRNA (Fig. 3*D*), we have concluded that TGFβ1 activates the transcription of the *Adam12* gene.

Smad2 and Smad3 are the main mediators of the transcriptional responses to TGFβ1. Smad3-Smad4 DNA binding elements contain a repeated AGAC sequence or its complement, GTCT. Activated Smad2-Smad4 complexes are recruited to the activin response elements with the help of FoxH1a or FoxH1b (40, 41). The ~5.1-kilobase genomic region upstream of the translation initiation site of mouse *Adam12* contains a promoter activity and comprises multiple AGAC (or GTCT) sequences, including a 9×GTCT repeat, and an inverted activin response element, AATAAACA (Fig. 4*A*). Despite the presence of these motifs, gene reporters containing different fragments of the *Adam12* promoter were not responsive to TGFβ1 (Fig. 4*B*). pA12.Luc1 reporter stably transfected into NIH3T3 cells was also unresponsive to TGFβ1 (result not shown), excluding the possibility that activation of the *Adam12* promoter by TGFβ1 occurs only when the promoter is integrated into chromatin. Nevertheless, the induction of ADAM12 by TGFβ1 in $Smad2^{-/-}$ or $Smad3^{-/-}$ MEFs was significantly impaired when compared with the wild-type MEFs (Fig. 4, *C* and *D*). These results indicate that Smad2/3 do play a role in regulation of ADAM12, but TGFβ-responsive elements are located outside the ~5.1-kilobase promoter region in the *Adam12* gene.

Two lines of evidence further suggest that induction of ADAM12 expression by TGFβ1 involves derepression of the *Adam12* gene. First, pretreatment of cells with cycloheximide, an inhibitor of translation, did not abolish the induction of ADAM12 mRNA by TGFβ1 (Fig. 5*A*). In fact, cycloheximide

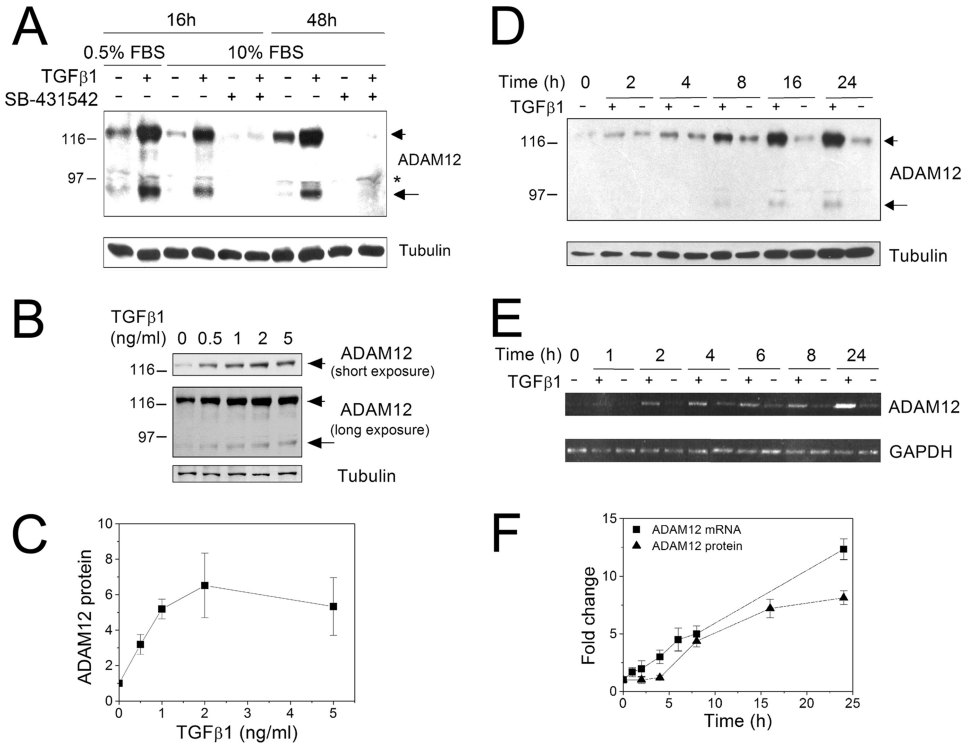

FIGURE 2. **Characterization of TGFβ1-induced expression of ADAM12.** *A*, the effect of serum on the induction of ADAM12 by TGFβ1. NIH3T3 cells were preincubated for 24 h in the presence of 0.5 or 10% FBS, as indicated, followed by treatment with 2 ng/ml of TGFβ1 for an additional 16 or 48 h. In some cases, 10 μM SB-431543, an inhibitor of TβRI, was added 30 min prior to TGFβ1 treatment. *B* and *C*, TGFβ1 dose-response of the induction of ADAM12. *B*, NIH3T3 cells were incubated for 24 h with the indicated doses of TGFβ1, and the levels of ADAM12 protein were analyzed as described in the legend to Fig. 1. Quantitative differences in the abundance of the full-length ADAM12 (*short arrow*) are best shown after short exposure of the immunoblot, the mature ADAM12 (*long arrow*) is visualized after long exposure. *C*, the intensities of the bands corresponding to the full-length ADAM12 protein in *panel B* were quantified using densitometry and ImageJ software. The data represent the mean ± S.E. (*error bars*) from three independent determinations. *D-F*, time course of ADAM12 induction by TGFβ1. NIH3T3 cells were incubated without or with 2 ng/ml of TGFβ1 for the indicated times. *D*, the levels of ADAM12 protein were analyzed by Western blotting, as described in the legend to Fig. 1. *E*, the levels of ADAM12 mRNA were analyzed by semi-quantitative RT-PCR, GAPDH is a gel-loading control. *F*, the intensities of the bands corresponding to the full-length ADAM12 protein in *D* and ADAM12 mRNA in *E* were quantified using densitometry and ImageJ software. The data represent the mean ± S.E. (*error bars*) from three independent determinations.

To determine whether SnoN is directly involved in TGFβ1-induced up-regulation of ADAM12, we first studied the effect of SnoN overexpression on the level of ADAM12 in TGFβ1-treated cells. NIH3T3 cells were transduced with SnoN or control retroviruses, and 24 h later they were incubated for 16 h with or without TGFβ1. As shown in Fig. 6, *A* and *B*, overexpression of SnoN caused ~50% reduction in ADAM12 expression in response to TGFβ1, and this effect was statistically significant. The lack of a stronger inhibition of ADAM12 expression by SnoN may be caused by the fact that the efficiency of viral transduction was only ~50–60% (as determined by GFP fluorescence, result not shown).

Next, we examined the effect of knocking down the expression of the endogenous SnoN in NIH3T3 cells on the expression levels of ADAM12. NIH3T3 cells were infected with lentiviral SnoN shRNA or control shRNA particles, and cells with stable incorporation of shRNA vectors were selected in the presence of puromycin. The level of the endogenous SnoN protein in shSnoN cells was reduced to an undetectable level (Fig. 7*A*). Induction of ADAM12 mRNA by TGFβ1 was more potent and occurred with faster kinetics in shSnoN cells than in shRNAControl, as revealed by qRT-PCR (Fig. 7*B*).

alone increased the level of ADAM12 mRNA (Fig. 5*B*). These results suggest that *de novo* protein synthesis is not required for the up-regulation of ADAM12 by TGFβ1 and that cycloheximide might block synthesis of a transcriptional repressor acting on the *Adam12* promoter/gene. Second, pre-treatment of cells with MG132, a proteasomal inhibitor, efficiently blocked the induction of the ADAM12 protein and mRNA by TGFβ1 (Fig. 5, *C* and *D*). This result, together with the effect mediated by cycloheximide, indicates that TGFβ1 signaling leads to proteasomal degradation of a repressor of the *Adam12* gene. SnoN, a negative regulator of TGFβ1 signaling, is known to be rapidly degraded in response to TGFβ1 stimulation, and thus may be a good candidate for a repressor of the *Adam12* gene. Indeed, analysis of SnoN protein levels in cells treated with TGFβ1 confirmed that SnoN, a 75-kDa protein, was degraded within 30 min of TGFβ1 treatment and remained at a low level for up to 4 h after adding TGFβ1 (Fig. 5*E*). As SnoN expression is up-regulated by TGFβ1 signaling as a part of the negative feedback loop mechanism that limits the duration and strength of the TGFβ1 signals (32), the level of SnoN began to rise and eventually returned to its initial level after 8–16 h of TGFβ1 treatment (Fig. 5*E*).

As the induction of ADAM12 by TGFβ1 was very efficiently blocked by MG132 (see Fig. 5, *C* and *D*), suggesting that degradation of a transcriptional repressor is required for ADAM12 expression, we asked whether MG132 would be equally potent in blocking the induction of ADAM12 in SnoN-deficient cells. If SnoN is the repressor of the *Adam12* gene, then in the absence of SnoN, MG132 should have little effect on ADAM12 expression. As shown in Fig. 7*C*, *right panel*, incubation of shSnoN cells with TGFβ1 in the presence of MG132 led to the induction of ADAM12 expression, whereas no induction was observed in control cells under the same conditions (Fig. 7*C*, *left panel*). The induction of ADAM12 in shSnoN cells in the presence of MG132 was more modest than in the absence of the inhibitor (Fig. 7*D*), but this can be explained by the actual levels of SnoN in these cells. Although no SnoN was detected in shSnoN cells in the absence of the inhibitor, MG132 treatment resulted in a significant accumulation of SnoN (Fig. 7*C*). This most likely was the result of incomplete double stranded mRNA degradation, active synthesis of SnoN protein, and inhibition of its degradation. The accumulation of SnoN in MG132-

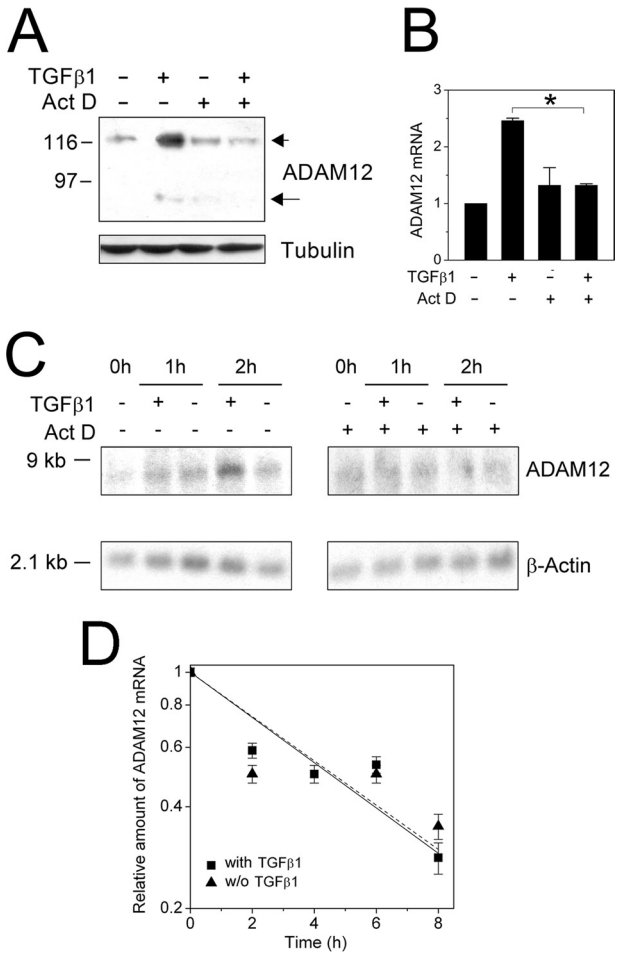

FIGURE 3. **Induction of ADAM12 expression by TGFβ1 occurs at the transcriptional level.** *A*, NIH3T3 cells were pretreated for 15 min with 5 μg/ml of actinomycin D or with DMSO, then incubated for 8 h with or without 2 ng/ml of TGFβ1, followed by Western blotting with anti-ADAM12 antibody, as described in the legend to Fig. 1. Tubulin is a gel loading control. The experiment was repeated 3 times with similar results. *B*, qRT-PCR analysis of ADAM12 expression. NIH3T3 cells were pretreated for 15 min with 5 μg/ml of actinomycin D or DMSO, and then incubated without or with 2 ng/ml of TGFβ1 for 4 h. The level of ADAM12 mRNA was normalized to GAPDH. The data represent the mean ± S.E. (*error bars*) from three independent experiments. *Asterisk* indicates the statistically significant effect ($p < 0.05$) of inhibitor treatment. *C*, Northern blot analysis of ADAM12 expression. NIH3T3 cells were pretreated for 15 min with 5 μg/ml of actinomycin D or with DMSO, and then treated without or with 2 ng/ml of TGFβ1 for the indicated times. Total mRNA was extracted and hybridized with ADAM12 and β-actin probes. The analysis was repeated 3 times with similar results. *D*, the effect of TGFβ1 on the stability of ADAM12 mRNA. NIH3T3 cells were incubated for 16 h with 2 ng/ml of TGFβ1 (*squares*) or with vehicle alone (*triangles*), and then 5 μg/ml of actinomycin D was added (time 0). Total mRNA was extracted at various time points and ADAM12 mRNA levels, normalized to GAPDH mRNA, were determined by qRT-PCR. The data represent changes in ADAM12 mRNA relative to the levels at time 0 (mean values ± S.E. (*error bars*), $n = 3$). The half-life of ADAM12 mRNA in the presence or absence of TGFβ1 was estimated as 6.5 ± 1.0 h^{-1} (*solid line*) and 6.6 ± 1.7 h^{-1} (*dashed line*), respectively.

treated shSnoN cells might be the reason why SnoN knockdown by shRNA did not fully bypass the inhibitory effect of MG132 on the induction of ADAM12 by TGFβ1. Alternatively, SnoN might not be the sole repressor of the *Adam12* gene. A possible candidate for ADAM12 repression may be a SnoN-related protein Ski, which has also been reported to undergo proteasomal degradation in response to TGFβ1 stimulation (42).

To determine whether Ski plays a role in repressing *Adam12*, we examined the effect of TGFβ1 on the level of ADAM12 protein in $Ski^{-/-}$ cells, in the presence or absence of MG132 (Fig. 8). As shown in Fig. 8*C*, inhibition of protein degradation completely blocked the induction of ADAM12 by TGFβ1 in $Ski^{-/-}$ cells. Thus, Ski does not appear to play a major role in repressing the *Adam12* gene.

To determine whether the role of SnoN in TGFβ1-induced expression of ADAM12 observed in NIH3T3 cells can be extended to other cell types, we examined the effect of TGFβ1 on ADAM12 expression in human cancer cell lines that typically express higher levels of SnoN than untransformed cells. For our analysis, we selected several cell lines in which the major components of the canonical TGFβ1 signaling pathway remain intact and which are responsive to TGFβ1 signals: MDA-MB-231, MDA-MB-435S, DU145, and HT1080 (43–45). The basal level of ADAM12 mRNA in all four cancer cell lines was higher than in MCF10A cells, but it was not inhibited by SB-431542 (results not shown), indicating that this was not a result of an elevated autocrine/paracrine TGFβ1 signaling. Upon TGFβ1 treatment, there was a ~7-fold increase in ADAM12 expression in MCF10A cells and a much lower (<2-fold) increase in MDA-MB-231, MDA-MB-435S, DU145, and HT1080 cells (Fig. 9*A*). As expected, no induction of ADAM12 was detected in Smad4-deficient MDA-MB-468 cells, or in T47D and MCF7 cells, which express very low levels of TβRII and do not respond to TGFβ1 signals (46) (results not shown). In agreement with previous reports, the level of SnoN in cancer cells was consistently higher than in the normal mammary epithelial cell line MCF10A, whereas the level of Ski showed more variation (Fig. 9*B*). Thus, it appears that among TGFβ1-responsive cells, there is an inverse correlation between the extent of ADAM12 induction by TGFβ1 and the level of SnoN, but not Ski (Fig. 9*C*). These results further support the role of SnoN in repression of the *ADAM12* gene.

DISCUSSION

The induction of ADAM12 expression by TGFβ1 was first reported in hepatic stellate cells (13, 24). Our studies demonstrate that induction of ADAM12 by TGFβ1 is a more general phenomenon and it takes place in fibroblasts and in epithelial cells. Recently, it has been shown that ADAM12 can enhance TGFβ signaling through its interaction with TβRII (47). Thus, TGFβ1-mediated induction of ADAM12 may be part of a positive feedback loop mechanism, leading to self-amplification of TGFβ1 signals.

TGFβ1 does not appear to cause general up-regulation of ADAM proteins, as at least two other ADAMs, ADAM9 and ADAM15, are not affected by the cytokine treatment (Ref. 13 and this report). According to our knowledge, the only other ADAM reported to be up-regulated by TGFβ1 is ADAM19, whose mRNA levels increase in alveolar epithelial cells treated with TGFβ1 (48). The same study showed that TGFβ1 treatment leads to down-regulation of the ADAM28 mRNA level, further highlighting the specificity of TGFβ1 in regulating the expression of individual ADAMs.

According to previous reports, the induction of ADAM12 by TGFβ1 in hepatic stellate cells occurred with slow kinetics, as the level of ADAM12 mRNA was not significantly changed before 24 h of TGFβ1 treatment (13). Furthermore,

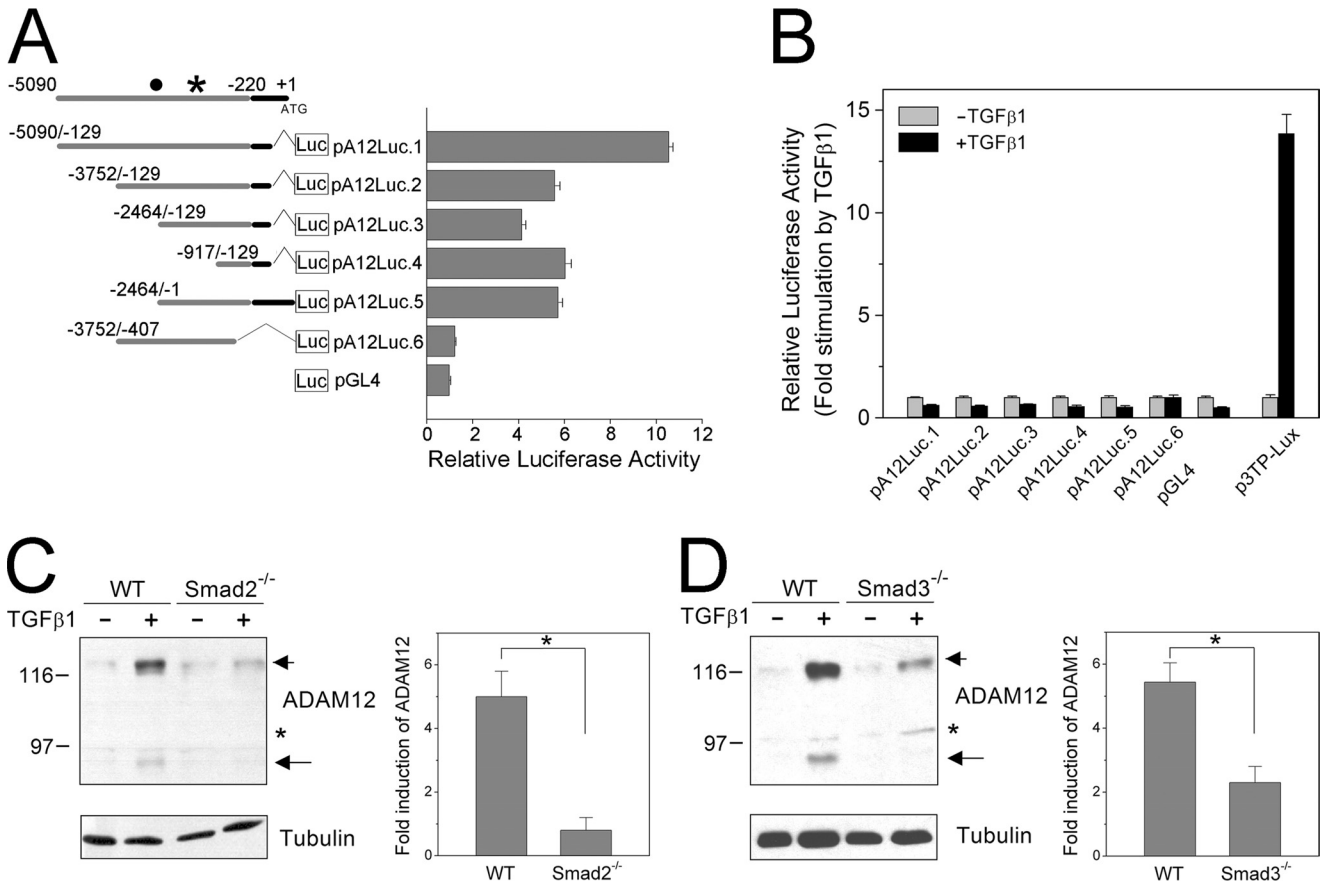

FIGURE 4. **Induction of ADAM12 by TGFβ1 is reduced in Smad2- or Smad3-deficient cells.** *A*, characterization of the mouse *Adam12* promoter. Schematic representation of gene reporter constructs is shown on the *left*. The translation initiation site is at nucleotide position +1, the putative transcription start site (*TSS*) is located at position −220, the 5′-untranslated region is shown in *black*, the genomic upstream sequence is in *gray*, the 9×GTCT repeat at −1368 to −1333 is indicated by the *asterisk*, the activin response element at −2685 to −2678 is indicated by the *circle*. NIH3T3 cells were co-transfected with individual ADAM12 reporters and pRL-TK, and analyzed 24 h later. After normalization to *Renilla* luciferase, firefly luciferase activity relative to that of pGL4 was calculated for each reporter. The data represent the mean ± S.E. (*error bars*) from two to three independent experiments. The core *Adam12* promoter is located between residues −407 and −129, and additional positive regulatory elements may be present between residues −5090 and −3752. *B*, the effect of TGFβ1 on the activity of the *Adam12* promoter; the p3TP-Lux vector is positive control. *C*, induction of ADAM12 in *Smad2*$^{-/-}$ cells. Wild-type (*WT*) or *Smad2*$^{-/-}$ MEFs were incubated for 24 h with or without 2 ng/ml of TGFβ1, followed by analysis of ADAM12 expression by Western blotting, as described in the legend to Fig. 1; tubulin is a loading control. The full-length ADAM12 protein is indicated by a *short arrow*, the mature form lacking the pro-domains is indicated by *long arrow*, the *asterisk* denotes a nonspecific band. Fold-induction of ADAM12 by TGFβ1 was quantified by densitometry and ImageJ; the data represent the means from three different determinations ± S.E. (*error bars*); the *asterisk* indicates statistically significant effect ($p < 0.05$). *D*, induction of ADAM12 in *Smad3*$^{-/-}$ cells. The experiments and quantification of the results were performed as in *C*.

the effect of TGFβ1 on ADAM12 expression was partially blocked by inhibitors of MAPKs, phosphoinositide 3-kinase, or p70S6 kinase (24), indicating that TGFβ1 might up-regulate ADAM12 via Smad-independent pathways. In this report, we show that the induction of ADAM12 by TGFβ1 in mouse fibroblasts is more rapid, and increased levels of ADAM12 mRNA are detected within ~2 h after TGFβ1 treatment. ADAM12 induction is completely blocked upon inhibition of transcription or proteasomal degradation, but is not blocked by cycloheximide, an inhibitor of translation. Based on these observations, we conclude that TGFβ1 induces ADAM12 expression by relieving a transcriptional repression of the *Adam12* gene. We show that SnoN, a transcriptional repressor that is efficiently degraded after TGFβ1 stimulation, is involved in the regulation of ADAM12 expression. Overexpression of SnoN reduces ADAM12 induction, and down-regulation of SnoN expression by shRNA leads to increased induction of ADAM12 by TGFβ1. Importantly, whereas pre-treatment of shControl cells with MG132, a proteasomal inhibitor, completely blocks the induction of ADAM12 by TGFβ1, the same pre-treatment of shSnoN cells still allows ADAM12 to be induced by TGFβ1. The extent of induction of ADAM12 by TGFβ1 in shSnoN cells in the presence of MG132 is not as potent as in the absence of the proteasomal inhibitor, but this is most likely due to the fact that MG132 treatment eventually leads to build up of SnoN protein in these cells. In contrast, incubation of *Ski*$^{-/-}$ MEFs with MG132 fully abolishes the effect of TGFβ1 on ADAM12 expression. Clearly, these results indicate that SnoN, rather than Ski, is a repressor that needs to be degraded to derepress the *Adam12* gene.

Although SnoN was first described as a negative regulator of TGFβ1 signaling, it is not a universal repressor of TGFβ1 responsive genes. Rather, SnoN acts in a gene-specific manner, and inhibition of the proteasome leads to abrogation of certain TGFβ1 target gene regulation, without any effect on other TGFβ1 target genes (49). Furthermore, recent reports indicate that under certain circumstances SnoN can act as a positive

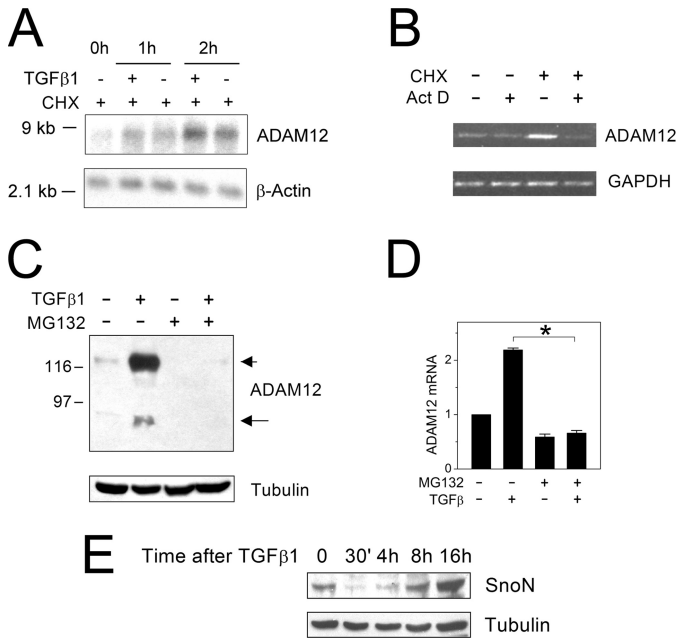

FIGURE 5. **Induction of ADAM12 expression by TGFβ1 involves derepression of the *Adam12* gene.** *A* and *B*, ADAM12 expression is induced in the absence of protein synthesis. In *A*, NIH3T3 cells were pretreated for 2 h with 5 μg/ml of cycloheximide (*CHX*), an inhibitor of translation, and then treated with or without 2 ng/ml of TGFβ1 for the indicated amounts of time, with the continuous presence of CHX. Total RNA was extracted and hybridized with ADAM12 or β-actin probes. In *B*, NIH3T3 cells were pre-treated for 15 min with 5 μg/ml of actinomycin D or DMSO, and then treated for 4 h with or without 5 μg/ml of CHX, as indicated. The level of ADAM12 mRNA was evaluated by semi-quantitative RT-PCR, GAPDH is an internal control. The experiments shown in *A* and *B* were repeated 3 times with similar results. *C* and *D*, induction of ADAM12 by TGFβ1 is blocked by a proteasomal inhibitor. NIH3T3 cells were pre-treated for 1 h with or without 10 μM MG132, and then treated for 16 (*C*) or 4 h (*D*) with or without 2 ng/ml of TGFβ1, in the presence or absence of MG132, as indicated. The level of ADAM12 protein (*C*) was analyzed by Western blotting, as described in the legend to Fig. 1; the level of ADAM12 mRNA was quantified by qRT-PCR (*D*). In *D*, ADAM12 mRNA was normalized to GAPDH mRNA; the data represent the mean $\pm$ S.E. (*error bars*) from two independent experiments. *Asterisk* indicate statistically significant effect ($p < 0.05$) of inhibitor treatment. *E*, the level of SnoN repressor at the indicated times after adding TGFβ1 was evaluated by Western blotting.

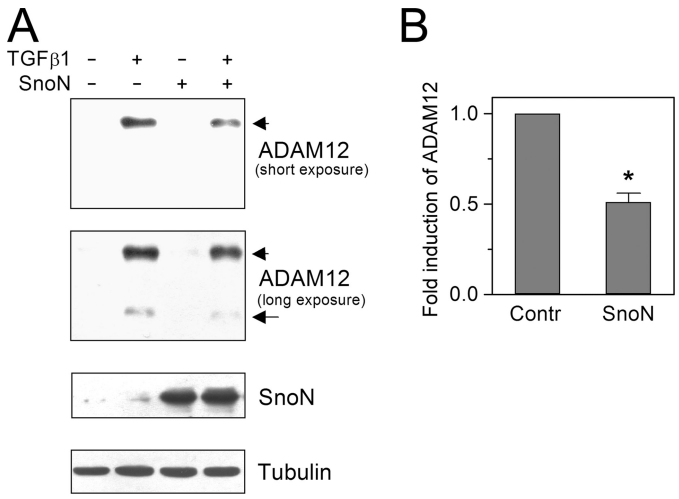

FIGURE 6. **Overexpression of SnoN partially inhibits the induction of ADAM12 by TGFβ1.** *A*, NIH3T3 cells were transduced with SnoN or control retroviruses, and 24 h later they were treated for an additional 16 h with or without 2 ng/ml of TGFβ1. ADAM12 and SnoN levels were examined by Western blotting. The full-length ADAM12 (*short arrow*) and the mature ADAM12 (*long arrow*) are best visualized at short and long film exposures, respectively. *B*, the experiment shown in *panel A* was repeated three times, and the relative changes in the level of ADAM12 protein were quantified by densitometry and ImageJ; the data represent the mean $\pm$ S.E. (*error bars*); *asterisk* indicates statistically significant effect ($p < 0.05$) of SnoN.

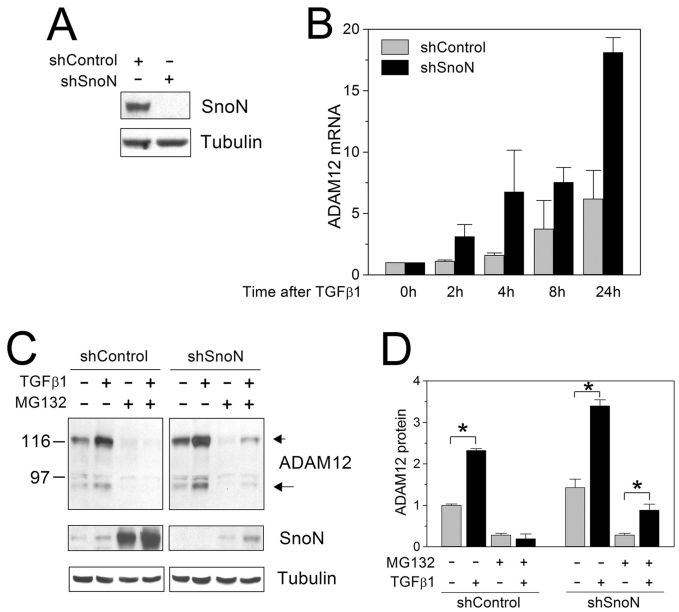

FIGURE 7. **Knockdown of SnoN expression increases ADAM12 expression in response to TGFβ1 stimulation.** NIH3T3 cells were stably transduced with SnoN shRNA or control shRNA lentiviruses. *A*, the level of SnoN protein in shSnoN or shControl cells was evaluated by Western blotting. *B*, shSnoN and shControl cells were incubated with 2 ng/ml of TGFβ1 for the indicated times. ADAM12 mRNA levels, normalized to GAPDH mRNA, were measured by qRT-PCR; fold-changes over the levels at time 0 are shown. *C*, shSnoN and shControl cells were incubated for 16 h with or without 2 ng/ml of TGFβ1, in the absence or presence of 10 μM MG132, followed by evaluation of ADAM12 and SnoN protein levels. *D*, the intensities of the bands corresponding to the full-length ADAM12 protein in *panel C* were quantified using densitometry and ImageJ software. The data represent the mean $\pm$ S.E. (*error bars*) from three independent experiments. *Asterisks* indicate statistically significant differences ($p < 0.05$).

mediator of transcription (50). Indeed, a microarray analysis of human lung cancer A549 cells demonstrated that a large set of genes is down-regulated in cells lacking SnoN, suggesting that SnoN may function as a transcriptional activator, in addition to acting as a transcriptional repressor of the Smad proteins (43). Thus, the effect of SnoN on a particular target gene is not easily predictable, and a negative or positive regulation is possible. As shown in this study, in the case of ADAM12, SnoN is a negative regulator of its expression.

ADAM12 expression is dysregulated in many cancers, cardiac hypertrophy, during aseptic loosening of hip replacement implants, and in experimental autoimmune encephalomyelitis (1, 19–23). Each of these pathological conditions is accompanied by increased levels of TGFβ1 and/or abnormal expression of SnoN (31, 51–54). It is tempting to speculate that up-regulation of ADAM12 expression in cardiac hypertrophy, in inflammatory responses related to osteolysis, or in experimental autoimmune encephalomyelitis is directly linked to activation of TGFβ1 signaling. Furthermore, during fibrotic kidney disease after obstructive injury, SnoN is down-regulated due to enhanced ubiquitin-mediated degradation, which in turn is a result of TGFβ1-induced expression of Smurf2 ubiquitin ligase (55, 56). It is possible that a similar chain of events leads to degradation of SnoN during fibrosis associated with cardiac

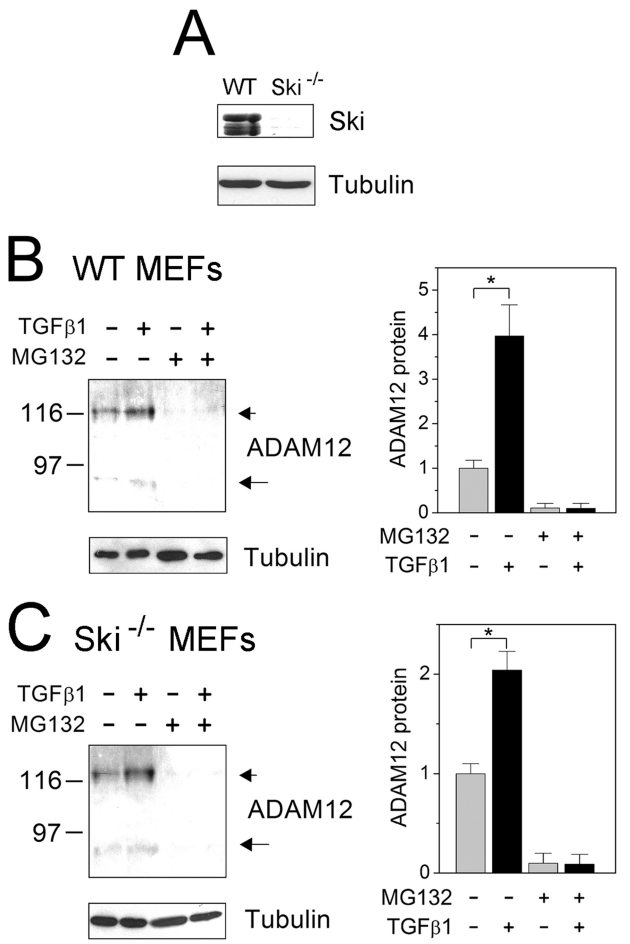

FIGURE 8. **Inhibition of proteasomal degradation blocks the induction of ADAM12 by TGFβ1 in Ski-null cells.** *A*, the absence of Ski expression in *Ski*$^{-/-}$ MEFs was confirmed by Western blotting. *B* and *C*, the effect of MG132 on ADAM12 induction by TGFβ1. Wild-type (*WT*) MEFs (*B*) or *Ski*$^{-/-}$ MEFs (*C*) were incubated for 12 h with or without 2 ng/ml of TGFβ1, in the absence or presence of 10 μM MG132, followed by evaluation of ADAM12 protein levels (*left panels*). The intensities of the bands corresponding to the full-length ADAM12 protein were quantified using densitometry and ImageJ software (*right panels*). The data represent the mean ± S.E. (*error bars*) from three independent experiments.

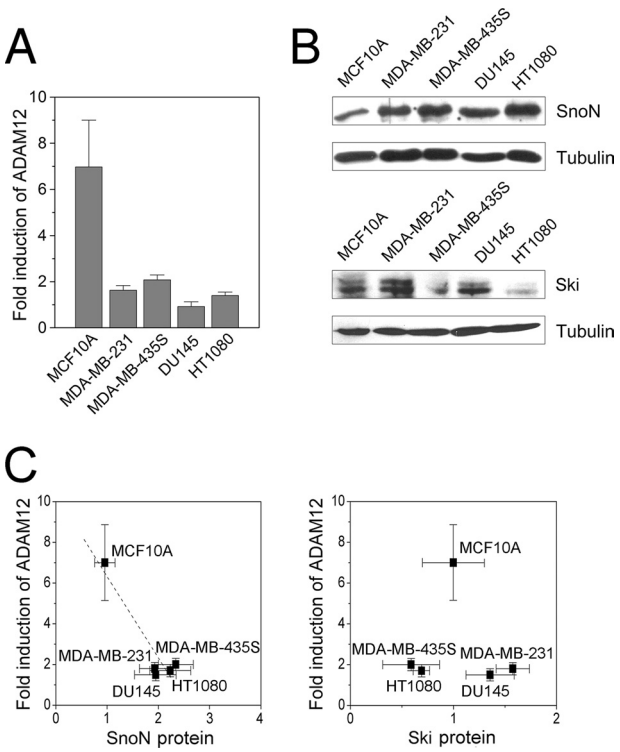

FIGURE 9. **Diminished induction of ADAM12 by TGFβ1 in cancer cell lines correlates with SnoN expression.** *A*, fold-induction of ADAM12 mRNA, normalized to β-actin mRNA, after 24 h treatment with 2 ng/ml of TGFβ1 was evaluated by qRT-PCR. The data represent the mean ± S.E. (*error bars*) from two independent experiments. MCF10A are untransformed human mammary epithelial cells, MDA-MB-231 is a breast cancer cell line, MDA-MB-435S are melanoma cells, DU145 is a prostate cancer line, and HT1080 is a fibrosarcoma cell line. *B*, the levels of SnoN and Ski expression in different cell lines were evaluated by Western blotting. *C*, fold-induction of ADAM12 in each cell line was plotted against the level of SnoN (*left*) or Ski (*right*); SnoN and Ski protein levels in MCF10A cells are set as 1. SnoN and Ski protein levels were determined by densitometric analysis of Western blots in *panel C* and ImageJ quantification. The data represent the mean ± S.E. (*error bars*) from three independent determinations. The linear regression analysis indicates an inverse correlation between ADAM12 induction by TGFβ1 and SnoN (*dashed line*, $R^2 = 0.91$, $p = 0.007$); no correlation is evident between ADAM12 induction and Ski.

hypertrophy, which would explain high levels of ADAM12 expression in hypertrophic myocardium. In cancer, the situation is more complex, as both TGFβ1, a positive ADAM12 regulator, and SnoN, a negative ADAM12 regulator, are elevated. Our studies in cancer cells show that increased basal expression of ADAM12 is not due to the autocrine/paracrine effects of TGFβ1 produced by some of these cell lines. Rather, it is an inherent feature of cancer cells and may be a result of genetic or epigenetic changes associated with the oncogenic transformation. Increased basal levels of ADAM12 expression also do not correlate with increased SnoN levels in cancer cells. However, cancer cells have many other components of the TGFβ1 pathway aberrantly expressed, including TGFβ receptors and SMADs, and direct correlations between absolute expression levels of ADAM12 and SnoN in different cell types may be difficult to establish. Importantly, our results indicate that there is an inverse correlation between the level of SnoN in cancer cells and the ability of TGFβ1 to induce ADAM12 expression.

TGFβ signaling plays dual roles during tumor development. During early phases of tumorigenesis, TGFβ acts as a tumor suppressor by limiting cancer cell proliferation and enhancing differentiation. In later stages, TGFβ promotes tumor growth by stimulating cell migration, invasion, and metastasis, by modifying tumor microenvironment, and by modulating host immune responses (51, 57). High expression of SnoN makes cancer cells resistant to the anti-proliferative effects of TGFβ (a pro-oncogenic role of SnoN) and limits their metastatic potential (an anti-tumorigenic activity of SnoN) (43). Interestingly, both tumor-promoting and tumor-suppressing roles have been postulated for ADAM12. Transgenic expression of ADAM12Δcyt (lacking the cytoplasmic domain) in mammary tumors of murine mammary tumor virus-PyMT mice accelerates tumor growth, which is consistent with the tumor-promoting function of ADAM12 (8). On the other hand, ADAM12 is frequently mutated in breast cancer (2, 3), and cancer-associated mutations cause mislocalization of the ADAM12 protein in cells and interfere with its function at the cell surface, suggesting that the wild-type ADAM12 may also play an anti-tumor role (4). We believe that identification of SnoN as a repressor of the *ADAM12* gene will contribute to advances in studies

on the role of ADAM12 in tumor progression and in the development of other pathological conditions.

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