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Role of SARA (SMAD Anchor for Receptor Activation) in Maintenance of Epithelial Cell Phenotype*

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By inducing epithelial-to-mesenchymal transition (EMT), transforming growth factor-β (TGF-β) promotes cancer progression and fibrosis. Here we show that expression of the TGF-β receptor-associated protein, SARA (Smad anchor for receptor activation), decreases within 72 h of exposure to TGF-β and that this decline is both required and sufficient for the induction of several markers of EMT. It has been suggested recently that expression of the TGF-β signaling mediators, Smad2 and Smad3, may have different functional effects, with Smad2 loss being more permissive for EMT progression. We find that the loss of SARA expression leads to a concomitant decrease in Smad2 expression and a disruption of Smad2-specific transcriptional activity, with no effect on Smad3 signaling or expression. Further, the effects of inducing the loss of Smad2 mimic those of the loss of SARA, enhancing expression of the EMT marker, smooth muscle α-actin. Smad2 mRNA levels are not affected by the loss of SARA. However, the ubiquitination of Smad2 is increased in SARA-deficient cells. We therefore examined the E3 ubiquitin ligase Smurf2 and found that although Smurf2 expression was unaltered in SARA-deficient cells, the interaction of Smad2 and Smurf2 was enhanced. These results describe a significant role for SARA in regulating cell phenotype and suggest that its effects are mediated through modification of the balance between Smad2 and Smad3 signaling. In part, this is achieved by enhancing the association of Smad2 with Smurf2, leading to Smad2 degradation.

Epithelial-to-mesenchymal transition (EMT)[3] describes a process by which cells lose their relatively differentiated epithelial characteristics and show increased migratory or synthetic properties. This transition is evidenced by loss of proteins involved in cell-cell junctions, such as E-cadherin and occludin, and by an increase in proteins, such as vimentin and fibronectin, as well as *de novo* production of smooth muscle α-actin (αSMA) (1).

Phenotypic transitions are important in the differentiation of tissues during morphogenesis. Dedifferentiation plays a role in tumor progression by increasing cellular invasiveness and in tissue fibrosis through the generation of extracellular matrix-producing myofibroblasts. The importance of EMT in kidney disease has been demonstrated in experimental models where blocking EMT attenuates fibrosis (2). Since TGF-β is a potent initiator of renal EMT, this represents one way in which aberrant TGF-β signaling stimulates fibrosis in the kidney.

The TGF-β superfamily consists of TGF-β1, TGF-β2, TGF-β3, activins, and bone morphogenic proteins. These proteins are widely expressed in virtually all mammalian cell types, as are their downstream signaling mediators, the Smad proteins. TGF-β signaling is initiated when ligand-bound TGF-β type II receptor binds to and phosphorylates the TGF-β type I receptor (3–5). Phosphorylation of the TGF-β type I receptor in its cytoplasmic GS region leads to its activation and its ability to activate the receptor-regulated Smads (R-Smads), Smad2 and Smad3, by C-terminal serine phosphorylation. Once phosphorylated, the R-Smads form a heteromultimeric complex with the common mediator (Co)-Smad (Smad4) and accumulate in the nucleus to regulate transcriptional responses (3–5).

Smad protein expression can be modulated through proteasomal degradation (6, 7). This ubiquitin-mediated proteolysis occurs via a cascade of enzymatic reactions of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), with E3 being the defining factor for substrate selectivity. Smurf2 (Smad ubiquitination regulatory factor-2) is one such E3 ligase. Smurf2 belongs to the HECT domain ubiquitin ligase family and has recently been suggested to be specific for the degradation of Smad2 (8, 9). In addition to ubiquitin ligases, numerous proteins have been identified that promote Smad expression, stability, activation, and assembly into transcription-regulatory complexes (10). Several of these proteins control the localization of Smads and TβRs and the interaction of Smads with the receptor complex (11, 12). One such protein, SARA (Smad Anchor for Receptor Activation), was initially described as a recruitment factor for R-Smad to the TβR (13). It contains both a Smad-binding domain, which interacts with Smad2 and Smad3 (14), and a C-terminal, TβR complex-interacting region (13). SARA was therefore proposed to play a role in presenting R-Smads to the receptor for phosphorylation.

The initial report describing this molecule suggested that SARA and Smad2 are associated basally and then dissociate upon receptor activation. In contrast, our previous studies of kidney cells demonstrated very little basal association of SARA with Smad2 but high levels of interaction upon TGF-β1 stimu-

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[3] The abbreviations used are: EMT, epithelial to mesenchymal transition; αSMA, smooth muscle α-actin; TGF, transforming growth factor; R-Smad, receptor-regulated Smad; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; siRNA, small interfering RNA; shRNA, short hairpin RNA; HKC-C, HKC-S, HKC-Sd2, HKC cells stably expressing control, SARA, or Smad2 shRNA, respectively.

lation (15). The specific role of SARA in TGF-β1-mediated Smad signaling remains poorly understood. A previous study in COS7 cells showed that SARA was required for Smad2-dependent signaling but not for Smad3-dependent signaling (16). Additionally, our previous work showed that in human kidney mesangial cells, which express nearly equal levels of Smad2 and Smad3, SARA preferentially interacts with Smad2 in response to TGF-β1 treatment (15). However, another report showed that a SARA binding-deficient mutant of Smad2 could still be activated by TGF-β receptors (17). Therefore, the specific role for SARA in Smad2- or Smad3-specific signaling is not fully determined.

To date, with only a small number of studies on SARA expression and function, there is little information linking it to any specific disease model. SARA expression has been shown to be increased in epithelial cells derived from patients with asthma compared with normal subjects (18) and in synovial fibroblasts from rheumatoid arthritis patients compared with osteoarthritis patients (19). SARA expression may decrease as liver fibrosis develops (20). There is also a study showing that SARA expression declines during the transdifferentiation of hepatic stellate cells in a model of liver fibrosis (21). However, in none of these reports was it determined whether the increase or decrease in SARA expression was a mediator of the disease process. We therefore examined whether the expression of SARA plays a functional role in potentially profibrotic activity of human kidney epithelial cells and how SARA participates in Smad2 or Smad3-specific functions related to this transition.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Active recombinant human TGF-β1, purchased from R&D Systems (Minneapolis, MN), was maintained as a stock solution of 4 μg/ml in 4 mM HCl and used at a final concentration of 1 ng/ml. SB431542 was purchased from Sigma and used at a final concentration of 5 μM. Antibodies to SARA, Hrs, Smurf2, and Smad1/2/3 are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); E-cadherin was from Transduction Laboratories (Lexington, KY); αSMA was from Dako (Carpinteria, CA); β-actin was from Sigma; Ubiquitin, phospho-Smad2, and phospho-Smad3 were from Cell Signaling (Danvers, MA); and ZO-1 (zonula occludens-1), Smad2, and Smad3 were from Zymed Laboratories Inc. (South San Francisco, CA).

Cell Culture—The renal tubular epithelial cell line HKC, obtained from Dr. L. Racusen (22), and HK-2 obtained from the ATCC (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum, penicillin/streptomycin, amphotericin B, HEPES buffer, and glutamine. Human primary cultures of neonatal foreskin fibroblasts were a generous gift from Dr. J. Varga, established as previously described (23). Fibroblasts were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 1% vitamins, 1% penicillin/streptomycin, and 2 mM L-glutamine and studied between passages 4 and 8.

Quantitative PCR—Total RNA was harvested from HKC-C or HKC-S, after 72-h treatment with TGF-β1 or vehicle, using the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's directions. Following RNA quantification with the Quant-it RiboGreen assay (Invitrogen), RNAs were reverse-transcribed to cDNAs with the iScript cDNA synthesis kit (Bio-Rad). Real time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) with the iCycler iQ real time PCR detection system (Bio-Rad). Real time data were collected for 40 cycles of; 95 °C for 10 s and 55.3 °C for 45 s, followed by melt curve analysis to verify the single peaks of amplicons. Primers were designed using software provided by Integrated DNA Technologies (Coralville, IA) and custom synthesized by the company. Primers used were as follows: COL1A1, 5′-CAATGCCCTTTCTGCTCCTTT-3′ (forward) and 5′-CACTTGGGTGTTTGAGCATTGCCT-3′ (reverse); Smad2, 5′-TCAAGAGAGAAAACAGCACTTGA-3′ (forward) and 5′-TTTCTAGACACTAATTTTCCCATCTAA-3′ (reverse); SARA, 5′-GGTGAGGTGGCTCCAGTATG-3′ (forward) and 5′-CTCTGCAGTGATGCCTCCTT-3′ (reverse); human β_2-microglobulin, 5′-TGTCTGGGTTTCATCCATCCGACA-3′ (forward) and 5′-TCACACGGCAGGCATACTCATCTT-3′ (reverse); mouse β_2-microglobulin, 5′-TTCTGGTGCTTGTCTCACTGA-3′ (forward) and 5′-CAGTATGTTCGGCTTCCCATTC-3′ (reverse). Relative expression of the gene of interest was estimated by correction with the expression of β_2-microglobulin, using the $\Delta\Delta Ct$ method.

Immunoprecipitation, Western Blot Analysis, and Immunocytochemistry—Prolonged TGF-β1 treatments were done in serum-free medium, and cells were refed and retreated every 48 h. For shorter TGF-β1 treatments, cells were serum-deprived overnight prior to treatment. Whole cell lysates were prepared by lysis in radioimmune precipitation buffer, and immunoprecipitations were performed as previously described (24). For separation of cytosol and nuclear fractions, cells were scraped in a detergent-free buffer, subjected to Dounce homogenization, and centrifuged at low speed. The supernatant of this spin was collected as the cytosolic cell fraction, and the pellet was lysed in radioimmune precipitation buffer, syringe-sheared, and centrifuged to remove insoluble material. This remainder was collected as the nuclear fraction. Western blots and densitometric analysis were performed as previously described (24).

For immunocytochemistry, cells on coverslips in serum-free medium were treated with vehicle or 2 ng/ml TGF-β1 for 72 h before paraformaldehyde fixation followed by permeabilization with Triton X-100. After blocking nonspecific sites with bovine serum albumin, cells were incubated with rabbit anti-ZO-1 at 1:150 for 2 h at room temperature, washed with PBS, and then incubated with 2.5 μg/ml ALEXA 594-conjugated goat anti-rabbit IgG for 30 min. Coverslips were mounted with Aqua-Poly/Mount (Polysciences, Warrington, PA) and viewed under a Zeiss Axiovert 200 M confocal microscope with a Zeiss plan-apochromat ×100/1.4 numerical aperture oil objective, and images were acquired using LSM 510 version 4.2 SP1 software. Digital images were converted to TIFF files, and a figure was prepared using Adobe Photoshop.

Transient Transfection and Luciferase Assay—The αSMA promoter-luciferase reporter construct was a generous gift from Dr. Robert Schwartz (Baylor College of Medicine) (25). The SARA, ARE-Luc reporter construct, and Fast-2 expression constructs were kindly provided by Dr. Jeffrey Wrana (13). The

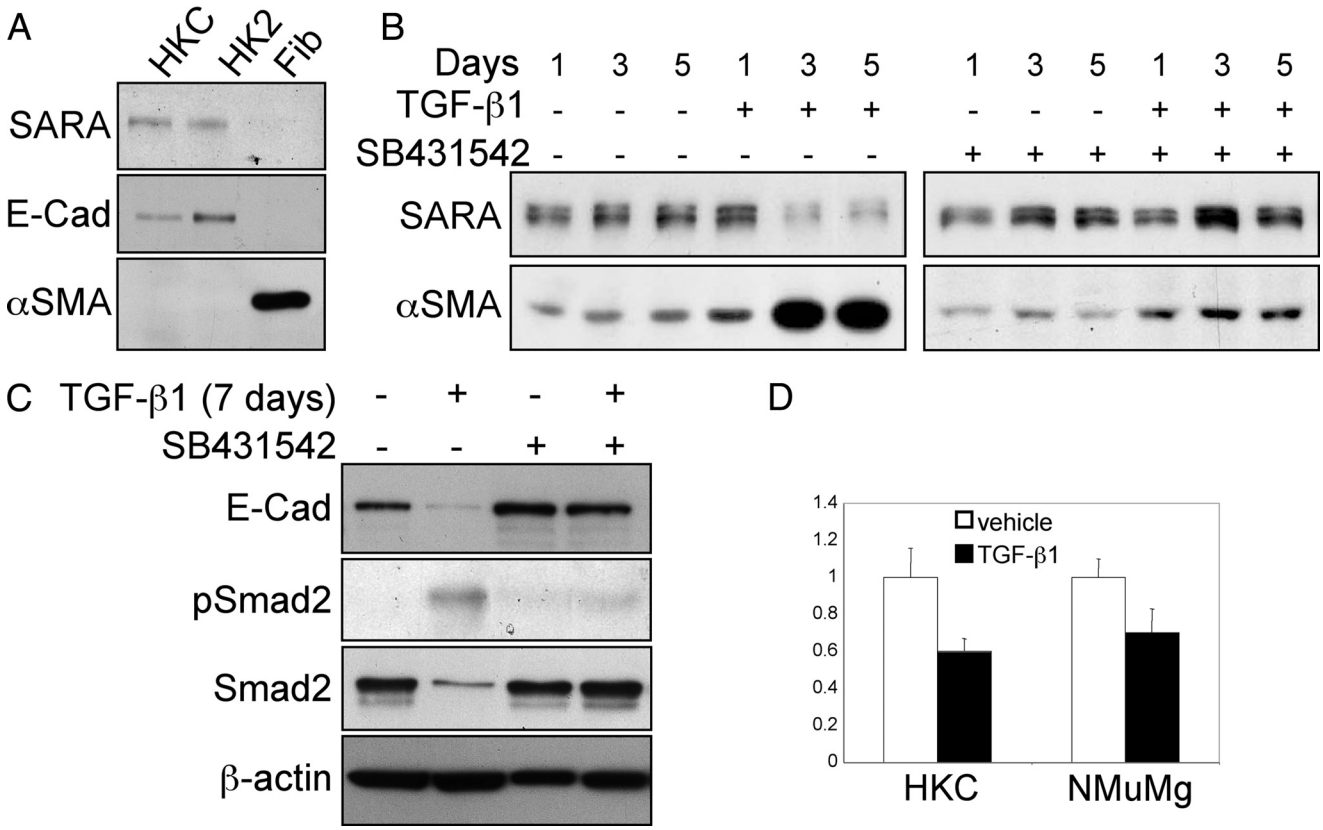

FIGURE 1. **SARA expression is associated with a mesenchymal phenotype.** *A*, Western blots show that human kidney epithelial cell lines HKC and HK-2 have relatively higher levels of SARA than human neonatal foreskin fibroblasts (*Fib*). E-cadherin (*E-Cad*) and αSMA are included as phenotypic controls. *B* and *C*, HKC cells were pretreated for 1 h with either vehicle control or the Alk5 kinase inhibitor SB431542 prior to TGF-β1 treatment for 1, 3, or 5 days (*B*) or 7 days (*C*) with change of media and retreatment every 48 h. *D*, TGF-β1 (*black bars*) also triggers a decline in SARA mRNA compared with vehicle-treated cells (*white bars*), as determined by quantitative PCR, in both HKC and NMuMg cells. Fold induction of SARA was calculated over reference gene (β_2-microglobulin) expression from triplicate measurements.

SBE-Luc reporter construct was generously provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, The Johns Hopkins University) (26). Transfection was performed with the FuGENE 6 transfection reagent (Roche Applied Science), and luciferase and β-galactosidase activities were measured as described previously (24).

Transient and Stable Knockdowns—SARA and fluorescein isothiocyanate-conjugated control siRNA were purchased from Santa Cruz Biotechnology. Lentiviral shRNAmir SARA (ZFYVE9 clone ID V2LHS_35329), shRNAmir Smad2 (clone ID V2LHS_251359), and non-silencing GIPZ shRNAmir control were purchased from Open Biosystems (Huntsville, AL). For siRNA, cells were seeded at 5×10^4 cells/well in 6-well plates and incubated overnight prior to transfection using 8 μl/well FuGENE 6 transfection reagent and 14.4 μl/well of 10 μM solutions of either control or SARA siRNA stocks. Three hours later, TGF-β1 or control vehicle was added, and cells were harvested 5 days later for Western blot analysis. To generate stable knockdown cell lines, pGIPZ clones, which are supplied as bacterial cultures in *E. coli*, were first subjected to $CaPO_4$ transfection for lentiviral packaging in HEK293 FT cells (Invitrogen) using psPAX2 and pMD2.G according to the Open Biosystems protocol. HKC cells were incubated with viral supernatants, and after 48 h, puromycin was added in a concentration previously determined to result in 100% cell death of non-infected HKC cells to select infected cells. Once cells reached confluence, they were frozen or used for experiments up to passages 4–6 in the continual presence of puromycin.

RESULTS

We previously studied SARA in human kidney mesangial cells but observed at that time that cells of a mesenchymal phenotype, such as mesangial cells, had far less SARA expression than cells of an epithelial phenotype, such as HeLa or mink lung epithelial cells. This finding is demonstrated in Fig. 1*A*, comparing SARA protein expression in E-cadherin-expressing cells of epithelial origin (human kidney HKC or HK-2) with that of αSMA-expressing fibroblasts (human skin fibroblasts). This suggested the possibility that the transition of epithelial cells to a mesenchymal/fibroblastic phenotype might include a reduction in the expression of SARA. To investigate this possibility, we treated HKC, a renal proximal tubular epithelial cell line, with 1 ng/ml (40 pM) TGF-β1 and assessed the levels of SARA expression at various times after treatment. As shown in Fig. 1*B*, TGF-β1 treatment for 3 and 5 days resulted in a reduction in SARA corresponding to the timing of an increase in TGF-β1-induced smooth muscle α-actin (αSMA) expression. HeLa cells and a mouse mammary gland epithelial cell line (NMuMg) also showed reduced SARA expression in response to prolonged TGF-β1 treatment (data not shown). Further, using quantitative PCR, we determined that the TGF-β1-mediated loss of SARA occurred at the level of mRNA expression (Fig. 1*D*). This

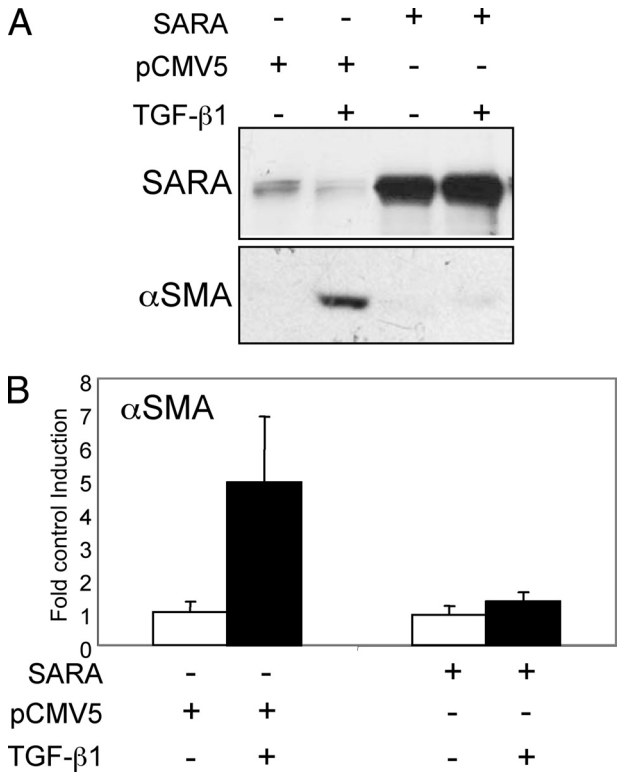

FIGURE 2. **High levels of SARA expression inhibit αSMA induction by TGF-β1.** *A*, HKC transfected with either pCMV5 empty vector or SARA were treated with TGF-β1 for 3 days and analyzed by Western blot. The blot shown is representative of three independent experiments. *B*, HKC were transfected with either pCMV5 or SARA along with an αSMA promoter-luciferase reporter construct and cytomegalovirus-β-galactosidase (*β-gal*). 3 h post-transfection, cells were treated with either vehicle control (*white bars*) or TGF-β1 (*black bars*) for an additional 48 h. Bars represent triplicate measurements of luciferase activity normalized to β-galactosidase and corrected to vehicle-treated controls from a representative of three separate experiments.

was true for HKC as well as for NMuMg cells. Therefore, depletion of SARA in response to TGF-β1 is not limited to kidney epithelial cells. Both the reduced SARA expression and the increased αSMA expression required active TGF-β1 signaling, because they were each inhibited in the presence of the type I TGF-β receptor kinase inhibitor SB431542 (Fig. 1*B*, *right*). Another marker of EMT, the loss of E-cadherin expression, was also affected by TGF-β1 within this time frame, and examination of the phosphorylation of Smad2 confirmed that SB431542 inhibits Alk5 kinase activity as expected (Fig. 1*C*). Interestingly, as seen in Fig. 1*C*, 7 days of TGF-β1 treatment also greatly reduced the total Smad2 expression.

To determine whether the alterations in SARA and αSMA expression induced by TGF-β1 were related, we overexpressed SARA in HKC epithelial cells so that the levels would remain high throughout 3 days of TGF-β1 treatment. We found that when SARA levels remained high, αSMA expression was no longer induced by TGF-β1 (Fig. 2*A*). Overexpression of SARA in NMuMg cells also disrupted TGF-β1-induced αSMA expression (data not shown), indicating that, like the loss of SARA expression, this response was not specific to kidney epithelial cells. Further, although TGF-β1 induced the activity of an αSMA promoter-luciferase reporter construct in the presence of an empty plasmid of the vector for SARA (pCMV5), this induction did not occur when SARA was overexpressed (Fig. 2*B*). Together these data suggest that the TGF-β1-dependent reduction in SARA expression is not simply coincident with an increase in αSMA but may play an important role in inducing its expression.

Since high levels of SARA expression appear to cause cells to retain epithelial characteristics, we questioned whether lowering levels of SARA expression was sufficient to cause phenotypic alterations associated with EMT. We first reduced the SARA expression levels in HKC using siRNA. As shown in Fig. 3*A*, compared with a scrambled fluorescein isothiocyanate-conjugated siRNA control, SARA siRNA reduced SARA expression to a level similar to the reduction caused by 3 days of TGF-β1 treatment. The lower levels of SARA expression caused by siRNA were associated with higher basal levels of αSMA expression, even without TGF-β1 treatment. Hrs (hepatic growth factor-regulated tyrosine kinase substrate), a Smad-binding protein that does not show altered expression in response to TGF-β1 treatment, was included as a loading control (Fig. 3*A*, *bottom*). A similar increase in αSMA expression was seen in HEK293 cells transfected with SARA siRNA (Fig. 3*B*). We confirmed this finding by creating a stable SARA knockdown cell line of HKC (HKC-S) using a lentiviral vector to express an shRNA to SARA. As shown in Fig. 3*C* (*left*), HKC-S cells showed greatly reduced expression of SARA (a greater than 90% reduction) compared with cells infected with a lentiviral GIPZ control vector (HKC-C). We investigated the effect of SARA reduction on multiple markers of EMT in HKC-S cells. As with SARA siRNA, HKC-S cells had higher basal expression of αSMA compared with the HKC-C cells (Fig. 3*C*, *middle*). Investigation of E-cadherin expression (Fig. 3*C*, *right*) or of junctional ZO-1 staining (Fig. 3*D*) in HKC-S cells showed that these were decreased. However, investigation of the basal expression of fibronectin showed little to no difference between HKC-C and HKC-S cells (data not shown). Therefore, although the loss of SARA causes alterations in expression of proteins consistent with EMT, it may not represent a complete transition.

We next examined whether the loss of SARA would enhance the fibrogenic response to TGF-β1 by performing quantitative PCR to examine the expression of the fibrosis-associated increase in type I collagen (COL1A1). As shown in Fig. 4, collagen mRNA was induced 3-fold by TGF-β1 in control cells but 15-fold in HKC-S cells. This 5-fold greater response in HKC-S than HKC-C cells suggests that, in addition to its TGF-β1-independent effects, the loss of SARA also enhances the fibrogenic potential of the cellular response to TGF-β1.

Although it was unclear why loss of SARA would be sufficient to induce markers of EMT, one intriguing possibility was that the reduced expression of this mediator of TGF-β1 signaling could alter the balance of Smad activity or expression. We therefore examined the receptor-dependent phosphorylation of Smad2 or Smad3 in HKC-C or HKC-S cells. As shown in Fig. 5*A*, without SARA expression in the HKC-S cells, the level of TGF-β1-dependent phosphorylated Smad2 was reduced, whereas the level of phosphorylated Smad3 appeared unaffected. Further, the nuclear translocation of Smad2 in response to TGF-β1 treatment was blocked in HKC-S cells (Fig. 5*B*). Activity of the transfected, Smad2-specific ARE-luciferase

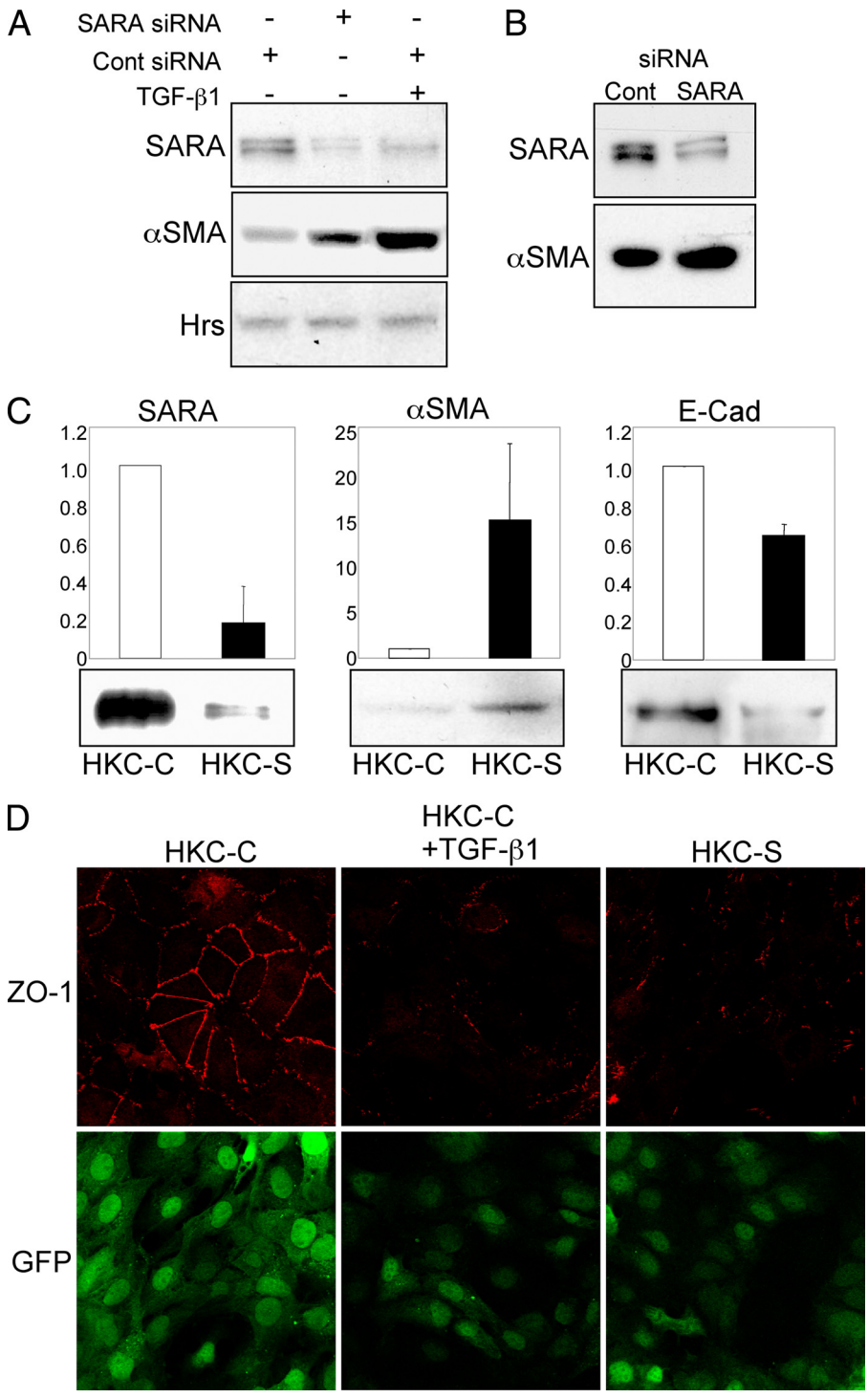

FIGURE 3. **Loss of SARA expression results in spontaneous phenotypic alterations independent of TGF-β1 treatment.** Western blots of HKC (*A*) or HEK293 (*B*) transfected with siRNA control or siRNA SARA and treated with vehicle or TGF-β1 for 5 days show that loss of SARA induces αSMA. Hrs is included as a loading control. *C* and *D*, HKC were infected with either lentiviral control green fluorescent protein-shRNA (HKC-C) or lentiviral SARA green fluorescent protein-shRNA (HKC-S), treated with puromycin to kill uninfected cells, and then cultured for multiple passages in the presence of puromycin. Western blot analysis for markers of EMT (*C*) shows that HKC-S cells have higher αSMA and lower SARA and E-cadherin (*E-Cad*) expression than HKC-C cells. The *graphs* show combined densitometric values from three separate experiments, normalized to HKC-C control expression. *D*, immunocytochemical analysis of green fluorescent protein (*GFP*) to detect infected cells or of ZO-1 junctional expression in HKC-C or HKC-S cells treated with either vehicle or TGF-β1 for 3 days shows a loss of ZO-1 expression in both HKC-C cells treated with TGF-β1 and HKC-S cells treated with vehicle only.

reporter construct showed a strong TGF-β1 induction in HKC-C cells (about 400-fold over untreated HKC-C cells). This response was greatly reduced in HKC-S cells (Fig. 5*C*). The TGF-β1-dependent activation of this Smad2-specific reporter could be reestablished by co-transfecting SARA back into the HKC-S cells, confirming that the loss of the TGF-β1 response in HKC-S cells was specifically due to the loss of SARA expression (Fig. 5*C*). In contrast, stable knockdown of SARA did not inhibit TGF-β1 activation of the Smad3-dependent SBE-luciferase reporter, and the response was unaffected by adding back SARA to the HKC-S cells (Fig. 5*D*). Taken together, these data suggest that SARA plays an important role in TGF-β1-stimulated Smad2 phosphorylation, nuclear translocation, and transcriptional activity but appears to be dispensable for Smad3 activation in response to TGF-β1.

In our studies of Smad2 activation in HKC-S cells, we noticed that these cells appeared to have less overall expression of Smad2 (Fig. 5*B*, *Wcl*). In fact, as shown in Fig. 5*E*, Smad2 expression was reduced by ~50% in HKC-S cells compared with HKC-C, but Smad3 expression was not altered. If TGF-β1 leads to the loss of SARA expression, and the loss of SARA expression were to lead to a loss of Smad2 expression, then prolonged periods of TGF-β1 treatment also ought to deplete the cells of Smad2. Therefore, the loss of Smad2 expression that we found in response to 7 days of TGF-β1 treatment (Fig. 1*C*) is consistent with that which might be caused by the loss of SARA under these conditions.

To address how a loss of SARA expression might specifically affect levels of Smad2, we first examined whether this was a transcriptional effect. As shown in Fig. 6*A*, quantitative PCR showed no difference in Smad2 mRNA, although SARA mRNA was depleted, between HKC-C and HKC-S cells.

This suggested that the loss of SARA expression might affect Smad2 at the protein level. Since Smads are known to be down-regulated through ubiquitin proteasome-mediated degradation, we investigated the ubiquitination level of Smad2 or Smad3 by performing immunoprecipitations using Smad-specific antibodies and blotting for ubiquitin. As shown in Fig. 6*B*, Smad2 was more highly ubiquitinated in HKC-S cells compared with control cells; however, the ubiquitination of Smad3 was similar in control and SARA-depleted cells.

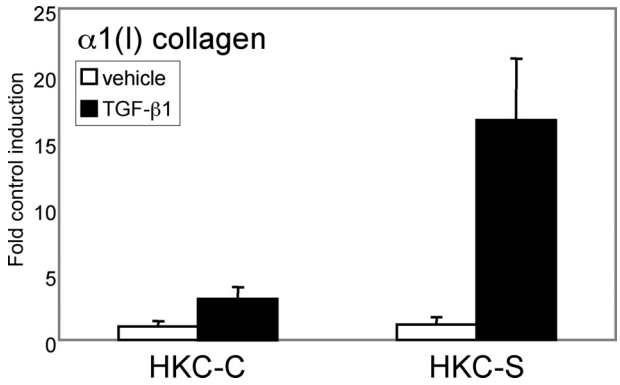

FIGURE 4. **TGF-β1 induction of α1(I) collagen is increased in HKC-S cells.** HKC-C or HKC-S cells were treated with either vehicle (*white bars*) or TGF-β1 (*black bars*) for 72 h, and mRNA levels were assessed by quantitative PCR. -Fold induction of α1(I) collagen was calculated over reference gene (β2-microglobulin) expression from triplicate measurements. Representative data of three independent experiments is shown.

To begin to determine how the loss of SARA might affect Smad2 ubiquitination, we examined the expression of Smurf2, which is an E3 ubiquitin ligase proposed to be specific for Smad2 over Smad3 (8, 9). Presumably, if SARA-depleted cells express higher levels of Smurf2, they might be expected to have less overall Smad2 expression. However, when we compared Smurf2 at the level of protein expression (Fig. 7*A*) or promoter activity (data not shown), we found no difference between HKC-C and HKC-S cells. We therefore examined the interaction between Smad2 and Smurf2 by co-immunoprecipitation. Pull-down of Smad2 yielded much more associated Smurf2 from SARA-depleted cells than from control cells (Fig. 7*B*, *left*). This enhanced interaction was also apparent in immunoprecipitations of Smurf2 from HKC-C or HKC-S cells (Fig. 7*B*, *right*). No interaction between Smurf2 and Smad3 was seen in Smurf2 immunoprecipitates from either control or SARA-depleted cells (data not shown), confirming that this is a Smad2-specific interaction. If the effects of decreased SARA expression resulted from decreased Smad2 expression and activity, then cells depleted of Smad2 should behave similarly to those depleted of SARA. To examine this paradigm, we used a lentiviral vector to stably express a Smad2 shRNA in HKC cells. As shown in Fig. 8*A*, the cells expressing Smad2 shRNA (HKC-Sd2) had greatly reduced Smad2 expression compared with cells expressing the control vector (HKC-C). The level of phosphorylated Smad2 was reduced in response to TGF-β1 treatment in the HKC-Sd2 cells; however, TGF-β1-induced Smad3 phosphorylation was retained in these cells (Fig. 8*B*). To determine whether HKC-Sd2 cells had altered basal expression of αSMA, we transfected them with the αSMA-luciferase reporter construct along with either empty vector or Smad2 add-back. As shown in Fig. 8*C*, HKC-Sd2 cells had a higher basal expression of αSMA, which could be reduced by re-expression of Smad2. An increase in basal αSMA expression could also be seen at the protein

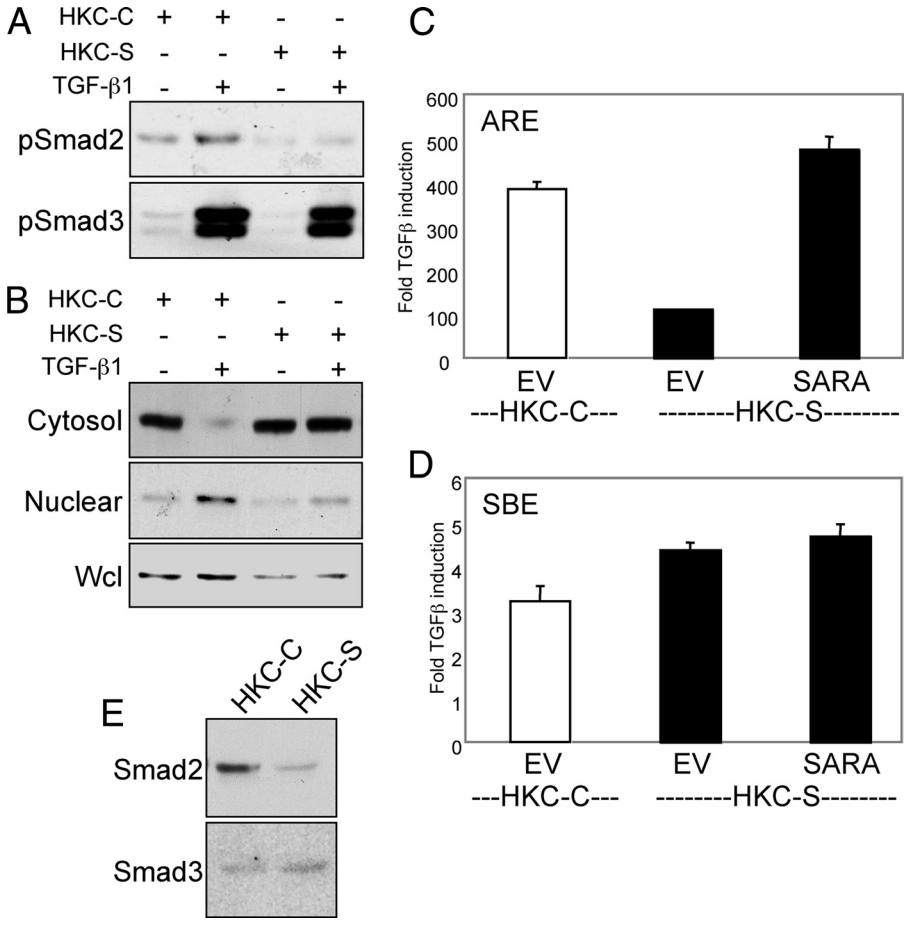

FIGURE 5. **Loss of SARA specifically impairs Smad2 signaling.** *A* and *B*, Western blots of HKC-C or HKC-S lysates from cells treated with either vehicle control or TGF-β1 for 30 min and either lysed as whole cell lysate (*Wcl*) (*A* and *B*) or separated into nuclear and cytosolic fractions (*B*) show a loss of phosphorylated Smad2 and nuclear translocation in HKC-S cells. *C* and *D*, HKC-C or HKC-S cells were transfected with either Fast-2 and a Smad2-responsive ARE-luc reporter (*C*) or a Smad3-responsive SBE-luc reporter (*D*), along with β-galactosidase and either pCMV5 empty vector or SARA. 3 h post-transfection, cells were treated with vehicle or TGF-β1 and assessed for luciferase and β-galactosidase activities after 24 h. *Bars*, TGF-β1 induction compared with untreated for each set of triplicate samples normalized to β-galactosidase activity. The graphs are representative of at least three independent experiments. *E*, Western blots of HKC-C or HKC-S lysate show that HKC-S cells have less Smad2 expression than HKC-C.

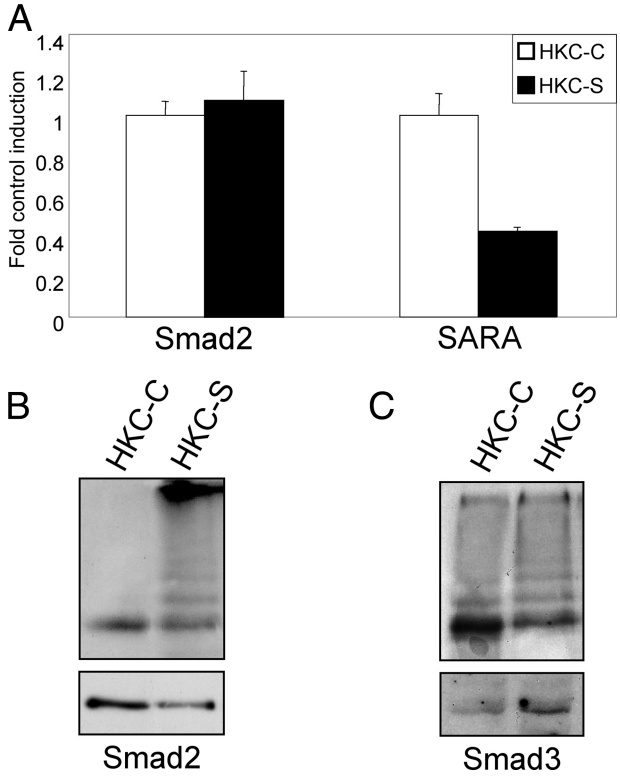

FIGURE 6. **Depletion of SARA does not affect Smad2 mRNA levels but does increase Smad2 ubiquitination.** *A*, quantitative PCR was performed on HKC-C (*white bars*) or HKC-S (*black bars*) for either Smad2 or SARA. -Fold induction of each was calculated over reference gene (β2-microglobulin) expression from triplicate measurements. Representative data of three independent experiments is shown. *B* and *C*, immunoprecipitation of either Smad2 (*B*) or Smad3 (*C*) were blotted for ubiquitin. The *lower panels* show the same lysates blotted for total Smad2 (*B*) or Smad3 (*C*).

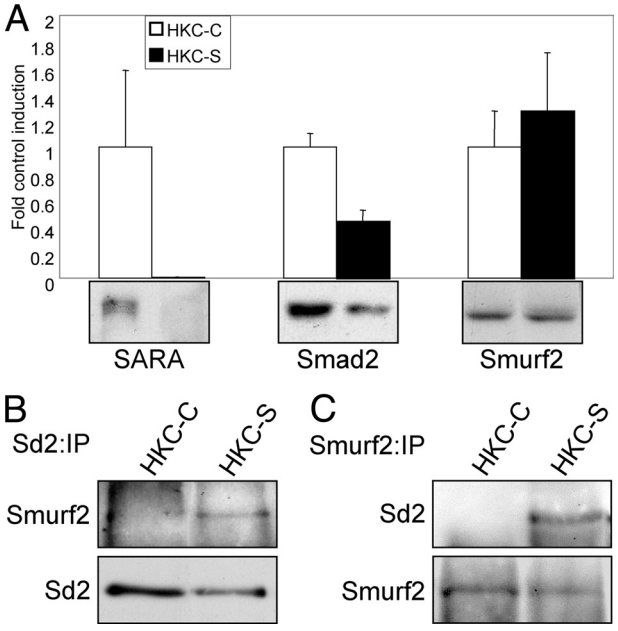

FIGURE 7. **Smurf2 levels are similar between HKC-C and HKC-S cells, but more Smurf2 is associated with Smad2 in HKC-S cells.** *A*, HKC-C (*white bars*) or HKC-S (*black bars*) were blotted for either SARA, Smad2, or Smurf2. Although SARA and Smad2 expression is reduced, Smurf2 expression is not altered. *Graphs* represent combined densitometric analysis from three independent experiments normalized to HKC-C. *B* and *C*, immunoprecipitations (*IP*) of either Smad2 (*B*) or Smurf2 (*C*) were blotted for associated Smurf2 (*B*) or Smad2 (*C*). These blots were then reprobed for either Smad2 or Smurf2 to examine total expression of each protein.

level in HKC-Sd2 cells (Fig. 8*D*). Together, our data suggest that a decline in SARA expression results in a reduction in Smad2 expression levels through enhanced Smad2-Smurf2 interaction as well as a defect in Smad2 signaling and increased expression of a mesenchymal marker.

DISCUSSION

Epithelial cells that are injured or subjected to stress may assume what is generally described as a fibrogenic phenotype, in which they lose cell-cell junctions and show increased migration. These morphological and functional changes, accompanied by changes in the cell's biochemistry, often conclude in a synthetic cell type that generates extracellular matrix. This process, referred to as EMT, is characterized by a number of specific changes in protein expression, including decreased expression of E-cadherin and increased expression of αSMA. Although these two molecular events do not define the cell as a myofibroblast, they are commonly accepted as markers of the process that leads to EMT. TGF-β is a common initiator of EMT. Therefore, our finding that certain aspects of EMT can be induced by the removal of SARA suggests that changes in the expression or function of this adaptor of TGF-β1 signaling may be an important mediating event in the development of the fibrogenic phenotype. Our data suggest that the loss of SARA leads to enhanced αSMA production in epithelial cells through the specific inhibition of Smad2 signaling and expression, which may occur through enhanced Smad2-Smurf2 interaction.

SARA has been demonstrated to bind both Smad2 and Smad3 (13). However, in our previous studies of human mesangial cells, we found that, although Smad2 and Smad3 were expressed at equal levels, immunoprecipitation of SARA showed predominantly SARA-associated Smad2, with a small amount of Smad3 (15). Our results here show that a loss of SARA expression does not affect Smad3-dependent signaling or Smad3 expression. This finding is consistent with previous studies demonstrating that a SARA binding-deficient mutant of Smad3 remained responsive to TGF-β1 (16).

The loss of Smad2 has been suggested in recent studies to mediate changes in marker expression consistent with EMT (12). In kidney proximal tubular epithelial cells, basal expression of αSMA increased as Smad2 was depleted via siRNA (27), and in studies using Cre/LoxP-mediated gene targeting, Smad2 knock-out hepatocytes spontaneously acquired mesenchymal cell features characteristic of EMT (28). How Smad2 ablation promotes increased EMT was investigated in a recent study using keratinocytes (29). In that study, it was shown that mice with keratinocyte-specific deletion of Smad2 had accelerated formation and malignant progression of skin tumors when compared with wild-type mice. The Smad2-null tumors were poorly differentiated and underwent EMT, and it was demonstrated that EMT mediated by a loss of Smad2 was due to an increase in expression of the E-cadherin transcriptional repressor, Snail. Therefore, the fact that the loss of SARA leads to a concomitant loss of

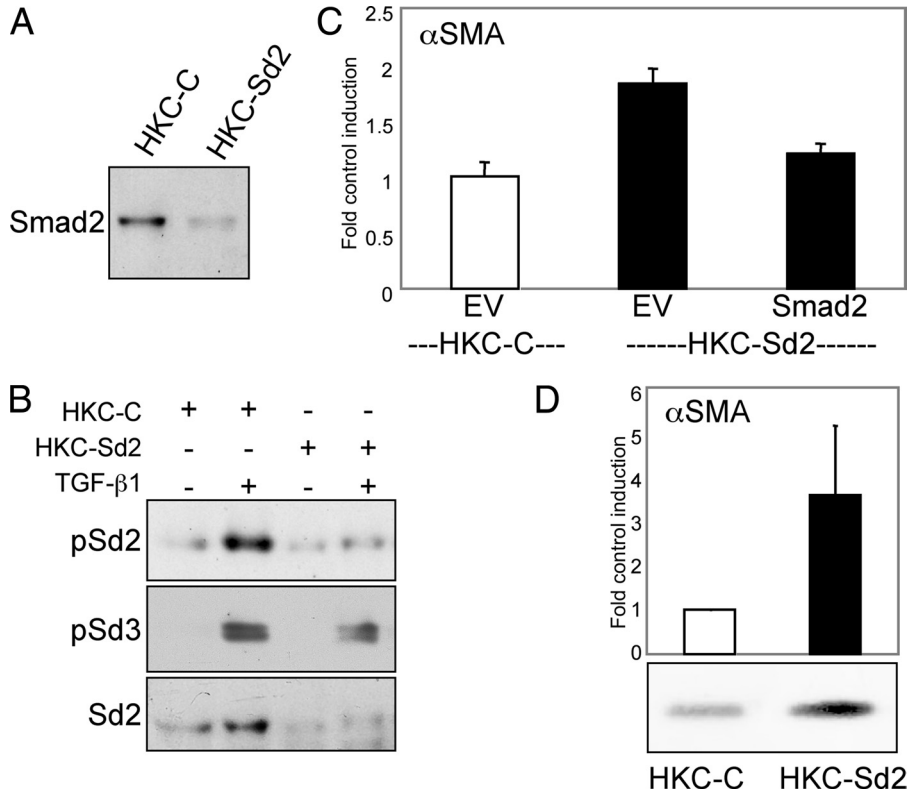

FIGURE 8. **Loss of Smad2 mimics the effects of loss of SARA on αSMA expression.** *A*, Western blots of lysates from either HKC-C cells or HKC infected with lentiviral shRNA against Smad2 (*HKC-Sd2*) show reduced Smad2 expression in HKC-Sd2 cells. *B*, HKC-Sd2 cells treated with vehicle or TGF-β1 for 30 min show reduced total and phosphorylated Smad2 compared with similar treatment of HKC-C cells. *C*, HKC-C or HKC-Sd2 cells were transfected with an αSMA promoter reporter construct, β-galactosidase, and either pCMV5 or Smad2 and assessed by luciferase assay 48 h post-transfection. *Bars*, triplicate luciferase measurements corrected for β-galactosidase from a representative experiment of three separate experiments. *D*, Western blots showing that αSMA protein expression is higher in HKC-Sd2 cells compared with HKC-C cells. The *graph* depicts combined densitometric values from three separate experiments normalized to HKC-C control expression.

Smad2 expression suggests that SARA down-regulation might contribute to EMT in part through indirect effects on Snail. However, to date, we have been unable to detect differences in Snail expression between HKC-C and HKC-S.[4]

The loss of SARA expression affecting Smad2 signaling is not entirely unexpected, given that SARA has been described by our laboratory and others as an adaptor protein for TGF-β-receptor-Smad2 interaction (13, 15, 30, 31). However, our finding that SARA also may affect Smad2 expression is novel. Using yeast two-hybrid analysis and *in vitro* binding assays, the E3 ubiquitin ligase Smurf2 has been shown to bind Smad1, Smad2, and Smad3. However, this interaction appears to reduce the steady-state levels of Smad1 and Smad2, without significantly affecting Smad3 expression (8, 9). How the loss of SARA might induce increased association of Smad2 and Smurf2 is not clear. TGF-β1 receptors have been shown to traffic through either endocytic vesicles or caveolar subcellular compartments (32). SARA is associated with the receptor in the early endosomes, and Smad2-dependent signaling is enhanced. In contrast, in the caveolar raft compartment, the receptor associates with Smad7 and Smurf2, resulting in inhibition of Smad2 signaling and reduced receptor expression (32). This study found that, if endocytic internalization were blocked, more of the receptor localized to rafts, whereas if rafts were disrupted, more of the receptor was associated with endosomes. Therefore, a possible mechanism by which SARA might cause more Smad2-Smurf2 complex formation is that the loss of endosomally localized SARA might drive more of the receptor, and possibly receptor-associated Smad2, to caveolar fractions, where it interacts with Smurf2. However, in the context of reduced SARA expression, the degree of Smad2-TβR association is uncertain, so the relative compartmentalization of the Smad2-receptor complex remains a matter of speculation. The nature of the interactions among SARA, the TGF-β receptors, Smad2, and Smurf2 is probably complex and is the focus of ongoing studies in our laboratory. Based on our current data showing that SARA expression is important in maintaining an epithelial cell phenotype, answers to these questions as well as the questions of how SARA is regulated and how TGF-β1 causes the depletion of SARA may help direct future studies in disease processes involving EMT.

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[4] C. E. Runyan, T. Hayashida, S. Hubchak, J. F. Curley, and H. W. Schnaper, unpublished data.

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