Pro tumorigenic actions of S100A2 involve regulation of PI3/Akt signaling and functional interaction with Smad3

**Introduction**

Members of S100 protein family are multifunctional proteins that are differentially expressed in a wide variety of cell types and have been reported to regulate inflammatory responses, cell cycle progression and differentiation. Several observations implicated S100 family of calcium-binding proteins in the initiation and progression of a variety of human cancers (1). In addition, S100 proteins have been shown to influence the pro-survival pathways during cancer progression (2). Expression of several S100 proteins such as S100A4, S100A9, S100A8 and S100B are commonly upregulated in several cancers (1,3–5). In contrast, S100A2, S100A11 and S100A9 have been documented to function as tumor suppressors or tumor promoters in different cancers (1,6). Moreover, S100A2, S100A4, S100A6, S100A8, S100A9 and S100A11 have been shown to be associated with metastasis (1,3–7).

Initially, S100A2 was identified as a downregulated gene in breast cancer (8). Later, reduced S100A2 expression was observed in lung cancer, oral squamous cell carcinoma and gastric cancer (6). Consequently, S100A2 was thought to be a putative tumor suppressor. Subsequently, overexpression of S100A2 was detected in basal type breast cancer (9), non-small cell lung cancer (NSCLC) (10), ovarian cancer (11), endometrial cancer (12), head and neck squamous cell carcinoma (13), lymphoma (14), gastric cancer (15) and bladder carcinoma (16). In pancreatic cancer, overexpression of S100A2 correlates with tumor progression, poor prognosis (17) and is an independent predictor of response to pancreatectomy (18). In NSCLC cell lines, ectopic expression of S100A2 enhanced transwell and transendothelial migration, suggesting its involvement in the metastatic process of NSCLC (19). In good agreement, high S100A2 expression in tissues correlated with increased metastasis of lung cancer (10).

Our earlier findings demonstrated that like S100A4, expression of S100A2 is induced by transforming growth factor-β (TGF-β), which is a potent inducer of epithelial-mesenchymal transition (EMT) and cancer progression (12,20,21). Very recently, circulating tumor cells from breast cancer patients exhibited dynamic changes in EMT markers with active TGF-β signaling (22), suggesting its role in tumor cell dissemination. Signaling through TGF-β involves two types of transmembrane serine/threonine kinase receptors, TβRI and TβRII. On ligand binding, TβRI and TβRII form a complex, resulting in the activation of TβRII, which then activates TβRI by phosphorylation on serine/threonine residues. The activated TβRI further phosphorylates Smad 2 and 3, two major intracellular effectors proteins for TGF-β signaling. Subsequently, phosphorylated Smad2/3 forms a complex with Smad4, which then gets translocated into the nucleus and interacts with myriads of coexpressors or coactivators to regulate target gene expression. Thus, availability of different types of Smad-binding proteins in different cellular contexts determines the diverse cell responses to TGF-β (23).

Cells undergoing TGF-β-induced EMT are characterized by (i) disassembly of cell–cell junctions; (ii) reorganization of the actin cytoskeleton; (iii) loss of epithelial polarity and (iv) remodeling of cell–matrix adhesive interaction, resembling differentiated fibroblasts or myofibroblasts (21,24). Intriguingly, EMT of malignant cells is associated with intravasation, extravasation and colonization to distant organs (24). Collectively, EMT is characterized as a prerequisite for cancer cell metastasis (25). Several findings have established members of S100 protein such as S100A4 as a common mediator of fibrosis, EMT and metastasis in experimental animal models (26). Additionally, loss of S100A2 has been shown to reduce lung metastasis in the murine xenograft model of NSCLC (10). Despite these findings, the exact role of S100A2 in carcinogenesis remains controversial and warrants further investigations.

Therefore, the aim of this study was to establish the role of S100A2 protein in tumor growth, EMT and TGF-β-mediated tumor promotion. Our findings highlight the involvement of S100A2 in the induction of EMT, enhanced cell invasion and TGF-β-mediated protumorigenic actions through physical interaction with Smad3.

**Materials and methods**

**Plasmids and constructs**

Control-short hairpin RNA (shRNA), S100A2-shRNA and S100A2-green fluorescent protein (GFP) plasmids were a kind gift from Dr Elmar Bulk (10). Smad3-Flag plasmid was a gift from Dr Lalage Wakefield. Full-length Smad3-glutathione-S-transferase (GST) and deletion mutants were a kind gift from Dr Elmar Bulk (10).
Isao Matsura (27), pRl-TK, p3TP-Luc, pSB-E-Luc and S100A2-GST constructs were described before (20). Human S100A2 open reading frame was amplified using complementary DNA derived from total RNA of HaCaT (normal skin keratinocytes) cells and cloned into pcDNA3.1(A) vector (Invitrogen, Carlsbad, CA) at HindIII and Xhol restriction sites. Primers used for amplifications are 5'-CGAAGCTTCCCATGATTTCCAGTCTTC-3' (forward) and 5'-ACTCGAGGTTCTGGTCAAGGTTGG-3' (reverse).

**RNA isolation, complementary DNA synthesis and semiquantitative RT–PCR**

Total RNA isolation, complementary DNA synthesis and reverse transcription–polymerase chain reaction (RT–PCR) were performed as described before (28). RPL-35a (ribosomal protein L35a) was used as an internal reference. The primer sequences used for RT–PCR are listed in Supplementary Table S1, available at Carcinogenesis Online.

**Detection of proteins by immunoblot**

Preparation of cell extract and immunoblotting was performed as described before (20). Briefly, equal amount of proteins were separated on 12.5–15% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membrane (Immobilin P; Millipore). Membranes were treated with 5% skimmed milk powder and incubated with polyclonal S100A2 (2 μg/ml), phospho-extracellular signal-regulated kinase 1/2 (1/2 μg/ml), total ERK, phosphorylated Akt (p-Akt) (ser-473) and total Akt antibodies at a dilution of 1:1000 (Cell Signaling Technology). Monoclonal anti-Flag (Sigma–Aldrich; dilution of 1:2000), p21 (Cell Signaling Technology; dilution of 1:1000), p-Akt, Smad3 (Abcam; dilution of 1:1000 each), polyclonal GFP (Abcam; dilution of 1:1000), E-cadherin (Becton Dickinson; dilution of 1:2000), vimentin (Sigma–Aldrich; dilution of 1:2000), N-cadherin (Abcam; dilution of 1:1000) and α-smooth muscle actin (α-Sma, Abcam; dilution of 1:750) were used in 5% bovine serum albumin in Tris-buffered saline and Tween 20. β-Actin (Sigma–Aldrich; dilution of 1:3000) served as a loading control.

**Immunohistochemistry**

Histological sections of xenograft tissues were examined by light microscopy using hematoxylin and eosin preparation. Paraffin sections (4 μm) from the xenograft tumors were collected on silane-coated slides and immunohistochemistry was performed. The antigen retrieval was achieved by heat treatment of the deparaffinized sections in a microwave oven at 600 W for 30 min for S100A2, N-cadherin, Ki-67, p-Akt and CD-31 in citrate buffer (10 mM, pH 6.0) or in Tris-ethylene diamine tetraacetic acid (pH 9.0) buffer, respectively. All sections were further treated with methanol and 5% hydrogen peroxide followed by washes with phosphate-buffered saline buffer (pH 7.4–7.6). Skimmed milk powder (5%) was used to block background staining for 45 min and incubated overnight with the primary antibody at 4°C (anti-Ki-67 monoclonal BGX 297, dilution of 1:30; anti-CD31, dilution of 1:25; BioGenex; antivimentin, dilution of 1:50; anti-N-cadherin, dilution of 1:50; anti-p-Akt-ser-473, dilution of 1:300; N-cadherin (Abcam; dilution of 1:1000) and α-smooth muscle actin (α-Sma, Abcam; dilution of 1:750) were used in 5% bovine serum albumin in Tris-buffered saline and Tween 20. β-Actin (Sigma–Aldrich; dilution of 1:3000) served as a loading control.

**Xenograft tumor growth in immunocompromised mice**

All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Indian Institute of Science. Briefly, 1.5 × 10^6 vector or S100A2 overexpressing A549 cells were resuspended in 100 μl of phosphate-buffered saline and injected subcutaneously on the flanks of 6–8 weeks old female nude mice (six mice per condition). Tumor growth was monitored every 5 days after 15 days of cell inoculation and measurements were made using vernier caliper. Forty-five days postinoculation, tumors were excised and weighed. The excised tumors were preserved in TRIZol at –80°C for subsequent RNA extraction (29) or in 10% formaldehyde for immunohistochemistry. Tumor volume was calculated using the formula L(W)^2/2, where ‘L’ is the length and ‘W’ is the width of the tumor.

**Extraction of proteins from cells and immunoprecipitation**

Cells were transfected with appropriate plasmids and 48 h posttransfection, cells were washed in ice-cold Dulbecco’s phosphate-buffered saline and lysed in modified radiomunoprecipitation assay (RIPA) buffer containing 30 mN-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid pH 7, 100 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 μg protease inhibitor complex, 1 mM sodium pyrophosphate and 10 mM sodium orthovanadate. Lysates were incubated on ice for 30 min and centrifuged at 13 000 r.p.m. for 30 min at 4°C. Total protein in the lysates was estimated by the Bradford method (Bio-Rad Laboratories). Clarified protein extracts were incubated with equilibrated protein A or G sepharose beads for 60 min. Precleared supernatant was incubated overnight at 4°C with S100A2, GFP, Flag (5 μg/ml) or Smad3 (2 μg/ml) antibodies, followed with addition of 30–50 μl of protein A or protein G sepharose beads for 1 h. The beads were washed three times in RIPA buffer and once in HBS (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid pH 7 and 100 mM NaCl) and subsequently prepared for immunoblottting. Calcium chloride (2 mM) and 5 mM ethylene glycol-bis(amoetethylyl)-tetraacetic acid (EGTA) were added as required in the interaction buffer.

**GST pull-down assays**

A549 or HEK293T cells transiently transfected with S100A2-GFP were lysed in modified RIPA buffer and diluted to 1:5 in interaction buffer (50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid pH 7, 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 5 mM ethylenediaminetetraacetic acid, 10 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate and protease inhibitors). GST-fused S100A2 or Smad3 proteins were expressed in Escherichia coli and purified using GST beads (GE Healthcare) as described before (20,27). Equal amounts of GST or GST fusion proteins bound to glutathione–Sepharose beads were incubated with lysates for 4h at 4°C, subsequently washed three times with RIPA buffer and interacting proteins were detected by immunoblotting. Purity of GST fusion proteins was confirmed by Coomassie blue staining. Calcium chloride (2 mM) and 5 mM EGTA were added as required in the interaction buffer.

**Statistical analysis**

All statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Statistical significance was evaluated by the unpaired Student’s t-test or one-way analysis of variance (ANOVA) using Fisher’s post hoc comparisons. Results are expressed as means ± standard error of the mean and differences are considered significant for P < 0.05.

**Results**

**Overexpression of S100A2 leads to EMT and enhanced invasion of A549 cells**

To examine the mechanism of S100A2 role in metastasis of lung cancer, stable clones overexpressing empty plasmid (vector) or human S100A2 protein (S100A2-2 and S100A2-20) were generated in A549, lung cancer cell line. Interestingly, S100A2 overexpressing A549 cells when seeded in a monolayer showed considerable alteration in the morphology of cells compared with the vector-expressing control cells. Elevated levels of S100A2 changed the polarized epithelial morphology of control cells to a scattered, elongated fibroblast-like morphology (indicated by arrows) as shown for S100A2-2 cells (Figure 1A) suggestive of EMT. To verify that these morphological changes seen upon overexpression of S100A2 were indeed associated with EMT, we examined the expression of EMT-related markers using RT–PCR, immunoblotting and immunofluorescence. Overexpression of S100A2 resulted in the increased expression of mesenchymal markers such as vimentin, N-cadherin and α-Sma with a concomitant decrease in the expression of epithelial markers such as E-cadherin. However, overexpression of S100A2 did not alter the levels of β-catenin (Figure 1B). Consistent with this observation, decrease in the messenger RNA expression of occludin (tight junction) and E-cadherin (adherens junction) was observed, whereas snail and vimentin transcript levels were significantly high in the S100A2-2 and S100A2-20 cells compared with the control (Supplementary Figure S1, available at Carcinogenesis Online). Overexpression of S100A2 in the indicated clones was confirmed using polyclonal antibody against S100A2 as shown by immunoblot (Figure 1B), semiquantitative RT–PCR and immunofluorescence analysis (Supplementary Figure S2A and B, available at Carcinogenesis Online). In the anchorage-independent growth assay, S100A2-2 and S100A2-20 cells formed large and ‘loose’ colonies as compared with the tight and compact colonies of the vector and parental A549 cells (Figure 1C). However, no significant difference in the number of soft agar colonies was observed between vector, S100A2-2 and S100A2-20 cells (data not shown). Consistent, with the observation made by Bulk et al., overexpression of S100A2 did not have any effect on A549 cellular proliferation as seen by bromodeoxyuridine cell proliferation assay and cell cycle analysis (Supplementary Figure S3A).
Fig. 1. S100A2 induces EMT and enhanced invasion of A549 cells. (A) Phase-contrast microphotographs of low density (upper panels) and high density (lower panels) of vector-transfected or S100A2 (S100A2-2 and S100A2-20)-expressing A549 cells. Arrows indicate spindle-shaped morphology of cells in S100A2-2 and S100A2-20 cells compared with cuboidal polar morphology of the control cells. Scale bar is 100 μm. Magnification is ×20. (B) Expression of EMT markers in A549 cells stably integrated with empty plasmid (vector) or S100A2-expressing plasmid (S100A2-2 and S100A2-20). Overexpression of S100A2 was assessed using polyclonal antibody against human S100A2 by western blot analysis. β-Actin served as a loading control. (C) Morphologies of the indicated cells in soft agar colony formation assay. Thirty thousand cells were plated in semisolid agar medium (0.6% soft agar) and allowed to grow for 7–14 days. The colonies were stained with crystal violet (0.05%), counted and photographed. (D) Immunofl orescence analysis of EMT markers in vector and S100A2 overexpressing cells. S100A2-2 and S100A2-20 cells showing diffused membrane staining for E-cadherin and cytosolic staining for vimentin (shown...
and B, available at Carcinogenesis Online). Immunocytochemistry of E-cadherin in S100A2-2 and S100A2-20 cells suggested disassembly of cell–cell junctions followed with an increase in vimentin expression and formation of actin stress fibers (F-actin) compared with the vector-expressing control cells (Figure 1D), suggestive of S100A2 role in mediating EMT. Since cells undergoing EMT acquire an invasive phenotype (30), we analyzed effect of S100A2 overexpression on the invasive capacity of A549 cells. S100A2 overexpression significantly enhanced invasion of A549 cells compared with the vector-expressing control cells in the matrigel-coated Boyden chambers (Figure 1E). Collectively, these data suggest a role for S100A2 in the induction of EMT followed by enhanced invasion of A549 cells.

Overexpression of S100A2 increased tumor growth and proliferation in vivo

We assessed the consequence of S100A2 overexpression on the tumorigenic potential of A549 cells in vivo, using a murine xenograft model. Subcutaneous administration of vector, S100A2-2 and S100A2-20 cells in immune-compromised animals generated palpable tumors. Notably, xenograft tumors expressing S100A2 showed significant increase in the tumor volume and tumor size over time (Figure 2A and B). Immunohistochemical examination showed higher nuclear expression of S100A2 in the xenograft sections from S100A2 tumors compared with the control tumors. Additionally, S100A2-positive xenograft tumors had increased positivity for Ki-67, CD31 (endothelial marker) and N-cadherin expression. Hematoxylin and eosin-stained sections characterized S100A2-positive tumor cells with fasciculate spindle-like elements, mimicking sarcomatous tumor compared with the control tumor (Figure 2C). In agreement with the in vitro data on the S100A2-mediated downregulation of E-cadherin, we observed an inverse correlation between high S100A2 expression and low or diffused membrane expression of E-cadherin in the xenograft tumors (Figure 2D). Interestingly, pooled RNA from the resected xenograft tumors showed upregulation of genes involved in extracellular matrix modulation and angiogenesis such as matrix metalloprotease (MMP)-1, MMP-7, transglutaminase 2, vimentin, urokinase plasminogen activator and vascular endothelial growth factor (VEGF) and a concomitant downregulation in the expression of E-cadherin and tissue plasminogen activator (Figure 2E). Collectively, these results confirmed induction of EMT and tumor growth upon overexpression of S100A2 in vivo.

Overexpression of S100A2 activates PI3/Akt signaling

To elucidate the signaling pathway(s) involved in the induction of EMT by S100A2, we examined the status of signaling molecules of various pathways implicated in tumor progression. Lysates from S100A2-2 and S100A2-20 showed elevated levels of p-Akt (Ser-473), compared with the control cells (Figure 3A). In concordance, increased nuclear staining of p-Akt (Ser-473), indicative of activated Akt signaling was also observed in the paraffin-embedded sections derived from S100A2-positive xenograft tumors compared with control tumors (Figure 3B). In good correlation, stable knockdown of S100A2 expression in Hep3B cells using S100A2-specific shRNA resulted in a drastic decrease in the amounts of p-Akt (Ser-473) followed by decrease in the expression of mesenchymal markers, N-cadherin and vimentin (Figure 3C and D). On the other hand, overexpression or knockdown of S100A2 had no effect on the phosphorylation of ERK1/2 (Figure 3A and C). These results suggested that S100A2 regulates activation of PI3/Akt signaling, an important signaling pathway involved in EMT of cancer cells, including hepatocellular carcinoma (31,32).

Loss of S100A2 or Smad3 attenuates TGF-β-induced EMT

With the established role of TGF-β in mediating EMT of cancer cells and our observations that increased expression of S100A2 can induce EMT in tumor cells, it is tempting to speculate that TGF-β-mediated EMT may involve S100A2, a known target of TGF-β (20). Towards this, Hep3B cells expressing high endogenous levels of S100A2 protein were used to achieve stable knockdown of S100A2 expression using shRNA. These cells were further monitored for their ability to undergo EMT in the absence (−) or presence (+) of TGF-β. As shown in Figure 4A, stable knockdown of S100A2 in Hep3B cells reduced N-cadherin and α-Sma expression in the absence of TGF-β, suggestive of its role in mediating EMT. Moreover, loss of S100A2 greatly attenuated TGF-β-induced expression of vimentin, N-cadherin and α-Sma (Figure 4A). Consistent with the established requirement of Smad3 in TGF-β-induced EMT of hepatocytes (33), stable knockdown of Smad3 using shRNA (Supplementary Figure S4, available at Carcinogenesis Online) downregulation of cells with a Smad3 inhibitor (SIS3) compromised TGF-β-induced EMT (Figure 4B). SIS3 is a specific inhibitor of Smad3. More importantly, reduced EMT by SIS3 was accompanied by reduced cell invasion of Hep3B cells even in the presence of TGF-β1 (Figure 4C). Our earlier finding also supported a role of S100A2 in mediating TGF-β-induced Hep3B cell migration and invasion (20). Taken together, these results suggest a strong dependence of S100A2 and Smad3 expression in TGF-β-induced EMT and invasion of Hep3B cells.

S100A2 interacts with the C-terminus MH2 domain of Smad3

Our study suggested induction of EMT upon S100A2 overexpression in A549 cells both in vitro and in vivo (Figures 1 and 2) and regulation of genes such as MMP-1, MMP-7, snail, vimentin and VEGF (Figure 2E and Supplementary Figure S1, available at Carcinogenesis Online). Incidentally, MMP-1, snail and vimentin are known targets of TGF-β/Smad3 signaling (34–36). Moreover, we demonstrate dependence of S100A2 and Smad3 in TGF-β-mediated EMT (Figure 4) and invasion/migration of cancer cells (20). S100A2 is known to function through physical interaction with proteins such as SMAD3 (37) Hence, it is plausible that S100A2 actions might involve its interaction with Smad3. To evaluate this, Smad3-Flag- and S100A2-GFP-expressing plasmids were cotransfected in HEK293T cells. As shown in Figure 5A, immunoprecipitation using GFP antibody was able to pull down S100A2 as detected by GFP antibody along with Smad3 whose presence was confirmed using Flag expression. To further establish this finding, we performed a GST pull-down assay in the presence or absence of Ca++. Partially purified recombinant S100A2-GST and GST proteins immobilized on GSH beads were incubated with lysates obtained from A549 cells. S100A2 interaction with Smad3 was detected using Smad3-specific antibody, as shown by immunoblotting (Figure 5B). Interestingly, unlike p53, S100A2 was able to bind Smad3 even in the absence of high Ca++. However, presence of 2 mM Ca++ enhanced S100A2 binding to Smad3, whereas addition of EGTA compromised this enhanced binding. Purified GST protein failed to show any binding of Smad3 with S100A2 even in the presence of 2 mM CaCl2 (Figure 5B). The interaction of S100A2 with Smad3 in Hep3B cells was also confirmed using coimmunoprecipitation assay (Figure 5C). Moreover, exposure of Hep3B to TGF-β enhanced S100A2 binding to Smad3 and addition of EGTA compromised S100A2–Smad3 interaction, suggesting influence of calcium on TGF-β-mediated S100A2–Smad3 interaction (Figure 5D). Collectively, these results indicated a positive influence of both calcium and TGF-β signaling in mediating intracellular S100A2–Smad3 interaction.

in green). Presence of stress fibers in the indicated cells were detected using antibodies against phallolidin conjugated to fluorescein isothiocyanate (shown in green). Nucleus was stained with propidium iodide, shown in red or 4′,6-diamidino-2-phenylindole, shown in blue. Magnification is x63. (E) Matrigel Boyden chamber assay. Twenty thousand cells overexpressing S100A2 or empty plasmid (vector) were plated in the matrigel-coated Boyden chambers. Percent-invaded cells in the lower chambers were determined as per the manufacturer’s instruction. Each bar represents mean ± standard error of the mean. All results are representative of two separate experiments performed in duplicates and analyzed by one-way ANOVA. **P < 0.01.
Overexpression or knockdown of S100A2 increased or decreased the expression of Smad3 protein, respectively (Figure 5E and F and Supplementary Figure S5A, available at Carcinogenesis Online). Interestingly, there was no influence of S100A2 on the Smad3 RNA expression (Supplementary Figure S5B and C, available at Carcinogenesis Online), indicating stabilization of Smad3 protein by S100A2. Further, S100A2 colocalized with Smad3 in the nucleus as shown in Hep3B and A549 cells overexpressing S100A2 in vivo. Total RNA was isolated from the excised xenograft tumors expressing vector and S100A2 as indicated. Messenger RNA expression of indicated genes were determined using semiquantitative RT–PCR. Primers specific for each gene is listed in Supplementary Table S1, available at Carcinogenesis Online.
S100A2–Smad3 interaction mediates EMT and invasion

Fig. 4. S100A2 and Smad3 are required for TGF-β-induced EMT and invasion of Hep3B cells. (A) Stable knockdown of S100A2 was performed in Hep3B cells using shRNA as described in Materials and methods. Lysates from control (sh-Control) or S100A2 knockdown (sh-S100A2) cells treated (+) or untreated (−) with TGF-β (5 ng/ml) for 48 h were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Expressions of the indicated proteins were detected using specific antibodies as described before. (B and C) Hep3B cells treated (+) or untreated (−) with SIS3 for 2 h and then exposed to TGF-β (5 ng/ml) for additional 48 h were subjected to immunoblot analysis with indicated antibodies (B) or invasion assay in Boyden chambers (C). Graph represents fold invasion of cells compared with the untreated (−) control. Each bar represents mean ± standard error of the mean. All results are representative of two separate experiments performed in duplicates and statistical significance was analyzed using one-way ANOVA. *P < 0.05, **P < 0.01.
was detected using GFP antibody in the immunoblot analysis. The binding of S100A2 with Smad3 was observed with full-length Smad3 (Smad3-wt) and Δ142 proteins, suggesting that the S100A2-binding site does not lie in the MH1 domain of Smad3 protein. Interestingly, further deletion of the entire MH1 domain and the linker region (Δ230-GST) retained bound S100A2 protein, whereas loss of MH2 domain (ΔC-GST) did not show pull down of S100A2, indicating involvement of MH2 domain of Smad3 in binding to S100A2 (Figure 5G). The purity of Smad3-wt and its various deletion mutants expressed in E. coli was established by Coomassie staining (Supplementary Figure S5D, available at Carcinogenesis Online).

S100A2 modulates TGF-β/Smad3-mediated transcriptional activity

To examine the influence of S100A2 binding with Smad3 on TGF-β-dependent gene transcription, luciferase reporter assays using Smad-responsive promoter reporter constructs (pSBE-Luc and p3TP-Luc) were performed. The reporter plasmids were cotransfected in HEK293T cells along with pRL-TK, empty vector, Smad3-Flag and S100A2-GFP expressing plasmids. Forty-eight hours posttransfection, S100A2 protein in the lysates was immunoprecipitated using 2 μg of polyclonal GFP antibody. Smad3 and S100A2 proteins were recovered and detected by western blot analysis using anti-Flag or anti-GFP antibodies, respectively, as described in the Materials and methods (upper panel). For input lanes, 2% of the lysates were used and expression of the transfected proteins were detected using indicated antibodies (lower panel). Immunoprecipitation using normal rabbit immunoglobulin G (NRIgG) was used as a negative control. (B) Ca²⁺ enhances S100A2 interaction with Smad3. Lysates from A549 cells were subjected to GST pull-down assays with purified GST-S100A2 or GST as a control. EGTA or CaCl₂ was included in the lanes as indicated. Smad3, GST and S100A2-GST proteins recovered on the beads were detected by western blot analysis using anti-Smad3 antibody. Coomassie stained partially purified GST or S100A2-GST protein with their indicated molecular masses are shown (lower panel). (C) Coimmunoprecipitation of Smad3 and S100A2 in Hep3B cells as described in the Materials and methods. Immunoprecipitation was performed using either Smad3 or S100A2 antibodies and lysates were subjected to western blot analysis with indicated antibodies. (D) Effect of TGF-β1 on the endogenous interaction of S100A2 with Smad3. Lysates from Hep3B cells treated with or without TGF-β1 for 2h were used for immunoprecipitation using anti-Smad3 antibody (lower panel). Immunoprecipitated S100A2 and Smad3 proteins were detected by western blot analysis. Control rabbit immunoglobulin G (NRIgG) served as a negative control. (E and F) S100A2 triggers stabilization of Smad3. (E) Lysates from A549 cells transiently overexpressing S100A2-GFP and control-GFP or (F) Hep3B cells stably expressing shRNA against S100A2 were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and expression of Smad3 was analyzed. β-Actin served as a normalizing control. (G) MH2 domain of Smad3 interacts with S100A2. Schematic diagram of Smad3 deletion mutants fused to GST and the major domains MH1, linker and MH2 are marked, left panel. The designation of each construct is indicated on the left and molecular sizes of each protein are indicated on the right. (Right panel) GST pull-down assay using Smad3 deletion mutants. Equal amounts of GST, GST-Smad3 and mutant Smad3 proteins fused to GST were incubated with cell lysates from HEK293T cells expressing S100A2-GFP. The expression of S100A2 was detected using anti-GFP antibody.
expression plasmids. As shown in Figure 6A, coexpression of S100A2 and Smad3 significantly (**p < 0.001) increased luciferase activity of pSBE-Luc or p3TP-Luc to 4-fold and 2-fold, respectively, compared with the untreated control. Exposure to TGF-β1 further enhanced pSBE-Luc activity to 17-fold and p3TP-Luc activity to 7-fold compared with the untreated control (Figure 6A and B, ***p < 0.001). Interestingly, depletion of S100A2 expression in Hep3B cells compromised TGF-β-stimulated pSBE-Luc reporter activity to ~2-fold (Figure 6C). However, depletion of S100A2 using shRNA did not have any significant effect on the pSBE-Luc activity in the absence of TGF-β (Figure 6C). These results indicated cooperation of S100A2 in Smad3-dependent transcription. We further confirmed these findings by evaluating the effect of S100A2 knockdown on the endogenous expression of TGF-β/Smad3 targets. As shown by RT-PCR and immunoblot analysis, stable loss of S100A2 in Hep3B cells abolished TGF-β-induced expression of direct Smad3 targets PAI-1 and p21 (Figure 6D and Supplementary Figure S7A, available at Carcinogenesis Online). However, stable overexpression of S100A2 did not alter the basal expression of p21 protein (Supplementary Figure S7B, available at Carcinogenesis Online). Similar results were obtained in endometrial cancer cell line, Ishikawa where transient knockdown of S100A2 attenuated TGF-β1-induced expression of p21 and vimentin (Figure 6E). Differential expression of p21, vimentin and PAI-1 has been shown to play an important role in TGF-β-mediated tumor progression (38). Taken together, these results highlighted an essential role of S100A2 as a cofactor in TGF-β/Smad3-dependent transcription.

Fig. 6. S100A2 modulates transcription of Smad-responsive promoters. (A and B) HEK293T cells cotransfected with pRL-TK, Smad3-Flag, pSBE-Luc (A) or p3TP-Luc (B) along with empty vector or S100A2 expression plasmid. Cells were either untreated (−) or treated (+) with TGF-β1 for 24 h before harvesting and subjected to luciferase assay. (C) TGF-β/Smad3-dependent transcription requires S100A2. Hep3B cells with stable S100A2 knockdown was cotransfected with pRL-TK and pSBE-Luc. Twenty-four hours posttransfection, cells were treated with TGF-β for additional 24 h before harvesting and subjected to luciferase assay. Values are mean ± standard error of the mean of two independent experiments performed in triplicates. (D and E) Silencing of S100A2 inhibits the endogenous expressions of TGF-β/Smad3 target genes. Hep3B cells with stable S100A2 knockdown were serum starved and treated with (+) or without (−) TGF-β1 (5 ng/ml) for 24 h. Ishikawa cells were transiently transfected with plasmids expressing control shRNA or S100A2-shRNA. Thirty-six hours posttransfection, cells were treated with TGF-β1 (5 ng/ml) for additional 24 h as described above. Messenger RNA expression of S100A2, p21, PAI-1 and vimentin were analyzed using semiquantitative RT–PCR. RPL-35a expression served as normalizing control. Bar graphs on the right represent fold differences over control of the expression of respective genes. Error bars represent mean ± standard error of the mean.

S100A2–Smad3 interaction mediates EMT and invasion
Discussion

Recently, S100 family of calcium-binding proteins, including S100A2 has emerged as valuable diagnostic and prognostic biomarkers in several types of human cancers (1). Altered expressions of S100A2 are associated with aggressive tumor behavior (9,10,17). S100A2 expression is found to be either downregulated or overexpressed in different types of tumors. S100A2 functions as a tumor suppressor in oral squamous cell carcinoma, whereas its protumorigenic actions have recently been documented in NSCLC (10,39). Overexpression of S100A2 is correlated with increased metastasis and poor prognosis of the patients in the early stage NSCLC (10,19). However, the exact role of S100A2 in the progression of cancer remains obscure. Few members of S100 proteins like S100A4 actions in the process of fibrosis, EMT and metastasis have just begun to unravel.

In the present study, the mechanism(s) by which S100A2 mediates its protumorigenic actions has been addressed. Using stable overexpression of S100A2 in A549 lung cancer cells, we demonstrate involvement of S100A2 in the induction of EMT followed by enhanced invasion and tumor growth in vivo. Effect of S100A2 in mediating EMT was shown by increased expression of mesenchymal markers such as vimentin, N-cadherin, α-Sma and a concomitant decrease in the expression of epithelial markers such as E-cadherin and occludin. In addition, the transcription factor snail, which is a negative regulator of E-cadherin expression, was also induced in cells overexpressing S100A2 protein. Further, loss of S100A2 in Hep3B cells also reversed the EMT phenotype, as seen by the loss in the expression of N-cadherin and vimentin levels. This unequivocally establishes the role of S100A2 in conferring EMT phenotype to transformed cells. Interestingly, overexpression of S100A2 resulted in the formation of distinct ‘spread out’ and loose colonies in anchorage-independent growth assay. This morphological feature of colonies in soft agar has been reported to be suggestive of increased metastatic potential of the cells (40,41). Activation of Akt signaling has been shown to be a critical step in promoting survival of epithelial cells under the anchorage-independent conditions, EMT, migration and invasion of tumor cells (31,40,41). Supporting this fact, overexpression of S100A2 preferentially increased the activation of p-Akt (Ser-473) in A549 cells, which was significantly reduced by knockdown of S100A2 expression in Hep3B cells.

To confirm the role of S100A2 in EMT and tumor growth in vivo, S100A2 overexpressing cells were injected subcutaneously in the flanks of immunocompromised mice and the phenotype of resultant tumors were examined. Subcutaneous injection of S100A2 clones formed significantly large tumors, which histologically appeared to have undergone EMT as seen by the ‘sarcomatous’ nature of the tumors. This was supported by the induction of EMT markers and enhanced VEGF expression. Activated p-Akt signaling was also observed in the resected tumors overexpressing S100A2. In light of the above findings, regulation of Akt signaling by S100A2 suggests a role for PI3-kinase/Akt pathway in S100A2-mediated protumorigenic actions.

It is of surprise to note that the S100A2-expressing tumors are larger although there was no growth promotion by S100A2 in culture conditions (Supplementary Figure S3, available at Carcinogenesis Online). This discordance could be due to the differences in the growth properties of these cells in vitro and in vivo. The enhanced proliferation in S100A2 tumors could be due to increased angiogenesis as seen by VEGF overexpression and CD31 positivity. This result is also in agreement with the report of Bulk et al. (10), where they did not observe any growth advantage of S100A2 overexpressing cells in monolayer but there was enhanced metastasis.

Intriguingly, S100A2 regulated expression of genes such as MMP-1, MMP-7, transglutaminase 2, urokinase plasminogen activator, vimentin, snail, E-cadherin involved in matrix modulation and EMT (21,42). Strikingly, TGF-β/Smad3-dependent signaling also regulates expressions of these genes. In addition, role of TGF-β and Smad3 in mediating EMT has been well documented for lung carcinoma and hepatocytes (33,43). Our experiments demonstrating involvement of S100A2 in Smad3-dependent regulation of gene expression and EMT prompted us to evaluate a possible interaction of S100A2 with Smad3. By GST pull-down and coimmunoprecipitation experiments, we demonstrate a physical interaction between S100A2 and Smad3 proteins. Furthermore, we demonstrate that Smad3–S100A2 interaction increased the Smad3 protein content without any change in the Smad3 RNA. This suggests stabilization of Smad3 protein in the presence of S100A2 protein. In the presence of high calcium and TGF-β, S100A2 binding to Smad3 was further enhanced. Moreover, S100A2 positively induced Smad3-dependent transcriptional activation. Supporting this, knockdown of S100A2 expression abolished TGF-β/Smad3-mediated regulation of its target genes namely p21, PAI-1 and vimentin. Most importantly, loss of S100A2 significantly reduced TGF-β-induced EMT and cancer cell migration/invasion (20). This is similar to the role of S100A4 in TGF-β actions where a physical interaction of S100A4 with Smad3 is required for MMP-9 regulation and invasion of breast cancer cells (27). All these point to an essential role of S100A2 as a cofactor in mediating protumorigenic actions of TGF-β. In addition, our findings highlight an indispensable role for S100 proteins in TGF-β-mediated EMT and invasion. Earlier, we established the transcriptional regulation of S100A2 by TGF-β independent of Smad3 signaling (20). In light of our previous findings, present study represents a unique example of novel interplay between non-canonical regulation of S100A2 by TGF-β that positively influence canonical TGF-β/Smad3 signaling in mediating its protumorigenic actions.

In summary, the findings from the present study have for the first time delineated the mechanism of S100A2 protumorigenic role and its involvement in TGF-β-mediated cancer cell invasion and EMT. Further, we established a protumorigenic role of S100A2 through induction of EMT. Moreover, this study highlights a novel interaction of S100A2 with Smad3, which may have an important implication in the progression of cancer. In our study, PI3-kinase/Akt signaling emerged as a major signaling pathway altered upon S100A2 overexpression. This observation substantiates the emerging role of Akt signaling in mediating EMT and increased survival of cancer cells (44,45). The PI3-kinase pathway has also been implicated as a contributing pathway to TGF-β-induced EMT. Finally, our study has identified S100A2 as a potential therapeutic target for combating EMT induced by activated TGF-β/Smad3 signaling.

Supplementary material

Supplementary Table S1 and Figures S1–S7 can be found at http://carcin.oxfordjournals.org/

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References

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