J Mol Med (2007) 85:753–762
DOI 10.1007/s00109-007-0180-7

ORIGINAL ARTICLE

Involvement of deterioration in S100C/A11-mediated pathway in resistance of human squamous cancer cell lines to TGFβ-induced growth suppression

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Received: 28 June 2006 / Revised: 5 January 2007 / Accepted: 10 February 2007 / Published online: 3 May 2007
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Abstract Recently, we demonstrated that S100C/A11 comprises an essential pathway for growth suppression by TGFβ in normal human keratinocytes. Nuclear transfer of S100C/A11 was a hallmark of the activation of the process. In the present study, we examined the possible deterioration in the pathway in human squamous cancer cell lines, focusing on intracellular localization of S100C/A11 and its functional partners Smad3 and Smad4. All four human squamous cancer cell lines examined (A431, BSCC-93, DJM-1, and HSC-5) were resistant to growth suppression by TGFβ. In BSCC-93, DJM-1, and HSC-5 cells exposed to TGFβ, S100C/A11 was not transferred to the nuclei, and p21(WAF1) was not induced. Overexpression of nucleus-targeted S100C/A11 partially recovered induction of p21 (WAF1) and p15(INK4B) and growth suppression by TGFβ1 in these cells. These results indicate that the deterioration in the S100C/A11-mediated pathway conferred upon the cancer cell lines resistance to TGFβ. In A431 cells, S100C/A11, Smad3, and Smad4 were simultaneously transferred to the nuclei, and p21(WAF1) was induced upon exposure to TGFβ. We provide evidence to indicate that refractoriness of A431 cells to TGFβ was probably because the amount of p21(WAF1) induced by TGFβ was insufficient to counteract cyclin A, which is highly overexpressed in A431 cells. Thus, the newly found S100C/A11-mediated pathway is at least partly involved in conferring upon human squamous cell cancers resistant to TGFβ-induced growth suppression, which is considered to play a critical role for the initiation and progression of many human cancers.

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Keywords S100C/A11 · TGFβ · Signal transduction · Skin · Cancer

Electronic supplementary material The online version of this article (doi:10.1007/s00109-007-0180-7) contains supplementary material, which is available to authorized users.

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Abbreviations

NHK	Normal human keratinocytes
DMEM	Dulbecco's modified Eagle's medium
Ad-p21(WAF1)	Adenovirus vector carrying p21(WAF1)
BCC	Basal cell carcinoma
SCC	Squamous cell carcinoma

Introduction

S100 proteins are characterized by two EF-hand-type Ca^{++}-binding motifs, and 20 family members have so far been identified in mammals [1]. Individual proteins show a cell type-specific and functional state-specific expression among different tissues and are thought to be involved in diverse pathological as well as physiological processes, including neurological diseases, cardiomyopathy, chronic inflammation, and cancer [2, 3]. Some of the S100 family member proteins have been shown to be up- or down-regulated in various human cancers [4, 5] and to play pivotal roles in the regulation of tumor growth and metastasis. For example, increased expression of S100A4 is an indication of poor prognosis in a number of human cancers [6–9], possibly because of enhancement of invasion/metastasis [10, 11] and angiogenesis [12, 13]. Up-regulation of S100A6 was also observed in colorectal cancer [14, 15] and pancreatic cancer [16, 17] and shown to be associated with poor survival in pancreatic cancer [18]. Relevance of S100A2 to human cancer remains controversial. S100A2 is often silenced by site-specific methylation in common human cancers and is thus thought to be a tumor-suppressor gene [19–21]. On the other hand, overexpression of S100A2 was shown to promote tumor-cell migration in culture and to be an indicator of poor prognosis for non-small cell lung cancer [22, 23].

S100C/A11 was first identified as a novel- Ca^{++}-binding protein from chicken gizzard smooth muscle and named calgizzarin [24]. Except for the binding of S100C/A11 to annexin I, its biological function remains largely unknown. In regard to cancer, S100C/A11 has been shown to be overexpressed in some human cancers, including colorectal cancer [25, 26], gastric cancer [27, 28], clear cell sarcoma of soft tissue [29], thyroid carcinoma [30], and uterine smooth muscle tumors [31]. On the other hand, expression of S100C/A11 was found to be reduced in bladder cancer [32] and some prostate cancer tissues [33] compared with that in the surrounding normal counterparts.

As S100C/A11 is encoded in the epidermal differentiation complex on human chromosome 1q21 and high Ca^{++} is a representative growth-suppressing agent for normal human keratinocytes (NHK) in culture, we studied the possible involvement of S100C/A11 in the growth regulation of NHK and found that S100C/A11 is actually a key element in the signal transduction triggered not only by high Ca^{++} but also by TGFβ, another potent inhibitor of epithelial cell growth [34–36]. When NHK cells were exposed to either high Ca^{++} or TGFβ, S100C/A11 was phosphorylated at 10Thr by protein kinase C and transferred to the nuclei through binding to the nucleolin. In the nuclei, S100C/A11 activates Sp1, which in turn induces transcription of p21(WAF1), an inhibitor of cell cycle progression. In addition to this common S100C/A11-mediated signal transduction pathway, unique pathways for individual agents, i.e., calcineurin-NFAT1 for high Ca^{++} and Smads for TGFβ, are also indispensable to the growth inhibition of NHK cells [35, 36]. Through these studies, it has become evident that the intracellular localization of S100C/A11 is more critical than the expression level per se for the growth regulation of NHK cells. In multicellular organisms, every normal cell is subjected to perpetual growth control exerted from systemic requirements, and cancer cells are usually refractory to signals for the growth regulation. TGFβ is a negative growth regulator acting on broad variety of epithelial cells, and loss of sensitivity to TGFβ-induced growth suppression is widely accepted as a critical event for initiation and/or progression of many human cancers [37–39]. In the present study, therefore, we aimed to clarify whether the possible deterioration in the newly found S100C/A11-mediated pathway is mechanistically involved in the resistance against the TGFβ-induced growth suppression of human squamous cell carcinomas (SCCs), particularly focusing on the intracellular localization of S100C/A11 and its functional partners Smad3 and Smad4.

Materials and methods

Cells, tissues, and reagents

Normal neonatal human epidermal keratinocytes (Cascade Biologics, Portland, OR) were cultured in the animal product-free medium EpiLife with the growth supplement HKGS (Cascade Biologics). The human vulvar epidermoid carcinoma cell line A431 and human cutaneous squamous carcinoma cell lines BSCC-93, HSC-5, and DJM-1 were purchased from ATCC. A431 was cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamate and 4.5 g/l glucose. BSCC-93 was cultured in DMEM, and HSC-5 and DJM-1 were cultured in MEM. The media were supplemented with 10% fetal bovine serum. Normal human skin tissues were obtained from volunteers under conditions approved by the institutional ethical review board. A tissue microarray (Skin Cancer Tissue AccuMax TM Array) was purchased from

ISU ABXIS, Seoul, Korea. TGFβ1 was provided by Sigma (St. Louis, MO).

Immunohistochemistry and immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then treated with ethanol. Normal human skin tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin under conventional conditions. The tissue microarray was provided after fixation with formalin. The samples were pretreated with a microwave and incubated with the first antibodies at 37°C for 1 h followed by the application of a second antibody, either Alexa594-conjugated goat antirabbit IgG antibody (Molecular Probes, Eugene, OR) or Alexa488-conjugated goat antimouse IgG antibody (Molecular Probes). The first antibodies used were polyclonal rabbit antihuman S100C/A11 raised in our laboratory [34], polyclonal rabbit antihuman p21 (WAF1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antihuman p15 (INK4B) antibody (Lab Vision, Westinghouse Drive Fremont, CA), polyclonal rabbit antihuman Id1H antibody (Santa Cruz Biotechnology), polyclonal rabbit antihuman Id2H antibody (Santa Cruz Biotechnology), monoclonal mouse antihuman Cdk2 antibody (Santa Cruz Biotechnology), polyclonal rabbit antihuman Cdk4 antibody (Santa Cruz Biotechnology), monoclonal mouse antihuman Cdc2 antibody (Santa Cruz Biotechnology), polyclonal rabbit antihuman cyclin A antibody (Santa Cruz Biotechnology), polyclonal rabbit antihuman cyclin B antibody (Santa Cruz Biotechnology), monoclonal mouse antihuman cyclin D1 antibody (BD Biosciences, San Jose, CA), polyclonal rabbit antihuman cyclin E antibody (Santa Cruz Biotechnology), polyclonal goat antihuman Smad2 antibody (Santa Cruz Biotechnology), polyclonal rabbit antihuman Smad3 antibody (Santa Cruz Biotechnology), monoclonal mouse antihuman Smad4 antibody (Lab Vision), polyclonal rabbit antihuman Sp1 antibody (Santa Cruz Biotechnology), monoclonal mouse anti-green fluorescent protein (GFP) antibody (Clontech Laboratories, Mountain View, CA), monoclonal mouse antihuman β-actin antibody (Sigma), mouse antihuman Keratin 14 antibody (Lab Vision), and monoclonal mouse antihuman Ki67 antibody (DakoCytomation, Kyoto, Japan). Elimination of the first antibodies consistently gave no signals.

Monitoring of DNA synthesis

DNA synthesis was monitored by the incorporation of ^{3}H-thymidine into DNA. NHK and skin cancer cell lines (A431, BSCC-93, DJM-1, and HSC-5) were inoculated at a density of 1×10^{5} cells/ml per well into 24-well plates and cultured overnight at 37°C. ^{3}H-Thymidine (1 μCi/ml; ARC, St. Louis, MO) was added to the cultures 1 h before harvesting the cells, and radioactivity in the trichloroacetic acid-insoluble fraction was measured.

Western blot analysis

Western blot analysis was performed under conditions reported previously [34]. The antibodies used were polyclonal rabbit antihuman p21(WAF1) (Santa Cruz Biotechnology), monoclonal mouse antihuman Cdk2 antibody (Santa Cruz Biotechnology), polyclonal rabbit antihuman cyclin A antibody (Santa Cruz Biotechnology), and monoclonal mouse antihuman β-actin antibody (Sigma). Antibodies against S100C/A11, Smad3, and Smad4 were the same with those used for immunocytochemistry.

In vitro assay of Cdk2 activity

Activity of Cdk2 was assayed in vitro using histone H1 as a substrate under essentially the same conditions describe previously [40]. Briefly, cell lysates were prepared with a buffer containing 0.5% NP-40, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 15% glycerol, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin, 50 mM *Tris*-HCl/pH 7.5. After centrifugation, the supernatants (500 μg protein) were incubated with 1 μg of polyclonal rabbit antihuman Cdk2 antibody (Santa Cruz Biotechnology) for 90 min at 4°C. The immune complex was collected on protein G-Sepharose beads (GE Healthcare Bio-Sciences, Piscataway, NJ) and washed three times with lysis buffer and twice with the kinase buffer (10 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg aprotinin, and 0.5 μg/ml leupeptin, 50 mM *Tris*-HCl/pH 7.5). Kinase reaction was performed in 30 μl of the kinase buffer, 4.5 μg of histone H1 (GIBCO BRL, Rockville, MD), and 10 μCi of [γ-32P] adenosine triphosphate (ICN, Costa Mesa, CA) at 30°C for 30 min. The reaction mixtures were electrophoresed in a 12% sodium dodecyl sulfate polyacrylamide gel, transferred onto a membrane, and subjected to autoradiography and Western blot analysis for Cdk2.

Overexpression of genes by an adenovirus vector

Adenovirus vector carrying p21(WAF1) [Ad-p21(WAF1)] was kindly provided by Dr. M. Kashiwagi (Showa University) [41]. Adenovirus vectors carrying nuclear localization signal (NLS)-GFP or NLS-GFP-S100C/A11 were prepared using BD Adeno-X™ Expression System 2 (BD Biosciences) under the conditions recommended by the manufacturer.

NLS derived from SV40 large T antigen (PKKKRKVEDP) conjugated with GFP was cloned by polymerase chain reaction using pEGFP-N2 (Clontech Laboratories) as a template and integrated into a donor vector together with S100C/A11 cDNA. Cells were infected with the adenoviruses at 10 MOI for 48 h.

Small interfering RNA

Small interfering RNAs (siRNAs) for cyclin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology and Ambion, respectively, and transfected to cells using LipofectAMINE 2000 (Invitrogen) at 20 nM.

Results

In the normal human epidermis, growing cells are confined to the basal layer, and the cells in the suprabasal layers stop growing and progressively undergo differentiation. As shown in Fig. 1a and b, S100C/A11 was found only in the cytoplasm of cells in the basal layer, which is characterized by expression of a specific cytokeratin K14 [42] (Fig. 1a-2) and Ki67, a marker for growing cells (Fig. 1b-2). On the other hand, S100C/A11 protein was partially translocated into the nuclei in the suprabasal layers. This observation accords well with our previously described growth-regulation mechanism, i.e., S100C/A11 inhibits the growth of NHK when transferred into the nuclei [36]. For S100C/A11, we immunostained a tissue microarray containing tissues from human basal cell carcinomas (BCCs) and SCCs (Supplementary Fig. 1 and Table 1). Contrary to the normal epidermis, the staining patterns of cancer tissues were not orderly and thus suggested possible deterioration of the S100C/A11-mediated pathway in some cancer cases.

We analyzed NHK and four representative human SCC lines with respect to sensitivity to TGFβ-induced growth suppression and intracellular localization of S100C/A11, Smad3, and Smad4. When the cells were exposed to TGFβ1 for 24 h, DNA synthesis was reduced to 4.4% of the control in NHK at 1 ng/ml (Table 1), whereas the four cancer cell lines were resistant to the growth suppression at varying degrees, i.e., 67.8–111%. Even at a higher dose of 10 ng/ml, DNA synthesis of the cancer cell lines remained higher than 50%.

To get an insight into mechanisms of TGFβ-resistance, we examined protein levels of representative cell cycle regulators (Fig. 2a). In accordance with the results of a previous study, p21(WAF1) and p15(INK4B) were remarkably induced in NHK by TGFβ1. p21(WAF1) is the principal executor of TGFβ-triggered growth suppression in NHK [35]. Large amounts of p21(WAF1) were detected in untreated A431 and DJM-1 cells, and the amount was further enhanced by TGFβ1 in A431 cells but not in DJM-1 cells. The induced amount of p21(WAF1) in A431 cells

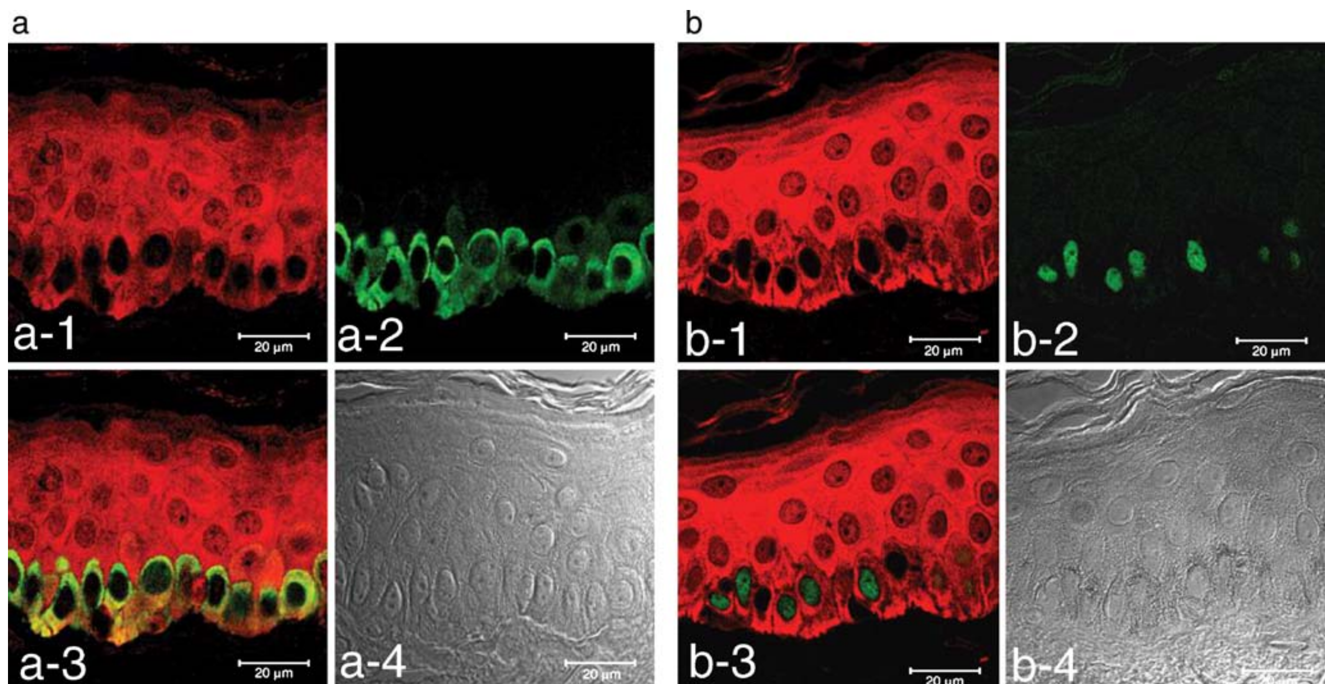

Fig. 1 Immunohistochemistry of normal human skin for S100C/A11 and Ki67. Normal human skin was immunostained for S100C/A11 (*a-1* and *b-1*; in red), cytokeratin K14 (*a-2*; in green), and Ki67 (*b-2*; in green). (*a-3* and *b-3)* Merged picture; (*a-4* and *b-4*) differential interference contrast picture. *Scale bars*, 20 μm

Table 1 Intracellular localization of S100C/A11 and Smads and DNA synthesis in cells exposed to TGFβ

	S100C/A11		Smad3		Smad4		Percent ^{3}H-TdR/TGFβ[a]	
	Cont	TGFβ	Cont	TGFβ	Cont	TGFβ	1 ng/ml	10 ng/ml
NHK	C[b]	N	C	N	C	N	4.4±0.7[c]	3.2±0.9
A431	C	N	C	N	C	N	82.6±6.7	51.9±6.4
BSCC-93	C	C	C	N	C	N	67.8±2.4	60.4±2.0
DJM-1	C	C	C	N	C	C	94.6±4.4	68.8±6.0
HSC-5	C	C	C	N	N	N	111.0±7.4	73.0±12.1

[a] Relative incorporation of ^{3}H-TdR into DNA compared with the untreated cells
[b] Intracellular localization: *C* cytoplasm; *N* nuclei.
[c] Mean + standard deviation

was comparable to that induced in NHK cells, but still, A431 was resistant to the growth inhibition by TGFβ1. In BSCC-93 and HSC-5 cells, the levels of p21(WAF1) protein were low and not induced by TGFβ1. p15(INK4B) was induced in NHK and A431 but not detected in the other cell lines. In NHK, all the other cell cycle regulator proteins examined were markedly down-regulated by TGFβ1 except for Cdk2 (Fig. 2a). The protein levels remained mostly unchanged in the cancer cell lines.

We previously demonstrated that p21(WAF1) is induced by a cooperative action of Sp1 activated by S100C/A11 and Smad proteins in NHK cells [35, 36]. Overexpression of S100C/A11 was observed in the cancer cell lines (Fig. 2b). No difference in the expression of Sp1 and Smad2 were observed among the cell types. Smad3 was also overexpressed, although moderately, in A431 and BSCC-93 cells. Smad4 protein level decreased in A431 and HSC-5 cells compared to that in NHK, and Smad4 protein was marginally detectable in BSCC-93 and DJM-1 cells. Thus, no consistent correlation was observed between the expression levels of S100C/A11, Sp1, Smad2, Smad3, and Smad4 and the inducibility of p21(WAF1).

Next, we examined intracellular localization of S100C/A11, Smad3, and Smad4 proteins before and after treatment with TGFβ1. In NHK, S100C/A11 protein was transferred from the cytoplasm to the nuclei together with Smad3 and

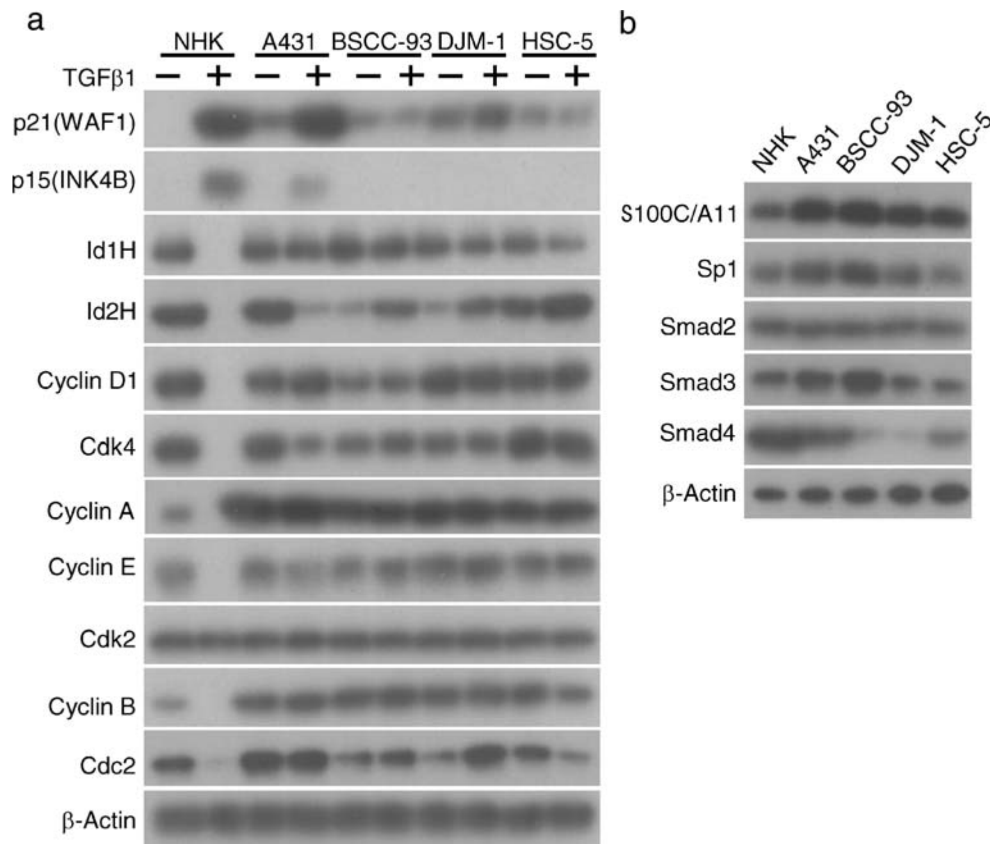

Fig. 2 Induction of cell cycle regulator proteins by TGFβ 1 and protein levels of S100C/A11, Sp1, Smad2, Smad3, and Smad4 in NHK and human squamous cell carcinoma cell lines. **a** Cells were cultivated with or without 1 ng/ml TGFβ1 for 24 h and 30 μg protein of each cell extract was used for Western blot analysis. **b** Western blot analysis of designated cell extracts was performed for S100C/A11, Sp1, Smad2, Smad3, and Smad4. β–Actin was used as a control for applied amounts of protein

Smad4 upon exposure to TGFβ1 (Fig. 3), as expected from the results of our previous study [35, 36]. This translocation mode of the three proteins was observed only in A431 cells. S100C/A11 remained in the cytoplasm in BSCC-93, DJM-1, and HSC-5 cells even in the presence of TGFβ1 (Fig. 3; Table 1), indicating deterioration in the S100C/A11-mediated pathway in these cell lines. Smad4 protein was observed in the cytoplasm and in the nuclei in DJM-1 and HSC-5 cells, respectively, irrespective of the treatment with TGFβ1. Smad3 was translocated into the nuclei in all of the cancer cells upon exposure to TGFβ1. Omission of the first antibodies consistently gave no detectable signals.

To address a question why A431 cells were resistant to TGFβ-induced growth suppression in spite that both S100C/A11- and Smads-mediated pathways appeared intact and p21 (WAF1) protein was induced, we examined amounts of p21(WAF1) and cyclin A proteins, activity of Cdk2, and DNA synthesis in cells exposed to TGFβ1 or infected with Ad-p21(WAF1) (Fig. 4). TGFβ1 inhibited DNA synthesis only in NHK cells (Fig. 4c). Application of Ad-p21(WAF1) resulted in the overexpression of p21(WAF1) to far higher levels than that induced by TGFβ1 (Fig. 4a), leading to the inhibition of DNA synthesis even in the cancer cell lines including A431 (Fig. 4c). Activity of Cdk2 determined in vitro well paralleled with the extent of DNA synthesis (Figs. 4b,c). Cyclin A that is known to compete with p21 (WAF1) in terms of regulating Cdk2 activity [43] was tremendously overexpressed in A431 cells compared with NHK and even with the other cancer cell lines (Fig. 4a). Down-regulation of cyclin A with siRNA (Fig. 5a) clearly sensitized A431 to growth suppression by TGFβ1 (Fig. 5b), although the down-regulation alone resulted in partial suppression of DNA synthesis. In A431, therefore, the p21 (WAF1) protein level induced by TGFβ1 was insufficient to overcome the overexpressed cyclin A, resulting in the resistance to growth suppression by TGFβ1.

If it is true that deterioration of S100C/A11-mediated pathway, i.e., lack of functional S100C/A11 in nucleus, is

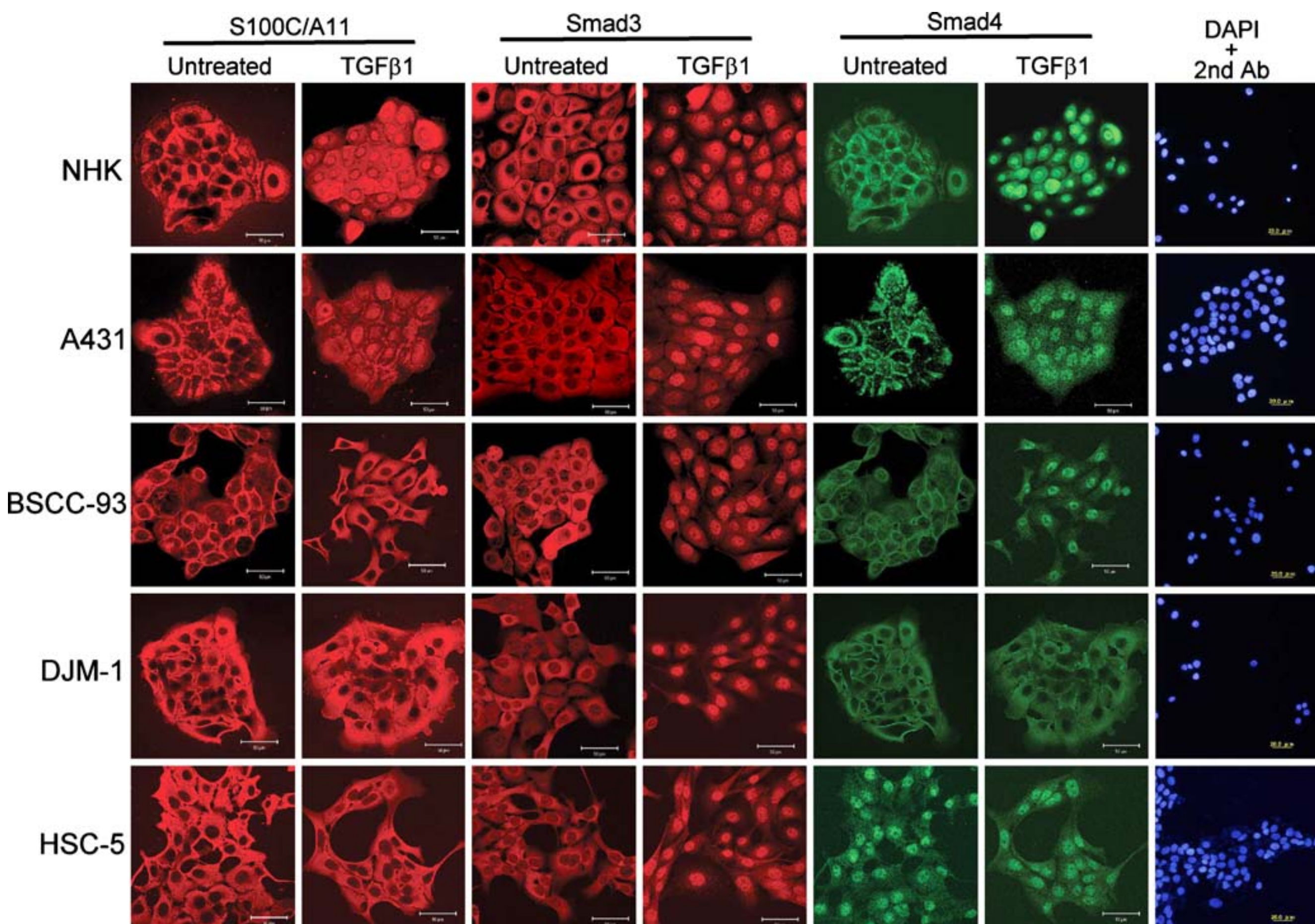

Fig. 3 Intracellular localization of S100C/A11 and Smads in the cells exposed to TGFβ1. NHK and four squamous cell carcinoma cell lines were immunostained for S100C/A11, Smad3, and Smads before and after treating with 1 ng/ml TGFβ1 for 24 h. S100C/A11 and Smad4 were detected by double-staining of the same cell samples. Negative control pictures were shown in which the cells were applied only with a second antibody against rabbit IgG with nuclear staining with DAPI. *Scale bars*, 20 μm

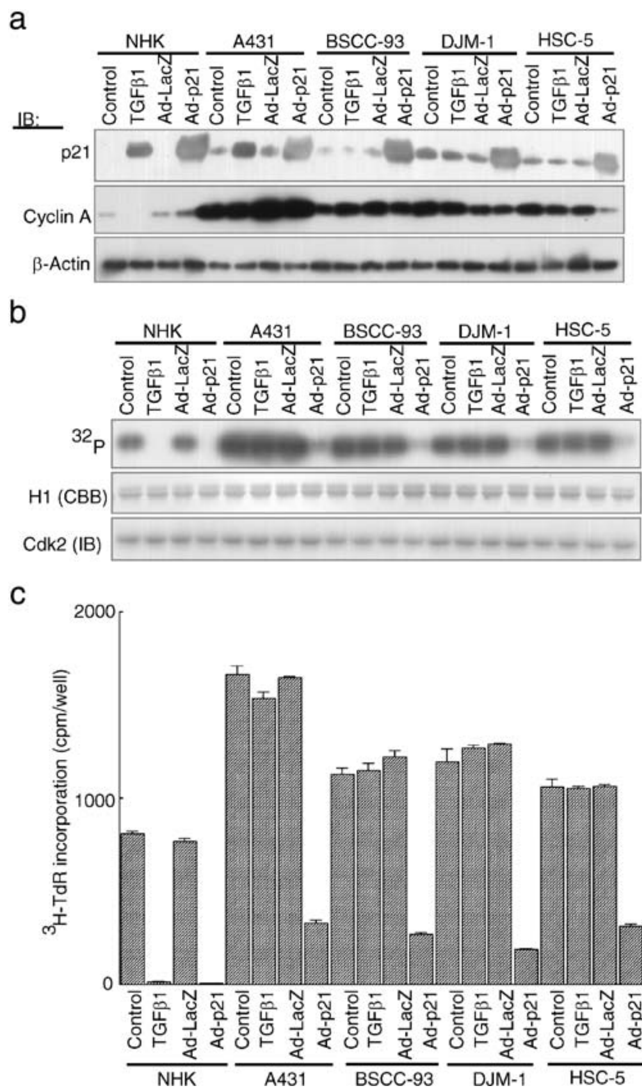

Fig. 4 Effects of TGFβ1 and overexpression of p21(WAF1) by an adenovirus vector. NHK and four squamous cell carcinoma cell lines were exposed to TGFβ 1 (1 ng/ml, 24 h) or infected with Ad-LacZ or Ad- p21(WAF1) (10 MOI, 48 h). **a** Cell extracts were analyzed for p21(WAF1), cyclin A, and β-Actin by Western blotting. **b** Activity of Cdk2 assayed in vitro using Cdk2 protein immunoprecipitated from cell extracts and histone H1 as a substrate. *H1 (CBB)*, histone H1 transferred onto a membrane stained with Coomassie Brilliant Blue; *Cdk2 (IB)*, Western blot analysis for Cdk2. c; Incorporation of ^{3}H-thymidine (1 μCi/ml, 1 h)

a cause of the resistance to TGFβ1-induced growth inhibition in some of the cancer cell lines, forced nuclear localization of S100C/A11 would at least partially recover the sensitivity of the cells to TGFβ1. To examine this notion, we overexpressed NLS-GFP-conjugated S100C/A11 by an adenovirus vector and confirmed nuclear localization of the expressed protein (data not shown). In NHK and A431 cells, protein levels of p21(WAF1) and p15 (INK4B) were enhanced by the infection itself, and the levels further increased upon exposure to TGFβ1 (Fig. 6a). p21(WAF1) and p15(INK4B)were induced by TGFβ1 even in BSCC-93, DJM-1, and HSC-5 cells. In accordance to the induction profile, incorporation of ^{3}H-thymidine was partially suppressed by TGFβ1 (1 ng/ml) in all the cancer cell lines (Fig. 6b).

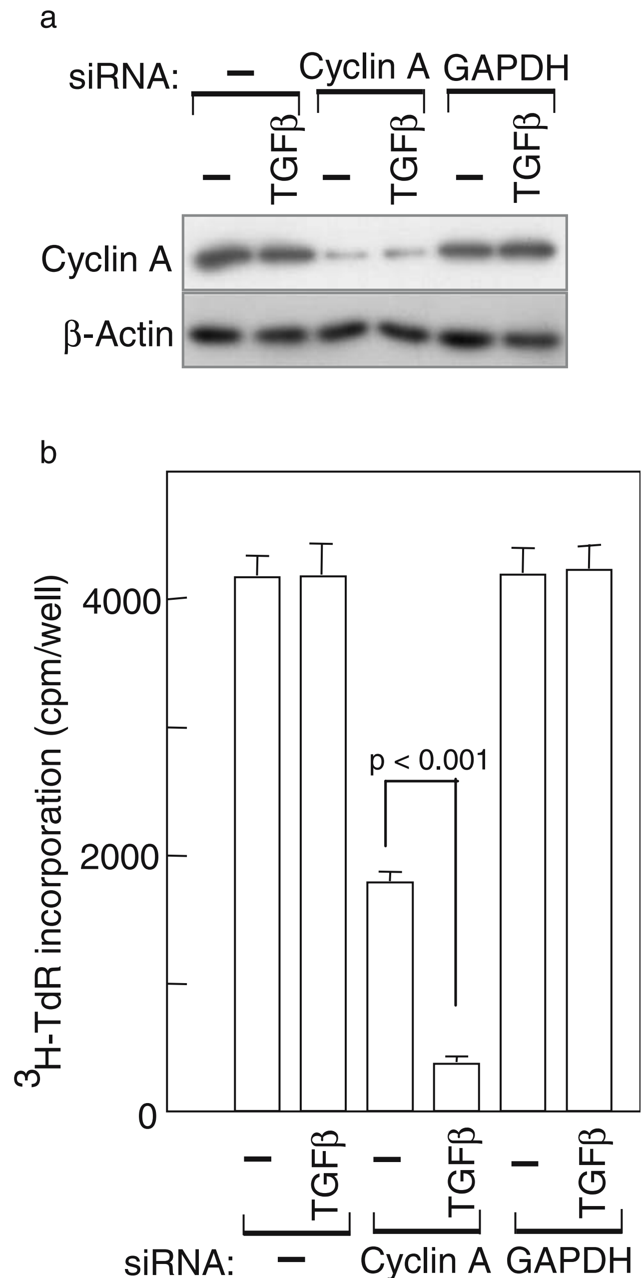

Fig. 5 Sensitization of A431 cells to TGFβ1 by down-regulation of cyclin A using siRNA. Forty-eight hours after transfection with siRNAs (20 nM), the cells were exposed to TGFβ1 (1 ng/ml) for another 24 h and harvested for analysis. **a** Western blot analysis for cyclin A and β-actin. **b** Incorporation of ^{3}H-thymidine (1 μCi/ml) added 1 h before harvest into an insoluble fraction

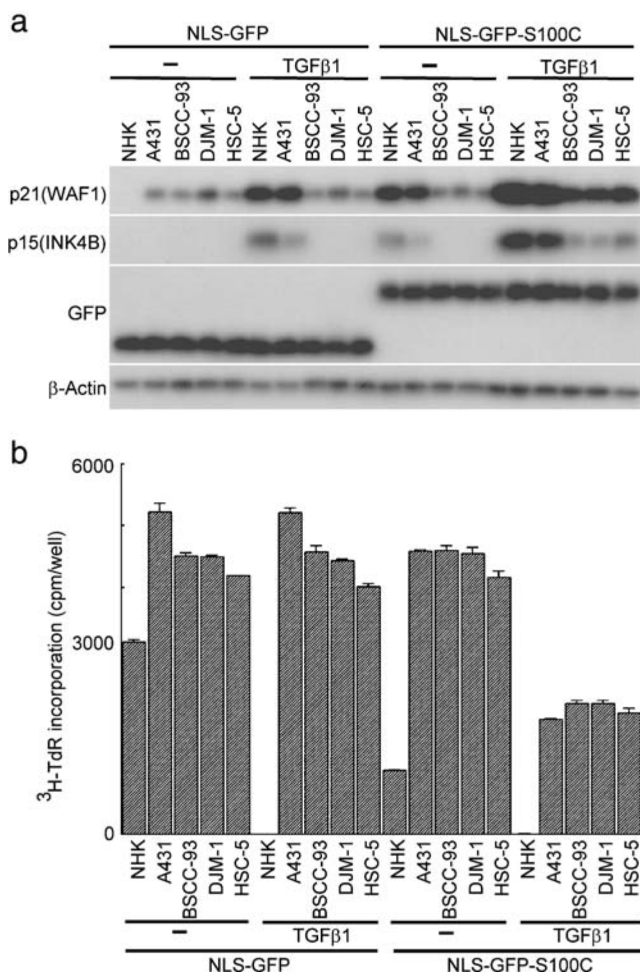

Fig. 6 Effects of overexpression of S100C/A11 conjugated with NLS on the expression of p21(WAF1) and p15(INK4B) and on the cell growth. Twenty-four hours after infection with Ad-NLS-GFP or Ad-NLS-GFP-S100C (10 MOI), the cells were exposed to TGFβ1 (1 ng/ml) for another 24 h and harvested for analysis. **a** Western blot analysis for p21(WAF1) and p15(INK4B). **b** Incorporation of ^{3}H-thymidine (1 μCi/ml) added 1 h before harvest into an insoluble fraction

Discussion

Progress in molecular and cellular biology has been strengthening the notion that functions and biological significance of a given molecule largely differ depending on different biological contexts. At the cell level, "biological contexts" can be regarded as an integrated functional state of entire molecules within a cell, and this can never be defined perfectly. It is important, therefore, to employ a clearly defined cell system as an alternate, if compromised. In this respect, the human epidermis provides a rare opportunity in a sense that one can repeatedly and stably cultivate normal human cells of epithelial origin and use them in early culture. We previously revealed molecular mechanisms of TGFβ-triggered and S100C/A11-mediated growth inhibition in detail using solely NHK cells in early culture [35, 36]. Based on this knowledge, we examined possible alteration in the S100C/A11-mediated growth regulation mechanism in malignant counterparts of NHK in the present study.

TGFβ is a potent growth inhibitor for most epithelial cell types, and inactivation of the intracellular signaling pathway contributes tumorigenesis [37–39]. For example, the TGFβ type II receptor is inactivated by mutation in most human gastrointestinal cancers, and Smad4 in nearly half of pancreatic cancers [44]. TGFβ also inhibits the growth of epidermal keratinocytes not only in culture but also in vivo. Glick et al. [45] reported that the labeling index of TGFβ1-null epidermis was three to five times higher than that of wild-type littermates. TGFβ1-deleted keratinocytes were shown to be more readily transformed into SCC [46], and Smad4-null mice developed spontaneous skin tumors with an incidence of 70% [47]. These results indicate that loss of TGFβ action plays a critical role in the development and progression of skin cancers. In accordance with this, all of the four squamous cancer cell lines examined were resistant to TGFβ1-induced growth suppression (Table 1; Figs. 4c and 6b).

In NHK cells, S100C/A11 is transferred to the nuclei by the help of nucleolin upon exposure to TGFβ1 [35, 36]. Depletion of nucleolin by siRNA resulted in the retention of S100C/A11 in the cytoplasm and concomitant failure in the growth suppression. S100C/A11-free nuclei, therefore, are a hallmark of the growing state at least in keratinocytes. In addition to the S100C/A11-mediated pathway, activated Smad proteins are also essential for the growth inhibition by TGFβ1. Depletion of Smad3 using siRNA abrogated the growth suppression by TGFβ1 in NHK cells [35, 36]. It is known that Smad3 activated by the ligand-bound receptor can move into nuclei after forming homomers or heteromers with Smad4 [48, 49]. In DJM-1 cells, in which Smad3 is transferred to nuclei (Fig. 3; Table 1), p21(WAF1) was not induced probably because of the lack of nuclear translocation of S100C/A11 and Smad4, which is essential for efficient transcriptional induction [35, 36, 50]. S100C/A11, Smad3, and Smad4 were translocated into the nuclei in NHK cells upon exposure to TGFβ1 (Fig. 3), resulting in the induction of p21(WAF1) and the cessation of growth. In BSCC-93, DJM-1, and HSC-5 cells exposed to TGFβ1, S100C/A11 was not transferred to nuclei, and p21(WAF1) was not induced (Figs. 2a, 3, and 4a). Overexpression of nucleus-targeted S100C/A11 partially recovered the induction of p21(WAF1) and p15(INK4B) and the growth suppression by TGFβ1 in these cells (Fig. 6). These results indicate that deterioration in the S100C/A11-mediated pathway conferred upon the cancer cell lines resistance to TGFβ-induced growth suppression. In A431 cells, S100C/A11, Smad3, and Smad4 were simultaneously transferred to the nuclei, and p21(WAF1) was induced upon exposure to TGFβ1 (Figs. 2a, 3, and 4a). Cyclin A was expressed at a

very high level in the cells (Fig. 4a). As cyclin A is known to activate Cdk2, competing with p21(WAF1)[37], excess amount of p21(WAF1) induced by Ad-p21(WAF1) inhibited DNA synthesis even in A431 cells (Fig. 4c), and down-regulation of cyclin A with siRNA resulted in the sensitization of A431 cells to TGFβ1-induced growth suppression (Fig. 5b), refractoriness of A431 cells to TGFβ1 was probably because the amount of p21(WAF1) induced by TGFβ1 was insufficient to counteract the overexpressed cyclin A.

In conclusion, the S100C/A11-mediated pathway newly found by us is at least partly involved in conferring upon human cancer cells resistant to TGFβ-induced growth suppression, which is considered to play a critical role for the initiation and progression of many human cancers.

Acknowledgments The authors sincerely thank Dr. Mariko Kashiwagi (Showa University) for providing an adenovirus vector expressing p21 (WAF1). This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to N. H., 14370260 and 17014065).

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