Gynecologic Oncology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Invasion of uterine cervical squamous cell carcinoma cells is facilitated by locoregional interaction with cancer-associated fibroblasts *via* activating transforming growth factor-beta

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HIGHLIGHTS

- TGF-β pathway is distinguished as a driver initiating invasion of cervical squamous cell carcinoma.
- TGF-β is activated through interaction of squamous cell carcinoma cells and cancer associated fibroblasts at tumor invasive front.

ARTICLE INFO

Article history:
Received 26 August 2014
Accepted 21 November 2014
Available online xxxx

Keywords:
TGF-beta
Invasion
Cancer associated fibroblasts
Cervical cancer

ABSTRACT

Objective. Local invasion is a common pattern of spread in uterine cervical squamous cell carcinoma (CSCC). Although transforming growth factor-beta (TGF-β) facilitates invasion of various types of cancer cells, the role of the TGF-β pathway in CSCC is unclear. In this study, we analyzed the role of TGF-β signaling in the progression of CSCC.

Methods. Immunohistochemistry was used to examine the expression of TGF-β pathway molecules in 67 CSCC samples with clinicopathological data. Activation of the TGF-β pathway was investigated following co-culture of CSCC cells and cervical cancer-associated fibroblasts (CCAFs).

Results. Clinicopathological analysis of CSCC samples revealed that prominent expression of TGF-β receptor-2 was more frequent in CSCC with lymphovascular space invasion (LVSI) than without LVSI ($p < 0.01$). Lymph node metastasis was more frequent in cases in which phosphorylated SMAD3 (pSMAD3) was localized exclusively at the boundary of tumor clusters ($n = 9$, $p < 0.05$). Recombinant TGF-β1 increased pSMAD3 expression and enhanced cellular invasion ($p < 0.005$) in CSCC cells, which was attenuated by an inhibitor of the TGF-β receptor ($p < 0.005$). Enhanced pSMAD3 expression and invasion was also observed when conditioned media from CSCC cells co-cultured with CCAFs were administered. Luciferase assays showed that this medium contained a large amount of active TGF-β. Along with TGF-β activation, thrombospondin-1 was upregulated in both CSCC cells and CCAFs, while thrombospondin-1 silencing in either CSCC cells or CCAFs repressed the activity of TGF-β. Thrombospondin-1 was prominently expressed in cases with pSMAD3 boundary staining ($p < 0.05$).

Conclusions. These results suggest that interaction between CSCC cells and surrounding CCAFs activates TGF-β *via* thrombospondin-1 secretion to facilitate CSCC invasion.

Introduction

Despite widespread vaccination against human papilloma virus and periodic cancer screening, cervical cancer remains one of the highest ranking diseases causing mortality in women, and new strategies to treat this disease are urgently needed. Squamous cell carcinoma, the most common histological subtype of cervical cancer, spreads

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http://dx.doi.org/10.1016/j.ygyno.2014.11.075
0090-8258/

principally by migrating into the lymphatics or by invading adjacent soft tissues. Eradication of locoregional lesions is critical but is not attainable in cases with tumor extension into the urinary tract and/or rectum for fear of impairing urinary or bowel function. Even if the extension is more limited, it is also difficult to obtain cancer-free-margins, leading to pelvic recurrence from residual cancer cells. Obstruction of the ureter(s) is not trivial and is observed in 55.8% of advanced cases [1]. It is, therefore, important to develop effective treatments for invasive extension of this disease, and to this end, it is essential to further elucidate the mechanisms of cervical cancer invasion.

Transforming growth factor-beta (TGF-β) is currently known to promote cancer invasion [2]. TGF-β serves as a potent growth inhibitor for normal epithelial cells [3], but malignant transformed cells acquire resistance to the growth inhibitory effect of TGF-β [4]. Moreover, as breast cancer advances, TGF-β acts as a promoter by inducing epithelial to mesenchymal transition (EMT), invasion, and metastasis [5,6]. In gynecologic cancers, activated TGF-β signaling promotes peritoneal dissemination of ovarian cancer [7]. Thus, TGF-β is widely recognized as a key molecule that drives cancer cell progression, but little is known about the role of TGF-β in uterine cervical cancer.

Growing evidence has shown that the stroma around cancer cells plays an important role in cancer progression. Cancer-associated fibroblasts (CAFs) exhibit morphological phenotypes of myofibroblasts and are known to promote cancer progression through interactions with adjacent cancer cells. TGF-β is a key cytokine mediating such interactions [8,9]. However, we presently lack clarification regarding the role of TGF-β in these interactions in cervical squamous cell carcinoma (CSCC).

In this study, we analyze the role of TGF-β signaling in the progression of CSCC. We show that activation of TGF-β induced by the interaction between CSCC cells and CAFs plays a key role in the initiation of tumor metastasis.

Materials and methods

Tissue samples and immunohistochemistry

Tissue samples and clinicopathological information were collected from sixty-seven patients (median age, 54 +/− 23 years) with stage IB–IIB CSCC who underwent radical hysterectomy or trachelectomy in Kyoto University Hospital from January 2003 to July 2010 with written consent under the approval of the ethics committee. Patient characteristics are described in Table 1.

Table 1
Clinicopathological analyses of 67 CSCC patients.

		n	5-year PFS	*p*
Age	<=50 yo	28 (42%)	85.302	—
	>50 yo	39 (58%)	85.322	0.8001
Stage	IB1	38 (57%)	88.610	—
	IB2	6 (9%)	100	0.4226
	IIA1	5 (7%)	100	0.4901
	IIA2	2 (3%)	50.000	0.0145
	IIB	16 (24%)	72.222	0.1667
LVSI	−	22 (33%)	100	—
	+	45 (67%)	78.714	0.0361
Stromal invasion	<1/2-	18 (27%)	100	—
	≥1/2	49 (73%)	79.564	0.4245
Size	<4 cm	55 (81%)	87.893	
	≥4 cm	12 (19%)	74.074	0.0967
Parametrium invasion	−	15 (22%)	87.569	—
	+	52 (78%)	77.037	0.4245
Vaginal margin	>1 cm	62 (93%)	83.826	—
	≤1 cm	5 (7%)	100	0.3502
LNM	−	45 (67%)	92.371	—
	+	22 (33%)	71.753	0.0252

Immunohistochemical staining was performed for six molecules: TGF-β, TGF-β receptor type 1 (TGFBR1), TGF-β receptor type 2 (TGFBR2), pSMAD3, α-smooth muscle actin (α-SMA) and thrombospondin-1 (TSP-1), using the streptavidin–biotin peroxidase complex method as previously reported [7]. The staining methodology is described for each molecule in Supplementary Table 1. Immunostaining was evaluated for intensity and distribution by semi-quantitative scoring as previously described [10] while blinded to clinical data: intensity: 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong); distribution: 0 (no staining), 1 (1–25%), 2 (26–50%) and 3 (51–100%). Staining of TGF-β, TGFBR1, TGFBR2, and TSP-1 was designated as high if cumulative scores were 4 or greater.

Cell lines and culture

Human CSCC cell lines, CaSki, ME-180, QG-U and SKG-IIIa, were obtained from the Riken BioResource Center (Tsukuba, Japan). Primary cervical cancer-associated fibroblasts (pri-CCAFs) were obtained by isolating and mincing the stromal tissue from surgically-removed CSCC with collagenase as previously described [11]. Immortalized cervical cancer-associated fibroblasts (im-CCAFs) and pri-CCAFs were maintained in DMEM (Nacalai Tesque) supplemented with 10% heat-inactivated fetal bovine serum (FBS, v/v; Biowest, France) and penicillin–streptomycin (100 IU/ml penicillin, 100 μg/ml streptomycin; Nacalai Tesque). CSCCs were pretreated with media containing A83-01 (0, 1, 2, or 10 μM, Tocris, Bristol, UK) for 30 min, and were subsequently treated with recombinant TGF-β1 (0, 1, 2, or 10 ng/ml, Peprotech, Rocky Hill, NJ). Protein was extracted from cultured cells using Pierce RIPA Buffer (Thermo, Rockford, IL) supplemented with Protease Inhibitor Cocktail (Nacalai Tesque) and Phosphatase Inhibitor Cocktail (Nacalai Tesque) for Western blotting. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and used for quantitative RT-PCR and microarray analysis.

Stealth RNAi™ siRNA against *TSP-1* (Invitrogen, Carlsbad, CA) or Stealth™ RNAi Negative Control Medium GC Duplex (Invitrogen, Carlsbad, CA) was used for RNA interference or non-targeting assays. Cells were transfected with siRNAs by using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. siRNA sequences were as follows: siTSP-1,1: CCA GAU CAG GCA GAC ACA GAC AAC A/U GUU GUC UGU GUC UGC CUG AUC UGG, siTSP-1,2: CCA CAG GCC AAA GAC GGG UUU CAU U/A AUG AAA CCC GUC UUU GGC CUG UGG, and siTSP-1,3: UGG CAU CCC UGA GGC AGA UGA AGA A/U UCU UCA UCU GCC UCA GGG AUG CCA.

Cellular functional assays

CSCC cells were treated with/without A83-01 (2 μM) +/− recombinant TGF-β1 (2 ng/ml). Viable cell numbers were determined using a colorimetric assay, and population doubling times were calculated after treatment with TGF-β1. Cellular migration and invasion was assessed by wound healing assays and Boyden-chamber assays as described below.

Wound healing assays

When CSCC cells were 90–100% confluent, "wounds" were introduced by dragging a sterile 1000 μl plastic pipette tip across the cell monolayer. The widths of the scratch lines were measured at five independent locations for each specimen after the treatment. The mean distance filled between the cells most closely spaced on each leading edge of the wound was calculated after 24 h, and the ratio of the distance filled by treated *versus* untreated cells was designated as the migration value.

Invasion assays

After pretreatment with TGF-β1, CSCC cells were collected and suspended in serum-free medium for Boyden-chamber assays. The

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lower chamber was filled with 0.8 ml medium with 10% FBS as a chemoattractant, and in the upper compartment, 8×10^4 cells in 0.2 ml medium were seeded and incubated at 37 °C for 18 h to allow for migration to the lower chamber. Cells that penetrated the membrane coated with BD Matrigel matrix (Becton Dickinson, Franklin Lakes, NJ) were visually counted under light microscopy, and the mean from the sums of five high power fields (×200) was designated as the measure of invasiveness.

Animal studies

Female BALB/cAJcl-nu, nu/nu mice were purchased from Nihon Clea (Kyoto, Japan). Animal care and experimental procedures under pathogen-free conditions were performed in accordance with the guidelines of the Institute of Laboratory Animals Graduate School of Medicine, Kyoto University. Subcutaneous xenografts were established in the flanks by inoculating 1×10^8 CaSki cells carrying the Lenti-FireTM construct. Lentiviral transduction of target cells was performed with firefly luciferase Luc2 (In Vivo Imaging Solutions, Cheyenne, WY). Thirty-three days later, subcutaneous tumors were harvested, cut into 3 mm pieces, and transplanted into the uterus of 28 independent anesthetized female nude mice to enable development of uterine tumors. Mice with tumor transplants were injected intra-abdominally with A83-01 (150 μg/body, n = 14) or DMSO control (n = 14) three times a week for 58 days. Growth of uterine tumors was tracked once a week using the Xenogen-IVIS Spectrum instrument (PerkinElmer, Waltham, MA) following intra-abdominal injection of D-luciferin (1 mg, Wako, Osaka, Japan). On day 59, remaining mice were euthanatized and lymph nodes (LNs) were sampled and processed using QIAshredder (Qiagen, Hilgen, Germany) to assess LN metastasis with human Luc2 expression by RT-PCR. The primer sequences used for Luc2 detection were: 5′-CAG CTG CAC AAA GCC ATG AA-3′ and 5′-AAG TCG TAC TCG TTG AAG CC-3′.

Co-culture of CSCC cells and CCAFs and TGF-β in conditioned media (CM)

CSCC cells and CCAFs were plated separately in mono-culture (1×10^4 cells/cm^2) or in a 1:1 mixture co-culture (5×10^3 cells/cm^2, respectively), and the supernatant of cultured media was reserved as "conditioned media" (CM) for further analysis. Total TGF-β1 in CM was quantified using the Quantikine ELISA Human TGF-β1 kit (B&D, Minneapolis, MN). For quantification of the activated form of TGF-β in the CM, plasminogen activator inhibitor-1 (PAI-1) promoter/luciferase-transfected mink lung epithelial cells (TMLECs) were used as previously reported [12]. The luminescence of TMLECs was evaluated as the amount of activated TGF-β in the CM using the Luciferase Assay System (Promega, Madison, WI).

Statistical analysis

In immunohistochemical analyses, Fisher's exact test was used to test for significant between-group differences. In all other analyses, a two-tailed Student's *t*-test was used to test for significance. All statistical analyses were done using GraphPad Prism 5.5 and R software. A *p*-value < 0.05 was considered statistically significant.

A complete description of the materials and methods, and any associated references are available in Additional supporting information.

Results

Clinicopathological analysis of CSCC in association with the TGF-β pathway

A total of 67 patients were treated for locally advanced CSCC (IB1; n = 38, IB2; n = 6, IIA1; n = 5, IIA2; n = 2, and IIB; n = 16). The 67 cases included 22 with lymph node metastasis and two with distant metastasis. Patients with lymphovascular space invasion (LVSI) or lymph node metastasis (LNM) exhibited worse clinical outcomes than those without these characteristics ($p < 0.05$ for each, Table 1). Immunohistochemical staining was conducted for TGF-β, TGFBR1, TGFBR2, pSMAD3, α-SMA, and TSP1. TGF-β was expressed in both tumor cells and stromal areas (Fig. 1A), while TGFBR1 and TGFBR2 were expressed mainly in tumor cells (Fig. 1B–C). The cases with high TGFBR2 expression showed more frequent LVSI than those with low expression ($p < 0.01$, Table 2). Immunostaining of pSMAD3 was more definitive. Among 67 cases, pSMAD3 expression was undetectable in 34 cases and weakly expressed in another 24 cases, while pSMAD3 showed dense staining exclusively in the boundary areas of tumor nests adjacent to stroma in the other nine cases (Fig. 1D). These nine cases with pSMAD3 boundary staining more frequently showed lymph node metastasis (LNM) as compared to the remaining 58 cases ($p < 0.05$, Table 2). Immunostaining of TSP-1 was found in both tumor and stromal cells (Fig. 1E). In the pSMAD3 boundary staining cases with high expression of TSP-1 was observed in 8 of 9 cases, which was more frequent than the other cases ($p < 0.05$, Table 2). α-SMA was enhanced in stromal areas adjacent to tumor nests (Fig. 1F), indicating that cancer stromal cells had the phenotype of myofibroblasts.

The impact of TGF-β signaling on cellular characters of CSCC cells

Western blotting revealed that pSMAD3 expression in CaSki, ME-180, and QG-U cells was induced by recombinant TGF-β1 even at 1 ng/ml, and that pretreatment with A83-01 tempered the induction of pSMAD3 (Fig. 2A). SKG-IIIa cells did not respond to treatment with recombinant TGF-β1 (data not shown).

Proliferative activity was not altered in CaSki cells by TGF-β signal induction with 2 ng/ml recombinant TGF-β1, or by TGF-β signal inhibition with A83-01 (Fig. 2B). Proliferative activity was also not altered in ME-180 cells following treatment with recombinant TGF-β1 or A83-01 (Supplementary Fig. 1A).

Next, *in vivo* tumor growth assays were conducted to investigate whether inhibition of TGF-β pathway signaling could suppress tumor growth or metastasis. After Luc2-transfected CaSki tumor blocks had been transplanted into each uterus of 29 nude mice, the mice were divided into two groups and treated weekly with A83-01 or control solution. Tumor luminescence in each mouse was tracked weekly for nine weeks. There was no significant difference in the luminescent signal, indicating uterine tumors grew with or without A83-01 treatment (Fig. 2C). Six mice treated with A83-01 and one mouse in the control group died prior to day 59, the planned experimental endpoint. Among the euthanized mice, *Luc2* expression in abdominal lymph nodes was confirmed for two in the A83-01 treatment group and eight in the control group. Death or node metastasis therefore occurred in a total of 8 mice with and 9 mice without A83-01 treatment (data not shown).

On the other hand, cellular migration and invasion were significantly increased following addition of recombinant TGF-β1 in CaSki cells (Fig. 2D–E, $p < 0.005$), but these effects of TGF-β were significantly attenuated by pretreatment with A83-01 (Fig. 2D–E, $p < 0.05$). In QG-U cells that are also highly responsive to TGF-β (Fig. 2A), migration was augmented by TGF-β but attenuated by A83-01 as well (Supplementary Fig. 1B, $p < 0.005$, <0.00005, respectively).

A gene expression microarray analysis for CaSki cells treated with recombinant TGF-β1 revealed that 211 probes (175 genes) and 160 probes (152 genes) were up- or down-regulated, respectively, as compared to mock treated controls ($p < 0.001$, Supplementary Table 2). The gene ontology term "cell migration" was enriched among the up-regulated genes (Supplementary Table 3, $p = 0.001$). Gene set enrichment analysis (GSEA) also showed that "Anastassiou Cancer Mesenchymal Transition Signature" was up-regulated following treatment with recombinant TGF-β1 (Fig. 2F, FDRq = 0.006).

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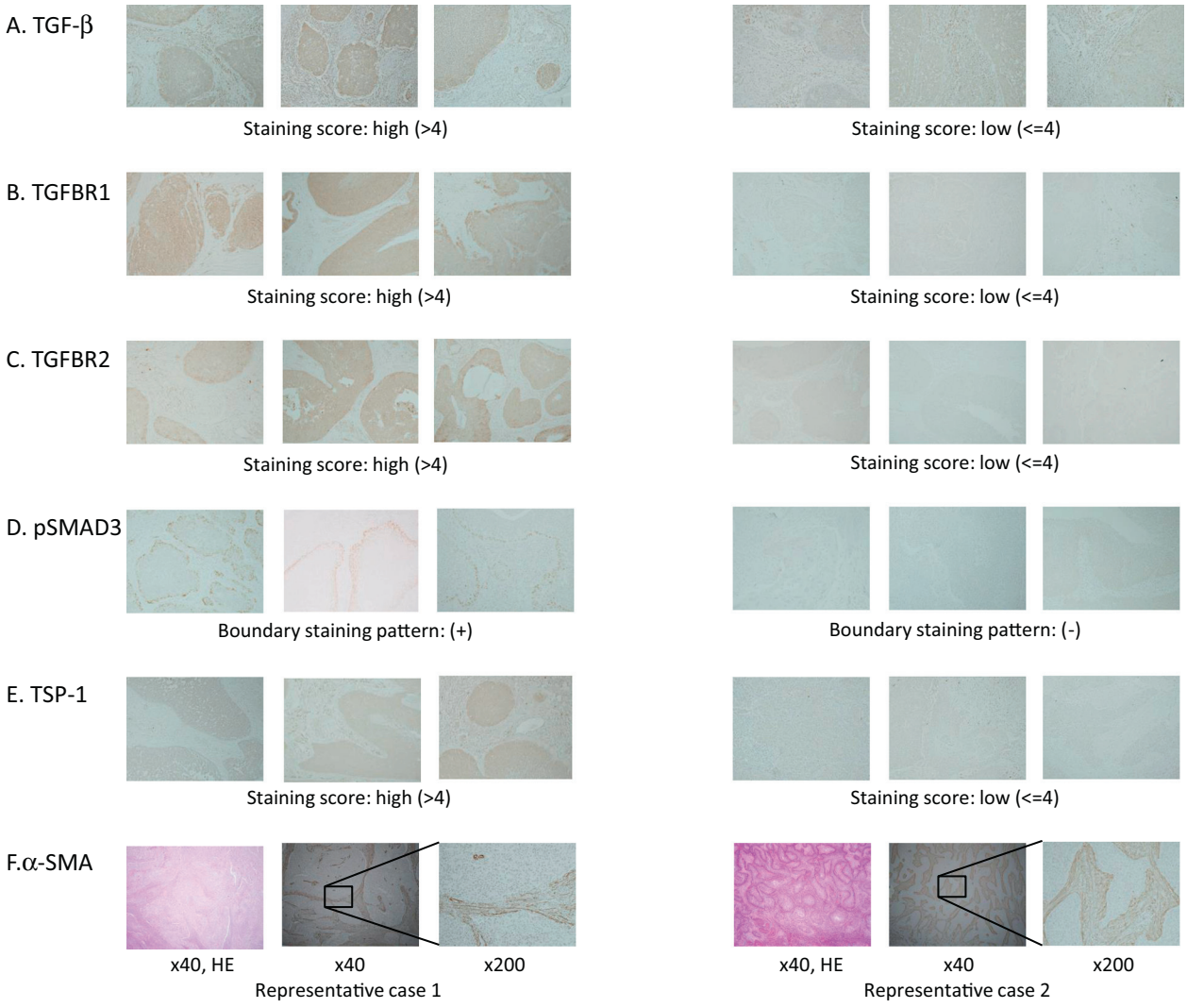

Fig. 1. Immunohistochemistry for TGF-β pathway related molecules. (A, B, C and E) Three representative cases with a high staining score (>4) and with a low staining score (<=4) are shown stained with antibodies against TGF-β, TGFBR1, TGFBR2 and TSP-1 respectively. (D) Three representative cases with and without a boundary staining pattern are shown following immunostaining using an anti-pSMAD3 antibody. All images are presented with a magnification power of ×200. (F) Two representative cases are shown after hematoxylin and eosin (HE) staining at ×40 magnification, and with α-SMA immunostaining at ×40 and ×200 magnification.

Interaction between CSCC cells and cervical cancer-associated fibroblasts (CCAFs)

As shown in Fig. 1F, α-SMA was expressed in stromal areas adjacent to tumor nests, indicating the existence of CCAFs with myofibroblast characteristics. Western blotting showed that α-SMA was expressed in both immortalized cervical cancer-associated fibroblasts (im-CCAFs) and primary cervical cancer-associated fibroblasts (pri-CCAFs), but not in CaSki cells (Fig. 3A).

To investigate whether interactions between CSCC cells and CCAFs influenced the characteristics of CSCC cells, CSCC cells and CCAFs were co-cultured in a 1:1 mixture (henceforth, "CSCC/CCAF") and we

Table 2
Immunohistochemical analyses of 67 CSCC patients.

		LVSI		LNM		TSP-1	
		+	−	+	−	High	Low
TGF-β	High	38 (57%)	17 (25%)	21 (31%)	34 (51%)	29 (43%)	26 (39%)
	Low	7 (10%)	5 (7%)	1 (1%)	11 (16%)	6 (9%)	6 (9%)
	p	0.5095		0.086		1	
TGFBR1	High	23 (34%)	4 (6%)	10 (15%)	27 (40%)	21 (31%)	16 (24%)
	Low	22 (33%)	8 (12%)	12 (18%)	18 (27%)	14 (21%)	16 (24%)
	p	0.4347		0.3029		0.4667	
TGFBR2	High	28 (42%)	6 (9%)	13 (19%)	21 (31%)	20 (30%)	14 (21%)
	Low	17 (25%)	16 (24%)	9 (13%)	24 (36%)	15 (22%)	18 (27%)
	p	0.0096		0.4372		0.3319	
pSMAD3	Boundary (+)	7 (10%)	2 (3%)	6 (9%)	3 (4%)	8 (12%)	1 (1%)
	Boundary (−)	38 (57%)	20 (30%)	16 (24%)	42 (63%)	27 (40%)	31 (46%)
	p	0.7066		0.0498		0.0285	

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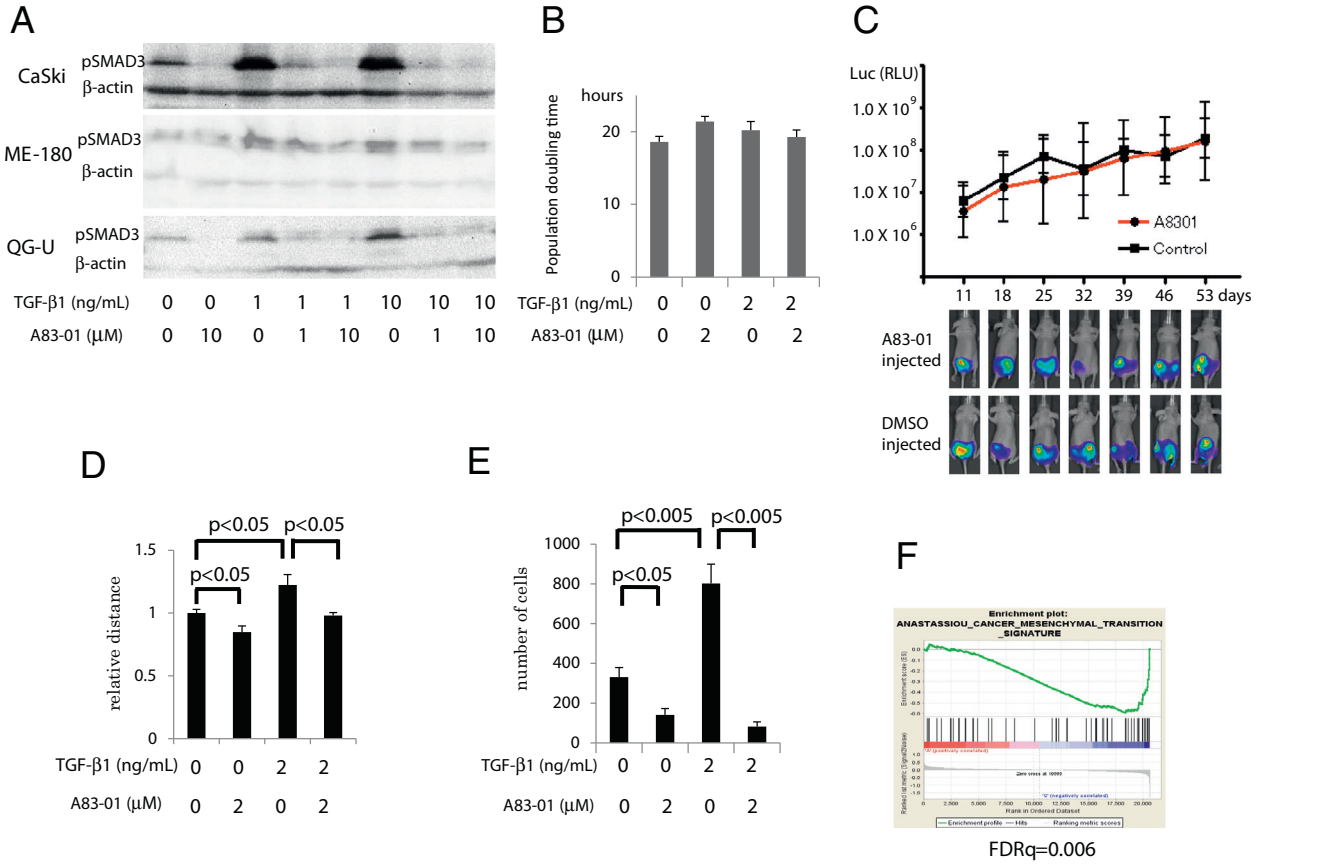

Fig. 2. The effects of TGF-β on CSCC cell behavior. (A) Western blotting revealed that recombinant TGF-β1 (1 or 10 ng/ml) induced pSMAD3 expression in CaSki, ME-180 and QG-U cells, and that A83-01 (1 or 10 μM) reduced this effect. (B) Population doubling time of CaSki cells was not significantly affected by recombinant TGF-β1 and/or A83-01 (n = 8 each). (C) Luciferase activity of uterine tumors derived from luciferase-expressing CaSki cells transplanted into nude mice was not significantly affected by trans-abdominal injection of A83-01. Lucs; relative light units of luminescence of tumor burdens. (D) Recombinant TGF-β1 promoted migration of CaSki cells, which was reduced by A83-01 (n = 5 each). Y-axis; relative migration distance measured in the scratch assay compared with non-treated cells. (E) Recombinant TGF-β1 promoted the invasiveness of CaSki cells, which was reduced by A83-01 (n = 6 each). Y axis; number of CaSki cells crossing the membrane in invasion assays. (F) Gene Set Enrichment Analysis. "Anastassiou Cancer Mesenchymal Transition Signature" was significantly up-regulated following treatment with recombinant TGF-β1 in CaSki cells (FDRq = 0.006).

collected the resultant CM. Both the CM of im-CCAFs and pri-CCAFs contained larger amounts of total TGF-β1 than did the CM of CaSki cells (Fig. 3B, $p < 0.0005$, $p < 0.0001$ respectively), and total TGF-β1 was higher in CM of im-CCAFs and pri-CCAFs than CaSki/im-CCAF and CaSki/pri-CCAF (Fig. 3B, $p < 0.00005$, $p < 0.00001$, respectively). Nevertheless, the induction of pSMAD3 expression in CaSki cells was observed more prominently with the CM of CaSki/im-CCAF or CaSki/pri-CCAF than CCAFs only or CaSki cells only (Fig. 3C). This induction was attenuated with A83-01 treatment (Fig. 3D). Cellular invasion was enhanced in CaSki cells treated with the CM of CaSki/im-CCAF as compared to treatment with CM from CCAFs only or CaSki cells only (Fig. 3E, $p < 0.05$ for both comparisons).

TGF-β has a latent form and an activated form. To determine whether the activated form of TGF-β is more abundant in CM of CSCC/CCAF than CSCC cells or CCAFs, we used plasminogen activator inhibitor-1 promoter/luciferase-transfected mink lung epithelial cells (TMLECs), which enabled us to determine the concentration of active TGF-β based on the linear positive correlation with luminescence from TMLECs (Fig. 3F). Significantly higher luminescence, indicating a larger amount of activated TGF-β, was observed in TMLECs treated with the CM of CaSki /imCCAF compared with CaSki cells or im-CCAF (Fig. 3F, $p < 0.00005$, $p < 0.005$, respectively). These results were reproduced in ME-180 cells (Fig. 3F, $p < 0.005$, $p < 0.05$, respectively).

Molecular mechanism underlying the interaction between CSCC cells and CCAFs

To determine which molecule activated TGF-β during the interaction between CSCC cells and CCAFs, gene expression in the mono- and co-cultured CSCC cells and im-CCAFs was examined by quantitative RT-PCR for thrombospondin-1 (TSP-1, Fig. 4A), integrin α_V and neuropilin-1 (Supplementary Fig. 2A). Of these three genes, *TSP-1* showed increased expression in CaSki/im-CCAF relative to CaSki and im-CCAF (Fig. 4A, $p < 0.0001$, $p < 0.001$, respectively). This up-regulation was most striking on the fourth day of incubation (Supplementary Fig. 2B), and was confirmed on the fourth day at the protein level as well (Fig. 4A). These results were reproduced in ME-180 cells (Fig. 4A).

siRNA-mediated knockdown of TSP-1 expression in either CaSki cells or im-CCAFs was conducted to investigate whether TSP-1 activates the latent form of TGF-β. TSP-1 expression was most prominently reduced by siRNA, "siTSP-1,3" among the three siRNAs tested (Supplementary Fig. 2C). Thus siTSP-1.3 was used for subsequent experiments. Total expression of *TSP-1* in CaSki/im-CCAF was markedly repressed, even with *TSP-1* knockdown in CaSki cells (Fig. 4B, $p < 0.0005$) or im-CCAFs (Fig. 4C, $p = 0.0005$). TMLEC assays consistently showed that the amount of active TGF-β1 in the CM of CaSki/im-CCAF was markedly reduced with *TSP-1* knockdown in either CaSki cells (Fig. 4B, $p < 0.05$) or im-CCAFs (Fig. 4C, $p < 0.001$).

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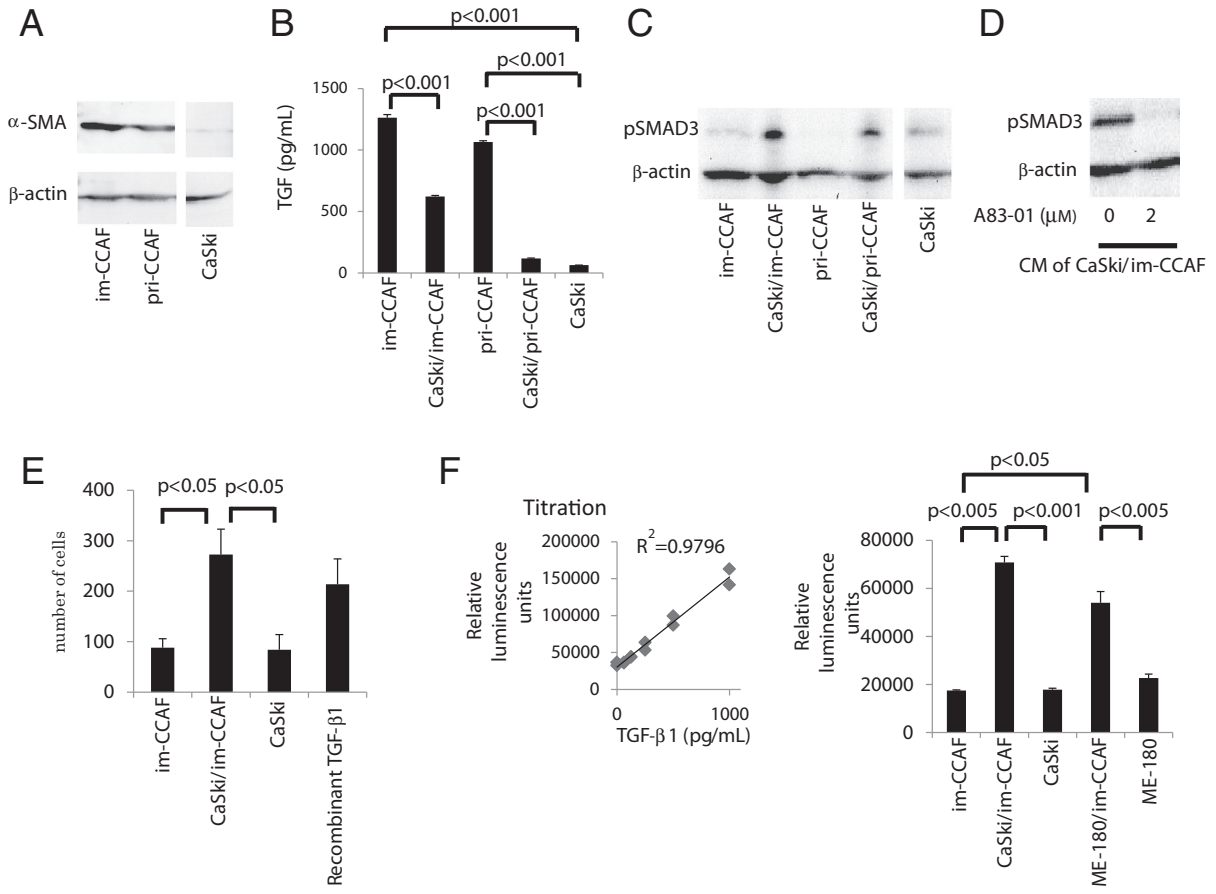

Fig. 3. Interaction between CSCC cells and CCAFs. (A) Western blotting showed α-SMA expression in im-CCAFs and pri-CCAFs, but not in CaSki cells. (B) ELISA revealed that both the CM of im-CCAFs and pri-CCAFs contained higher total TGF-β1 than the CM of CaSki cells, and that total TGF-β1 was higher in the CM of im-CCAFs and pri-CCAFs than in CaSki/im-CCAF and CaSki/pri-CCAF. "/" indicates co-culture of cell types. (C) High pSMAD3 expression was observed in CaSki cells treated with CM of CaSki/im-CCAF and CaSki/pri-CCAF, as compared with that of im-CCAF, pri-CCAF, and CaSki. (D) Pre-treatment with A83-01 reduced pSMAD3 expression induced by CM of CaSki/im-CCAF in CaSki cells. (E) Invasion of CaSki cells was enhanced by CM of CaSki/im-CCAF compared with that of im-CCAFs and CaSki cells (n = 3 each). Y-axis; number of invaded cells. (F) Left: Luminescence from TMLECs was correlated with the concentration of recombinant TGF-β1 in the media (R2 = 0.9796). Right: Higher luminescence was found in TNLECs treated with the CM of CaSki/im-CCAF or ME-180/im-CCAF as compared with im-CCAFs, CaSki cells, or ME-180 cells (n = 3 each). Y-axis; luminescence of TMLECs.

Immunostaining of surgically excised specimens showed that TSP-1 was expressed in both tumor and stromal cells (Fig. 1E). Tumoral TSP-1 staining scores were not significantly related to the clinical data for the 67 patients, but the nine cases with pSMAD3 boundary staining were more likely to have a high TSP-1 staining score (Table 2, $p < 0.05$), indicating that TSP-1 activates the TGF-β pathway when CSCC cells and CCAFs interact.

Discussion

TGF-β is known to play various roles in malignant progression. It inhibits growth of normal epithelial cells, and conversely, it promotes EMT, invasion and metastasis in cancer cells, thus eluding tumor-suppressive effects [2–4]. With regard to CSCC, the role of TGF-β signaling is more complicated and not well understood. TGF-β pathway functions in an inhibitory manner for pelvic lymph node metastasis [13], while TGF-β1 expression increases through progression from cervical intraepithelial neoplasia to invasive CSCC [14]. Furthermore, TGF-β1 promotes chromosomal instability in human papillomavirus 16 E6E7-infected cervical epithelial cells [15]. Thus in CSCC, TGF-β signaling appears to function in the promotion of tumorigenesis but does not proceed node metastasis although it is known to promote invasion and metastasis in other cancers. In this study, we investigated the role of TGF-β signaling at the initial phase of CSCC progression, which had not previously been well clarified.

We began this study with careful evaluation of the expression status of TGF-β signaling-associated molecules in 67 CSCCs, and revealed that cases with boundary-specific expression of pSMAD3 were more likely to have node metastasis, which was a noteworthy poor prognostic factor. Boundary pSMAD3 expression in tumor clusters was also more frequently accompanied by stromal expression of TSP-1 ($p < 0.05$), which is an activator of TGF-β signaling [16]. These results suggest that to assess the actual tumoral activity resulting from the TGF-β signal TGF-β signaling activity in boundary areas of tumor clusters should be investigated, and pSMAD3 boundary staining could be an indicator not only of active TGF-β signaling, but may also serve as a biomarker of progression.

Tumor progression consists of local proliferation and extra-organ spread. The proliferation of the CSCC cell lines was not affected by TGF-β1 or A83-01 both *in vitro* and *in vivo*, while TGF-β enhanced migration and invasiveness *in vitro*. GSEA analysis revealed that the gene expression profile of CaSki cells was shifted toward the mesenchymal phenotype by TGF-β administration, and the up-regulated genes included *FN1*, *COL5A1*, *SNAI2* and *SOX4* that are known to be involved with EMT [17,18]. These results suggest that TGF-β may not have a role in local proliferation but rather in extra-organ spread of CSCC, resulting in a high rate of node metastasis among the cases with boundary pSMAD3 expression.

There was a correlation between node metastasis (LNM) and boundary pSMAD3 expression, but not between LNM and pSMAD3 expression in whole tumor nests. This result led us to investigate the role of the

Please cite this article as: Nagura M, et al, Invasion of uterine cervical squamous cell carcinoma cells is facilitated by locoregional interaction with cancer-associated fibro..., Gynecol Oncol (2014), http://dx.doi.org/10.1016/j.ygyno.2014.11.075

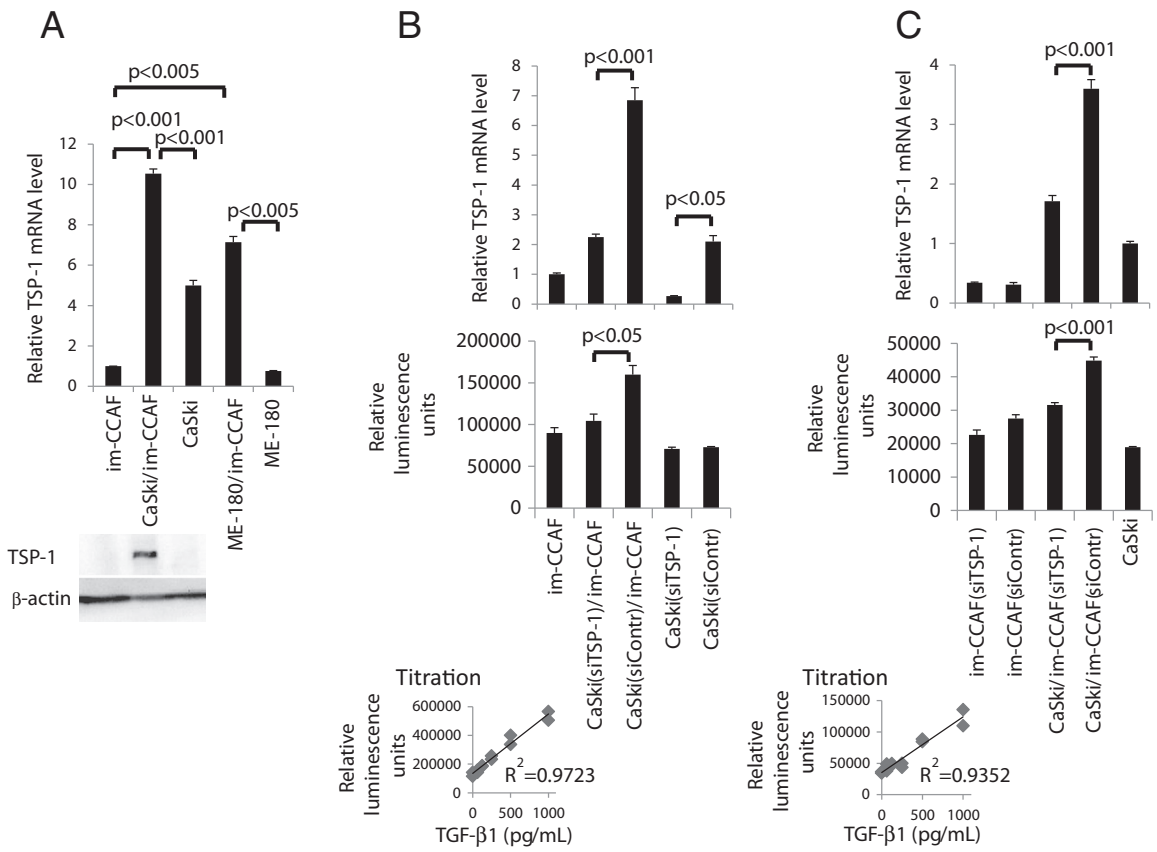

Fig. 4. Molecular mechanism of TGF-β activation in the interaction between CSCC cells and CCAFs. (A) *TSP-1* was up-regulated in both CaSki/im-CCAF and ME-180/im-CCAF. The enhanced production of TSP-1 in CaSki/im-CCAF was also confirmed by Western blotting. Y-axis; normalized mRNA level relative to im-CCAF. TSP-1 analyzed by quantitative RT-PCR. (B) Silencing of *TSP-1* in CaSki cells with siRNA, the efficiency of which was confirmed by quantitative RT-PCR relative to *TSP-1* expression in the im-CCAF control (n = 3), led to reduced luminescence from TMLECs treated with the CM of CaSki/im-CCAF (n = 3). The luminescence from TMLECs was correlated with the concentration of recombinant TGF-b1 (R2 = 0.9723). (C) Silencing of *TSP-1* in im-CCAFs with siRNA, the efficiency of which was confirmed by quantitative RT-PCR relative to *TSP-1* expression in the CaSki cells control (n = 3), also led to reduced luminescence of TMLECs treated with the CM of CaSki/im-CCAF (n = 3). The luminescence from TMLECs was correlated with the concentration of recombinant TGF-b1 (R2 = 0.9352).

microenvironment on TGF-β signal regulation. CAFs surrounding tumor clusters are generally thought to have a myofibroblast phenotype and secrete TGF-β to facilitate cancer progression [8,9,11]. The secretion of TGF-β was confirmed in CCAFs. Furthermore, when CaSki cells were co-cultured with CCAFs, active TGF-β in the CM was increased, and consequently, expression of pSMAD3 was up-regulated and cellular invasion was remarkably augmented. As active TGF-β in the CM was also high and pSMAD3 expression increased in breast cancer cells when co-cultured with fibroblasts [19], our results suggest that the amount of active TGF-β, the biologically relevant form, is regulated during activation through the interaction between CSCC cells and CCAFs.

TGF-β is secreted as a latent form and activated by several molecules, such as thrombospondin-1 (TSP-1), integrin α_V and neuropilin-1 [16]. Of these molecules, TSP-1 was markedly up-regulated when CSCC and CCAF cells were co-cultured, and the amount of activated TGF-β in the co-cultured CM was reduced when TSP-1 was silenced either in CSCC cells or in CCAFs. As there was a positive correlation between tumoral TSP-1 expression and pSMAD3 boundary staining, TSP-1 is likely a key factor activating TGF-β in CSCC. An increase in TSP-1s is associated with poor survival in liver cancer [20], and the amino-terminal domain of TSP-1 functions to directly promote the invasiveness of papillary thyroid cancer cells [21]. Our results suggest that TSP-1 could increase the invasiveness of CSCC cells through activation of TGF-β.

In conclusion, we found that interaction between CSCC and stromal cells activates TGF-β and that this was mediated by TSP-1 resulting in up-regulation of pSMAD3 in CSCC cells, ultimately promoting invasion. Expression of pSMAD3 in the boundary area of CSCC indicates TGF-β activation at the tumor front, and is highly likely to be accompanied by lymph node metastasis. Although we do not yet know what determines distant metastasis *via* initial invasion, understanding the molecular mechanisms of TGF-β activation at the tumor front in cervical carcinoma sheds new light on the mechanism of CSCC progression which will help in developing new treatment strategies.

Conflict of interest statement

All authors declare that they have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2014.11.075.

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