Med Mol Morphol (2015) 48:129–137
DOI 10.1007/s00795-014-0089-5

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ORIGINAL PAPER

SPARC is associated with carcinogenesis of oral squamous epithelium and consistent with cell competition

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Received: 21 August 2014 / Accepted: 22 September 2014 / Published online: 14 October 2014
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Abstract The matricellular protein, secreted protein acidic and rich in cysteine (SPARC) is thought to be involved in cell competition. The objective of this study is to investigate the role of SPARC in cancerization of oral squamous epithelium. Clinical specimens from 57 pre- and early cancerous lesion, 66 invasive squamous cell carcinoma (SCC) and controls were immunostained with SPARC. Clinical features and SPARC expression were evaluated. Furthermore, effects of SPARC knockdown and overexpression were examined in oral cancer and keratinocyte cell lines. Leukoplakia, carcinoma in situ, and early invasive SCC had more SPARC-positive cells than normal mucous epithelium. However, there were no significant differences between leukoplakia, carcinoma in situ, and early SCC, and there were no correlations between SPARC immunoreactivity and prognosis of invasive oral SCCs. Cell proliferation was down-regulated by SPARC siRNA, and enhanced by SPARC transformed keratinocytes. But SPARC overexpression did not enhance cell migration activity. SPARC is induced by dysplastic cells in the early stage of cancerization, and may improve survival capability, but is not involved in malignancy. SPARC may act to escape from elimination by cell competition.

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Keywords SPARC · Carcinogenesis · Oral cancer · Leukoplakia · Dysplasia

Introduction

In cancerization of oral mucosa, the concept of field cancerization is widely accepted, in which an accumulation of mutations by various carcinogens cause multiple precancerous lesions resulting in cancer of the oropharyngeal-upper digestive tract field [1, 2].

In recent years, phenomena that mutant cells were extruded by surrounding more adapted cells, were observed and called "cell competition" in drosophila [3]. Furthermore, failure of cell competition can be associated with carcinogenesis [4]. Secreted protein acidic and rich in cysteine (SPARC) is expressed in "loser cells" and performs a self-protecting function in cell competition [5]. Though there are few reports on human cancer and cell competition, SPARC has been reported in various human cancers, and it plays positive or negative roles in tumor malignancy depending on tumor cell types [6].

In this study, to clarify SPARC association with cancerization of oral squamous epithelium, SPARC expression in oral precancerous or early cancerous lesions in vivo and proliferation and survival of SPARC-transfected cells in vitro were investigated.

Materials and methods

Cell line and patient samples

OSC-2 cells were established in our laboratory from patients with oral squamous cell carcinoma (OSCC) and

Table 1 Patients with oral leukoplakia, CIS, and early invasive SCC

Parameters	*n*	%
Diagnosis		
Leukoplakia		
Hyperplasia	12	16.4
Mild dysplasia	4	5.5
Moderate dysplasia	5	6.8
Severe dysplasia	1	1.4
PVL	5	6.8
Early cancer		
CIS	15	20.5
Early invasive SCC	16	21.9
Control		
Normal healthy oral mucosa	15	20.5
Site		
Tongue	37	50.7
Gingiva	12	16.4
Buccal mucosa	11	15.1
Lip	5	6.8
Oral floor	5	6.8
Palate	3	4.1

PVL proliferative verrucous hyperplasia

Table 2 Patients with invasive carcinoma

	n	%
Gender		
Male	47	75.0
Female	19	25.0
Site		
Tongue	40	63.9
Cheek	13	19.4
Mouth floor	13	16.7
T classification		
T1	11	11.1
T2	38	58.3
T3	6	16.7
T4	11	13.9
N classification		
N0	44	58.3
≥N1	22	41.7
M classification		
M0	66	100.0
M1	0	0.0
Clinical type		
Superficial	5	13.9
Exophytic	11	30.6
Endophytic	20	55.6

Mean age 67.0 ± 11.7 years (42–86 years)

cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10 %(v/v) fetal bovine serum, 10 mM glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen Co., Carlsbad, CA). Primary human keratinocytes (PHK) were obtained from JCRB Cell Bank (Osaka, Japan). Those cells were cultivated in keratinocyte-SFM, supplemented with BPE and rEGF (all from Invitrogen) according to the manufacturer's recommendation.

Oral leukoplakia, carcinoma in situ (CIS), and early invasive squamous cell carcinoma (SCC) samples were obtained from surgically excised tissues of 58 patients (Table 1) to evaluate intraepithelial SPARC expression. For the control group, 15 normal mucous membrane tissues were obtained from benign diseases, such as fibroma. Additionally, sixty-six patients with invasive OSCC were enrolled in the present study (Table 2) to analyze SPARC expression in cancer cells and clinicopathological characteristics.

Immunohistochemistry

The protein expression of SPARC was examined in precancerous and cancerous lesions for immunohistochemical staining. All samples were fixed with 10 % buffered formalin solution and embedded in a paraffin block. Paraffin sections of 4 μm thickness were obtained, deparaffinized, and dehydrated using a graded ethanol series. For immunostaining, the sections were transferred to a 10 mM citrate buffer solution (pH 6.0) and heated twice in a microwave oven for 10 min. Endogenous peroxidase activity was quenched by exposure of the sections to 0.3 % H_2O_2 in methanol for 5 min. The sections were incubated overnight at room temperature with primary antibodies to SPARC (1:300, TAKARA BIO Inc., Otsu, Japan). Thereafter, the sections were incubated with EnVision horseradish peroxidase-labeled polymer (Dako, Glostrup, Denmark) for 30 min, followed by development with diaminobenzidine + chromogen (Dako) for 5 min. Next, the sections were counterstained with hematoxylin staining solution and examined under a light microscope. The percentage of positively stained cells was calculated in randomly examined five high-power fields (200×) covering entire epithelial thickness.

siRNA and plasmid construct

Cells were seeded in an antibiotic-free medium for 24 h before transfection. SPARC-specific siRNA (5′-CAAGACCUUCGACUCUUCCUU-3′) was synthesized by Ambion (Austin, TX, USA). Plasmids containing the SPARC cDNA were constructed using pcDNA6.2/C-

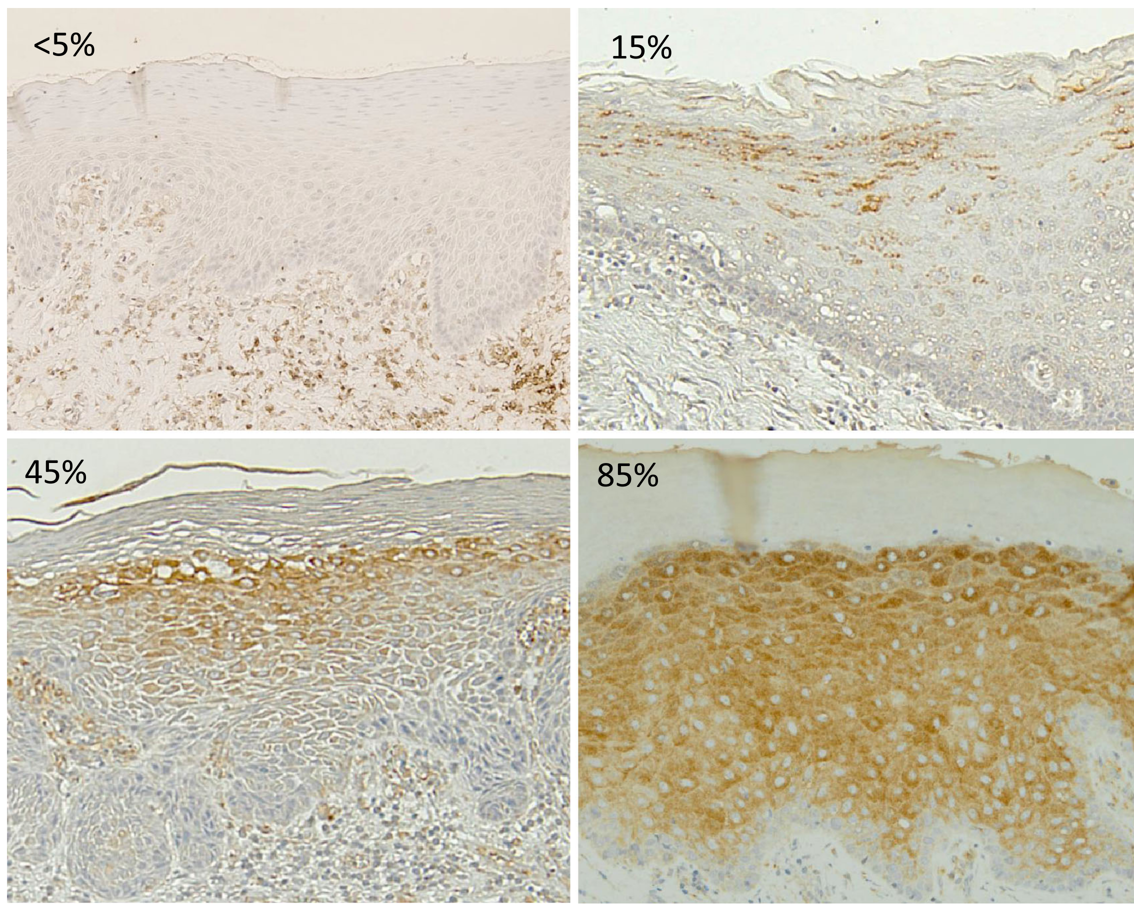

Fig. 1 Representative immunohistochemical staining of SPARC in leukoplakia. SPARC immunoreactivity was evaluated by positive cell rate in total epithelium. Representative staining specimens, negative (<5 %), weak (15 %), moderate (45 %), strong (85 %), were shown

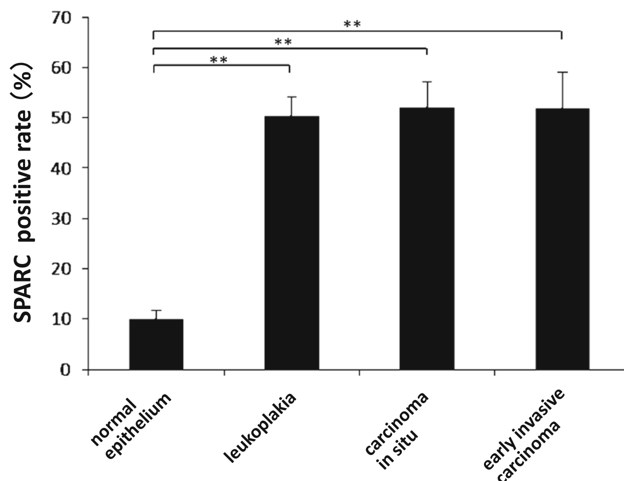

Fig. 2 SPARC-positive cell ratio in each epithelial lesion. SPARC-positive cell ratio in leukoplakia, carcinoma in situ, and early SCC was much higher than in normal mucosa. However, there were no significant differences between leukoplakia, carcinoma in situ, and early SCC

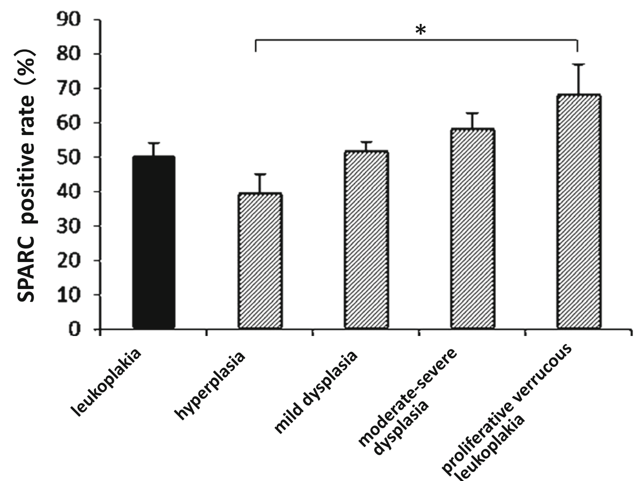

Fig. 3 SPARC-positive cell ratio in white precancerous lesion (leukoplakia). PVL had the highest SPARC-positive cell ratio of leukoplakia, followed by moderate–severe dysplasia, mild dysplasia, and hyperplasia. There was a tendency that the stronger potential the lesion had, the higher ratio of SPARC they had

EmGFP-DEST (Invitrogen). Transfection was performed with the oligofectamine and lipofectamine reagent (Invitrogen).

Cell proliferation assay (MTT assay)

Cell proliferation was estimated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay. Cells (1.0×10^4 cells/well) were cultured in a 96-well microplate for 24 h. After each treatment, the cells in each well were washed with 200 µl of phosphate-buffered saline (PBS) and incubated with 5 mg/ml MTT solution (Sigma-Aldrich Inc.) at 37 °C for 4 h. The supernatants were then removed and the formazan crystals in each well were solubilized by the addition of 200 µl of dimethyl sulfoxide for 30 min. The colored formazan product was measured using a plate reader at a wavelength of 570 nm. Experiments were repeated three times with triplicate samples for each experiment.

Wound healing assay

Migration assay was carried out according to the manufacturer's manual of CytoSelect 24-well wound healing assay (Cell Biolabs, Inc). In brief, 24-well cell culture plates were coated with fibronectin and wound healing inserts were put into the wells with the inserts aligned in the same direction and in firm contact with the bottom of the wells. Cell suspension (250 µl) was added to either side of the open ends at the top of the insert, and incubated overnight to form a monolayer. Then the inserts were removed to begin the wound healing assay. For each well, pictures were taken on a dissection microscope at a magnification of 40×. Cell migration was quantified by the migrated distance.

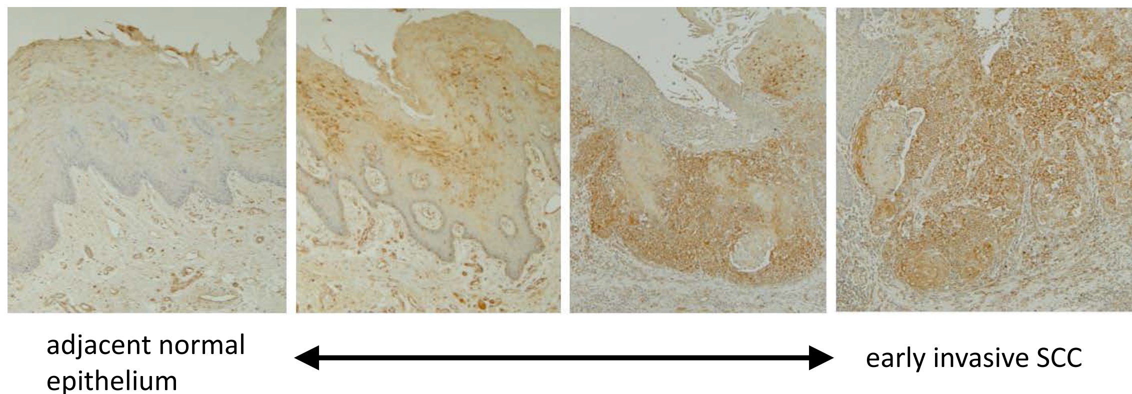

Fig. 4 Representative immunohistochemical staining of SPARC in early stage of tongue squamous cell carcinoma. SPARC immunoreactivity was slight in adjacent normal epithelium, but became intense in thickened dysplastic epithelium and tumorous tissue

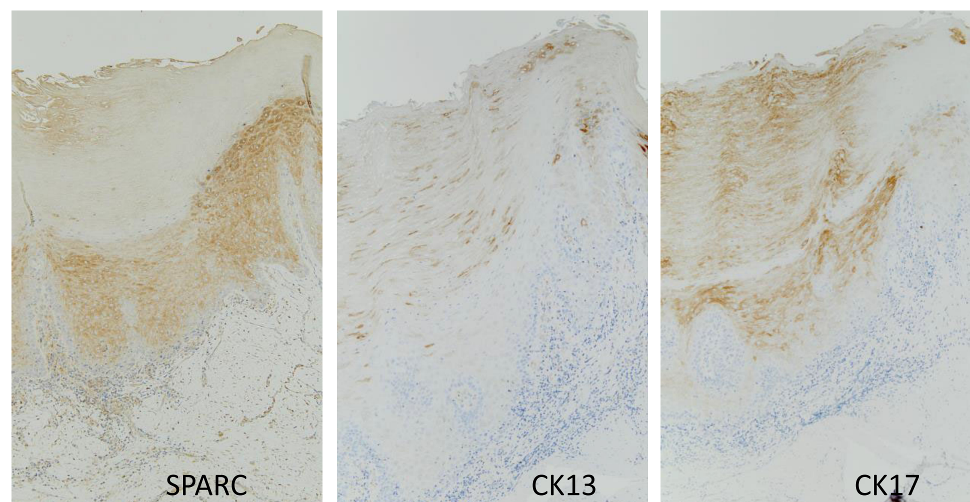

Fig. 5 Representative immunohistochemical staining of SPARC and CK13/17. SPARC was mainly localized in the stratum spinosum of a leukoplakia with moderate dysplasia, and had little immunoreactivity in the basal or suprabasal layer. CK13 was little stained in SPARC-positive region, however, CK17 was almost positive in SPARC-positive region

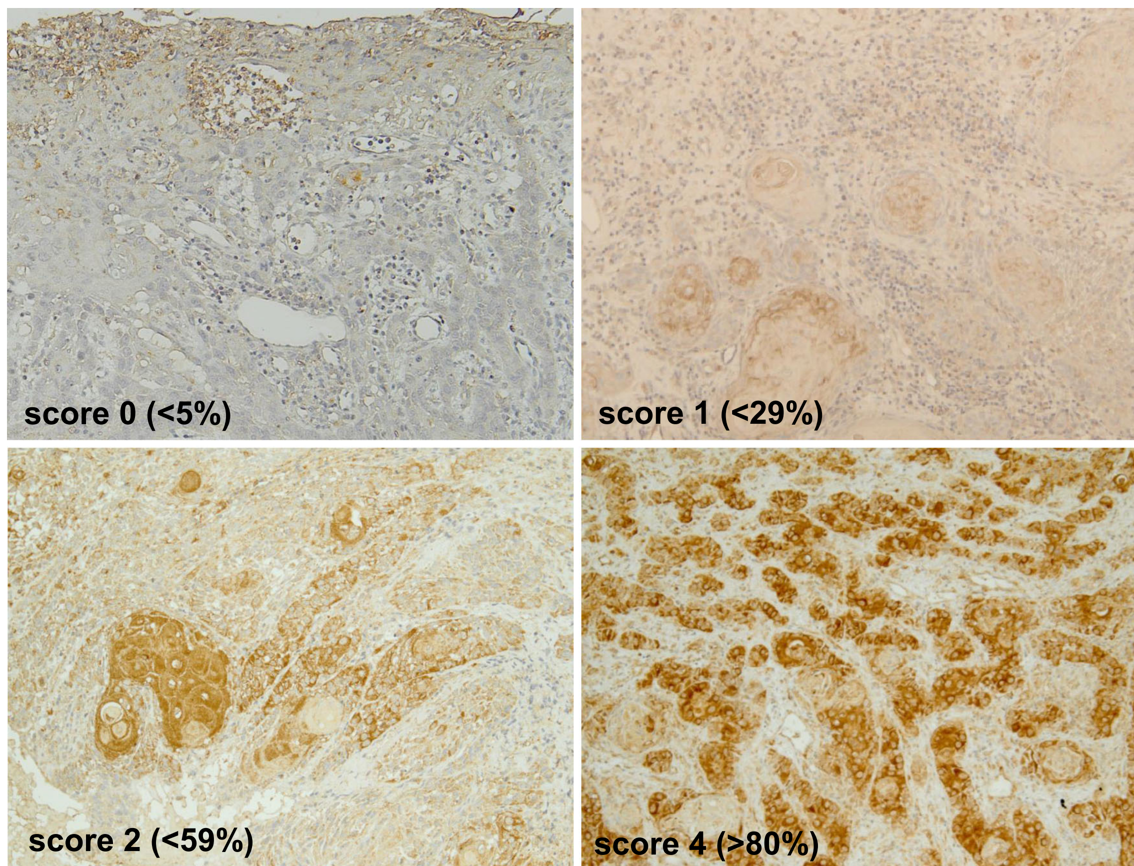

Fig. 6 Representative immunohistochemical scoring of SPARC in invading oral SCCs. Immunoreactivity was scored in one of 5 grades (0–4)

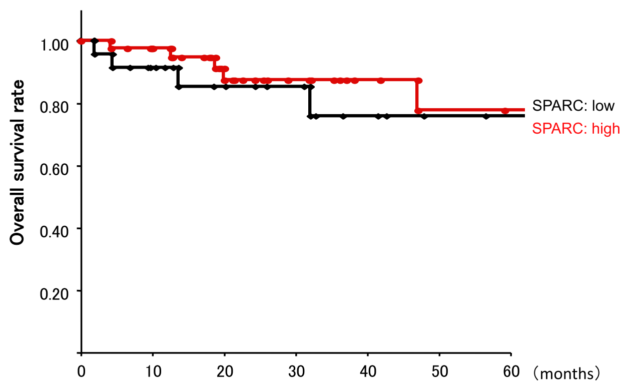

Fig. 7 Overall survival rate in low and high SPARC expression groups. There were no correlations between SPARC immunoreactivity and prognosis of invasive oral SCCs

Co-culture of SPARC overexpression cells and parent cells

OSC-2 cells and SPARC-transfected cells were co-plated in the 24-well plate at a ratio of 1:1, or 2:1 with a total density of 1×10^5 cells/well, and cultured at 37 °C under 5 % CO_2. After 48 h co-culture, the cell dominance was assessed with a FV1000D confocal laser microscope (Olympus, Tokyo, Japan).

Western blot analysis

Extracted proteins (50 µg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto an Immobilon-P membrane (Immobilon, Millipore Corporation, Bedford, MA). Blocking was performed in Tris-buffered saline containing 5 %(w/v) skim milk powder and 0.1 %(v/v) Tween-20. The membranes were probed with the anti-SPARC monoclonal antibody (Haematologic Technologies Inc, Essex Junction, VT) at 1:5,000. Detection was performed with an ECL system (Amersham, Piscataway, NJ).

Statistics

The differences between the mean values were compared by means of Mann–Whitney *U* tests. All statistical analyses were performed using Excel Statistics 2008 (SSRI Co., Ltd, Tokyo, Japan): p values of <0.05 were considered to be statistically significant (two tailed).

Results

SPARC expression in oral precancerous and early cancerous lesions

The clinical specimens from 57 cases and 15 controls were immunostained by SPARC. The details of the cases were 27 leukoplakias and 15 carcinomas in situ, and 16 early invasive SCC (Table 1). SPARC immunoreactivity was evaluated by positive cell rate in total epithelium. Representative staining specimens are shown in Fig. 1. In immunohistochemical staining, leukoplakia, carcinoma in situ, and early SCC, SPARC-positive cell ratio was much higher than in normal mucosa (Fig. 2). However, there were no significant differences among leukoplakia, carcinoma in situ, and early SCC. Among white precancerous lesions (leukoplakia), i.e. hyperplasia, mild dysplasia, moderate–severe dysplasia, and proliferative verrucous leukoplakia (PVL), PVL had the highest SPARC-positive cell ratio of leukoplakia, followed by moderate–severe dysplasia, mild dysplasia, and hyperplasia. There was a tendency that the stronger potential the lesion had, the higher ratio of SPARC they had (Fig. 3). In early stages of tongue SCC, SPARC immunoreactivity was slight in adjacent normal epithelium, but became intense in thickened dysplastic epithelium and tumorous tissue (Fig. 4).

SPARC is expressed in stratum spinosum of dysplastic epithelium

In immunohistochemical staining of SPARC and CK13/17, SPARC was localized mainly in the stratum spinosum of a leukoplakia with moderate dysplasia, and had little immunoreactivity in the basal or suprabasal layer (Fig. 4,5). CK13 was little stained in SPARC-positive region, however, CK17 was almost positive in the SPARC-positive region (Fig. 5).

SPARC expression in invasive oral SCC does not reflect clinical prognosis

The clinical specimen from 66 invasive SCC cases was immunostained with SPARC (Table 2). As representative immunohistochemical scoring of SPARC in invasive oral SCC is shown in Fig. 6, immunoreactivity was scored in

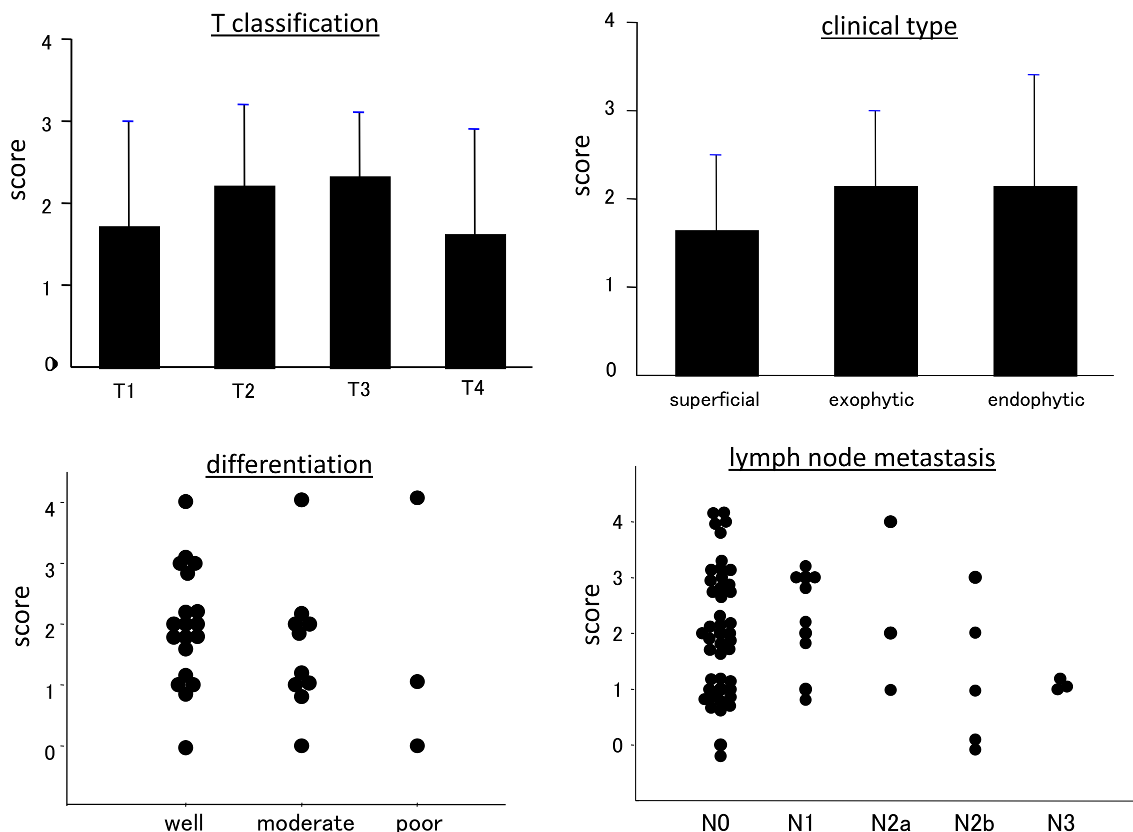

Fig. 8 SPARC immunoreactivity and clinical manifestations. There were no correlations between SPARC immunoreactivity and clinical manifestations (T classification, clinical type, differentiation, and lymph node metastasis) of invasive oral SCCs

one of 5 grades (0–4). Overall survival rate in low (score 0 and 1) and high (score 2, 3 and 4) SPARC expression groups, is analyzed by Kaplan–Meier estimate (Fig. 7). There were no correlations between SPARC immunoreactivity and prognosis of invasive oral SCC. Similarly, there were no correlations between SPARC immunoreactivity and clinical manifestations (T classification, clinical type, differentiation, and lymph node metastasis) of invasive oral SCC (Fig. 8).

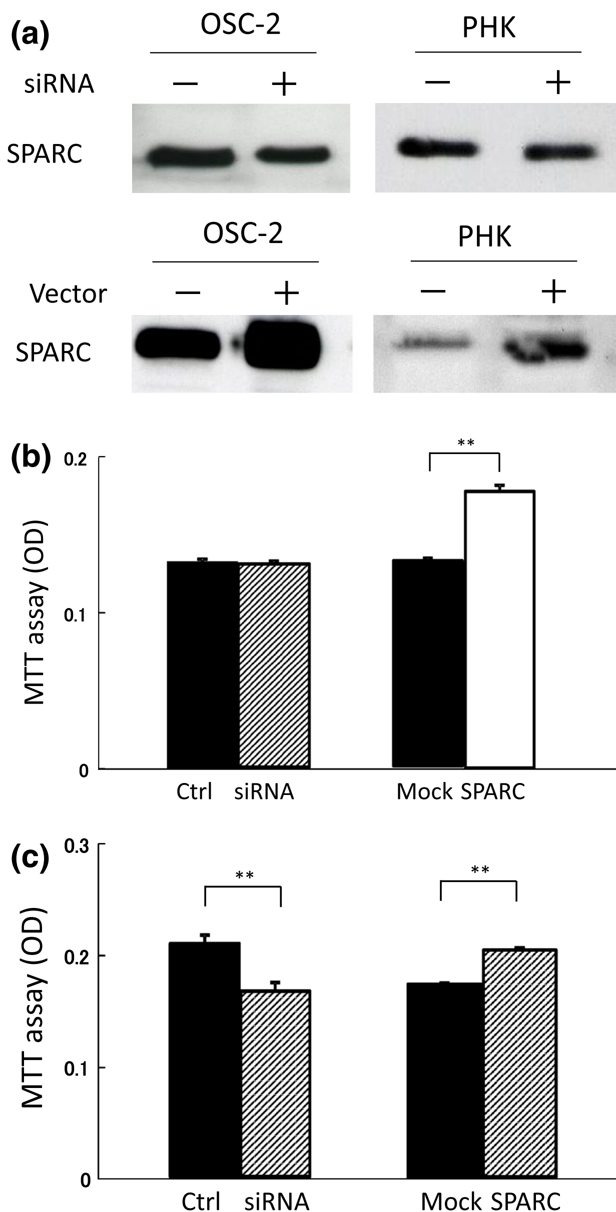

Fig. 9 SPARC enhances tumorigenicity in OSC and PHK cells. **a** SPARC expression decreased by siRNA in both OSC-2 and PHK cell lines, and increased by SPARC transfection. **b** Cell proliferation was enhanced by SPARC transformed OSC-2 cells. **c** Cell proliferation was down-regulated by SPARC siRNA, and enhanced by SPARC transformed PHK cells

Tumorigenic potency in cell lines after knockdown or forced expression of SPARC

SPARC expression was confirmed by western blot analysis (Fig. 9a). SPARC expression decreased by siRNA in both OSC-2 and PHK cell lines, and increased by SPARC transfection. In OSC-2 cell, cell proliferation was enhanced by SPARC overexpression, but was not inhibited by knockdown SPARC (Fig. 9b). In PHK cells, non-oncogenic keratinocytes, cell proliferation was down-regulated by SPARC siRNA, and enhanced by SPARC transformed PHK cells (Fig. 9c), while migration ability was not affected by SPARC overexpression or repression of SPARC in either OSC-2 or PHK cell lines(data not shown).

SPARC is advantageous to cell survival

When two variants of OSC-2 cells, with or without SPARC overexpression, were co-cultured, SPARC overexpression cells became dominant when they were confluent in 48 h (Fig. 10).

Discussion

SPARC, also known as osteonectin or BM-40, is a matricellular glycoprotein, which modulates cell–matrix interactions, remodeling, and repair. SPARC interacts with several extracellular matrix components and functions as a de-adhesive molecule, as a cell cycle inhibitor, and a modulator of cytokine and growth factor activities [7]. Furthermore, SPARC can be a biomarker of several types of cancer [8].

It has been reported that SPARC associates with various cancers, however, SPARC promotes malignancy in some types of cancer and it is tumor suppressor in other types of cancer [9]. For example, SPARC expression is correlated with malignancy, in breast cancer [10, 11], melanoma [12, 13], osteosarcoma [14], glioblastoma [15], and bladder cancer [16], and has a tumor-suppressing effect in medulloblastoma [17] and ovarian cancer [18, 19]. In colon cancer [20], pancreatic adenocarcinoma [21, 22], and non-small cell lung cancer [23], stromal SPARC expression is related with prognosis.

In cancers of the head and neck including oral cancer, there are some reports that SPARC expression correlates with poor prognosis [24, 25]. However, in invasive oral squamous cell carcinoma (OSCC) specimen, immunohistochemical expression of SPARC was not correlated with survival rate and clinicopathological conditions such as T stage, clinical type, differentiation, and lymph node metastasis. Therefore, SPARC may express at an early stage and is consistent with some signals as a result of competition with surrounding normal cells.

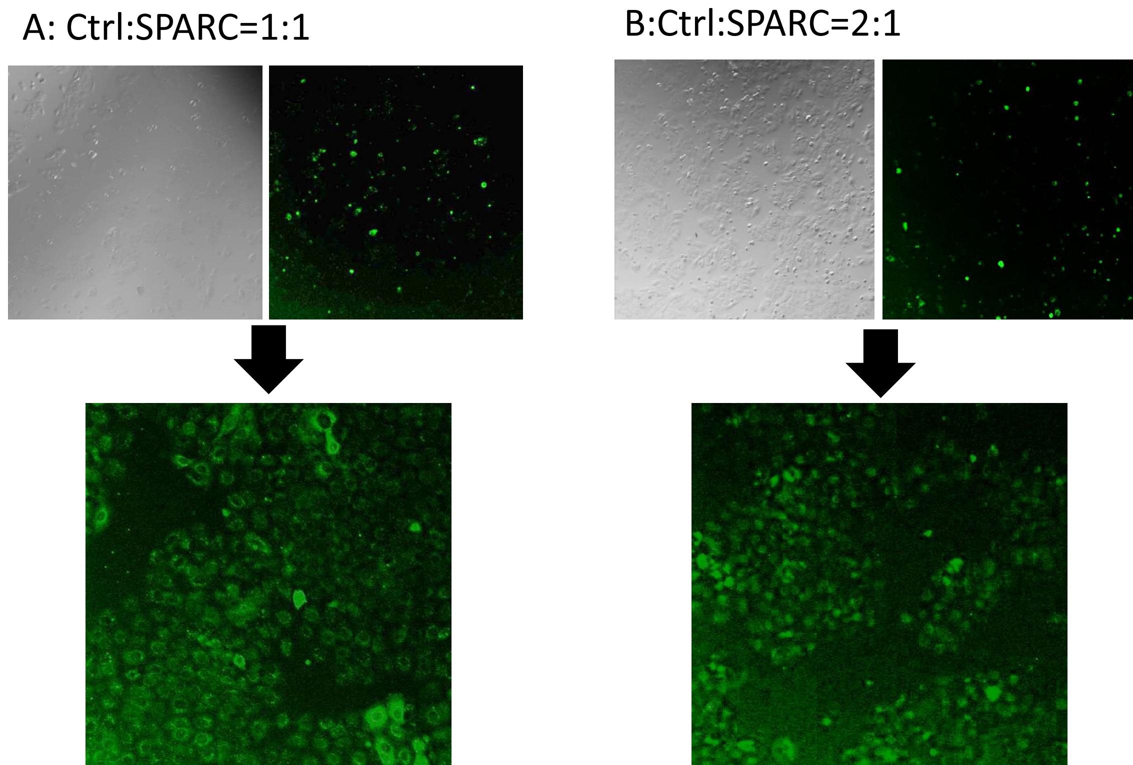

Fig. 10 Co-culture of two variant of OSC-2 cells, with or without SPARC overexpression. SPARC overexpression cells were labeled *green*, and became dominant when they were confluent in 48 h

Although SPARC does not seem to be a biomarker of invasive OSCC, we suspected a relationship between SPARC and cell competition and investigated SPARC expression in pre- and early cancerous lesions. In the results, leukoplakia, carcinoma in situ, and early invasive SCC had more SPARC-positive cells than normal mucous epithelium. This may suggest that some transformation of cells provoke SPARC expression. While leukoplakia has more severe dysplasia, it has a high positive cell rate of SPARC, cancerization did not further increase the SPARC-positive cell rate. SPARC is thought to be associated with the early stages of the multi-step cancerization mechanism.

SPARC localized mainly in the stratum spinosum layer, but little in the suprabasal and basal layer. The localization was consistent with that of CK-17, but not CK-13. CK-17 is thought to be stained in OIN/CIS and suggest malignant transformation [26]. As cancer stem cells exist in the basal layer and outer layer of cancer cell nests [27], SPARC-positive cells do not reflect stem cells. D2-40, one of the cancer stem cell markers [28], usually localizes in the basal layer and outer layer of cancer cell nests.

In cell competition, SPARC is expressed in "loser cells" and performs a self-protecting action to avoid apoptosis [5]. Therefore, SPARC may be expressed by the cells acquiring malignant transformation before selection, or by "loser cells" around the tumor cells.

In the in vitro study, although SPARC regulated cell proliferation positively, it was not involved in cell migration. This implies SPARC can help cell survival but it has no relationship with tumor malignancy and metastatic ability. It is consistent with our clinical data that showed SPARC expression had no relationship with survival rate and lymph node metastasis.

Thus, SPARC is induced by dysplastic cells in the early stages of cancerization, and improves survival capability, but is not involved in malignancy. SPARC may act to escape from elimination by cell competition. SPARC may be useful to detect epithelial mutative change in the early stages of cancerization, although it is unsuitable as a prognosis predictive biomarker.

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