Biochemical and Biophysical Research Communications 439 (2013) 96–102

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Small proline-rich protein-1B is overexpressed in human oral squamous cell cancer stem-like cells and is related to their growth through activation of MAP kinase signal

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ARTICLE INFO

Article history:
Received 3 August 2013
Available online 13 August 2013

Keywords:
Oral cancer
Cancer stem cell
SPRR1B
RASSF4
ALDEFLUOR assay

ABSTRACT

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are considered to be essential for tumor maintenance, recurrence and metastasis. Therefore, eradication of CSCs/CICs is essential to cure cancers. However, the molecular mechanisms of CSCs/CICs are still elusive. In this study, we investigated the molecular mechanism of the cell growth of oral CSCs/CICs. Oral CSCs/CICs were isolated as aldehyde dehydrogenase 1 bright ($ALDH1^{br}$) cells by the ALDEFLUOR assay. *Small proline-rich protein-1B* (*SPRR1B*) gene was shown to be overexpressed in $ALDH1^{br}$ cells by a cDNA microarray and RT-PCR. SPRR1B was shown to have a role in cell growth and maintenance of $ALDH1^{br}$ cells by SPRR1B overexpression and knockdown experiments. To elucidate the molecular mechanism by which SPRR1B regulates cell growth, further cDNA microarray analysis was performed using SPRR1B-overexpressed cells and cells with SPRR1B knocked down by siRNA. Expression of the tumor suppressor gene Ras association domain family member 4 (*RASSF4*) was found to be suppressed in SPRR1B-overexpressed cells. On the other hand, the expression of *RASSF4* was enhanced in cells in which SPRR1B expression was knocked down by SPRR1B-specific siRNA. RASSF4 has an RA (Ras association) domain, and we thus hypothesized that RASSF4 modulates the MAP kinase signal downstream of the Ras signal. MAP kinase signal was activated in SPRR1B-overexpressed cells, whereas the signal was suppressed in SPRR1B knocked down cells. Taken together, the results indicate that the expression of SPRR1B is upregulated in oral CSCs/CICs and that SPRR1B has a role in cell growth by suppression of RASSF4.

1. Introduction

Head and neck cancer including oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and is a serious growing health problem all over the world [1]. Despite recent progress in treatments, survival rates of patients with the disease have not greatly improved. The poor prognosis is due to resistance to treatment, recurrence and distant metastasis.

The theory of cancer stem-like cells/cancer-initiating cells (CSCs/CICs) has recently emerged [2]. This theory is based on the idea that cancers are composed of heterogeneous cell populations and only a small fraction of cancer cells can give rise to a tumor again. Therefore, CSCs/CICs are thought to be responsible for recurrence and distant metastasis, and, importantly, CSCs/CICs have been shown to be resistant to chemotherapy and radiotherapy [3]. CSCs/CICs were initially reported in hematopoietic malignancies and later in solid tumors [4,5]. CSCs/CICs have also been isolated from primary head and neck squamous cell carcinomas (HNSCC) using the CSC/CIC marker CD44, which is a common CSC/CIC marker [6,7]. $CD44^{+}$ cells were also reported to express chemoresistance genes, including *ABCB1*, *ABCG2* and *CYP2C8* [8].

The aldehyde dehydrogenase (ALDH) family of enzymes is comprised of cytosolic isoenzymes that oxidize intracellular aldehydes and contribute to the oxidation of retinol to retinoic acid in early stem cell differentiation [9]. High ALDH1 activity has been used to isolate normal hematopoietic and central nervous system stem cells [10–13]. ALDH1 activity has also been found in stem cells

Abbreviations: CSC, cancer stem-like cell; CIC, cancer-initiating cell; OSCC, oral squamous cell carcinoma; SPRR1B, small proline-rich protein-1B; RASSF4, ras association domain family member 4; NOD/SCID, non-obese diabetic/severe combined immunodeficiency.

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http://dx.doi.org/10.1016/j.bbrc.2013.08.021

derived from hematopoietic malignancies including multiple myeloma and acute myeloid leukemia [12,14]. Ginestier et al. successfully isolated breast CSCs/CICs by using ALDH1 activity for the first time [15]. Chen et al. reported isolation of CSCs/CICs from head and neck squamous cell carcinoma (HNSCC) by using ALDH1 activity [16]. These reports indicate that ALDH expression may be an important new marker for the isolation of CSCs/CICs.

In this study, we isolated oral CSCs/CICs by the ALDEFLUOR assay and isolated a novel oral CSC/CIC-specific genes. One of the oral CSC/CIC-specific genes was *small proline-rich protein-1B (SPRR1B)*, and we analyzed the molecular mechanisms of SPRR1B in oral CSCs/CICs.

2. Materials and methods

2.1. Cell lines and cell culture

Human oral squamous cell carcinoma (OSCC) cell lines, OSC19, OSC20, OSC30, OSC40, OSC70 and POT1, were established in our laboratory (Table S1). The OSCC cell line HSC-2 was purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). The OSCC cell line SAS was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Tohoku, Japan). All of these cell lines were cultured in RPMI-1640 medium (Sigma–Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Technologies Japan, Tokyo, Japan) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Isolation and culture of OSCC-derived ALDH1^{br} cells

Identification of aldehyde dehydrogenase 1 (ALDH1)-positive OSCC cells was carried out using the ALDEFLUOR assay (StemCell Technologies, Durham, NC, USA) and fluorescence-activated cell sorting as described previously [15,17,18].

2.3. Reverse transcriptase PCR (RT-PCR)

RT-PCR analysis was performed as described previously [19]. The PCR mixture was initially incubated at 98 °C for 2 min, followed by 35 cycles of denaturing at 98 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. Primer pairs used for RT-PCR analysis were 5-CCAGTTCTAAGGGACCATACAGA-3′ and 5′-CTCCTTGGTTTTGGGGATG-3′ for *SPRR1B* with an expected PCR product size of 181 base pairs (bp), 5′-CATGATGGAGACG GAGCTGA-3′ and 5′-ACCCCGCTCGCCATGCTATT-3′ for *SOX2* with an expected PCR product size of 410 bp, 5′-TGGAGAAGGAGAAGCT GGAGCAAAA-3′ and 5′-GGCAGATGGTCGTTTGGCTGAATA-3′ for *POU5F1* with an expected PCR product size of 163 bp, 5′-TGTTAGC

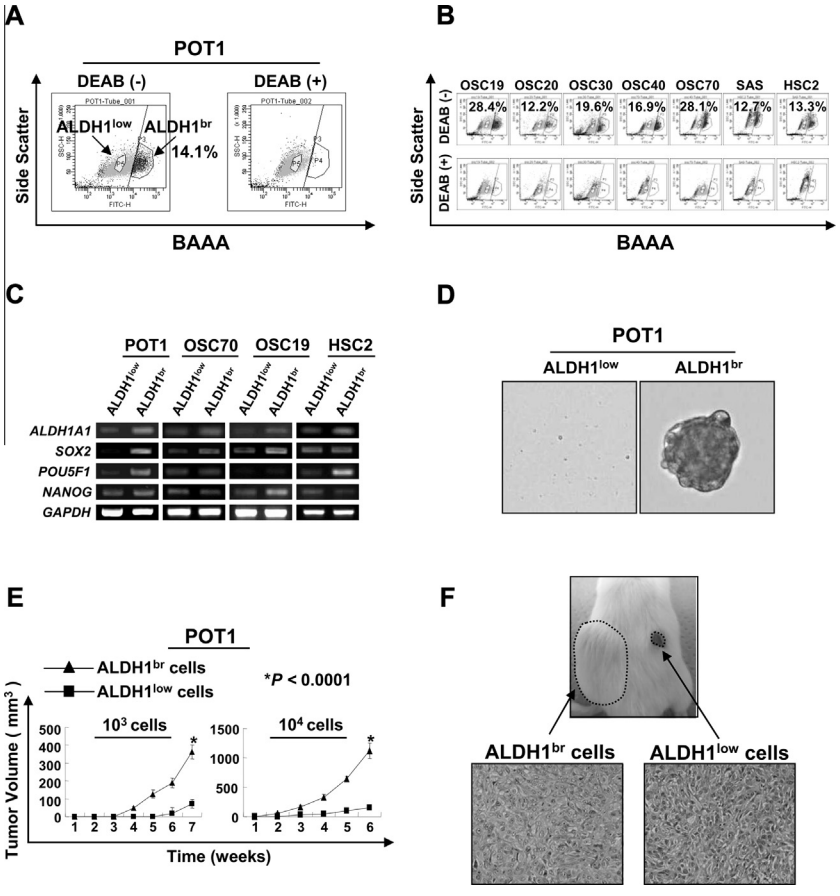

Fig. 1. Isolation of CSCs/CICs from OSCC cells by ALDEFLUOR assay. (A, B) ALDH1^{br} cells were isolated using several OSCC cell lines (POT1, OSC19, OSC20, OSC30, OSC40, OSC70, SAS and HSC2). Percentage represents the proportions of ALDH1^{br} cells. (C) RT-PCR of stem cell markers. ALDH1^{br} and ALDH1^{low} cells derived from POT1, OSC70, OSC19 and HSC2 cells were examined for expression of stem cell markers (*SOX2*, *POU5F1* and *NANOG*). *GAPDH* was used as an internal control. (D) Representative picture of tumor sphere. ALDH1^{br} and ALDH1^{low} cells derived from POT1 cells were cultured in DMEM/F12 media containing EGF and bFGF. After 2 weeks of culture *in vitro*, a picture of a tumor sphere was taken. (E) Tumor formation ability of POT1 ALDH1^{br} and ALDH1^{low} cells. ALDH1^{br} and ALDH1^{low} cells derived from POT1 cells were inoculated into the backs of NOD/SCID mice subcutaneously with serial dilution (10^2–10^5). Graphs show the tumor growth curves of ALDH1^{br} (closed triangle) – and ALDH1^{low} (closed circle) – injected groups with injections of 10^3 and 10^4 cells. Data represent means ± SD. Differences between ALDH1^{br} cells and ALDH1^{low} cells were examined for statistical significance using Student's *t*-test. $^*P < 0.0001$. (F) Histology of ALDH1^{br} cell-derived and ALDH1^{low} cell-derived tumors. Tumors derived from POT1 ALDH1^{br} cells and POT1 ALDH1^{low} cells were stained by hematoxylin and eosin. Magnification, ×200.

Table 1
Tumorigenicity of POT1-ALDH1^{br} and ALDH1^{low} cells at 5-weeks after inoculation into NOD/SCID mice.

		Cell numbers of inoculation			
Population		100	1000	10,000	10,00,00
ALDH1^{br}	Incidence	0% (0/5)	80% (4/5)	100% (5/5)	n.d.
	Volumes (mm^3)	–	127.0 ± 18.6*	645.0 ± 35.7*	
ALDH1^{low}	Incidence	0% (0/5)	40% (2/5)	60% (3/5)	100% (5/5)
	Volumes (mm^3)	–	19.0 ± 26.1*	97.0 ± 24.4*	570.0 ± 45.6

Data shown are for xenografted samples.
NOD/SCID mice were injected with live ALDH1^{br} and ALDH1^{low} cells isolated by ALDEFLUOR assay and mixed with matrigel.
Numbers indicate the ratio of tumour incidence relative to the number of injections.
* $P < 0.0001$.

TGATGCCGACTTG-3′ and 5′-TTCTTAGCCCGCTCAACACT-3′ for *ALDH1A1* with an expected PCR product size of 154 bp, 5′-GCTGAG ATGCCTCACACGGAG-3′ and 5′-TCTGTTTCTTGACCGGGACCTTGTC-3′ for *NANOG* with an expected PCR product size of 161 bp, 5′-ACCGTGAGG AAGAAGGGACT-3′ and 5′-CCTTTAGAGGGCAGCTAGG C-3′ for *RASSF4* with an expected PCR product size of 156 bp and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTG-TA-3′ for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* with an expected product size of 452 bp. *GAPDH* was used as an internal control.

2.4. Gene expression profiling using cDNA microarrays

The total RNAs from ALDH1^{br} cells were labeled with Cy5 dye and those from ALDH1^{low} cells were labeled with Cy3 dye and were hybridized to a 29138-spot Human Panorama Micro Array (Sigma–Aldrich) for 16 h at 45 °C. The intensities of Cy5 and Cy3 fluorescence were measured with a GenePix 4000B scanner (Axon Instruments, Austin, TX) and were analyzed with GenePix Pro 5.0 software (Axon Instruments). Global normalization of the resultant data was carried out using Excel 2004 (Microsoft, Redmond, WA). A dye-swap experiment (labeling ALDH1^{br} and ALDH1^{low} cells with Cy3 and Cy5, respectively) was also performed. An average ratio of more than 2.0, reproducible in two experiments, was determined to indicate differential upregulation in ALDH1^{br} cells.

2.5. Xenograft transplantation

Xenogtaft transplatation was performed as described previously [17,18]. Sorted ALDH1^{br} and ALDH1^{low} cells from POT1 cells were collected and re-suspended at concentrations of 1×10^2 to 1×10^5 cells per 100 μl of PBS and then mixed with 100 μl of Matrigel (BD Biosciences).

2.6. siRNAs and transfection

SPRR1B and *RASSF4* specific small interfering RNA (siRNA) were desined and synthesised using the BLOCK-it RNAi designer system (Life Technologies). Cells were seeded at 50% confluence, and transfections were carried out using Lipofectamine RNAi max (Life Technologies) according to the manufacturer's instructions. Control siRNAs were obtained from Life technologies.

2.7. SPRR1B stable transformant

Full-length *SPRR1B* cDNA was amplified from cDNA of POT1 with PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The PCR product was inserted into pMXs-puro expression vector (a kind gift from Prof. T Kitamura, Tokyo, Japan). Genes were transduced by a retrovirus packaging cell PLAT-A (a kind gift from Prof. T Kitamura, Tokyo, Japan) as described previously [20].

2.8. Western blot

Western blotting was performed as described previously [19]. For detection of ERK and phosphorylated ERK, rabbit anti-ERK polyclonal antibody (K-23; 1:200 dilution; Santa Cruz Biotech.) and rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr 202/Tyr 204) monoclonal antibody (D13.14.4E, 1:2000 dillution; Cell Signaling Tech.) were used.

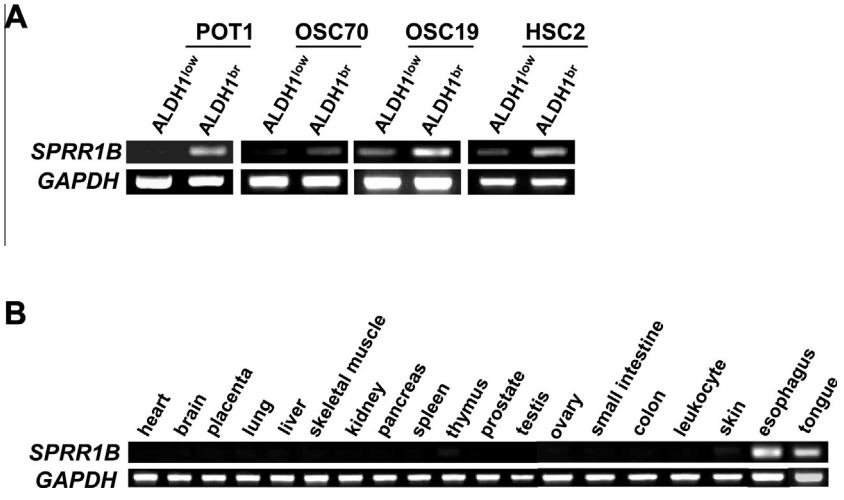

Fig. 2. Expression of *SPRR1B* in various oral cancer cell lines and human normal adult tissues. (A) Expression of *SPRR1B* in various oral cancer cell lines. *SPRR1B* expression was assessed by RT-PCR using ALDH1^{br} and ALDH1^{low} cells derived from POT1, OSC70, OSC19 and HSC2 cells. *GAPDH* was used as an internal control. (B) Expression of *SPRR1B* in normal adult tissues. *SPRR1B* expression was assessed by RT-PCR using the heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocytes, skin, esophagus and tongue. *GAPDH* was used as an internal control.

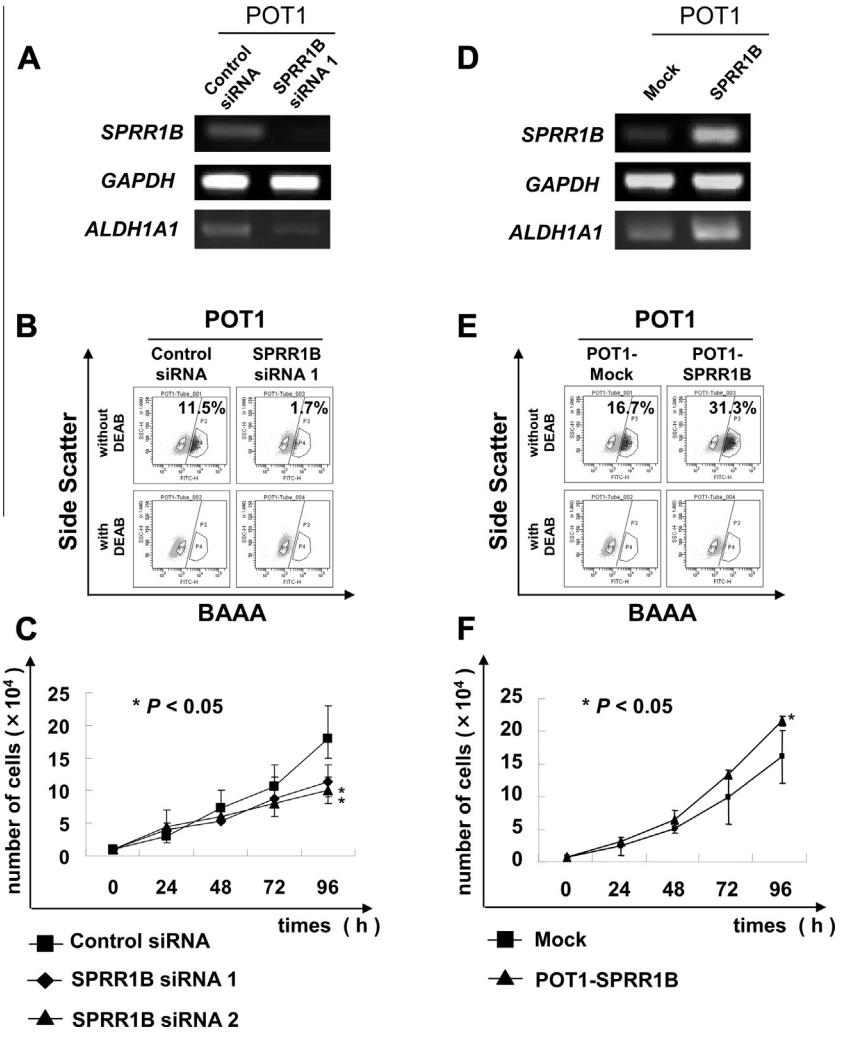

Fig. 3. SPRR1B has a role in cell growth of OSCC. (A) RT-PCR of SPRR1B knockdown cells. *SPRR1B* siRNA was transfected into POT1 cells. Twenty-four hours after transfection, total RNAs were purified and the expression of SPRR1B and ALDH1A1 was evaluated by RT-PCR. *GAPDH* was used as an internal control. (B) ALDEFLUOR assay. *SPRR1B* knockdown cells were analyzed by the ALDEFLUOR assay. Percentage represents the proportion of ALDH1^{br} cells. (C) *In vitro* cell growth. *In vitro* growth of *SPRR1B* siRNA-transfected POT1 cells were evaluated by cell count. Differences between control siRNA-transfected POT1 cells and *SPRR1B* siRNA-transfected POT1 cells were examined for statistical significance using Student's *t*-test. $^{*}P < 0.05$. (D) RT-PCR of SPRR1B-overexpressed cells. Cells stably transfected with SPRR1B and control vector-transfected cells were used. The expression of SPRR1B and ALDH1A1 was evaluated by RT-PCR. *GAPDH* was used as an internal control. (E) ALDEFLUOR assay. *SPRR1B*-overexpressed cells were analyzed by the ALDEFLUOR assay. Percentage represents the proportion of ALDH1^{br} cells. (F) *In vitro* cell growth. *In vitro* growth of *SPRR1B*-overexpressed POT1 cells was evaluated by cell count. Differences between control vector-transfected POT1 cells and *SPRR1B*-transfected POT1 cells were examined for statistical significance using Student's *t*-test. $^{*}P < 0.05$.

2.9. Statistical analysis

In the xenograft model and *in vitro* cell growth data, samples were analyzed using Student's *t*-test, with $P < 0.05$ conferring statistical significance.

3. Results

3.1. Isolation of OSCC stem-like cells

We used the ALDEFLUOR assay to isolate CSCs/CICs from OSCC cell lines. An ALDEFLUOR bright (ALDH1^{br}) population was detected in all of the cell lines (Fig. 1A and B). The proportions of ALDH1^{br} cells ranged from 12.2% for OSC20 to 28.4% for OSC19. To confirm that CSCs/CICs were enriched in ALDH1^{br} cells, we performed RT-PCR, sphere formation and tumor formation *in vivo* using NOD/SCID mice. We examined the expression of stem/progenitor cell genes including, *SOX2*, *POU5F1* and *NANOG* [21–24]. The ALDH1^{br} population expressed *ALDH1A1*, *SOX2*, *POU5F1* and *NANOG* at higher levels than those in the ALDH1^{low} population (Fig. 1C). Spheroid-like bodies were observed in POT1 ALDH1^{br} cells under the condition of culture in DMEM/F12 serum-free medium containing bFGF and EGF, whereas spheroid-like bodies were not observed in POT1 ALDH1^{low} cells (Fig. 1D).

To evaluate the *in vivo* tumor-initiating ability of ALDH1^{br} and ALDH1^{low} cells, we injected 10^2, 10^3, 10^4 and 10^5 cells into the backs of NOD/SCID mice. Tumor growth was initiated in 80% (4/5) and 100% (5/5) of the 10^3 and 10^4 ALDH1^{br}-injected mice, respectively (Table 1). On the other hand, tumor growth was initiated in 40% (2/5) and 60% (3/5) of the 10^3 and 10^4 ALDH1^{low}-injected mice, respectively. The tumors derived from 10^3 and 10^4 of ALDH1^{br} cells grew faster than those derived from ALDH1^{low} cells (Fig. 1E). The histologic findings showed no differences (Fig. 1F). These results indicate that CSCs/CICs were enriched in POT1 ALDH1^{br} cells.

3.2. Isolation of an OSCC stem-like cell-specific gene, SPRR1B

To analyze the molecular mechanisms of ALDH1^{br} cells, we screened for ALDH1^{br} cell-specific genes using a micro-array. The

overexpressed genes (rate > 2.0) in POT1 ALDH1^{br} cells are summarized in Table S2. To isolate a common OSCC CSC/CIC gene, we screened for an ALDH1^{br}-specific gene by RT-PCR using cDNAs derived from OSC70 ALDH1^{br} cells and ALDH1^{low} cells (Fig. S1). *Small proline-rich protein-1B (SPRR1B)* showed ALDH1^{br} cell-specific expression, and we therefore extended the RT-PCR study to other OSCC cells. *SPRR1B* showed ALDH1^{br} cell-specific expression in all cell lines (Fig. 2A). *SPRR1B* was expressed in esophagus, tongue and skin, which contain squamous cell epithelia, but was not expressed in other organs including the heart, brain, placenta, lung, liver, kidney, skeletal muscle, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocytes and skin (Fig. 2B).

3.3. Regulation of ALDH^{br} cell growth by SPRR1B

To further investigate the role of *SPRR1B*, SPRR1B knockdown and overexpression experiments were performed. Three SPRR1B gene-specific siRNAs were confirmed for suppression of SPRR1B mRNA by RT-PCR. *SPRR1B* gene knockdown reduced the expression of *ALDH1* compared to that in control siRNA-transfected POT1 cells (Fig. 3A). *SPRR1B* gene knockdown reduced the ALDH1^{br} population compared to that in control siRNA-transfected POT1 cells (11.5–1.7%) (Fig. 3B). SPRR1B gene knockdown suppressed POT1 cell growth significantly compared with the growth of control siRNA-transfected POT1 cells (Fig. 3C).

On the other hand, the expression of ALDH1 was enhanced in SPRR1B-overexpressed POT1 cells compared with the expression in control vector-transfected POT1 cells (Fig. 3D). The proportion of ALDH1^{br} cells and cell growth rate were enhanced in SPRR1B-overexpressed POT1 cells compared with those in control vector-transfected POT1 cells (Fig. 3E and F).

3.4. Role of SPRR1B in cell grow by suppression of the tumor suppressor RASSF4

To search for a downstream gene of SPRR1B, we performed further microarray screening. *RASSF4* and *STEAP1* genes were downregulated in SPRR1B-overexpressed cells and *RASSF4* and *STEAP1* genes were upregulated in SPRR1B knockdown cells (Fig. S2). Ras association domain family member 4 (*RASSF4*) contains an RA (Ras association) domain and works as a Ras effector and it has role as a tumor suppressor gene. We therefore hypothesized that

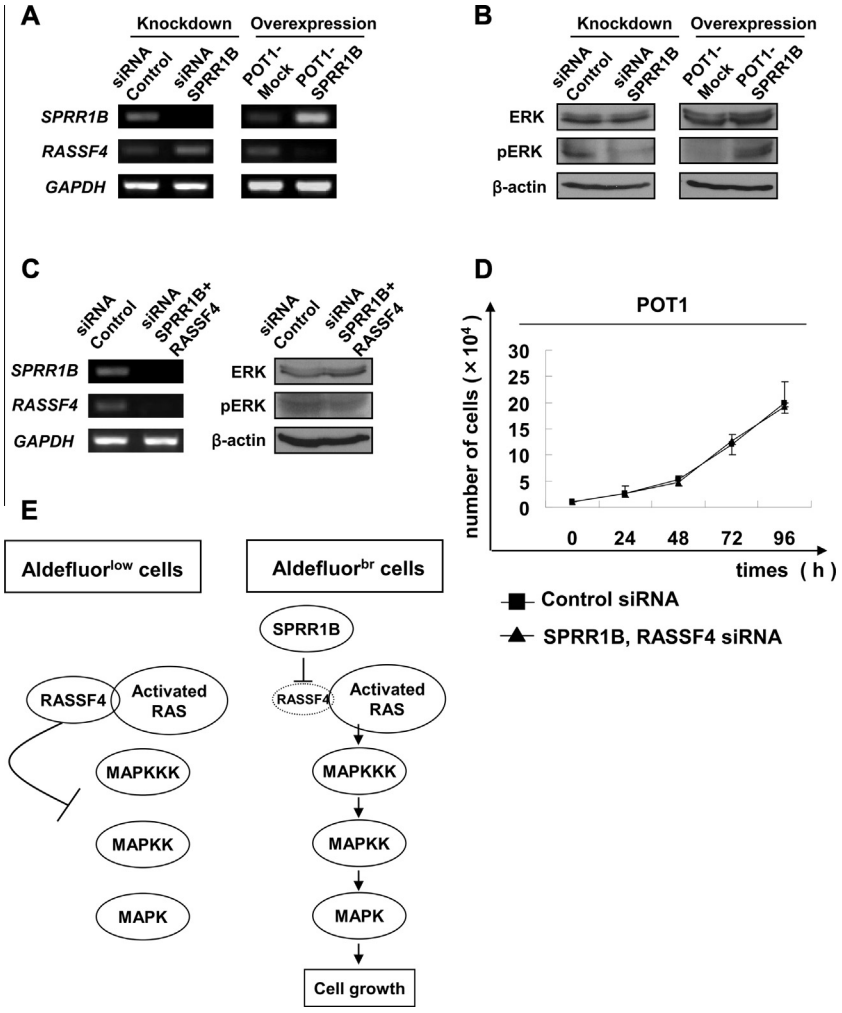

Fig. 4. *RASSF4* is negatively regulated by SPRR1B and has a role in inhibition of MAP kinase signal and cell growth. (A) RT-PCR of SPRR1B knockdown cells and SPRR1B-overexpressed cells. SPRR1B knockdown cells and SPRR1B-overexpressed cells were analyzed by RT-PCR. The expression of *SPRR1B* and *RASSF4* was evaluated. *GAPDH* was used as an internal control. (B) Western blot of MAP kinase signals using *SPRR1B* knockdown cells and SPRR1B-overexpressed cells. Total ERK protein and the phosphorylated form of ERK protein were detected by Western blotting. β-Actin was used as an internal control. (C) RT-PCR and Western blot analysis of SPRR1B and RASSF4 siRNA-transfected cells. Control siRNA or SPRR1B and RASSF4 siRNA was transfected into POT1 cells. The expression of SPRR1B and RASSF4 was evaluated by RT-PCR. Phosphorylation of ERK was evaluated by Western blot analysis. (D) *In vitro* growth of SPRR1B siRNA- and RASS4 siRNA-transfected POT1 cells. Data represent means ± SD. (E) Schematic model of SPRR1B related to growth of ALDH1^{br} cells.

downregulation of the *RASSF4* gene is one of the mechanisms of the enhancement of cell grow by SPRR1B overexpression.

To confirm the gene expression regulation, RT-PCR was performed. Expression of *RASSF4* was upregulated in SPRR1B siRNA-transfected cells compared with that in of control siRNA-transfected cells. On the other hand, expression of RASSF4 was downregulated in SPRR1B-overexpressed cells (Fig. 4A). Since RASSF4 has an RA domain and has a role as a tumor suppressor, we hypothesized that RASSF4 suppresses the MAP kinase signal, which is one of the major cell growth signals. The phosphorylated form of ERK was downregulated by transfection of SPRR1B siRNA compared with that in control siRNA-transfected cells. On the other hand, the phosphorylated form of ERK was upregulated by overexpression of SPRR1B compared with that in control vector-transfected cells (Fig. 4B).

To verify that the ERK inhibition effect is due to RASSF4, we transfected RASSF4-specific siRNA into SPRR1B siRNA-transfected cells. The amount of the phosphorylated form of ERK was the same as that in control siRNA-transfected cells (Fig. 4C). The growth rates of SPRR1B siRNA- and RASSF4 siRNA-transfected cells were same as the growth rate of control siRNA-transfected cells (Fig. 4D).

4. Discussion

In this study, we isolated oral CSCs/CICs as ALDH1^{br} cells. The tumor-initiating ability of ALDH1^{br} cells were confirmed by using NOD/SCID mice that ALDH1^{br} cells derived from POT1 cells showed higher tumor initiation ability than that of ALDH1^{low} cells. Furthermore, ALDH1^{br} cells more efficiently produced tumor spheres than did ALDH1^{low} cells and showed higher levels of stem cell markers including *SOX2*, *POU5F1* and *NANOG*; thus, POT1 ALDH1^{br} cells are likely to be enriched with a CSC/CIC population and should be suitable for analysis of OSCC CSCs/CICs.

The *SPRR* gene family includes 17 genes comprising four subfamilies [25,26]. SPRRs are structural proteins and they form a cornified cell envelop (CE) with other structural proteins such as involucrin and loricrin and are thought to work as a barrier [27]. SPRRs, especially SPRR3, have been reported to be up-regulated in malignant diseases [28,29]. Cho et al. reported that SPRR3 activated AKT and decreased the expression of p53 through the activation of MDM2 in colon cancer cells. On the other hand, overexpression of SPRR1B was reported to induce G0 cell cycle arrest in Chinese hamster ovary (CHO) cells [30]. In this study, overexpression of the *SPRR1B* gene enhanced cell grow *in vitro* and increased the ALDH1^{br} population in POT1 cells. Thus, the *SPRR1B* gene has a role to enhance cell growth of OSCC but not in CHO cells. One of the mechanisms may be suppression of the tumor suppressor RASSF4 by SPRR1B (Fig. 4E).

Human *RASSF4* binds to K-Ras in a GTP-dependent manner via the effector domain and synergizes with K-Ras to induce apoptotic cell death in 293T cells. Expression of *RASSF4* inhibits the growth of human tumor cells. *RASSF4* is broadly expressed in human tissues but is frequently down-regulated in human tumor cell lines and primary tumors. The expression RASSF4 correlates with methylation of the promoter. RASSF4 induces apoptosis in 293T cells in a Ras-associated manner [31,32]. Induction of apoptosis is considered to one of the mechanisms by which RASSF4 acts as a tumor suppressor. In this study, we found that RASSF4 suppresses the MAP kinase signal by suppression of ERK phosphorylation. Since RASSF4 has an RA domain and associates with Ras, RASSF4 may directly suppress the MAP kinase signal.

In summary, we successfully isolated OSCC CSCs/CICs by using the ALDEFLUOR assay. The *SPRR1B* gene was expressed more highly in the ALDH1^{br} population than in the ALDH1^{low} population and has a role in OSCC cell growth by suppression of the tumor suppressor RASSF4. Overexpression of SPRR1B might be related to carcinogenesis of OSCC and maintenance of OSCC CICs/CSCs.

Acknowledgments

The authors thank Dr Kitamura for kindly providing a retrovirus system. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant Nos. 16209013, 17016061 and 15659097) for Practical Application Research from the Japan Science and Technology Agency, and for Cancer Research (15–17 and 19–14) from the Ministry of Health, Labor and Welfare of Japan, Ono Cancer Research Fund (to N. S.) and Takeda Science Foundation (to Y. H.). This work was supported in part by the National Cancer Center Research and Development Fund (23-A-44).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.021.

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