

Fasudil, a Rho kinase inhibitor, suppresses tumor growth by inducing CXCL14/BRAK in head and neck squamous cell carcinoma

Chihiro MIYAMOTO^{1,2*}, Yojiro MAEHATA^{1,2*}, Kazuhiko MOTOHASHI^{1*}, Shigeyuki OZAWA^{2,3}, Takeharu IKOMA^{2,3}, Kouki HIDAKA⁴, Satoko WADA-TAKAHASHI¹, Shun-Suke TAKAHASHI¹, Fumihiko YOSHINO¹, Ayaka YOSHIDA¹, Eiro KUBOTA^{2,3}, Ryu-ichiro HATA^{1,2}, and Masaichi-Chang-il LEE⁵

¹Department of Oral Science, Graduate School, Kanagawa Dental University, Japan; ²Oral Health Science Research Center, Graduate School, Kanagawa Dental University, Japan; ³Department of Oral Surgery, Graduate School, Kanagawa Dental University, Japan; ⁴Department of Periodontal Regeneration, Kanagawa Dental University, Japan; and ⁵Yokosuka-Shonan Disaster Health Emergency Research Center, Graduate School, Kanagawa Dental University, Japan

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ABSTRACT

CXCL14/BRAK (BRAK) is a secreted chemokine with anti-tumor activity, and its expression is suppressed in tumor cells. We previously reported the anti-tumor activity of BRAK in cell lines of head and neck squamous cell carcinoma (HNSCC) and the suppression of BRAK secretion in these cells. BRAK secretion in fibrosarcoma cells is restored by Fasudil, which is a Rho-kinase (ROCK) inhibitor. In this study, we examined the anti-tumor effect of BRAK by evaluating its gene expression and protein secretion in HNSCC cell lines. We found that BRAK mediated the suppressive effect of Fasudil against HNSCC cells. Tumor development in female BALB/cAJcl-nu/nu mice was suppressed by Fasudil. Also secretion of BRAK protein by tumor cell lines *in vitro* was significantly stimulated by Fasudil treatment. Similarly, the production of BRAK protein was significantly increased by the addition of Fasudil to cultured tumor cells. Furthermore Fasudil significantly increased BRAK gene expression at the mRNA level in HNSCC cell line. Inhibition of the RhoA/ROCK pathway by siRNAs significantly stimulated BRAK gene expression. These results show that the tumor-suppressive effect of Fasudil was mediated by BRAK, suggesting that Fasudil may therefore be useful for the treatment of HNSCC.

Head and neck squamous cell carcinoma (HNSCC) is the most frequent form of cancer in the head and neck region, with an estimated 644,000 cases worldwide in 2012 and increasing every year (27, 28). HNSCC is generally treated surgically; however, large excisions require tissue transplantation and can affect the patients' appearance and their ability to converse and eat, thus reducing their quality of life

(QOL) (4, 26, 33). To improve QOL, it is important to reduce the tumor size with radiation or molecular targeted therapy prior to surgery (2, 34).

Abbreviations

CXCL14/BRAK, CXC Chemokine Ligand 14/Breast-and Kidney-Expressed Chemokine; DMEM, Dulbecco's modified Eagle's medium; DMEM-10, DMEM supplemented with 10% fetal bovine serum; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HNSCC, head and neck squamous carcinoma; PBS, phosphate buffered saline; *i.p.*, intraperitoneal; RhoA, Ras-homologous small GTPase; ROCK, Rho kinase; QOL, quality of life; qPCR, real-time quantitative polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction

*These authors contributed equally to this work.

Address correspondence to: Dr. Yojiro Maehata, Department of Oral Science, Kanagawa Dental University, 82 Inaoka-cho, Yokosuka 238-8580, Japan
Tel: +81-46-822-9600, Fax: +81-46-822-8868
E-mail: maehata@kdu.ac.jp

We previously reported that chemokine CXCL14/BRAK (BRAK) suppresses vascularization and suppresses tumor formation in cell lines of HNSCC (20–22). BRAK was first identified in breast and kidney tissues, from which the protein got its name (7). Subsequently, BRAK was found to activate B cells and monocytes (29), as well as to attract dendritic cells to tumor tissues (24). BRAK also has anti-angiogenic activity, and induces apoptosis of tumor cells (20). BRAK transcripts are abundant in normal tissues but are minimally expressed or absent in certain carcinoma cell lines (7). BRAK expression inhibits tumor vascularization and suppresses tumor development in BRAK transgenic mice (9, 13). We also showed that although BRAK is a secretory protein that possesses an N-terminal signal peptide sequence, BRAK secretion is suppressed in carcinoma cells (12). These findings suggest that induction of BRAK secretion in tumor cells may be an effective means for therapeutic treatment of tumors.

We previously reported that the Ras-homologous small GTPase (RhoA) and its downstream effector Rho-kinase (ROCK) inhibit BRAK secretion in mouse fibrosarcoma cells (13). RhoA and ROCK play a central role in smooth muscle contraction, cytoskeleton rearrangement, cell migration, cell proliferation, and gene expression (8, 11, 18). Secretory proteins and receptors are transported along microtubules and actin fibers, which are downstream effectors of the RhoA/ROCK pathway (23). RhoA gene expression is enhanced in several tumors, including HNSCC (1); thus, in general, excess activation of RhoA/ROCK causes aberrant localization of proteins and receptors in HNSCC.

The ROCK-specific inhibitor Fasudil suppresses the growth of fibrosarcomas by increasing BRAK secretion (13). Also Fasudil modifies myosin light chain phosphorylation in smooth muscle cells (3, 23). It was approved in Japan in 1995 for the clinical treatment of vascular spasms in the brain (15). The safety of Fasudil has been confirmed in more than 1400 patients with subarachnoid hemorrhage (15). Fasudil may be useful as a molecular targeted therapy with few side effects for the treatment of cancers.

In this study, we demonstrated that the ROCK-specific inhibitor Fasudil suppresses HNSCC growth by stimulating BRAK expression and secretion.

MATERIALS AND METHODS

Materials and animals. The experimental procedures were performed in accordance with the guidelines of

the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985), and the protocols were approved by the Institutional Animal Care Committee (Kanagawa Dental University, Yokosuka, Japan).

BALB/cAJcl-nu/nu female mice were from Clea Japan (Tokyo, Japan). Fasudil (1-[5-isoquinolinesulfonyl]-homopiperazine) from Tocris Bioscience (Bristol, UK) was used for *in vitro* experiments, and Fasudil hydrochloride from Asahi Kasei Pharma Co. (Tokyo, Japan) was used *in vivo* ones. Other reagents and their sources were as follow; bovine serum albumin, HEPES, trypsin, gentamicin, and EDTA, from Wako Pure Chemical Industries (Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM), from Nissui Seiyaku (Tokyo, Japan); Fungizone, from Gibco (San Diego, CA, USA); siRentfect was from BIO-RAD (Hercules, CA, USA); fetal bovine serum (FBS), from Trance Scientific (Melbourne, Australia); SuperScript II reverse transcriptase, TRIzol total RNA isolation reagent, SuperScript First-strand Synthesis system, and Zeocin, from Invitrogen (Carlsbad, CA, USA); PrimeSTAR DNA polymerase and EX Taq polymerase were from TaKaRa (Otsu, Japan); siRNA (RhoA: Hs_RHOA_7 HP Validated siRNA, ROCK1: Hs_ROCK1_9 HP Validated siRNA, ROCK2: Hs_ROCK2_6 HP Validated siRNA, and AllStars negative control siRNA), from QIAGEN (Venlo, Netherlands); SYBR Green qPCR Master Mix, from Agilent Technologies (Santa Clara, CA, USA); CXCL14 Duo Set ELISA Development System kit were from R&D Systems (Abington, UK); QuantaBlu Fluorogenic Peroxidase Substrate Kit, from Pierce (Rockford, IL, USA); Lumi-Light Western blotting substrate, from Roche Diagnostics (Mannheim, Germany); and physiological saline, from Otsuka Pharmaceutical (Tokyo, Japan).

Cells and cell cultures. HNSCC cells such as HSC-2, HSC-3 and HSC-4 were obtained from Riken Bioresource Center (Ibaraki, Japan). The cells were cultured in DMEM supplemented with 50 µg/mL gentamicin sulfate, 250 ng/mL Fungizone, 12.6 mM HEPES, and 10% FBS at 37°C under 5% CO₂, and were subcultured following treatment with 0.25% trypsin. The cells were used within 3 to 4 passages and cell number was counted by using a Coulter Counter from Beckman Coulter (Brea, CA, USA).

Tumor growth in vivo. HSC-3 cells (2.0×10^6 cells/site) were subcutaneously injected into both sides of the dorsolateral regions of 12 BALB/cAJcl-nu/nu fe-

male mice. Tumor burden was established under the skin 2 days after injection; then, Fasudil dissolved in physiological saline (50 mg/kg/day) or vehicle was administered intraperitoneally (*i.p.*). Tumor volume was calculated according to the formula $(a \times b^2) / 2$, where *a* is the longest diameter of the tumor and *b* is the shortest diameter. Fifteen days after the start of Fasudil treatment, all mice were weighed and killed. Each tumor was subsequently removed and weighed.

Protein quantification. The culture medium was collected and cells were dissolved in radio-immunoprecipitation assay (RIPA) buffer. BRAK protein was quantified with a CXCL14 Duo Set ELISA Development System kit as described by the manufacturer, with minor modifications (9).

Reverse transcription and real-time quantitative PCR (qPCR) analyses. Total RNA was extracted with TRIzol reagent, reverse-transcribed with the SuperScript First-strand Synthesis System, and amplified with ExTaq DNA polymerase. qPCR was performed with Brilliant SYBR Green qPCR Master Mix and the following primers: BRAK, 5'-AATGAAGCCAAAGTACCCGC-3' (forward) and 5'-AGTCCTTTGCAACAAGTCTCC-3' (reverse), which yielded a 232-bp product; and beta-actin, 5'-AAAGACCTGTACGCCAACAC-3' (forward) and 5'-CTCGTCATACTCC TGCTTGG-3' (reverse), which yielded a 224-bp product. qPCR thermal cycling conditions were as follow; denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s.

RESULTS

Inhibitory effect of Fasudil on tumor growth of HNSCC cells

To examine the tumor-suppressive effect of Fasudil on HNSCC, first we inoculated female BALB/cAJcl-*nu/nu* mice with HSC-3 cells. Tumor burden under the skin was established 2 days after injection. Then, Fasudil or vehicle was administered. Nine days after the beginning of Fasudil treatment, tumor growth was significantly suppressed by Fasudil treatment (Fig. 1A, C). After 15 days, all mice were weighed and killed. The weight of tumors from mice treated with Fasudil was significantly less than that of the vehicle treated tumors (Fig. 1D). Meanwhile, we observed peritumoral vascularization and found that Fasudil treatment suppressed the neovascularization (Fig. 1B).

Fasudil stimulated BRAK secretion in HNSCC cells
Cultured HSC-3 cells were treated with Fasudil at doses of 5–50 μ M and cultured in serum-containing medium. After 24 h, BRAK secretion into the medium was determined by performing an ELISA. BRAK secretion increased with an increase in the dose of Fasudil (Fig. 2A). The level of BRAK protein in the cell layer also increased with increase in dose (Fig. 2B). The amount of total (BRAK in the cell layer and that secreted into the medium) BRAK protein increased at the dose of 25 μ M of Fasudil or higher (Fig. 2C). The BRAK secretion rate was calculated as the ratio of secreted to cell-layer BRAK protein, and this rate increased when the cells treated with 25 μ M of Fasudil or higher (Fig. 2D).

Fasudil increased mRNA levels of BRAK in HNSCC cells

Total BRAK protein levels increased with Fasudil treatment (Fig. 2C). To investigate the reason for this increase, we examined the effect of Fasudil in the gene expression of BRAK at the mRNA level. After 24 h treatment of cultured HSC-3 cells treated with Fasudil, we prepared RNA from the cells and analyzed their BRAK expression by performing qPCR. The results showed that BRAK gene expression in HSC-3 cells was stimulated with an increase in the Fasudil dose (Fig. 3), thus demonstrating that Fasudil promoted BRAK mRNA levels in these cells.

Inhibition of ROCK suppressed BRAK gene expression in HNSCC cells

We assessed the effect of ROCK and RhoA on the expression of BRAK mRNA in HSC-3 cells by performing qPCR. Gene knockdown of RhoA, ROCK1, and ROCK2 by use of their respective siRNA was evaluated by comparison to the expression in control siRNA-transfected cells. In HSC-3, BRAK gene expression was induced by transfection with siRNA for RhoA, ROCK1, or ROCK2 (Fig. 4A). Similar results were obtained with HSC-2 cells derived from oral floor carcinoma and with HSC-4, tongue carcinoma cell line (Fig. 4B, C).

DISCUSSION

In this study, we showed that Fasudil, a ROCK-specific inhibitor may be used for targeted molecular therapy of HNSCC. We previously reported that Fasudil suppresses tumor growth by increasing BRAK secretion in fibrosarcoma cells (13). Fasudil also suppresses metastasis of and invasion by breast adenocarcinoma cells (35). However, the mechanism by

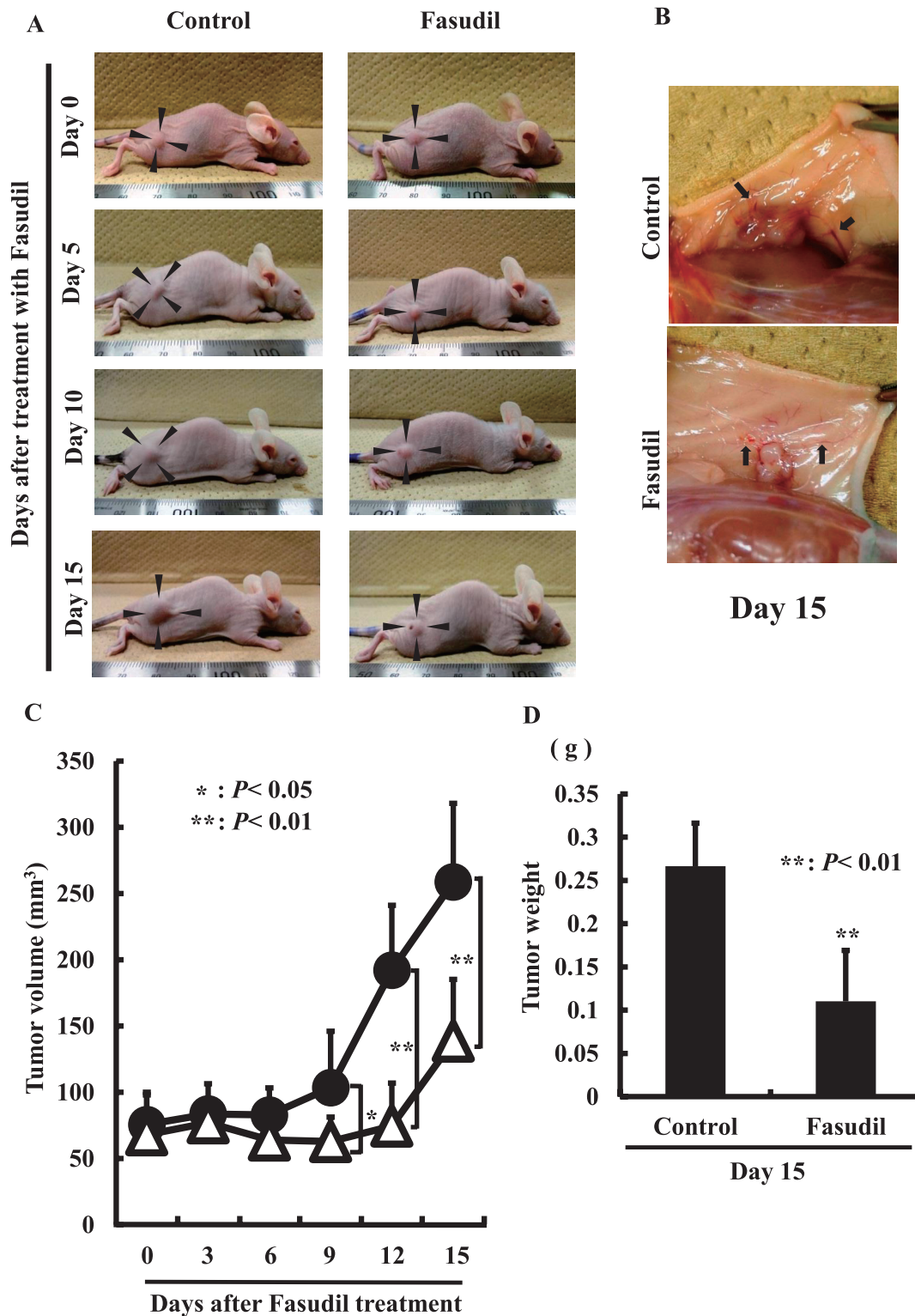


Fig. 1 Inhibition of growth of HSC-3 tumor cells by Fasudil. **(A)** Representative photographs of Fasudil- and vehicle-treated (Control) mice. Areas surrounded with arrowheads indicate the position of tumors. **(B)** Vascular structure in tumor transplants. Arrows indicate blood vessels. **(C)** Time-dependent effect of Fasudil on tumor growth. Vehicle-treated HSC-3 cells are indicated by closed circles; and those treated with Fasudil by open triangles. **(D)** Tumor weights after a 15-day treatment with Fasudil. All values are shown as mean \pm S.D. for 8 tumors. Significant differences between the vehicle and Fasudil were determined by performing Student's *t*-test * $P < 0.05$ and ** $P < 0.01$.

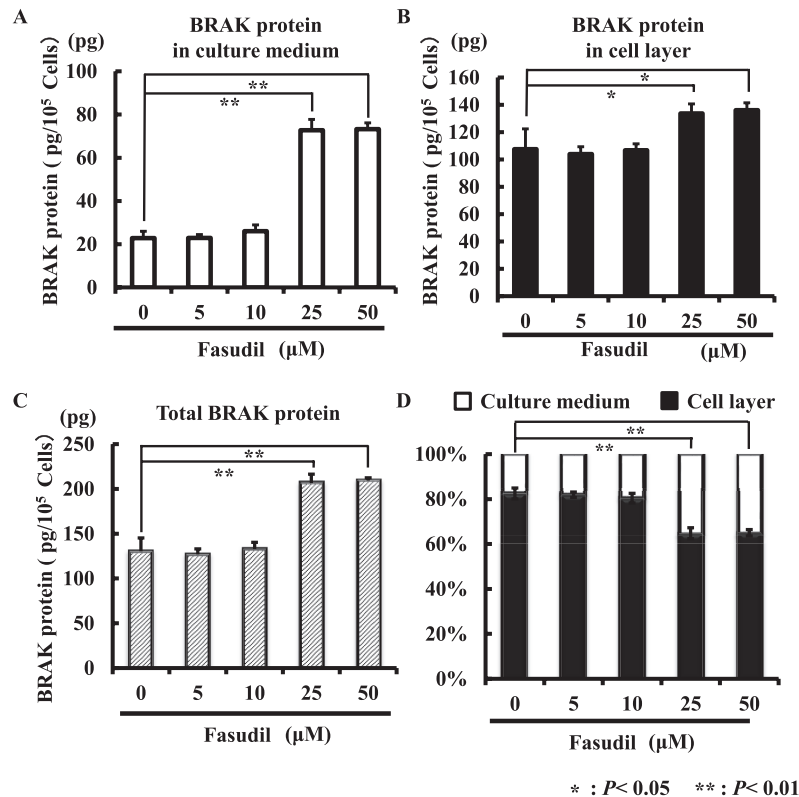


Fig. 2 Dose-dependent effects of Fasudil on the production and secretion of BRAK. HSC-3 cells (2.0×10^5 cells/35-mm dish) were treated with varying concentrations of Fasudil (0, 5, 10, 25, and 50 μM) for 24 h. (A–C) BRAK protein secreted in the culture medium (A), BRAK protein remaining in the cell layer (B), and total BRAK protein (culture medium plus cell layer (C) are shown. (D) Relative amounts of BRAK protein in the culture medium and cell layer. The columns and bars represent means \pm S.D. of triplicate assays. Significant differences between treatments were determined by performing Student's *t*-test: * $P < 0.05$ and ** $P < 0.01$.

which Fasudil suppresses tumor development has not been defined in HNSCC. We first examined the tumor-suppressive effect of Fasudil on HNSCC after injecting HSC-3 cells into the dorsolateral regions of female BALB/cAJcl-*nu/nu* mice. Fasudil suppressed HNSCC growth and peritumoral vascularization (Fig. 1A–D). We previously reported that peritumoral BRAK inhibits vascularization and suppresses tumor growth (9). These findings suggest tumor progression would be accelerated by inhibition of BRAK protein secretion by tumor cells.

To characterize the mechanism of tumor suppression by Fasudil treatment, we investigated the effect of Fasudil treatment on the secretion of BRAK protein. HSC-3 cells were treated with Fasudil and incubated in serum-containing culture medium for 24 h, and then the amounts in the cell layer and medium were determined. BRAK protein secretion from HSC-3 cells was increased by Fasudil used at a 25 μM of concentration or higher (Fig. 2A). Similarly, the levels of BRAK protein in the cell layer

and the total amount (in both cell layer and medium) were increased at dose of Fasudil 25 μM and higher (Fig. 2C). These results show that the total amounts of BRAK protein increased by Fasudil at dose of 25 μM or higher (Fig. 2D). These results suggest that Fasudil increased both the total amount of BRAK protein and secretion of BRAK protein in cell lines of HNSCC.

We previously demonstrated that the ROCK-specific inhibitor Fasudil increases BRAK secretion in fibrosarcoma cells (13); however, the effect of Fasudil on BRAK gene expression had not been characterized. We previously reported that activation of epidermal growth factor receptor (EGFR) signaling pathway suppresses BRAK gene expression in HNSCC (20–22), and others have shown that endocytosis of EGFR is inhibited by activation of RhoA/ROCK pathway (17). This might be one of the reasons that EGFR was overexpressed in several tumor cells, including HNSCC cells (10, 20, 31). Thus, we hypothesized that inhibition of RhoA/ROCK by Fasudil

may stimulate expression of the anti-tumor chemokine BRAK. Therefore, we examined BRAK gene expression in cell lines of HNSCC treated with Fa-

sudil and found that HSC-3 cells were induced amount of BRAK at the mRNA level in the presence of Fasudil (Fig. 3).

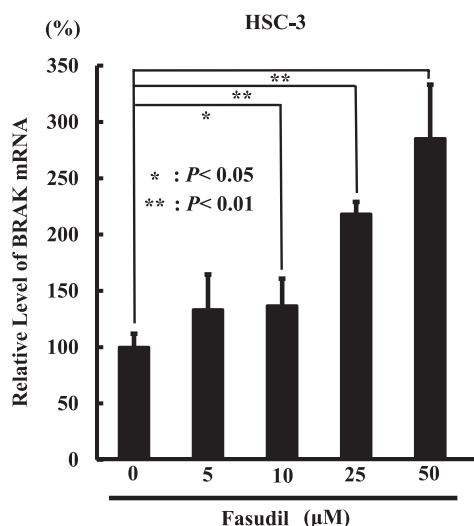


Fig. 3 Effect of Fasudil concentration on BRAK gene expression. HSC-3 cells (2.0×10^5 cells/35 mm dish) were treated with varying concentrations of Fasudil (0, 5, 10, 25, and 50 μ M) for 24 h. Levels of BRAK mRNA per GAPDH mRNA (endogenous standard) were calculated, and the value obtained from the cultured cells without Fasudil was set 100%. Columns and bars represent means \pm S.D. of triplicate assays. Significant differences were determined by performing Student's *t*-test * $P < 0.05$ and ** $P < 0.01$.

The results suggest that ROCK and RhoA regulate BRAK gene expression in cell lines of HNSCC (Fig. 3). Therefore, we examined BRAK gene expression in cells transfected with siRNAs for ROCK1, ROCK2, and RhoA. In HSC-3 cells, BRAK gene expression was elevated by suppressing expression of RhoA, ROCK1, and ROCK2 (Fig. 4A). Similar results were obtained with HSC-2 and HSC-4 cells (Fig. 4B, C). Fasudil is known to suppress activation of ROCK1 and ROCK2 in several tumor cells (14). Our results show that BRAK gene expression was induced by siRNA-mediated inhibition of the RhoA/ROCK pathway.

Tumor progression has multiple stages and depends on the balance of tumor suppressing and tumor-promoting genes (5, 25, 32). Overexpression of the RhoA/ROCK pathway occurs and is involved in invasion by and metastasis of several types of tumor cells (16, 19). Our present study showed that inhibition of the RhoA/ROCK pathway suppressed tumor progression by increasing the expression of the anti-tumor chemokine BRAK gene and the level of its secreted protein. In Japan, Fasudil is used for the clinical treatment of cerebral vasospasms after subarachnoid hemorrhage, and its safety has been confirmed in more than 1,400 patients (3, 6), with no

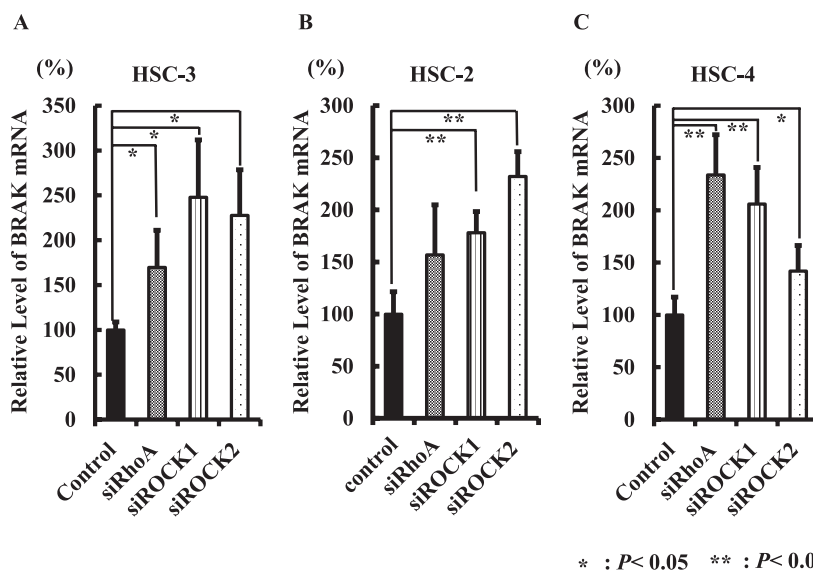


Fig. 4 Stimulation of BRAK expression by siRNA treatment. HNSCC cells (2.0×10^5 cells/35 mm dish) were inoculated into culture dishes containing DMEM-10, cultured for 12 h, and then transfected with siRNAs for RhoA, ROCK1, ROCK2, or control siRNA for 24 h. BRAK gene expression in HSC-3 cells (A), HSC-2 cells (B) and HSC-4 cells (C) are shown. Columns and bars represent means \pm S.D. of triplicate assays. Significant differences were assessed by performing Student's *t*-test * $P < 0.05$ and ** $P < 0.01$.

reported drug-associated deaths (30). These reports support the potential clinical utility of Fasudil in HNSCC.

In conclusion, the ROCK-specific inhibitor Fasudil promoted BRAK gene expression and protein secretion in HNSCC cells. Furthermore, Fasudil showed anti-tumor activity via angiostatic activity *in vivo*. These results suggest that Fasudil may offer therapeutic benefit via targeted RhoA/ROCK signaling in HNSCC. In the future, Fasudil can be used as a molecular target drug that has only a few side effects for the treatment of HNSCC. Further studies will define the mechanism of the anti-tumorigenic activity of Fasudil.

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Conflict of interest

The authors have no conflict of interest to report. Although the study was supported by external grants, there was no direct benefit (commercial/non-commercial) to the sponsors.

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