Full Paper

Fasudil Suppresses Fibrosarcoma Growth by Stimulating Secretion of the Chemokine CXCL14/BRAK

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Abstract. We previously reported that chemokine CXCL14/BRAK (BRAK) has antitumor activity in several carcinoma cells indicating that BRAK secretion suppresses carcinoma cells. Ras-homologous small GTPase (RhoA) and Rho-associated coiled-coil-containing protein kinase (ROCK) are important regulators of secretory processes, and activation of the RhoA/ROCK signaling pathway stimulates tumor invasion and metastasis. We investigated the effects of fasudil, a specific ROCK inhibitor, on BRAK secretion and tumor progression in mesenchymal fibrosarcoma cells (MC57). We demonstrated the antitumor activity of secreted BRAK using MC57 transplantation of BRAK in overexpressed transgenic mice. Further, to eliminate the influence of change in the mRNA expression of endogenous BRAK, we produced stable MC57 cell lines expressing BRAK (MC57-BRAK) or mock vector (MC57-MOCK). Fasudil significantly increased BRAK secretion by MC57-BRAK cells in a dose-dependent manner. To determine the effect of fasudil on tumor growth, MC57-BRAK and MC57-MOCK cells were transplanted into wild-type mice. Fasudil treatment suppressed tumor growth only in mice that had received MC57-BRAK cell transplants. These results indicate that fasudil inhibits fibrosarcoma growth by stimulating BRAK secretion and suggests that fasudil therapy might have clinical efficacy.

[Supplementary Figures: available only at <http://dx.doi.org/10.1254/jphs.12177FP>]

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Introduction

The chemokine CXCL14/BRAK (BRAK) was first found to be expressed in breast and kidney tissues, and thus it is called as BRAK (1). Later, it was reported to attract B cells, monocytes, and dendritic cells to tumor tissues (2 – 4). BRAK also has anti-angiogenic activity, stimulates migration of natural killer cells, and induces apoptosis (5 – 7). In general, BRAK mRNA expression is abundant in normal tissues but is less or absent in certain carcinoma cell lines (1). We previously reported

that BRAK suppressed growth of xenografted human head and neck squamous carcinoma (HNSCC) cells in athymic nude mice or SCID mice when the cells had been transfected with BRAK expression vectors (8 – 10). We also demonstrated that although BRAK is a secretory protein that possesses an N-terminal signal peptide sequence, secretion of BRAK protein was suppressed in carcinoma cells (1, 11). Therefore, it might be possible that the stimulation of BRAK secretion may induce tumor suppression.

Ras-homologous small GTPase (RhoA) is a member of the small GTPase family that controls cell adhesion and motility through reorganization of the actin cytoskeleton and regulation of actomyosin contractility (12). Rho-associated coiled-coil-containing protein kinases (ROCKs) are downstream targets of RhoA (13) and also

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participate in stress fiber formation as well as tumor growth and invasion (14). In various tumors, overexpression of Rho kinases leads to uncontrolled activation of the RhoA/ROCK signaling pathway (15). Vesicles containing secretory proteins are transported to release sites along microtubules and actin filaments, which are regulated by downstream molecules of the RhoA/ROCK signaling pathway (16). These findings suggest that the RhoA/ROCK signaling pathway plays a pivotal role in the regulation of the intracellular trafficking of secretory proteins in tumor cells. Further, the uncontrolled activation of the RhoA/ROCK signaling pathway may also induce suppression of the antitumor chemokine BRAK secretion in the tumor cells.

Fasudil (1-[5-isoquinolinesulfonyl]-homopiperazine) is a specific ROCK inhibitor, that has been proved to modify myosin light chain phosphorylation in smooth muscle cells. It is widely used in the clinical treatment of cerebral vasospasms that occur after subarachnoid hemorrhage and associated cerebral ischemic symptoms (17, 18). In addition, fasudil also inhibits tumor proliferation, metastasis, and invasion in several tumor cells (17, 19, 20). However, there are no studies that report the effect of fasudil on the secretion pathway of BRAK. Thus, we hypothesized that inhibition of the RhoA/ROCK signaling pathway using fasudil may stimulate the secretion of antitumor chemokine BRAK. Therefore, we investigated the effects of fasudil on BRAK secretion and tumor progression via inhibition of the RhoA/ROCK signaling pathway in fibrosarcoma cells in this study.

Materials and Methods

Materials and animals

C57BL/6 mice (Clea Japan, Tokyo) overexpressing the BRAK gene under the control of a β -actin promoter and CMV enhancer were generated as described previously (4). The procedures used in this study were 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 Institutional Animal Care Committees (Kanagawa Dental College, Yokosuka).

Fasudil (5-(1,4-diazepane-1-sulfonyl) isoquinoline) purchased from Tocris Bioscience (Bristol, UK) was used in the *in vitro* experiments, and fasudil hydrochloride obtained from Asahi Kasei Pharma Co. (Tokyo) was used in the *in vivo* experiments. Other reagents and their sources were as follows: bovine serum albumin, HEPES, trypsin, gentamicin, and EDTA (Wako Pure Chemical Industries, Osaka); Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku, Tokyo); fungizone (Gibco, San Diego, CA, USA); pTARGET Mammalian Expres-

sion Vector System (Promega, Fitchburg, WI, USA); FuGene 6 (Roche Applied Science, Indianapolis, IN, USA); RhoA G-LISA™ Activation Assay kit (Cytoskeleton, Inc., Denver, CO, USA); TetraColor One (Seikagaku Biobusiness Co., Tokyo); fetal bovine serum (Trance Scientific, Melbourne, Australia); fungizone, Super Script II reverse transcriptase, TRIzol total RNA isolation reagent, SuperScript First-strand Synthesis system, pTracer-CMV Vector, and Zeocin (Invitrogen, Carlsbad, CA, USA); PrimeSTAR DNA polymerase and EX Taq polymerase (Takara Bio, Inc., Otsu); SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA); CXCL14 Duo Set ELISA Development System kits (R&D Systems, Abington, UK); QuantaBlu Fluorogenic Peroxidase Substrate Kit (Pierce, Rockford, IL, USA); Lumi-Light Western blotting substrate (Roche Diagnostics, Mannheim, Germany); and physiological saline (Otsuka Pharmaceutical, Tokyo).

Cells and cell cultures

Methylcholanthrene-induced fibrosarcoma (MC57) cells were obtained from Riken Bioresource Center (Ibaraki). Mouse dermal fibroblast cells were obtained from DS Pharma Biomedical (Osaka). The cells were cultured with DMEM in the presence of 50 μ g/mL gentamicin sulfate, 250 ng/mL Fungizone, 12.6 mM HEPES, and 10% FBS at 37°C under 5% CO₂, and they were subcultured following treatment with 0.25% trypsin. The cells were used within 3 to 4 passages and counted using a Coulter Counter from Beckman Coulter (Brea, CA, USA).

Construction and transfection of the BRAK expression vector and cloning of BRAK-expressing and control empty (MOCK) vector-transfected cells

The full-length cDNA of human BRAK was cloned using a TA cloning strategy with the pTARGET Mammalian Expression Vector System to obtain pCL-BRAK as described previously (8). We used the human gene instead of the mouse gene because the amino acid sequences of both species are quite similar (two homologous substitutions out of 77 amino acids) and no difference in BRAK function was observed (21). However, its RNA sequences were easily differentiated by RT-PCR; full-length BRAK cDNA was isolated from pCL-BRAK vectors by digestion with *Eco*RI restriction endonuclease and ligated into the pTracer expression vector that had been cut with the same enzyme to create pTracer-BRAK. The sequence and sense direction of the inserted BRAK gene were verified by DNA sequencing. MC57 cells (2.0×10^5) were transfected with pTracer-BRAK or a MOCK vector using FuGene 6, and stable transformants were selected by culturing the cells in DMEM-10 con-

taining 400 $\mu\text{g/mL}$ ZeocinTM until all non-transfected cells had died. Cells that survived after this treatment were used as MOCK-MC57 cells. BRAK protein expression from the transfected pTracer-BRAK vector was detected by western blotting using anti-BRAK monoclonal antibodies as described previously (8). BRAK-expressing cells were cloned according to the limiting dilution method, and BRAK expression was confirmed by RT-PCR and western blotting.

Assay for RhoA activation

RhoA activation assays were performed with the RhoA G-LISATM Activation Assay kit according to the manufacturer's instructions.

Protein quantification

Cells were isolated with RIPA (Radio-Immunoprecipitation Assay) buffer, and the culture media was collected. BRAK protein levels in the media were quantified using the CXCL14 Duo Set ELISA Development System kits following the manufacturer's instructions, with minor modifications (4).

Western blotting

Sample solutions were heated for 10 min at 100°C prior to electrophoresis through 4%–20% gradient polyacrylamide gels. Proteins were transferred onto PVDF membranes using the iBlot dry blotting system at a constant voltage of 20 V for 6 min. The blots were blocked with 3% non-fat dry milk in Tris-buffered saline (TBS) with 0.05% Tween 20 for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C in a blocking solution of bovine serum albumin in TBS with 0.05% Tween 20. Both the p-cofilin (phosphorylated cofilin) and cofilin primary antibodies were diluted 1:200. Anti-rabbit horseradish peroxidase-linked secondary antibodies were diluted 1:1000 in blocking solution (3% non-fat dry milk in TBS containing 0.05% Tween 20). The blots were washed and incubated with secondary antibodies for 1 h at room temperature in the blocking solution. Immunoreactive bands were visualized using the Lumi-Light Western blotting substrate.

Tumor growth in vivo

The dorsolateral regions of BRAK transgenic (TG) mice and wild-type (WT) mice were shaved using an electric shaver and depilation mousse was applied to completely remove all of the body hair. MC57 cells (7.5×10^6 cells/site) were subcutaneously injected into both sides of the dorsolateral regions of 6 BRAK TG mice and 6 WT mice. Tumor burden was established under the skin 4 days after the injection of tumor cells, and tumor volume measurements were begun. MC57-

BRAK or MC57-MOCK cells (7.5×10^6 cells/site) were subcutaneously injected into both sides of the dorsolateral regions of 14 female C57BL / 6 mice / group. When the tumor burden had been established under the skin (7 days after the injection of tumor cells), fasudil (50 mg/kg per day, i.p) diluted in physiological saline or vehicle was administered to the tumor-bearing mice. 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.

Reverse-transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR analysis

Total cellular RNA was extracted using the TRIzol total RNA isolation reagent following the manufacturer's instructions and was analyzed for BRAK, BMAC (mouse CXCL14), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression by RT-PCR. An aliquot of each sample was denatured and reverse transcribed using a SuperScript First-Strand Synthesis system according to the manufacturer's protocols. PCR amplification was performed on the RT reaction product using primers and 0.5 units of EX Taq polymerase. The primer sets used were as follows: BRAK (192 bp), 5'-ACG GGTCCAAATGCAAGTGC-3' (forward) and 5'-TGA TGAAGCGCTTGCTGCTC-3' (reverse); BMAC (208 bp), 5'-TGAAGCTCTTCTTACCAGGG-3' (forward) and 5'-CATCTGGCAACCCTACAACA-3' (reverse). GAPDH mRNA was co-amplified as an internal control [176 bp, 5'-CATGGCCTTCCGTGTTCTTA-3' (forward) and 5'-TGCTGTTGAAGTCGCAGGAG-3' (reverse)]. The PCR products were separated by 2% agarose gel electrophoresis and visualized using ethidium bromide staining as reported previously (11). Brilliant SYBR Green qPCR Master Mix was used for quantitative PCR.

Cell viability assay

MC57-MOCK and MC57-BRAK cells were seeded (4×10^3 cells/well) in 96-well plates. Nearly confluent cells were serum starved overnight and cultured in DMEM-0 with varying concentrations of fasudil (0–500 μM) for 24 h. For the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, TetraColor One, a water-soluble derivative of MTT, was reduced to a formazan product by active mitochondrial enzymes in living cells. After adding 10 μL of the dye solution to each well, the plates were incubated for 120 min at 37°C, and a microplate reader was used to measure absorbance at 450 nm. Absorbance values were expressed as a percentage of the absorbance of untreated cells, and the fasudil concentration that resulted in 50% growth inhibition (IC_{50}) was calculated.

Statistical analyses

Results are expressed as the mean $\pm$ standard deviation (S.D.). Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA). *P*-values less than 0.05 were considered to be statistically significant.

Results

Growth suppression of mouse fibrosarcoma cell allograft in BRAK TG mice

In order to investigate the antitumor activity of secreted BRAK, mouse fibrosarcoma cells (MC57) (22) were injected to both sides of the dorsolateral regions of BRAK TG and WT mice. The size of the transplanted tumors formed in BRAK TG mice were significantly smaller than those in WT C57BL/6 mice ($P < 0.05$), irrespective of the number of MC57 cells present (Fig. 1: A, B).

BRAK protein secretion is inhibited by activation of the RhoA/ROCK signaling pathway in fibrosarcoma cells

In order to eliminate the influence of change in the mRNA expression of endogenous BRAK, we developed a stable cell line expressing BRAK (MC57-BRAK) and a control cell line (MC57-MOCK) by transfecting BRAK-expressing or MOCK vectors into MC57 cells that were not expressing endogenous BRAK protein. Furthermore, we performed RT-PCR to measure the gene expressions of human BRAK/CXC14, mouse BMAC/cxc14, found as the mouse orthologue of human BRAK (23), and mouse GAPDH in normal mouse fibroblasts (mFBs) among MC57, MC57-MOCK, and MC57-BRAK

cells (Supplementary Fig. 1A: available in the online version only). In addition, we measured RhoA activation levels in mouse fibroblasts (mFB), parent MC57 cells, and the engineered MC57 cell lines (MC57-MOCK and MC57-BRAK) using the G-LISA assay. Relative RhoA activation levels in MC57, MC57-MOCK, and MC57-BRAK were significantly higher than those in mFBs. No significant difference in the RhoA activation was observed among MC57, MC57-MOCK, and MC57-BRAK (Supplementary Fig. 1B). To investigate the relationship between RhoA activation levels and the amount of secreted BRAK in MC57-MOCK and MC57-BRAK cells, we determined RhoA activity in the presence of serum because serum contains lysophosphatidic acid (LPA), a major activator of the RhoA/ROCK signaling pathway (24, 25). The relative levels of RhoA activities in MC57-MOCK and MC57-BRAK cells were significantly increased under serum-containing culture conditions (Fig. 2: A, B). The amount of BRAK protein in the culture medium of MC57-BRAK cells was measured after serum treatment, and the levels of secreted BRAK protein in the culture medium were significantly decreased under serum-containing culture conditions compared to those from cells cultured under serum-free conditions (Fig. 2C). No significant change in the levels of BRAK mRNA expression by MC57-BRAK cells was observed under serum-containing culture conditions (Supplementary Fig. 2A: available in the online version only).

Dose-dependent effect of fasudil treatment on the secretion of BRAK protein in mouse fibrosarcoma cells

MC57-BRAK cells were treated with fasudil and cultured under serum-containing culture conditions to deter-

Fig. 1. Difference in tumor growth rate between MC57 cells transplanted into BRAK transgenic (TG) mice and wild-type (WT) mice. A: Representative photographs of tumors (indicated by arrow heads) 18 days after transplantation of MC57 cells. B: Tumor volumes in WT mice (open circles) and BRAK TG mice (closed circles). The circles represent the mean of six tumors each and the bars, the standard deviation. Significant differences TG vs. WT mice according to Student's *t*-test are indicated by * $P < 0.05$ or ** $P < 0.01$.

Fig. 2. Activation of RhoA regulates secretion of BRAK protein in MC57-BRAK cells. After serum starvation, MC57-MOCK (A) and MC57-BRAK (B) cells were cultured in DMEM-0 (control) or DMEM-10 (10% serum) for 15 min. The RhoA activation level was determined by G-LISA™. C: After serum starvation, MC57-BRAK cells were cultured in DMEM-10 for 24 h. The levels of secreted BRAK protein were quantified using CXCL14 Duo Set ELISA Development System kits. The columns and bars represent means $\pm$ S.D. in triplicate assays. * $P < 0.05$, significantly different vs. control, (Student's *t*-test).

mine the amount of BRAK protein that had been secreted into the culture medium. An ELISA assay showed that the levels of BRAK secreted from MC57-BRAK cells were increased by fasudil treatment in a dose-dependent manner (Fig. 3A). In addition, no significant change in BRAK mRNA expression was observed after addition of fasudil to MC57-BRAK cell cultures (Supplementary Fig. 2A). The amount of secreted BRAK increased after 12 h of fasudil treatment and exhibited a further time-dependent increase for up to 24 h (Fig. 3B). The IC_{50}

values of MC57-BRAK and MC57-MOCK cells were determined by the MTT assay after 24 h of fasudil treatment. The average IC_{50} values of fasudil were $398 \pm 24 \mu M$ in MC57-BRAK cells and $401 \pm 19 \mu M$ in MC57-MOCK cells (Supplementary Fig. 2B). No significant difference was observed in cell proliferation between MC57-BRAK and MC57-MOCK cells in the in vitro culture conditions (Supplementary Fig. 2B). To confirm the inhibitory effect of fasudil on the RhoA/ROCK signaling pathway, we used western blotting to evaluate the dephosphorylation of cofilin, a downstream target molecule of the RhoA/ROCK signaling pathway in MC57-BRAK cells. Cofilin dephosphorylation was inhibited 15 min after fasudil treatment, with a further time-dependent increase in inhibition up to 360 min (Fig. 3C).

Inhibitory effect of fasudil on tumor growth of BRAK-expressing fibrosarcoma cells

Because fasudil stimulated the secretion of the anti-tumor chemokine BRAK in fibrosarcoma cells, we hypothesized that a pharmacological agent that induces BRAK secretion might exhibit BRAK-mediated antitumor activity. To further clarify the effect of fasudil on tumor growth, MC57-BRAK and MC57-MOCK cells were subcutaneously inoculated into both sides of the dorsolateral regions of 14 female WT mice. Half of the mice were administered fasudil on a daily basis (50 mg/kg per day, i.p.). The growth of BRAK-expressing MC57-BRAK cells was significantly suppressed in these mice compared with that of the MC57-MOCK cells, which had been transfected only with a MOCK vector. Fasudil administration further reduced tumor growth in mice that had received MC57-BRAK cell transplants, while no significant effect on tumor growth was observed in mice that received MC57-MOCK cell transplants (Fig. 4: A, B).

Discussion

Tumors develop in multiple steps (26 – 28), and tumor progression is dependent on the balance between expression of genes that promote tumor progression and genes that suppress tumor progression (29, 30). RhoA/ROCK signaling, which is also involved in tumor growth and invasion, has received significant attention as a potential target for the treatment of a wide range of pathological conditions including tumors (31, 32). In the present study, we demonstrated that inhibition of uncontrolled activation of the RhoA/ROCK signaling by fasudil treatment stimulates the secretion of the antitumor chemokine BRAK and suppresses the growth of mouse fibrosarcomas in vivo.

First, we investigated the effects of secreted BRAK on

Fig. 3. Effect of fasudil treatment on BRAK secretion. **A:** Dose-dependent effect of fasudil treatment on BRAK secretion and cofilin phosphorylation. MC57-BRAK cells were treated with varying concentrations of fasudil (0, 5, 10, 25, and 50 μM) for 24 h. **B:** Time-dependent effect of fasudil treatment on BRAK secretion (0, 6, 12, and 24 h). The levels of secreted BRAK protein were quantified using CXCL14 Duo Set ELISA Development System kits. **C:** Dose-dependent effect of fasudil treatment on dephosphorylation of cofilin. MC57-BRAK cells were treated with fasudil (25 μM) for varying incubation times (0, 15, 30, 60, 180, and 360 min). The columns and bars represent means $\pm$ S.D. in triplicate assays. Significant differences TG vs. WT mice according to Student's *t*-test are indicated by * $P < 0.05$ or ** $P < 0.01$.

tumor growth of fibrosarcoma cells. We then produced BRAK TG mice that express BRAK from a vector containing a β -actin promoter and CMV enhancer. These TG mice expressed the BRAK gene ubiquitously, and the level of BRAK protein in their blood (10 ng/mL blood plasma) was 10 times higher than in WT C57BL/6 mice (1 ng/mL blood plasma) (4). After we transplanted mesenchyme-derived MC57 sarcoma cells into BRAK-TG mice, we found that the growth of tumors was also completely suppressed (Fig. 1). This was not due to an immunological response to a new epitope that might be expressed in the MC57 cells because tumors grew very rapidly when the same cells were transplanted to WT C57BL/6 cells. These results indicate that secreted BRAK has an antitumor effect on fibrosarcoma cells.

Recently, Rho family GTPases have gained much attention because of their ability to regulate a variety of cell activities, including tumorigenesis (33, 34). For ex-

ample, RhoBTB2, an atypical Rho GTPase, plays a very important role in BRAK gene transcription in both normal and cancerous epithelial cells (35). Further, RhoA belongs to a subfamily of typical Rho GTPases (31, 32). RhoA is frequently overexpressed in clinical cancer specimens and in tumor cell lines, thereby RhoA/ROCK signaling is constitutively activated (14, 32, 36). In addition, the RhoA/ROCK signaling plays a major role in the regulation of secretory protein trafficking. To investigate the levels of endogenous RhoA activation in fibroblasts and fibrosarcoma cells, we examined RhoA activation levels in mFBs, parent MC57 cells, and engineered MC57 cell lines (MC57-MOCK and MC57-BRAK). Relative RhoA activation levels in MC57, MC57-MOCK, and MC57-BRAK were significantly higher than those in mFBs (Supplementary Fig. 1B), indicating that endogenous RhoA is constitutively activated in fibrosarcoma cells.

Fig. 4. Inhibitory effect of fasudil on tumor growth in BRAK-expressing MC57 cells. **A:** Representative photographs of mice after 5 days of treatment with fasudil or vehicle. **B:** Time-dependent effect of fasudil on the growth of tumors. MC57-MOCK cells treated with vehicle are shown as open circles, and those treated with fasudil are shown as closed circles; MC57-BRAK cells treated with vehicle are shown as open triangles, and those treated with fasudil are shown as closed triangles. All values are shown as the mean $\pm$ S.D. for 14 tumors. Significant differences vs. WT mice according to Student's *t*-test are indicated by ** $P < 0.01$ or *** $P < 0.001$.

Although BRAK is a secretory protein that possesses an N-terminal signal peptide sequence, secretion of BRAK protein was suppressed in carcinoma cells (1, 11). Further, we determined the relationship between RhoA activation levels and the amount of secreted BRAK protein in fibrosarcoma cells (MC57-MOCK and MC57-BRAK) by culturing these cells in serum-containing or serum-free media. It was well known that LPA contained in serum is a major activator of the RhoA/ROCK signaling pathway (24, 25), and it promotes tumor progression and metastasis by stimulating expression of pro-inflammatory genes in several tumor types (37, 38). RhoA activity in MC57-BRAK cells was significantly increased under serum-containing culture conditions compared

with that in controls (Fig. 2: A, B). Furthermore, when MC57-BRAK cells were cultured in the presence of serum in the growth medium, the levels of secreted BRAK protein were significantly decreased compared to that when the same cells were cultured in the absence of serum (Fig. 2C). These results indicate that the RhoA/ROCK signaling is closely associated with BRAK protein secretion.

To further investigate the effect of RhoA/ROCK signaling activation on BRAK secretion in mouse fibrosarcoma cells, we cultured MC57-BRAK cells treated with fasudil under serum-containing culture conditions. We determined the amount of secreted BRAK protein in the culture medium using an ELISA assay. The amount of

BRAK protein secreted by MC57-BRAK cells increased after the fasudil treatment in a dose-dependent manner (Fig. 3A) and the levels of secreted BRAK protein continued to increase significantly 12–24 h after fasudil treatment (Fig. 3B). These results indicate that the ROCK inhibitor fasudil stimulates BRAK secretion in MC57-BRAK cells.

Cofilin, a downstream molecule in the RhoA/ROCK signaling pathway, regulates depolymerization of actin filaments during intracellular vesicle trafficking (39–41). Furthermore, actin-based myosin may drive vesicle recruitment in regulated exocytotic pathways (15, 42–45). We also showed that cofilin dephosphorylation was inhibited 15 min after the fasudil treatment. This inhibition progressed in a time-dependent manner up to 360 min (Fig. 3C). Thus, the inhibition of cofilin dephosphorylation by fasudil treatment induces accumulation of the actin cytoskeleton and promotes the secretion of the antitumor BRAK protein using MC57-BRAK cells.

In the present study, we have demonstrated that inhibition of RhoA/ROCK signaling by fasudil treatment stimulates the secretion of the chemokine BRAK and suppresses the growth of mouse fibrosarcoma cells in vivo. Tumor growth was significantly suppressed in mice with MC57-BRAK transplants and no significant differences in tumor sizes were observed in MC57-MOCK-transplanted mice by the treatment with fasudil (Fig. 4: A, B). These results suggest that fasudil suppresses fibrosarcoma growth by stimulating the secretion of the antitumor chemokine BRAK, and inhibition of the RhoA/ROCK signaling pathway could be a useful target for antitumor therapy.

Taken together, the results of our study suggest that the specific ROCK inhibitor fasudil might be useful as an antitumor drug for the treatment of fibrosarcoma. The BRAK-expressing TG mice showed no apparent abnormalities when observed for up to 2 years of age (4). In addition, a human case has also been described in which the person had a similar level of BRAK in the blood as the TG mice without any apparent abnormalities (46). These data support the possibility that stimulation of BRAK secretion is not associated with severe side effects. Additionally, the safety of fasudil has been confirmed in more than 1400 patients with subarachnoid hemorrhage (47). Overall, it is reasonable to speculate that fasudil, which stimulates BRAK secretion, might have clinical efficacy.

In conclusion, our results indicate that fasudil, a specific inhibitor of RhoA/ROCK signaling increases the secretion of BRAK in fibrosarcoma cells. Furthermore, fasudil also shows anti-tumorigenic effects in vivo by promoting the secretion of BRAK. However, it is necessary to further study and identify the precise mechanism

of action for the anti-tumorigenic effects of fasudil. The findings of our study suggest the possible use of fasudil for the treatment of several types of tumor in combination with other chemotherapeutic drugs.

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