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Original Research Article

Strong radioprotective FGF1 signaling down-regulates proliferative and metastatic capabilities of the angiosarcoma cell line, ISOS-1, through the dual inhibition of EGFR and VEGFR pathways

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ABSTRACT

Background and purpose: Angiosarcoma is associated with a poor prognosis and is treated with radiotherapy. Although FGF1 is a potential radioprotector, the influence of FGF1 on the malignancy of angiosarcoma remains unknown.

Materials and methods: Highly stable FGF1 mutants, which exhibit stronger mitogenic activity than wild-type FGF1, were examined as strong radioprotectors and signaling agonists to clarify the effects of FGF1 on the murine angiosarcoma cell line ISOS-1.

Results: FGF1 mutants reduced colony formation by and the *in vitro* invasion and migration of ISOS-1 cells, in addition to an increase in radiosensitivity to X-rays. In contrast, an FGFR inhibitor blocked the inhibitory effects of FGF1 mutants on colony formation, invasion, and migration. siRNA targeting the *Fgfr1* gene showed that strong FGFR1 signaling reduced colony formation by ISOS-1 cells. However, the FGF1 mutant reduced the activation of VEGFRs and EGFRs in ISOS-1 cells more strongly than wild-type FGF1. Moreover, the inhibition of VEGFRs and EGFRs synergistically reduced colony formation by and invasion and migration of ISOS-1 cells.

Conclusion: These results suggest that strong FGF1 signaling exerts not only radioprotective effects, but also inhibitory effects on proliferative and metastatic capacities of angiosarcoma through the dual inhibition of EGFR and VEGFR pathways.

Angiosarcoma is a malignant tumor of vascular or lymphatic origin that accounts for 4.1% of soft tissue sarcomas, and approximately 50% of angiosarcoma occurs in the head and neck; particularly the scalp [1]. Skin-derived angiosarcoma has an extremely poor prognosis, and thus angiosarcoma of the scalp and face is regarded as one category of malignant tumors. The Japanese Dermatological Association recently published management guidelines for scalp angiosarcoma [2]. In accordance with the Japanese guidelines, combination therapy including surgery, chemotherapy, and radiotherapy is strongly recommended as a primary treatment because of the invasive and multifocal nature of angiosarcoma, which results in local recurrence and a poor prognosis. However, radiotherapy is applied at high doses (>50 Gy) and in wide treatment fields in combination with other therapies, and thus is associated with a risk of adverse reactions.

Several fibroblast growth factors (FGFs) have been found to protect against radiation-induced damage [3,4]. FGF1 may exert potent effects on radiation-induced intestinal damage [4]. However, FGF1 has poor thermal stability and a relatively short half-life *in vivo* [5]; therefore, a number of efforts have been made in order to increase its stability, such as the creation of mutants [5,6]. Among these FGF1 mutants, Q40P, S47I, H93G, and K112N have been identified as strongly stabilizing substitutions, and combinations of each single mutation were found to enhance thermal stability, with the stability of FGF1 mutants increasing in parallel with the number of mutations (Fig. 1A) [6,7]. The high stability

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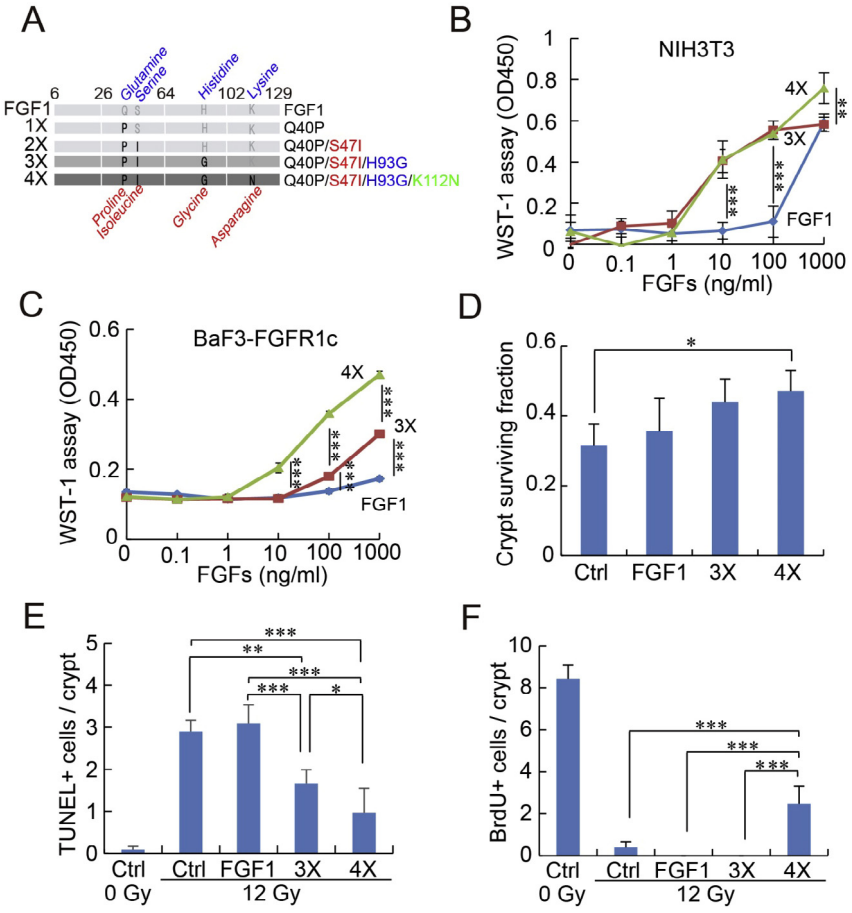

Fig. 1. The strong mitogenic activity of FGF1 mutants correlated with the level of their radioprotective effects. (A) Four stable FGF1 mutants were created by introducing multiple mutations into FGF1 (1X–4X). (B and C) NIH3T3 or BaF3-FGFR1c cells were cultured for 24 or 42 h with FGF1, 3X, or 4X at the indicated concentrations without heparin. An index of the cell number based on optical absorbance at 450 nm was obtained using WST-1 reagent. (D) Ten micrograms of each FGF without heparin was administered intraperitoneally to BALB/c mice 24 h after total body irradiation (TBI). The relative number of surviving crypts in the jejunum was assessed 3.5 days after irradiation at 10 Gy. (E and F) BALB/c mice received TBI with γ-rays at 12 Gy 24 h after the intraperitoneal administration of 100 μg of each FGF with heparin. A TUNEL and BrdU assays were performed in paraffin-embedded sections to evaluate apoptosis and proliferating cells in the crypts of the jejunum 24 h after irradiation. Values of colony formation rates are means ± SD (n = 3). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

of 3X was shown to enhance its protective effects against radiation-induced intestinal damage [8]; however, the effects of 4X, which has the highest stability, remain unknown.

Aberrant FGF signaling has been reported to promote tumor development by enhancing cell proliferation, cell survival, and tumor angiogenesis [9]; therefore, FGF radioprotectors may promote the progression and metastasis of tumors. On the other hand, FGF signaling has tumor suppressive functions under certain conditions [9]. For example, FGFR1b expression in human pancreatic cancer cells inhibited single-cell movement, *in vitro* invasion, and *in vivo* tumor formation and growth, whereas FGFR1c expression in non-malignant pancreatic ductal cells resulted in cellular transformation and *in vivo* tumor formation [10]. However, human FGF signaling is a very complex system that comprises 22 ligands and 4 transmembrane tyrosine kinase receptors (FGFR 1, 2, 3 and 4). Thus, the effects of signaling by each FGF on cancer cells need to be clarified in order to establish the appropriate clinical usage of FGF radioprotectors for cancer radiotherapy.

This study investigated the influence of FGF1 on the malignancy of an angiosarcoma cell line and demonstrated that strong FGF1 signaling inhibited the proliferative and metastatic capabilities of angiosarcoma through the dual inhibition of the EGFR and VEGFR signaling pathways.

Materials and methods

Cell line and reagents

The murine angiosarcoma cell line ISOS-1 was established from a tumor formed by the transplantation of human angiosarcoma into mice with severe combined immunodeficiency (SCID), as described previously [11], and was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS). Antibodiesand other reagents are listed in Supplementary Table 1.

Colony formation assay

A colony formation assay was performed to quantify the proliferative capability of ISOS-1 cells after exposure to ionizing radiation, FGF treatment, and growth factor receptor inhibition as described in the Supplementary materials and methods.

In vitro siRNA assay

Stealth RNAi is a type of chemically modified siRNA obtained from Invitrogen (Carlsbad, CA, USA). The synthesized oligonucleotides for the target site of each gene were as listed in Supple-

mentary Table 2. Each stealth RNAi duplex was transfected at a final concentration of 50 nM using Lipofectamine™ RNAiMAX in accordance with the manufacturer's protocol (Invitrogen).

Invasion and migration assays

The invasive and migration capabilities of ISOS-1 cells were examined using Transwell chambers containing a 6.5-mm filter with a pore size of 8 μm (Corning, Horseheads, NY, USA), as described previously [12] (Supplementary materials and methods).

Quantitative RT-PCR assay

The amount of each transcript in ISOS-1 cells was measured by a quantitative RT-PCR assay using LightCycler 480 (Roche Diagnostics, Mannheim, Germany) (Supplementary materials and methods). TaqMan probes used for the measurement of each transcript were as listed in Supplementary Table 3.

Irradiation

The cells were irradiated with X-rays using the X-ray generator Pantak HF-320S (Shimazu, Kyoto, Japan) at a dose rate of approximately 2.4 Gy/min.

Statistical analysis

All values represent the mean ± standard deviation of results obtained from more than 3 samples in each group, and values were compared using ANOVA and Fisher's protected least significant difference ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$).

Results

The strong mitogenic activity of FGF1 mutants correlated with the level of their radioprotective effects

Constructs of FGF1 mutants were created as described in Fig. 1A. The stability of FGF1 mutants increased in parallel with the number of mutations; therefore, Q40P/S47I/H93G/K112N (4X) was the most stable FGF1 [6,7]. His-tagged recombinant wild-type FGF1 and its mutant proteins were produced as described in Supplementary materials and methods [8]. In order to assess the *in vitro* mitogenic activity of recombinant FGF1 mutants, the proliferation of the mouse embryonic fibroblast cell line NIH3T3 was examined using WST-1 reagent 24 h after the culture with wild-type FGF1, 3X, or 4X in the absence of heparin (Fig. 1B). The mitogenic activities of 3X and 4X were significantly stronger than that of FGF1; however, 3X and 4X exhibited similar activities at less than 100 ng/ml (Fig. 1B). Therefore, BaF3 transfectants expressing FGFR1c (BaF3-FGFR1c) were used to estimate differences in mitogenic activities because this transfectant had high sensitivity and resolution for the reactivity of FGF with FGFR1c. [8]. As a result, the mitogenic activity of 4X was at least 10 times stronger than 3X, and 100-fold stronger than FGF1 (Fig. 1C), which was consistent with previous findings showing that the activity of FGF1 mutants increased in parallel with their structural stability [6–8]. Furthermore, the *in vivo* radioprotective effects of recombinant FGF1 mutants in the jejunum of BALB/c mice were examined using crypt, TUNEL, and BrdU assays (Supplementary materials and methods). Although FGF1 did not significantly increase crypt survival, the 3X or 4X treatment was effective for crypt regeneration, and 4X increased the number of crypts significantly more than 3X (Fig. 1D). In addition, 3X and 4X significantly inhibited radiation-induced apoptosis in the crypts 24 h after irradiation, with the level of apoptosis declining to 59% and 32%, respectively, of that in non-treated crypts (Fig. 1E). Moreover, the incorporation of BrdU into crypts was only detected in 4X-treated mice 24 h after irradiation, suggesting that 4X promoted cell proliferation in crypts, leading to enhanced tissue regeneration (Fig. 1F).

FGF1 mutants diminished the proliferative capability of the murine angiosarcoma cell line ISOS-1 and increased its radiosensitivity

In order to evaluate the effects of FGF1 mutants on the murine angiosarcoma cell line ISOS-1 through FGFR signaling pathways, the phosphorylation of Erk1/2 was examined using a Western blot analysis (Supplementary materials and methods). The normalized phosphorylation of Erk1/2 increased in ISOS-1 cells 1 h after the incubation with FGF1, 3X, or 4X; however, the activation of Erk1/2 by FGF1 decreased to control levels 6 h after the culture (Fig. 2A). In contrast, the phosphorylation of Erk1/2 by 3X or 4X was maintained until 24 h after the culture. The normalized phosphorylation of Erk1/2 increased in parallel with the activity of FGF1 mutants 24 h after the incubation, and 4X was the strongest activator of Erk1/2 in ISOS-1 cells (Fig. 2A), suggesting that FGF1 mutants enhance the signaling pathway through FGFRs in ISOS-1 cells. However, neither the wild-type nor the FGF1 mutants, 3X and 4X, increased the proliferation rate of ISOS-1 cells (Supplementary Fig. 1). In contrast, FGF1 mutants reduced colony formation by ISOS-1 cells in the order of their mitogenic activity (Fig. 2B and C). Thus, the strong mitogenic activity of FGF1 mutants actually decreased the proliferative capability of ISOS-1 cells. FGF1 mutants also increased the radiosensitivity of ISOS-1 cells. Survival curves for ISOS-1 cells derived from colony assays after X-ray irradiation were shifted downward by the 3X or 4X treatment (Fig. 2D). Moreover, 3X and 4X reduced D_{10} values, which were the radiation doses needed to reduce colony numbers to 10%, leading to the enhanced radiosensitivity of ISOS-1 cells (Fig. 2E).

Strong FGF1 signaling through FGFR1 decreased the proliferative capability of ISOS-1 cells

The transcript levels of Fgf receptors (*Fgfr*) in ISOS-1 cells were examined in angiosarcoma cells using quantitative RT-PCR to assess *Fgfr* expression profiles. ISOS-1 cells expressed extremely large amounts of *Fgfr1* transcripts, at least 10-fold more than other *Fgfrs* (Fig. 3A). *Fgfr2c* transcript levels were similar to those of *Fgfr3*, and ISOS-1 cells expressed low *Fgfr4* transcript levels; however, *Fgfr2b* was not expressed in ISOS-1 cells. In contrast, ISOS-1 cells expressed a number of *FGFs* such as *Fgf1*, *Fgf2*, *Fgf7*, *Fgf10*, *Fgf18*, and *Fgf22* (Fig. 3B). *Fgf1*, *Fgf7*, and *Fgf10* were abundantly expressed in ISOS-1 cells; however, FGF7 and FGF10 did not react with ISOS-1 cells because they did not express FGFR2b, to which FGF7, FGF10, and FGF22 specifically bind. On the other hand, FGF1 activated all of the FGFR subtypes including FGFR1; therefore, FGF1 may regulate ISOS-1 cells by an internal autocrine loop mechanism and FGF1 mutants may show the effectiveness of strong agonist against ISOS-1 cells. The pan-FGFR inhibitor, AZD4547, completely blocked the suppression of colony formation by 3X or 4X (Fig. 3C), suggesting that FGF1 mutants inhibited the proliferative capability of ISOS-1 cells through FGFRs. Moreover, siRNA-mediated *Fgfr1* repression canceled the inhibitory effects of 4X for colony formation (Fig. 3D and E); therefore, 4X suppressed the proliferative capability of ISOS-1 cells through FGFR1. The siRNA-mediated repression of *Fgfr1* by itself increased colony formation by ISOS-1 cells, whereas that of *Fgfr2* reduced it without the 4X treatment (Fig. 3D). In addition, the inhibition of FGFR2 increased FGFR1 expression (Fig. 3E). Therefore, FGFR2 signaling may support the survival of ISOS-1 cells through the down-regulation of FGFR1 expression. These results suggested that FGFR1 played a critical

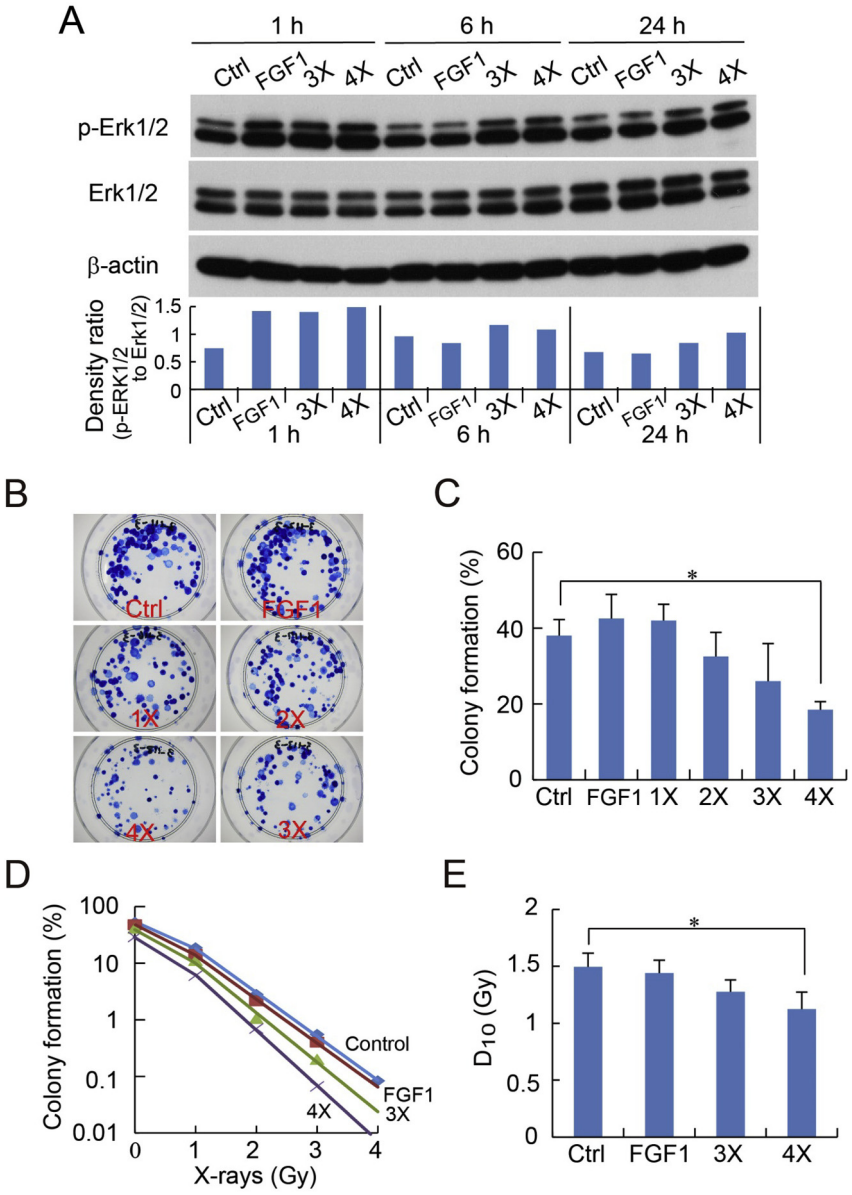

Fig. 2. FGF1 mutants diminished the proliferative capability of the murine angiosarcoma cell line ISOS-1 and increased its radiosensitivity. (A) ISOS-1 cells were incubated for 1, 6, or 24 h with 100 ng/ml of FGF1, 3X, or 4X, and Erk1/2 and its phosphorylation in ISOS-1 cells were then examined by a Western blot analysis. The density of p-Erk1/2 was divided by that of Erk1/2 for normalization. (B and C) The proliferative capability of ISOS-1 cells was examined 8 days after the culture with 100 ng/ml FGF mutants by colony assay. (D) Survival curves for ISOS-1 cells irradiated with X-rays were assessed by colony assay 8 days after the culture with 100 ng/ml FGF mutants. (E) D_{10} values, the radiation dose needed to reduce the colony number to 10%, were obtained by plotting on radiation survival curves. All values are means ± SD (n = 3). $^{*}P < .05$.

role in the anti-proliferative effects of strong FGF1 signaling on ISOS-1 cells.

Strong FGF1 signaling inhibited the in vitro invasion and migration of ISOS-1 cells

Several growth factors such as FGFs have the potential to cause the metastasis of malignant tumors. In order to estimate the involvement of FGF1 mutants in the metastasis of angiosarcoma, the *in vitro* invasiveness of ISOS-1 was examined using invasion assay after the culture with FGF1 mutants. 4X significantly reduced the number of ISOS-1 cells, which invaded through Matrigel-coated membranes, although wild-type FGF1 slightly decreased the number of invaded cells (Fig. 4A). The decrease observed in the *in vitro* invasion by FGF1 mutants was suppressed by the FGFR inhibitor, thereby restoring the invasiveness of ISOS-1 cells (Fig. 4B). Moreover, the migrated number of ISOS-1 cells was decreased by 4X treatment; however, FGF1 did not reduce the migrated cell number (Fig. 4C). Migration speed was also decreased by 4X, but not by FGF1 (Fig. 4D). The 4X-induced inhibition of the migration number and speed of ISOS-1 cells were canceled by the FGFR inhibitor (Fig. 4C and D). These results suggest that FGF1 signaling does not enhance the *in vitro* invasion and migration of ISOS-1 cells, but strong FGF1 signaling through FGFR inhibits the *in vitro* metastatic capabilities of angiosarcoma cells.

Strong FGF1 signaling suppressed the activation of VEGFR and EGFR, resulting in a decrease in the proliferative and metastatic capabilities of ISOS-1 cells

Angiogenesis or growth factors are suspected to be a key driver of angiosarcoma. Therefore, the expression profile of the phospho-

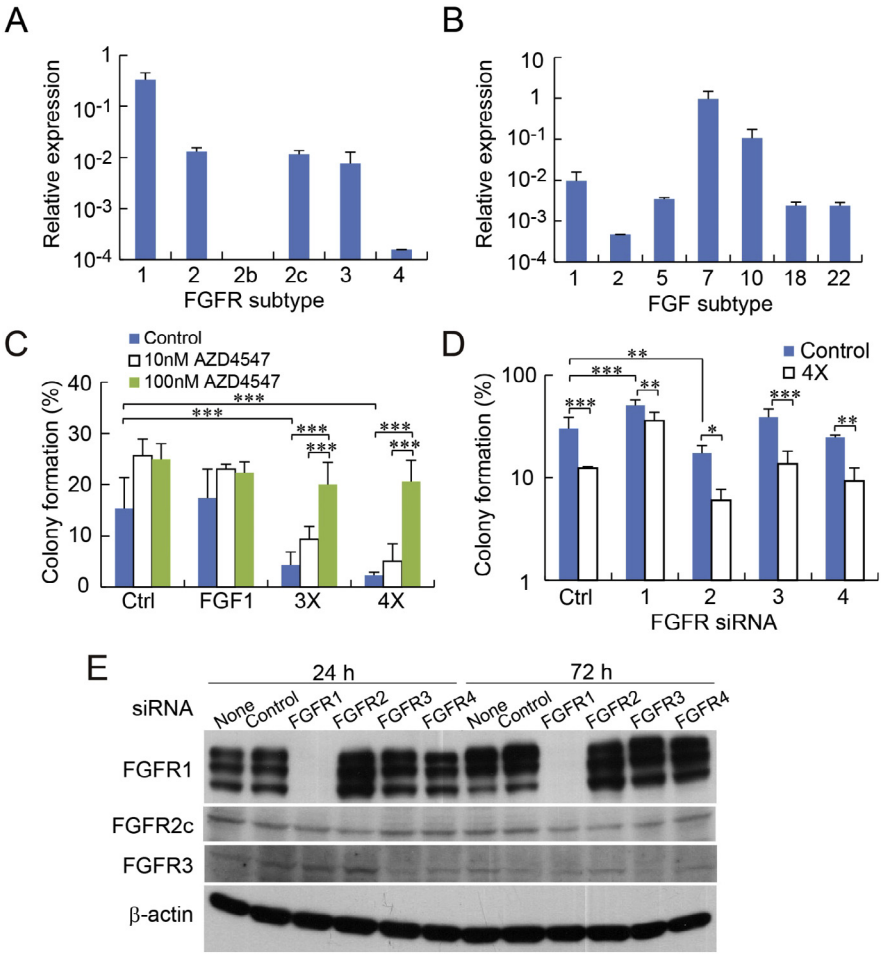

Fig. 3. Strong FGF1 signaling through FGFR1 decreased the proliferative capability of ISOS-1 cells. (A and B) The levels of *Fgf* and *Fgfr* transcripts in ISOS-1 cells were measured by quantitative RT-PCR. (C) The proliferative capability of ISOS-1 cells was examined 8 days after the culture with 100 ng/ml FGF mutants and 10–100 nM AZD4547 by colony assay. (D) The expression of each Fgfr subtype was repressed in ISOS-1 cells using siRNA transfection targeted against the *Fgfr* gene. The proliferative capability of FGFR-repressed ISOS-1 cells was examined 8 days after the culture with 100 ng/ml 4X by colony assay. (E) The expression level of FGFR1, 2c, or 3 in ISOS-1 cells was examined by Western blot analysis 24 or 72 h after siRNA transfection. All values are means ± SD (n = 3). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

rylated mouse receptor tyrosine kinases (RTKs) of ISOS-1 cells was examined after 20-h incubation with FGF1 or 4X to evaluate the influence of FGF1 mutants on other receptor signaling pathways using antibody arrays (Supplementary materials and methods). FGF1 treatment decreased the levels of p-VEGFRs, p-EGFRs, p-FGFRs, and p-HGFRs (Fig. 5A). 4X reduced the levels of p-VEGFRs (VEGFR2 and 3) and p-EGFRs (ErbB2, DrbB3, and ErbB4) more than FGF1, whereas p-FGFRs and p-HGFRs were not additionally reduced by 4X treatment. Moreover, Western blot analysis showed that the expression of EGFR decreased in ISOS-1 cells 1 h after the incubation with 3X or 4X, and its decrease continued for up to 24 h after the culture, (Fig. 5B). The phosphorylation of EGFR decreased in ISOS-1 cells 6 h after the incubation with 3X or 4X, and its phosphorylation decreased in parallel with the activity of FGF1 mutants 24 h after the incubation (Fig. 5B). In addition, the phosphorylation of VEGFR2 decreased in ISOS-1 cells 1 h after the incubation with 3X or 4X, although the expression of VEGFR2 decreased in ISOS-1 cells in parallel with the activity of FGF1 mutants 24 h after the incubation (Fig. 5B). In contrast, the transcript levels of *Egfrs* and *Vegfrs* were increased in ISOS-1 cells after the 20-h incubation with FGFs; however, these transcript levels were lower after 4X treatment than after FGF1 treatment (Supplementary Fig. 2). Colony formation, invasion, and wound healing assays were performed after the cultures with an EGFR inhibitor (AZD8931) and a VEGFR inhibitor (axitinib) to evaluate the effects of blockade of EGFR and VEGFR signaling on the proliferative and metastatic capacity of ISOS-1 cells. The single inhibition with AZD8931 or axitinib did not significantly decrease colony formation, invasion, or migration rate, whereas the dual inhibition with AZD8931 and axitinib significantly suppressed them (Fig. 5C and D and Supplementary Fig. 3). These results suggest that the dual blockade of EGFR and VEGFR activation by strong FGF1 signaling resulted in the inhibition of the proliferative, invasive, and migration capabilities of the angiosarcoma cell line.

Discussion

Highly stable FGF1 mutants, 3X and 4X, activate cell surface receptors to initiate strong and persistent intracellular signaling (Fig. 2A) [6,7]. siRNA targeting the *Fgfr1* gene showed that colony formation by ISOS-1 cells was suppressed through FGFR1 signaling pathway by 3X and 4X (Fig. 3D). In addition, the suppression of the proliferative and metastatic capabilities of pancreatic cancer cell line PANC-1 was previously reported in FGFR1b-overexpressing cells [10]; however, 3X and 4X did not suppress colony formation by non-transfected PANC-1 cells (data not shown). Therefore, high expression of FGFR1 in tumor cells might be required for inhibiting its malignancy through FGFR1 signaling. In the present study, *Fgfr1*

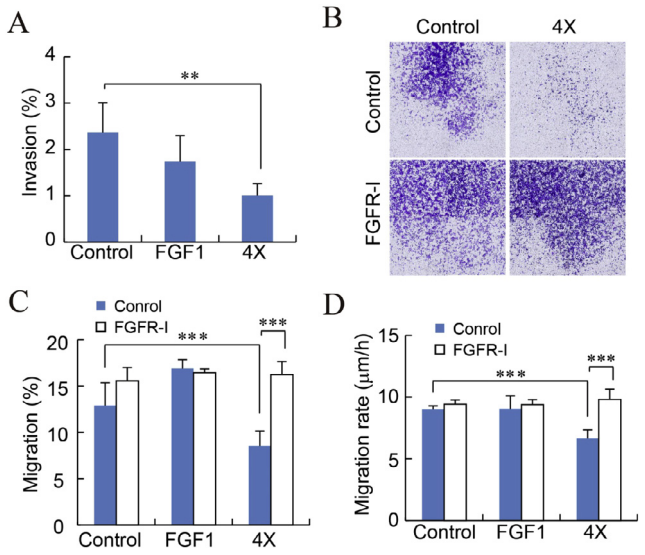

Fig. 4. Strong FGF1 signaling inhibited the *in vitro* invasion and migration of ISOS-1 cells. (A) The invasiveness of ISOS-1 cells was examined by invasion assay 24 h after the incubation with 100 ng/ml of FGF1 or 4X. The ratio of invaded cells was obtained by dividing them with the total number of seeded cells. (B) An invasion assay was performed with 100 ng/ml of 4X and/or 100 nM AZD4547 (FGFR inhibitor). Invaded cells on the Transwell membrane are shown. (C) The migration ratio of ISOS-1 cells was assessed by the migration assay using Transwell chambers. Cells were incubated for 24 h in Transwells with 100 ng/ml of FGF1 or 4X, and 100 nM AZD4547. (D) The migration of ISOS-1 cells was tracked by the wound healing assay using IncuCyte with 100 ng/ml of FGF1 or 4X and/or 100 nM AZD4547. The migration rate of ISOS-1 cells was assessed after a 12-h culture. All values are means ± SD (n = 3–6). $^{**}P < .01$, $^{***}P < .001$.

transcript levels in ISOS-1 cells were ten-fold higher than those of *Fgfr2* (Fig. 3A), and FGFR1 protein levels were also markedly higher than those of FGFR2 (Fig. 3E). In contrast, the repression of FGFR2 reduced colony formation by ISOS-1 cells (Fig. 3D) with a marked increase in FGFR1 expression (Fig. 3E), suggesting that FGFR2 signaling was involved in the down-regulation of FGFR1 expression. The significant difference observed between the expression of FGFR1 and FGFR2, suggests that 3X and 4X stimulate FGFR1 more strongly than FGFR2 in order to inhibit the malignancy of ISOS-1 cells. The Geo database in the National Center for Biotechnology Information (NCBI) revealed an inverse correlation between *FGFR1* and *FGFR2* expression in human angiosarcoma cases, with almost half of cases of angiosarcoma showing the more abundant expression of *FGFR1* than *FGFR2* transcripts (Supplementary Fig. 4) [13]. Thus, strong FGF1 signaling appeared to suppress the progression or metastasis of angiosarcoma in some human angiosarcoma cases strongly expressing FGFR1.

FGFs were originally discovered as angiogenic growth factors that may stimulate the growth, migration and differentiation of endothelial cells, and FGF signaling was previously linked to VEGF signaling [14]. For example, VEGFR2 expression was regulated in endothelial cells by FGF via the activation of Erk1/2 [15]. In contrast, there are several inhibitory feedback pathways that may suppress FGF signaling in endothelial cells. Sprouty family proteins (Spry) function in the inhibitory feedback of receptor tyrosine kinase, and one member of the family, Sprouty-4, has been shown to inhibit the FGF- and VEGF-mediated *in vitro* proliferation and migration of endothelial cells [16]. In the present study, the transcript levels of Sprouty family members were increased in ISOS-1 cells after the 20-h treatment with each FGF1 (data not shown). Moreover, siRNA-mediated regression of FGFRs increased the expression of EGFR and VEGFR2, suggesting that VEGFR and EGFR are down-regulated through FGFR signaling (Supplementary Fig. 5).

Angiosarcoma is a malignant tumor derived from the endothelium of vascular vessels or lymphatics, and expresses endothelial markers including Factor VIII, CD34, CD31, UEA-1, and VEGF. The murine tumorigenic endothelial cell line, ISOS-1, was established from a tumor induced by the transplantation of a human angiosarcoma tissue fragment into a SCID mouse [11]. They showed the active uptake of Dil-Ac-LDL, active binding activity with UEA-1 and GSA-I lectins, and expression of VEGFs as an endothelial marker (Fig. 5). Although the roles of angiogenic signaling in the pathogenesis of angiosarcoma remain unknown, anti-angiogenic molecules, such as a VEGFR kinase inhibitor [17,18] or anti-VEGF monoclonal antibody [19], are now being investigated for the treatment of angiosarcoma. However, the effectiveness of these biological agents against angiosarcoma is still limited [18]. In the present study, 100 nM axitinib (VEGFR inhibitor) did not inhibit the proliferative or metastatic capacities of ISOS-1 cells, although the IC_{50} of axitinib was 1–2 nM (Fig. 5C and D and Supplementary Fig. 3).

EGFR plays an important role in the development and progression of various epithelial cancers and is closely related to the VEGF pathways [20]. EGFR expression levels are not generally associated with endothelial malignant tumors, and EGFRs are expressed at a low density in tumors other than epithelial cancers; therefore, EGFR expression levels in sarcomas may not be sufficiently high for therapy by EGF targeting [21]. In the present study, the EGFR inhibitor (AZD8931) alone did not significantly decrease colony formation by and the *in vitro* invasion and migration speed of ISOS-1 cells (Fig. 5C and D and Supplementary Fig. 3). However, EGF targeting with accompanying second ligand targeting successfully killed canine hemangiosarcoma cells [21]; therefore, the blockade of the EGFR pathway has potential in the treatment of angiosarcoma. In contrast, EGFR and VEGFR share common downstream signaling pathways, and several preclinical studies provided evidence for the angiogenic effects of EGFR signaling [20].

A close relationship exists between EGFR and VEGFR signaling pathways and is useful for anticancer therapy. VEGF signaling is promoted by the expression of EGFR, whereas enhanced VEGF expression may be involved in resistance to anti-EGFR therapy as alternative survival pathways [20]. Therefore, preclinical studies showed that the dual inhibition of EGFR and VEGFR signaling was superior for tumor growth inhibition, decreased tumor vascularity, and tumor cell apoptosis to a single treatment with each inhibitor [22,23]. In addition, clinical trials have provided promising data to show that combined inhibition increases benefits over anti-EGFR agents alone [24]. Therefore, decreases in EGFR and VEGFR signaling by strong FGF1 signaling may inhibit the proliferative and metastatic capabilities of angiosarcoma cells.

One of the stable FGF1 mutants, 3X sufficiently repaired intestinal damage and improved gastrointestinal syndrome to enhance the duration of mouse survival, as reported previously [8]. In contrast, the other mutant 4X was more potent than 3X for mitogenic activity through FGFR1c, and 4X exerted stronger radioprotective effects than 3X (Fig. 1). However, 4X suppressed the proliferative capability of ISOS-1 cells and increased their radiosensitivity more than 3X, although this radiosensitization of tumor cells may be too low to increase the clinical effect of radiotherapy for angiosarcoma. In addition, the higher stability of 4X is useful for its manufacture and daily usage as a medicine. Accordingly, the highly stable mutant 4X is the most promising candidate for a radioprotector among the FGF1 mutants tested to prevent the side effects of radiotherapy for angiosarcomas.

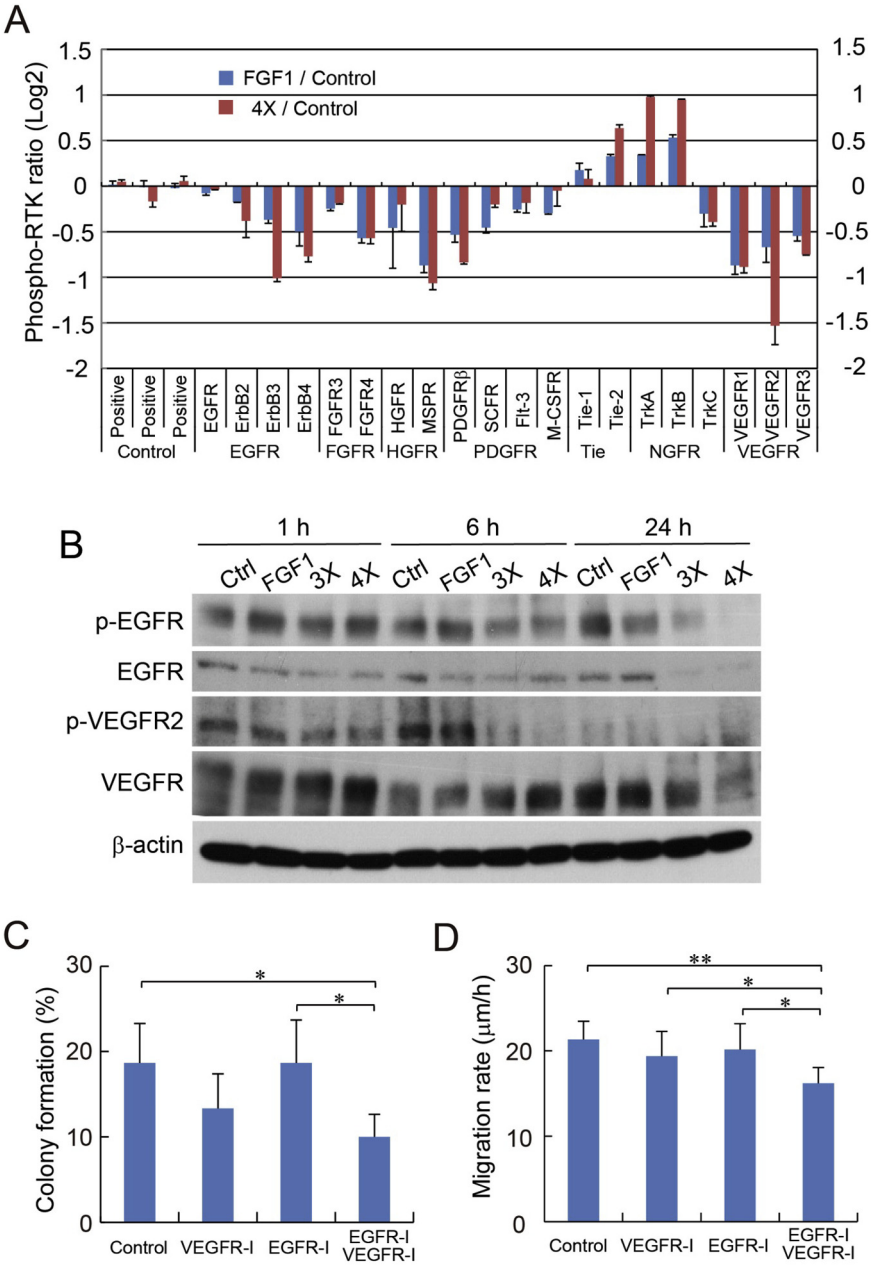

Fig. 5. Strong FGF1 signaling suppressed the activation of VEGFR and EGFR, resulting in a decrease in the proliferative and metastatic capabilities of ISOS-1 cells. (A) Expression profiling of different phosphorylated mouse RTKs was performed in ISOS-1 cells using a Mouse Phospho-RTK array after 20-h incubation with 100 ng/ml of FGF1 or 4X. Each expression value is shown as a Log2 ratio relative to the value of non-treated control cells. (B) ISOS-1 cells were incubated for 1, 6, or 24 h with 100 ng/ml of FGF1, 3X, or 4X, and EGFR, VEGFR2, and their phosphorylation in ISOS-1 cells were examined by Western blot analysis. (C) The proliferative capability of ISOS-1 cells was examined 8 days after the culture with 100 nM axitinib (a VEGFR inhibitor) and/or 100 nM AZD8931 (an EGFR inhibitor) using a colony assay. (D) The migration rate of ISOS-1 cells was assessed by a wound healing assay using IncuCyte after a 12-h culture with 100 nM axitinib and/or 100 nM AZD8931. All values are means ± SD (n = 3–6). $^{*}P < .05$, $^{**}P < .01$.

Conclusions

We herein demonstrated that stable FGF1 mutants with strong mitogenic activity not only exerted strong radioprotective effects, but also reduced the proliferative, invasive and migration capabilities of the murine angiosarcoma cell line ISOS-1 by strong FGF1 signaling through FGFR1. Our results also suggest that this strong signaling was related to the down-regulation of the VEGFR and EGFR pathways, resulting in the inhibition of their growth and metastasis. We concluded that highly stable FGF1 mutants are potential radioprotectors and their effects provide a new tool for the treatment or prevention of the adverse effects of angiosarcoma radiotherapy without promoting tumor progression and metastasis.

Conflict of interest

The authors state that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ctro.2017.10.006.

References

[1] Carsi B. Angiosarcoma. https://emedicine.medscape.com/article/276512-overview:, Medscape; 2012.

[2] Masuzawa M, Takenaka H, Murata T, Murakami S, Taguchi M, Asakoshi K, et al. The clinical practice guidelines for the management of angiosarcoma of the scalp. Nihon Hifuka Gakkai Zasshi 2015;125:1871–88.

[3] Okunieff P, Mester M, Wang J, Maddox T, Gong X, Tang D, et al. *In vivo* radioprotective effects of angiogenic growth factors on the small bowel of C3H mice. Radiat Res 1998;150:204–11.

[4] Hagiwara A, Nakayama F, Motomura K, Asada M, Suzuki M, Imamura T, et al. Comparison of expression profiles of several fibroblast growth factor receptors in the mouse jejunum: suggestive evidence for a differential radioprotective effect among major FGF family members and the potency of FGF1. Radiat Res 2009;172:58–65.

[5] Culajay JF, Blaber SI, Khurana A, Blaber M. Thermodynamic characterization of mutants of human fibroblast growth factor 1 with an increased physiological half-life. Biochemistry 2000;39:7153–8.

[6] Zakrzewska M, Krowarsch D, Wiedlocha A, Olsnes S, Otlewski J. Highly stable mutants of human fibroblast growth factor-1 exhibit prolonged biological action. J Mol Biol 2005;352:860–75.

[7] Zakrzewska M, Wiedlocha A, Szlachcic A, Krowarsch D, Otlewski J, Olsnes S. Increased protein stability of FGF1 can compensate for its reduced affinity for heparin. J Biol Chem 2009;284:25388–403.

[8] Nakayama F, Umeda S, Yasuda T, Asada M, Motomura K, Suzuki M, et al. Structural stability of human fibroblast growth factor-1 is essential for protective effects against radiation-induced intestinal damage. Int J Radiat Oncol Biol Phys 2013;85:477–83.

[9] Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer 2010;10:116–29.

[10] Liu Z, Neiss N, Zhou S, Henne-Bruns D, Korc M, Bachem M, et al. Identification of a fibroblast growth factor receptor 1 splice variant that inhibits pancreatic cancer cell growth. Cancer Res 2007;67:2712–9.

[11] Masuzawa M, Fujimura T, Tsubokawa M, Nishiyama S, Katsuoka K, Terada E, et al. Establishment of a new murine-phenotypic angiosarcoma cell line (ISOS-1). J Dermatol Sci 1998;16:91–8.

[12] Fujita M, Otsuka Y, Imadome K, Endo S, Yamada S, Imai T. Carbon-ion radiation enhances migration ability and invasiveness of the pancreatic cancer cell, PANC-1, *in vitro*. Cancer Sci 2012;103:677–83.

[13] Andersen NJ, Nickoloff BJ, Dykema KJ, Boguslawski EA, Krivochenitser RI, Froman RE, et al. Pharmacologic inhibition of MEK signaling prevents growth of canine hemangiosarcoma. Mol Cancer Ther 2013;12:1701–14.

[14] Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci 2001;22:201–7.

[15] Murakami M, Nguyen LT, Hatanaka K, Schachterle W, Chen PY, Zhuang ZW, et al. FGF-dependent regulation of VEGF receptor 2 expression in mice. J Clin Invest 2011;121:2668–78.

[16] Lee SH, Schloss DJ, Jarvis L, Krasnow MA, Swain JL. Inhibition of angiogenesis by a mouse Sprouty protein. J Biol Chem 2001;276:4128–33.

[17] Maki RG, D'Adamo DR, Keohan ML, Saulle M, Schuetze SM, Undevia SD, et al. Phase II study of sorafenib in patients with metastatic or recurrent sarcomas. J Clin Oncol 2009;27:3133–40.

[18] Ray-Coquard I, Italiano A, Bompas E, Le Cesne A, Robin YM, Chevreau C, et al. Sorafenib for patients with advanced angiosarcoma: a phase II trial from the French Sarcoma Group (GSF/GETO). Oncologist 2012;17:260–6.

[19] Agulnik M, Yarber JL, Okuno SH, von Mehren M, Jovanovic BD, Brockstein BE, et al. An open-label, multicenter, phase II study of bevacizumab for the treatment of angiosarcoma and epithelioid hemangioendotheliomas. Ann Oncol 2013;24:257–63.

[20] Ciardiello F, Troiani T, Bianco R, Orditura M, Morgillo F, Martinelli E, et al. Interaction between the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF) pathways: a rational approach for multi-target anticancer therapy. Ann Oncol 2006;17. vii109-vii14.

[21] Schappa JT, Frantz AM, Gorden BH, Dickerson EB, Vallera DA, Modiano JF. Hemangiosarcoma and its cancer stem cell subpopulation are effectively killed by a toxin targeted through epidermal growth factor and urokinase receptors. Int J Cancer 2013;133:1936–44.

[22] Shaheen RM, Ahmad SA, Liu W, Reinmuth N, Jung YD, Tseng WW, et al. Inhibited growth of colon cancer carcinomatosis by antibodies to vascular endothelial and epidermal growth factor receptors. Br J Cancer 2001;85:584–9.

[23] Jung YD, Mansfield PF, Akagi M, Takeda A, Liu W, Bucana CD, et al. Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model. Eur J Cancer 2002;38:1133–40.

[24] Tabernero J. The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. Mol Cancer Res 2007;5:203–20.