Pharmacologically targeting the myristoylation of the scaffold protein FRS2α inhibits FGF/FGFR-mediated oncogenic signaling and tumor progression

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Fibroblast growth factor (FGF)/FGF receptor (FGFR) signaling facilitates tumor initiation and progression. Although currently approved inhibitors of FGFR kinase have shown therapeutic benefit in clinical trials, overexpression or mutations of FGFRs eventually confer drug resistance and thereby abrogate the desired activity of kinase inhibitors in many cancer types. In this study, we report that loss of myristoylation of fibroblast growth factor receptor substrate 2 (FRS2α), a scaffold protein essential for FGFR signaling, inhibits FGF/FGFR-mediated oncogenic signaling and FGF10-induced tumorigenesis. Moreover, a previously synthesized myristoyl-CoA analog, B13, which targets the activity of N-myristoyltransferases, suppressed FRS2α myristoylation and decreased the phosphorylation with mild alteration of FRS2α localization at the cell membrane. B13 inhibited oncogenic signaling induced by WT FGFRs or their drug-resistant mutants (FGFRsDRM). B13 alone or in combination with an FGFR inhibitor suppressed FGF-induced WT FGFR- or FGFRDRM-initiated phosphoinositide 3-kinase (PI3K) activity or MAPK signaling, inducing cell cycle arrest and thereby inhibiting cell proliferation and migration in several cancer cell types. Finally, B13 significantly inhibited the growth of xenograft tumors without pathological toxicity to the liver, kidney, or lung in vivo. In summary, our study suggests a possible therapeutic approach for inhibiting FGF/FGFR-mediated cancer progression and drug-resistant FGF/FGFR mutants.

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This article contains Table S1, Figs. S1–S9, and supporting Materials and Methods.

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2The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; ERK, extracellular signal–regulated kinase; NMT, N-myristoyltransferase; MAPK, mitogen-activated kinase; p-, phosphorylated; PLCγ, phospholipase Cγ; IHC, immunohistochemistry; qRNA, guide RNA; MTT, organ (1). However, a large body of research has demonstrated that this signaling axis is highly deregulated in numerous cancers, including amplification of FGFs in epithelial or stromal cells and/or aberrant expression or activation of FGFRs resulting from genetic translocation, mutation, or amplification in tumorigenic cells (2). Pathological FGF/FGFR signaling promotes cross-talk of tumorigenic cells with their microenvironment, which drives tumor proliferation, angiogenesis, and metastasis in cancer progression (3–8).

FGF/FGFR signaling requires recruiting a scaffold protein called the fibroblast growth factor receptor substrate 2 (FRS2) to initiate downstream signaling. The FRS2 family is composed of two members, FRS2α and FRS2β. Both proteins contain the phosphotyrosine-binding domain and multiple tyrosine phosphorylation sites (9, 10). FRS2α mainly associates with FGF/FGFR signaling. It binds to the juxtamembrane region of FGFRs through its phosphotyrosine-binding domain (10, 11), and the activation of FGFRs phosphorylates several tyrosine sites of FRS2α. Whereas four sites mediate the binding with Grb2, which activates phosphotyrosinolinositol 3-kinase/AKT signaling and to a lesser extent Ras/ERK signaling, the other two sites facilitate binding with SHP2, which activates mainly the Ras/ERK pathway (10). Therefore, FRS2α is essential for FGF/FGFR-induced signaling and facilitates cancer cell proliferation and migration (12). The aberrant expression of FRS2α is also observed in some cancers (13). Therefore, targeting FRS2α is considered an important therapeutic approach in the inhibition of FGF/FGFR-mediated tumorigenesis (14).

Myristoylation of FRS2α is essential for its anchoring to the plasmatic membrane. FRS2α contains the MGXXX(S/T) consensus sequence at the N terminus for N-myristoylation modification (10). N-Myristoyltransferase (NMT) catalyzes the myristoylation modification process by transferring the myristoyl group from myristoyl-CoA to the glycine at the N terminus of a protein (15). In this study, we investigated a therapeutic approach to inhibit FGF/FGFR-mediated oncogenic signaling and proliferation of cancer cells by blocking myristoylation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; UGSM, urogenital sinus mesenchyme; H&E, hematoxylin and eosin.
FRS2α. We demonstrate that genetic knockout of FRS2α expression inhibited FGF/FGFR-mediated signaling. Signaling was rescued by the re-expression of the WT FRS2α, but not FRS2α(G2A), a mutation that prevents FRS2α myristoylation and FGF10-induced tumorigenesis. Additionally, a previously synthesized myristoyl-CoA analog inhibitor (16), B13, inhibited FRS2α myristoylation, resulting in reduced FGF/FGFR-mediated oncogenic signaling and suppression of cell proliferation and migration of cancer cells by causing cell cycle arrest. The compound suppressed oncogenic signaling mediated by FGFR drug-resistant mutants and inhibited growth of xenograft tumors without observed pathological toxicity to host organs in vivo. Our study provides a potential therapeutic approach for the treatment of FGF/FGFR-mediated tumor progression.

Results

Myristoylation of FRS2α is required for FGF2-induced signaling

Upon FGF induction, FRS2α is phosphorylated by FGFRs and further activates associated downstream signaling, such as PI3K/AKT and MAPK (9). To confirm the essential role of FRS2α in FGF/FGFR signaling, FRS2α was knocked out using the CRISPR/Cas9 system in mouse fibroblast cells NIH-3T3 (Fig. 1A). Two single colonies, FRS2αΔ-1 and FRS2αΔ-2, were identified and confirmed by PCR analysis (Fig. 1B) and Western blotting (Fig. 1C). Loss of FRS2α indeed impaired FGF2-induced AKT phosphorylation and MAPK signaling in both selected null mutants without alteration of PLCγ1 phosphorylation, which is not directly regulated by FRS2α activity (Fig. 1D). It should be noted that levels of p-ERK were much less inhibited than those of p-AKT due to the loss of FRS2α. It has been reported that the activated PLCγ induced by FGF/FGFR signaling could enhance MAPK signaling through the protein kinase C-Raf-MAPK pathway (17). As a result, although MAPK signaling is inhibited by knockout of FRS2α, activation of PLCγ could maintain MAPK signaling under the induction of FGF2.

The N terminus of FRS2α has been reported to be myristoylated (18). To examine whether myristoylation of FRS2α is crucial for FGF-induced signaling and its membrane localization, FRS2αΔ knockout cells were transduced with FRS2α(WT) or FRS2α(G2A) mutant by lentiviral infection (Fig. 1E). FRS2α(WT), but not FRS2α(G2A), a mutant unable to be myristoylated, rescued FGF2-induced AKT phosphorylation and MAPK signaling (Fig. 1F). Of note, levels of p-AKT and p-ERK were not able to be restored in the cells expressing FRS2α(WT) (without FGF2 induction) (Fig. 1F, lane 5 versus lane 1). Higher expression levels of FRS2α tended to have a more inhibitory effect on p-ERK levels (Fig. S1). This is probably because the ectopic expression of FRS2α leads to negative feedback mediated by MAPK signaling, as reported previously (19).

Loss of FRS2α myristoylation inhibits FRS2α tyrosine phosphorylation and interaction with downstream proteins, SHP2 and GRB2

FRS2α contains numerous tyrosine and threonyne phosphorylation sites (10). We examined whether loss of myristoylation leads to changes in phosphorylation status. Indeed, the levels of FRS2α tyrosine phosphorylation were significantly inhibited in cells expressing FRS2α(G2A) compared with FRS2α(WT) (Fig. 1G). In contrast, levels of FRS2α threonine phosphorylation were largely maintained in cells expressing FRS2α(G2A) (Fig. 1G). Of note, FRS2α(G2A) runs at a lower molecular weight in the gel compared with FRS2α(WT) which is probably due to loss of tyrosine phosphorylation sites. In addition, loss of myristoylation resulted in reduced protein-protein interactions between FRS2α and GRB2 or SHP2 (Fig. 1H). This result suggests that overexpression of FRS2α(G2A) is incapable of restoring FGF2-induced signaling in FRS2αΔ knockout cells (Fig. 1I) due to an interference with signal transduction.

Loss of FRS2α myristoylation suppresses paracrine FGF10-induced tumorigenesis

We further reasoned that loss of FRS2α myristoylation should inhibit FGF/FGF-induced tumorigenesis. Prostate primary cells isolated from BL6 mice prostate tissue were transduced with vector control, FRS2α(WT), or FRS2α(G2A) by lentiviral infection (Fig. S2A). The cells were mixed with FGF10-UGSM or GFP-UGSM cells, and the mixture was implanted subcutaneously in SCID mice (Fig. 2A) (20). Grafts derived from control vector, FRS2α(WT), or FRS2α(G2A) with GFP-UGSM cells (control group) showed normal prostate tubules with no difference in tissue regeneration (Fig. S2B). As expected, grafts derived from the FGF10-UGSM group (PrEC-control vector or PrEC-FRS2α(WT)) showed adenocarcinoma (Fig. 2B) (20, 21). Tumorigenic cells showed expression of androgen receptor and proliferation of CK8+ luminal cells without substantial changes in CK5+ basal cells. However, grafts derived from PrEC-FRS2α(G2A) with FGF10-UGSM showed normal tubules. The RFP signal indicated successful transduction of the exogenous genes. Overexpression of FRS2α was confirmed in the regenerated tissues derived from PrEC-FRS2α(WT) or FRS2α(G2A) + GFP-UGSM (Fig. S2B) or FGF10-UGSM (Fig. 2B). Immunohistochemistry analysis of the grafts derived from FRS2α(G2A) + FGF10-UGSM showed CK8+ luminal and CK5+ basal cells as the control groups (Fig. 2B and S2B). The results indicate that overexpression of FRS2α(G2A) inhibits FGF10-induced tumorigenesis.

A myristoyl-CoA analog, B13, suppresses the myristoylation of FRS2α and its cell membrane localization

Protein myristoylation is catalyzed by N-myristoyltransferases (NMT1 and NMT2) (22). Based on the NMT1 crystal structure (15), a myristoyl-CoA analog, B13, was identified from a panel of previously synthesized small-molecule compounds (23). An N-terminal truncated mutant of NMT1 was purified to examine the inhibitory effect of B13 (15). Compound B13 showed an inhibition of NMT1 enzymatic activity with I_{50} of 79.1 μM (Fig. 3B). Of note, it has been reported that the NMT1 mutant lacking the N terminus shows an approximately 3-fold increase in enzymatic activity compared with the full-length enzyme (25). Therefore, the obtained I_{50} might lead to an underestimation of the inhibitory efficacy of B13 on protein myristoylation in the cell-based assay (Fig. 3C). B13 showed dose-dependent inhibition of FRS2α myristoylation detected by click chemistry (Fig. 3C). For the click chemistry
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reaction, cells were incubated with myristic acid–azide (as a probe of labeling myristoyl-proteins) as the source of myristate for the myristoylation reaction. Cell lysates underwent a click reaction in which the azide functional group on myristoylated proteins was coupled to an alkyne tagged with biotin, allowing detection with streptavidin (Fig. 3C, lane 2 versus lane 1) (26). Myristoylation is essential for the localization of FRS2α. FRS2α was mainly detected in the membrane fraction in FRS2α(WT) cells, but mainly in the cytosol fraction in FRS2α(G2A) cells (Fig. 3D).

FRS2α was predominantly expressed in the cell membrane fraction (Fig. 3F), probably because it has multiple acylation sites in the N terminus (2GSCCSCP). FRS2α contains multiple potential S-palmitoylation sites (cysteines 4, 5, and 7) in addition to a myristoylation site (27). FRS2α levels increased about 2-fold in the cytosol fraction of the cells treated with B13 com-
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pared with the control (Fig. 3E). Given the total amount of FRS2α in the cell membrane, loss of myristoylation only mildly affects membrane protein levels. However, consistent with the genetic results, B13 suppressed endogenous levels of p-FRS2α at the membrane and inhibited the phosphorylation of FRS2α under FG2 induction (Fig. 3E). These data suggest that B13 regulates FRS2α activity and thus FGF/FGF signaling.

B13 inhibits dysregulated FGFR oncogenic signaling in a variety of cancer cells

Similar to the genetic loss of FRS2α in NIH-3T3 cells (Fig. 1, D and F), B13-mediated inhibition of FRS2α myristoylation reduced the levels of p-FRS2α, p-AKT, and p-ERK in a dose-dependent manner (Fig. 4A) and to a lesser extent p-PLCγ1 (Figs. 1D and 3G). Additionally, the inhibition required more than 12 h of treatment at a concentration of 15 μM (Fig. 4B).

Dysregulation of FGF/FGF signaling, including mutations resulting in FGFR activation and/or FGFR gene amplification, has been demonstrated to drive proliferation and survival of cancer cells. B13 reduced the levels of p-FRS2α and p-AKT with or without induction by FG2 in NIH-3T3 cells ectopically expressing FGFR2 but did not affect the level of p-FGFR (Fig. 4C). These data suggest that B13 inhibits FGF-induced signaling by targeting FRS2α and bypasses the activation of FGFRs.

Dysregulated FGFR signaling due to overexpression or activation of FGFR1/2 has been reported in a variety of cancer types. Bioinformatics analysis on expression levels of FGFR1/2 in 947 cancer cell lines indicated that FGFR1 expression levels were elevated in numerous cancer cell lines, including MDA-MB-134-VI breast cancer cells, and FGFR2 expression levels were elevated in SNU16 and KATO III stomach cancer cells (Fig. S3, A and B). PC-3, 22Rv1, and other prostate cancer cells expressed unaltered levels of FGFR1/2 compared with other cancer cell lines (28) (Fig. S3, A and B). The expression levels of FGFR1/2 were confirmed in a panel of selected cancer cells, including MDA-MB-134-VI, SNU16, KATO III, AGS, HCT116, and prostate cancer cells LNCaP, 22Rv1, PC-3, DU145, and normal prostate cells PNT2 (Fig. S3C). FRS2α expression was also detected in MDA-MB-134-VI, SNU16, KATO III, AGS, and HCT116 cells, which are also recommended by ATCC for anti-FGFR drug discovery analysis (Fig. S3C).

We further examined the inhibitory effect of B13 on endogenous FGF/FGFR-meditated signaling in some of these cancer cell lines. The levels of p-FRS2α, p-AKT, and/or p-ERK were elevated by FG2 induction in MDA-MB-134-VI and SNU16 cells, but not KATO III, probably due to high expression of FGFR2 (Fig. S3C). B13 significantly inhibited the levels of pFRS2α, p-AKT, and/or p-ERK under basal levels or exogenously added FG2 in the examined cancer cells (Fig. 4, D–F), suggesting that the compound inhibits FGF/FGFR-mediated oncogenic signaling in a variety of cancer cells.

B13 collaborates with FGFR inhibitors to reduce FGF-induced signaling

Because B13 inhibits the FRS2α scaffolding function, we further examined whether B13 collaborated with FGFR inhibitors, such as PD173074 or dovitinib (a receptor tyrosine kinase inhibitor), which target FGFR1/2 activity. Because cancer cells were under FGFR induction, the inhibitory effect was specifically due to FGFR-induced signaling. Whereas PD173074 or dovitinib alone reduced the levels of p-FRS2α, p-AKT, and p-ERK in cells with endogenous (Fig. S4, A and B) or ectopic expression of WT FGFR2 (Fig. 5, A and B), the combination of B13 with PD173074 (Fig. S4A and Fig. 5A) or dovitinib (Fig. S4B and Fig. 5B) further suppressed phosphorylation. The data suggest collaboration between B13 and FGFR inhibitors in the suppression of FGF/FGF signaling.

B13 overcomes oncogenic signaling by FGFR2 drug-resistant mutants (FGFR2DORM)

Because B13 targets myristoylation of FRS2α and inhibits WT FGFR signaling, we hypothesized that B13 may also inhibit FGFR2DORM-mediated oncogenic signaling. The mutants FGFR2(N549K) and FGFR2(V564I) have been reported to cause drug resistance in human endometrial cancers (29, 30). The inhibition of p-AKT and/or p-ERK was compromised in the cells harboring these FGFR2DORM compared with those expressing control vector or FGFR2(WT) under FGFR2 induction and treatment with PD173074 or dovitinib (Fig. S4, C and D). Ectopic expression of FGFR2(V564I) or FGFR2(N549K) led to FGFR2 independent activation of p-FRS2α, p-AKT, or p-ERK (under no FGFR2-induced condition) (Fig. S4E). Additionally, FGFR2(V564I) confered dovitinib resistance in cell migration assays (Fig. S5). In contrast, B13 reduced the levels of p-AKT and p-ERK in the cells with ectopically expressed FGFR2(N549K) (Fig. 5, C and D) or FGFR2(V564I) mutants (Fig. 5, E and F) compared with PD17304 or dovitinib. These

Figure 1. Loss of FRS2α myristoylation inhibits FRS2α tyrosine phosphorylation, interaction of FRS2α with downstream proteins SHP2 and GRB2, and FGFR/FGFR signaling. A, schematic diagram of CRISPR/Cas9-mediated FRS2α knockout. Cas9 protein, gRNA vector expressing guide RNA that targets downstream of the FRS2α translational start codon, and donor vector containing homologous arms and functional cassette (GFP-Puro) were introduced into NIH-3T3 cells by co-transfection. gRNA vector expressing scrambled RNA was used as control. The locations of primers are indicated for PCR analysis. G and RR primers were used for detection of WT, and RF and RR primers were used for detection of knockout cells. B, confirmation of homozygous knockout by PCR. Two individual single colonies named FRS2αΔ-1 and FRS2αΔ-2 were selected. Genomic DNA was extracted for use as a template for PCR analysis. C, confirmation of FRS2αΔ-1 and FRS2αΔ-2. Total lysate from two isolated cell lines were analyzed by Western blotting. D, loss of FRS2α inhibits FGFR2-mediated signaling. NIH-3T3 WT, FRS2αΔ-1, and FRS2αΔ-2 cell lines were stimulated with/without FG2 (50 μg/ml, 10 min). Protein lysates were analyzed for FRS2αα, p-FRS2αα, p-AKT(Thr-385; p-AKT(Ser-473), total AKT, p-ERK1/2, ERK2, p-PLCγ1, PLCγ1, and γ-tubulin. The red arrow indicates FRS2αα shifting toward higher molecular weight with FGFR2 induction. E, the protein sequence at the N terminus of WT FRS2αα and point mutation in the myristoylation (Myr) site (G2A) resulting in loss of myristoylation. F, FRS2αΔ-1 cells were transduced with control vector, FRS2αα(WT), or FRS2αα(G2A). Cells were treated with/without FGFR2 induction (50 μg/ml, 10 min). Western blot analysis for FRS2αα and p-FRS2αα, p-AKT, total AKT, p-ERK1/2, ERK2, p-PLCγ1, and γ-tubulin. Expression levels of p-AKT and p-ERK1/2 with FG2 induction were quantified by optical density, as shown in the bar graphs. The level of the WT group was set as 1. Values are mean ± S.D. (error bars) from three independent experiments. **, p < 0.01. G and H, the lysates from cells treated with FG2 (50 μg/ml, 10 min) were immunoprecipitated with the FRS2αα antibody, and phosphotyrosine (pY), phosphothreonine (pT), and FRS2αα levels were detected by immunoblotting (G). The immunoprecipitates pulled down by the FRS2αα antibody were also immunoblotted with GRB2 or SHP2 antibody (H).
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A

B

PrEC-Control vector + FGF10-UGSM

PrEC-FRS2(WT) + FGF10-UGSM

PrEC-FRS2(G2A) + FGF10-UGSM

H&E

RFP

FRS2α

AR

CK5/CK8 /DAPI
data indicate that B13, albeit at high concentrations, blocks FGFR2DRM-mediated oncogenic signaling.

B13 inhibits cancer cell proliferation and migration through cell cycle arrest

FGF/FGFR oncogenic signaling promotes cancer cell proliferation, survival, migration, invasion, and angiogenesis (6). Therefore, we examined whether proliferation of cancer cells via FGF/FGFR-mediated signaling depends on the expression of FRS2α. Indeed, shRNA-FRS2α inhibited the proliferation of AGS and KATO III cancer cells (Fig. S6, A and B), both of which possess elevated FGF/FGFR oncogenic signaling. Next, we examined whether cell proliferation depended on the myristoylation of FRS2α by treating cells with B13. B13 significantly inhibited the proliferation of SNU16, KATO III, and AGS cells (Fig. 6, A–C); however, B13 or genetic knockdown of FRS2α had limited inhibition on HepG2 (liver cancer cells) or 293T cells (Fig. S7, A–D). SNU16 and AGS cancer cell lines were

Figure 2. Loss of FRS2α myristoylation inhibits paracrine FGF10-induced tumorigenesis. A, diagram of the in vivo prostate regeneration assay. Freshly isolated prostate epithelial cells were transduced with control vector, FRS2α(WT), or FRS2α(G2A) by lentiviral infection. UGSM cells isolated from 16.5-day-old mouse embryos were transduced with FGF10 or GFP (control) by lentiviral infection. The transduced UGSM cells were mixed with the transduced prostate epithelial cells and collagen and implanted under the kidney capsule of SCID mice. The regenerated prostate tissues were harvested after an 8-week incubation. The regenerated tissues derived from GFP-UGSM (control) are presented in Fig. S2. B, H&E staining, RFP signal, and IHC staining of FRS2α, AR, and CK5 (green)/CK8 (red)/DAPI (blue) in regenerated tissue derived from PrEC-control vector + FGF10-UGSM, PrEC-FRS2α(G2A) + FGF10-UGSM, or PrEC-FRS2α(WT) + FGF10-UGSM groups. These three groups represent grafts containing prostate epithelial cells transduced with control vector, FRS2α(WT), or FRS2α(G2A) mixed with FGF10-UGSM, respectively. Scale bar, 100 μm.

Figure 3. B13, an analog of myristoyl-CoA, inhibits the myristoylation and cell membrane localization of FRS2α. A, structures of myristoyl-CoA and B13. B, B13 inhibits NMT1 enzymatic activity. Purified human NMT1 (70 nM) was incubated with a synthesized peptide substrate and myristoyl-CoA (1 μM) with different concentrations of B13. NMT1 activity was calculated according to fluorescence intensity. IC50 of B13 is 79.1 μM. C, myristoylation of FRS2α is inhibited by B13. FRS2αΔ-1 cells were transduced with FLAG-tagged FRS2α(WT). Cells were pretreated with 0, 10, 15, 20, and 25 μM B13 for 2 h. The treated cells were cultured with myristic acid–azide (12-azidododecanoic acid) (30 μM) for 16 h. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies. Immunoprecipitated proteins underwent the click chemistry reaction with biotin-alkyne, and myristoylated proteins were detected using horseradish peroxidase–conjugated streptavidin via immunoblotting. D, genetic mutation leading to loss of myristoylation inhibits the association of FRS2α with the cell membrane. FRS2αΔ cells were transduced with control vector (V), FRS2α(WT), or the myristoylation-deficient mutant FRS2α(G2A), and the expression levels of FRS2α were detected in the cytosol and cell membrane fractions. E, the cell membrane protein fractions were isolated from NIH-3T3 cells treated with/without B13 (15 μM) and with/without FGF2 induction (50 μg/ml, 10 min). 100 μg of the cytosol proteins (60 μl) and 20 μg of the cell membrane proteins (20 μl) were loaded. The protein levels of total FRS2α and p-FRS2α were measured in each fraction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and caveolin-1 were markers of the cytosol and cell membrane fractions, respectively. Immunoblots of FRS2α at long and short exposure times are displayed. Error bars, S.D.
particularly sensitive to B13 treatment with IC$_{50}$ values of 4.0 and 8.5 μM, respectively. Additionally, B13 also inhibited cell migration of MDA-MB-134-VI (Fig. 6D). We further analyzed the role of B13 on the cell cycle and found that B13 significantly arrested the cell cycle of SNU16 cells at the G2/M phase, KATO III cells at the S phase, and AGS cells at the G1 phase (Fig. 6, E–G). The inhibition of the cell cycle was further confirmed by up-regulation of p21 and/or p27 expression or down-regulation of p27 expression or down-regulation.
of CDK2, CDK4, and/or CDK6 (Fig. 6H). The cells arresting at different phases of the cell cycle by B13 and the alteration of different master regulator proteins might represent the diversity of cancer cell characteristics. Nevertheless, the myristoyl-CoA analog inhibitor suppressed proliferation of cancer cells by cell cycle arrest.
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To evaluate the effect of B13 on the growth of xenograft tumors, we first examined which cancer cells might be more dependent on FRS2/H9251 expression for tumor growth in vivo. SNU16 (higher expression levels of FGFR2) and PC-3 cells (lower expression level of FGFR2) were chosen (Fig. S3C). These two cell lines expressing shRNA-control or shRNA-FRS2/H9251 were subcutaneously injected into SCID mice. The size and weight of SNU16 and PC-3 xenograft tumors expressing shRNA-FRS2/H9251 were significantly reduced compared with the control group (Fig. S8, A and B). IHC staining showed that expression levels of Ki-67 and CD34 in the FRS2/H9251 knockdown tumors were reduced compared with the control group (Fig. S8, C and D). These data indicate that FRS2α is important for tumor growth in vivo. Additionally, the extent of inhibition of xenograft tumors by shRNA-FRS2α was higher in SNU16 xenograft tumors compared with PC-3 xenograft tumors (Fig. S8, A and B), probably due to higher expression levels of FGFR2 in SNU16 cells, which is more dependent on FGF/FGFR-mediated oncogenic signaling (Fig. S3C).

After confirming the dependence on FRS2α for the growth of SNU16 xenograft tumors, we evaluated the inhibitory effect of B13. The inhibitor had no significant effect on the body weight of SCID mice (Fig. 7A) or histological structure of the liver, kidney, and lung (Fig. 7B). The size and weight of tumors were significantly reduced in the B13 group compared with the vehicle control (Fig. 7C). IHC staining showed that expression levels of Ki-67 and CD34 in tumors were lower in the B13-treated group compared with the control (Fig. 7D).

The results indicate that B13, the myristoyl-CoA analog inhibitor, has no observed toxicity to the major organs of the host mice but is effective for the treatment of cancer progression in a mouse model.

Discussion

Our study demonstrates a novel approach in targeting FGF/FGFR-mediated oncogenic signaling and tumor progression. The co-translational myristoylation modification of FRS2α, a scaffold protein of FGFRs, plays an essential role in regulating FGF/FGFR signaling. Genetic ablation of FRS2α myristoylation
suppresses FGF/FGFR-mediated AKT and/or MAPK activation (Fig. S9). Myristoylation promotes the association of FRS2α with FGFRs. It is well documented that FGF/FGFR signaling facilitates the cross-talk of the epithelium with its microenvironment (9). For example, FRS2α has been illustrated as an important node in FGF/FGFR signaling in embryonic development (12). Additionally, FGF/FGFR is also one of the oncogenic driver signaling pathways in numerous cancers (31). Therefore, targeting myristoylation will provide a therapeutic strategy in FGFR-mediated cancer (32).

Protein myristoylation is catalyzed by NMTs (33, 34). We have illustrated that B13 effectively inhibits NMT enzymatic activity and suppresses FRS2α myristoylation with mild alteration of FRS2α localization in the cell membrane, subsequently suppressing FGF/FGFR-mediated oncogenic signaling (Fig. S9). Additionally, the compound effectively suppresses prolif-
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eration and migration of a variety of cancer cells. Given the fact that the dysregulation of FGF/FGFR signaling (8,9) and amplification of FRS2α are associated with numerous high-grade cancer types (13,35,36), B13 will provide a therapeutic approach to inhibit FGF/FGFR-mediated tumor progression.

Targeting FRS2α myristoylation exhibits benefits over FGFR inhibitors in the suppression of FGF/FGFR-mediated tumorigenesis. Currently, numerous FGFR inhibitors, including PD173074, dovitinib, and ponatinib, that block the tyrosine kinase domain of FGFRs are undergoing clinical trials for cancer treatment (37–39). Although these drugs exhibit substantial clinical responses, nonsynonymous mutations have been identified among the FGFRs. A majority of tumors develop drug-resistant mutants with elevated FGFR activity (30,40–43). Among those, mutations of the gatekeeper residues, such as FGFR1(V561M) and FGFR3(V555M), have been shown to confer resistance to the multikinase inhibitor PP58 and the FGFR inhibitor AZ12908010, respectively (44). Because FRS2α is an immediate downstream node of FGFRs, the FRS2α myristoylation inhibitor will avoid a selection pressure on FGFRs but will exhibit a similar inhibitory effect on FGF/FGFR signaling. In particular, targeting FRS2α myristoylation will potentially bypass FGFRdorm-induced tumor progression. Additionally, our data indicate that the combination of a FRS2α myristoylation inhibitor together with FGFR-targeting drugs shows a synergistic effect for inhibiting FGF/FGFR signaling.

NMTs regulate the myristoylation of an array of oncogenic proteins. Many oncogenic proteins, including c-Src, AMPK (AMP-activated protein kinase), and MARCKS (myristoylated alanine-rich C kinase substrate) require myristoylation modification to carry out their cellular functions (45–47). Therefore, B13 might target tumor progression in which myristoylation is essential for the “addicted” oncogenic protein in the tumors (48). Whereas cancer cells, such as SNU16, KATO III, and MDA-MS-134-VI, possess aberrant expression of FGFR1/2, other cancer cells, such as PC-3, are dependent on Src kinase activity (26). B13 might serve as an agent for targeting tumor progression driven by the oncogenic pathways in which a myristoylated protein is essential to mediate the oncogenic signaling. Promisingly, our data show that normal cells are different from cancer cells in responding to B13 inhibition. Cancer cells usually up-regulate their fatty acid metabolism, which potentially provides an elevated amount of myristoyl-CoA to support protein myristoylation (49–51). Additionally, B13 has also been reported as having an inhibitory effect on the proliferation of cancer cells via the regulation of ceramide biogenesis. The elevation of the ceramide to sphingosine ratio promotes the apoptotic pathway (52,53). Therefore, B13 might possess a dual effect by targeting myristoylation and ceramide functions in cancer cells.

Numerous NMT inhibitors have been reported to inhibit fungal growth due to low amino acid sequence similarity of the NMT protein between fungi or parasites and humans (54,55). However, only a limited number of agents have been identified so far as anti-cancer agents (15,22). B13 provides a possible therapeutic avenue to target oncogenic signaling pathways that require myristoylation, including FRS2α-mediated FGF/FGFR tumor progression, and block the cross-talk of cancer cells with their microenvironment, leading to metastatic disease (8). Of note, FRS2α also mediates other receptor tyrosine kinases, such as vascular endothelial growth factor receptor, nerve growth factor receptor, and brain-derived neurotrophic factor receptor (56–58), suggesting that B13 could potentially inhibit these receptor-mediated diseases as well.

Experimental procedures

Plasmid construction and lentiviral production

The coding sequence of mouse FGFR1, FGFR2 (FGFR2c isoform), or mutants was subcloned into the lentiviral vector FUCRW (59). The FRS2α mutant, FRS2α(G2A) (loss of myristoylation site), was also cloned into FUCRW. The primer sequences used for cloning are listed in Table S1. The cloning details are described in the supporting Materials and Methods. Lentivirus was generated by co-transfecting plasmids expressing the gene of interest and the packaging vectors MDL, VSV, and REV into HEK293T cells. Virus infection was performed as described previously (24). All lentivirus procedures followed the guidelines and regulations of the University of Georgia.

Cell culture

PNT2 cells were purchased from Sigma (catalog no. 95012613). NIH-3T3, 293T, and prostate cancer cell lines, including LNCaP, 22Rv1 DU145, and PC-3, were purchased from the American Type Culture Collection (ATCC) in September 2013. The FGFR genetic alteration cell panel, including SNU16, KATO III, AGS, HCT116, and MDA-MB-134-VI, was purchased from ATCC in October 2014. These cell lines carrying aberrant expression of FGFRs were recommended by ATCC for anti-FGFR drug discovery research. HepG2 was purchased from ATCC in March 2016. All of the cell lines purchased were defined as passage 1 (the first thawing) upon arrival. All cell lines were cultured in ATCC-recommended medium and temperature. Cell lines from ATCC and Sigma had a certificate of mycoplasma-free and authentication when purchased. All of the cell lines were used within no more than 8–10 passages and were periodically examined for mycoplasma contamination by using the ATCC detection kit.

CRISPR/Cas9-mediated gene knockout

Plasmids for FRS2α knockout were purchased from OriGene. One of the two guide RNA (gRNA) vectors (or scramble control vector) and donor vector (1:1 ratio) were co-transduced into NIH-3T3 cells by transient transfection using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h post-transfection, cells were split at a 1:10 ratio and cultured for an additional 3 days. After splitting cells seven times (at a 1:10 ratio), 2 μg/ml puromycin was applied to select drug-resistant cells. The medium was changed every 3 days. Single colonies were isolated by the limiting dilution method. The genomic DNA and proteins were extracted from the single colonies. Genomic PCR was performed to check whether the fragment between the two homologous arms in the donor vector was integrated into the genome properly. The primer sequences used for genomic PCR are listed in Table S1. Additionally, the
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genetic knockout of FRS2α in isolated colonies was confirmed by Western blot analysis.

**Examination of myristoylation by immunoprecipitation and click chemistry**

Cells were grown in 10-cm dishes to 80–90% confluence. To metabolically label myristoylated proteins, cells were further cultured in medium containing 12-azidododecanoic acid (Thermo Fisher Scientific, C10268) and 2% BSA (fatty acid-free) for 24 h. Cells were washed twice with PBS, and then 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing protease inhibitors was added. Protein lysates were extracted by incubation with the lysis buffer for 30 min on a shaker and collected by scraping. The lysates were centrifuged for 10 min at 12,000 × g. The supernatant was collected, and protein concentration was measured. Protein lysate (1 mg) was incubated with 40 µl of prewashed anti-FLAG® M2 affinity gel (Sigma-Aldrich, A2220) overnight. After immunoprecipitation, the resin was washed three times with lysis buffer and twice with M-PER buffer (Thermo Fisher Scientific). The Click-IT chemistry reaction was carried out for 1 h at room temperature in freshly made Click-IT reaction buffer (2 mM CuSO4, 1 mM bis(tert-butyl)-tris(2-mercaptoethanol), 10 mM sodium L-ascorbate, 50 µM biotin-alkyne). The mixture was then suspended in 4× SDS-loading buffer containing 2-mercaptoethanol. Samples were heated at 98 °C for 5 min and separated on 10% Tris-glycine SDS-polyacrylamide gels. Myristoylation was detected by probing the membrane with streptavidin-horseradish peroxidase (Thermo Fisher Scientific).

**Immunoprecipitation**

Cells were lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA) containing protease inhibitors and phosphatase inhibitors. One mg of total protein was used for immunoprecipitation with the anti-FRS2α antibody (Santa Cruz Biotechnology, sc-8318). After overnight incubation, protein A beads (50 µl; Cell Signaling Technology) were added to the lysate. After 1.5 h of incubation, the beads were washed with lysis buffer three times following by the addition of 2× SDS-loading buffer for immunoblotting. SHP2 (Santa Cruz Biotechnology, sc-7384), GRB2 (Santa Cruz Biotechnology, sc-8034), FRS2α (Santa Cruz Biotechnology, sc-8318), phosphothreonine (detected by anti-phosphothreonine antibody) (Abcam, ab9337), and phosphotyrosine (detected by anti-phosphotyrosine antibody) (Millipore, clone 4G10®, catalog no. 05-321) were examined by Western blotting.

**Subcellular fractionation**

Cells from 10-cm dishes were lysed in subcellular fractionation buffer (250 mM sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor mixture). The lysates were passed through a 25-gauge needle 10 times using a 1-ml syringe and then left on ice for 20 min. The nuclear fraction was collected by centrifugation at 720 × g for 5 min. The supernatant was transferred to a new tube and centrifuged at 10,000 × g for 5 min to pellet the mitochondrial fraction. The new supernatant containing cytosolic and cell membrane fractions was centrifuged in an ultracentrifuge at 100,000 × g for 1 h. The supernatant (cytosolic fraction) was transferred to a new tube. The pellet was washed with 400 µl of the fractionation buffer and resuspended by pipetting. The sample was recentrifuged for 45 min. The cell membrane pellet was resuspended in lysis buffer with 10% glycerol and 0.1% SDS.

**Fluorescence-based NMT activity assay**

The assay was performed in 96-well black microplates (Greiner Bio-One, Germany). Each well contained 31.6 µl of 2× RB buffer (100 mM HEPES, 1 mM EDTA), 4 µl of 2.8 µM NMT1 (in 1 mM EDTA, 250 mM NaCl, and 20 mM Tris, pH 8.5), 4 µl of 100 µM peptide (Gly-Ser-Asn-Lys-Ser-Lys-Pro-Lys, derived from the N terminus of Homo sapiens pp60src tyrosine kinase), 28.4 µl of H2O, and 4 µl of vehicle or different concentrations of B13. The reaction mix was incubated at 30 °C for 10 min. The reaction was started by adding 8 µl of 20 µM myristoyl-CoA (Avanti Polar Lipids) and incubated at 30 °C for 60 min. The released CoA was detected by adding 80 µl of 30 µM profluorescent probe 7-diethylamino-3-(4-maleimido-phenyl)-4-methylcoumarin (Sigma-Aldrich) to each well and incubated in the dark for 12 min. The final reaction volume was 160 µl/well. The fluorescence intensity was measured by a Flex Station 3 microplate reader by end point mode at excitation 390 nm and emission 479 nm.

**Cell proliferation, migration assay, and cell cycle analysis**

For the proliferation assay with drug treatment, cells were seeded in 96-well plates at a density of 3000–5000 cells per well depending on the growth rate of each cell line. After overnight incubation, cell numbers were measured by the MTT assay as day 0. Cells were then treated with different B13 concentrations or DMSO (a control) with each treatment having five independent wells as repeats. Cell numbers were measured after 24 h of treatment. The remaining wells were replaced with fresh medium containing B13 or DMSO every 24 h for 5 consecutive days. For the MTT assay, the growth medium was replaced with 100 µl of fresh culture medium without phenol red along with 10 µl of 12 mM MTT (Thermo Fisher Scientific) stock solution (dissolved in PBS) or 100 µl of the medium as a blank control. After incubation at 37 °C for 4 h, 100 µl of 10% SDS (dissolved in 0.01 M HCl) was added to each well. The plate was incubated at 37 °C for 4 h in a humidified chamber. Each sample was mixed by pipetting, and the absorbance at 570 nm was measured.

Cells were transduced with control vector or shRNA by lentiviral infection, and proliferation was measured. The transduced cells were cultured for 3 days and then plated at the same density (day 0). The cell numbers were measured by the MTT assay for 5 consecutive days. Cancer cell migration was evaluated by the wound-healing or the Transwell assay as described in the supporting Materials and Methods.

For cell cycle analysis, cells were grown in a medium containing 50 ng/ml FGF2 with B13 or DMSO for 3 days. The culture medium was replaced with fresh medium (with/without compound and FGF2) every 24 h. Cells (1 × 106) were collected and washed once with PBS and fixed with ice-cold 70% ethanol for
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30 min at 4 °C. After washing with PBS twice, the fixed cells were stained by FxCycle™ PI/RNase solution (Thermo Fisher Scientific) for 30 min in the dark at room temperature. Cells were analyzed using flow cytometry (CyAn ADP) (488-nm excitation and 585-nm emission).

Prostate regeneration assay

Primary prostate cells were isolated from 8–12-week-old male C57BL/6J mice and were transduced with FRS2α(WT) or FRS2α(G2A) by lentiviral infection. Urogenital sinus mesenchyme (UGSM) cells were isolated from 16.5-day embryos of C57BL/6J mice and transduced with control vector or FGFI0 by lentiviral infection. FRS2α(WT)- or FRS2α(G2A)-transduced prostate primary cells (2 × 10^5 cells/graft) were combined with FGFI0 or control UGSM (2 × 10^5 cells/graft). The cell mixture was resuspended in 20 μl of collagen type I (pH 7.0) (BD Biosciences). After overnight incubation, grafts were implanted under the kidney capsule of SCID male mice. Grafts were harvested after an 8-week incubation. C57BL/6J and CB.17SCID/SCID (SCID) mice were purchased from Tac- onic (Hudson, NY).

Xenograft tumors

To examine the inhibitory activity of B13 on the growth of xenograft tumors, 2 × 10^6 SNU16 cells were subcutaneously inoculated in the flank side of SCID female mice. After 2 weeks, mice carrying xenografts were randomly separated into two groups. B13 was dissolved in the vehicle solution containing 30% Kolliphor, 65% saline (0.9% NaCl), and 5% ethanol. One group received 160 μl of the drug solution at a concentration of 65 mg/kg body weight via tail vein injection twice a week for 6 weeks. The other group received 160 μl of vehicle via tail vein injection twice a week for 6 weeks. Body weight and tumor size were measured (length × width × width/2) weekly. Xenograft tumors, liver, lungs, and kidneys were harvested for immunohistochemistry analysis.

For examining the effect of FRS2α knockdown on xenograft tumors, SNU16 cells and PC-3 cells were transduced with shRNA targeting FRS2α or control shRNA by lentiviral infection. The infected SNU16 cells (2 × 10^6) or PC-3 cells (5 × 10^5) were subcutaneously inoculated in the flank side of SCID female and male mice, respectively. After 8 weeks (SNU16) or 6 weeks (PC-3), xenograft tumors were harvested. All animals were maintained according to the surgical and experimental procedures of the protocol A2013 03-008 approved by the institutional animal use and care committee at the University of Georgia.

Immunohistochemistry

Formalin-fixed/paraffin-embedded specimens were sectioned at 5-μm thickness and mounted on positively charged slides. Sections were stained by H&E or IHC staining as described previously (20). The following antibodies were used for immunohistochemistry: AR (Santa Cruz Biotechnology, sc-816; 1:200), CK5 (BioLegend, 905501; 1:500), CK8 (Bio- Legend, 904801; 1:1000), Ki67 (Novus Biologicals, NB500–170; 1:400), and CD34 (Abcam, ab81289; 1:1000).

Statistical analysis

Prism software was used to carry out statistical analyses. The data are presented as mean ± S.D. or mean ± S.E. and analyzed using Student’s t test. All t tests were performed at the two-sided 0.05 level for significance. *, p < 0.05; **, p < 0.01.

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