siRNA mediated knockdown of fibroblast growth factor receptors 1 or 3 inhibits FGF-induced anchorage-independent clonogenicity but does not affect MAPK activation

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Abstract. Supplementation with exogenous growth factors such as fibroblast growth factors (FGFs) is essential for anchorage-independent growth of the SW-13 human adrenal adenocarcinoma cell line. We have found that SW-13 cells express mRNAs for FGFRs 1, 3, and 4, but not FGFR2. To assess the roles of individual FGFRs, in anchorage-independent growth, we determined the effects of down-regulation of each FGFR on FGF2- and FGF4-mediated soft agar colony formation in these cells. Using RNAi strategies we found that knockdown of either FGFR1 or FGFR3 leads to inhibition of FGF2- or FGF4-induced soft agar clonogenicity without affecting that induced by heregulin ß1. However, this inhibition is independent of ERK1/2 activation as levels of FGF-induced phospho-ERK 1/2 remain unchanged upon knockdown of either FGFR1 or FGFR3. Conversely, RNAi-mediated knockdown of FGFR4 appeared to have no significant effect on either FGF2- or FGF4-induced anchorage-independent colony formation, or ERK1/2 phosphorylation. These results suggest that constitutive levels of both FGFR1 and FGFR3, but not FGFR4 are essential for FGF-stimulated anchorage-independent growth of SW-13 cells.

Introduction

Fibroblast growth factors (FGFs) belong to a family of 23 structurally-related polypeptides that play critical roles in both physiological and pathological pathways. FGFs are involved in a wide variety of cellular functions such as proliferation, differentiation, and migration, in addition to performing biologically important roles in angiogenesis, and early development such as limb patterning and proper bone development (1,2). FGFs elicit their effects by binding to one of four fibroblast growth factor receptors (FGFRs), which belong to a family of cell surface receptor tyrosine kinases characterized by an extracellular ligand-binding domain composed of three immunoglobulin (Ig)-like domains and an intracellular region that includes a split tyrosine kinase-signaling domain (3). Ligand-binding induces receptor dimerization resulting in trans-autophosphorylation of intracellular kinase domains and activation of downstream signaling pathways. Binding of FGFs to their receptors results in activation of the MEK-ERK1/2 and other signaling pathways. FGFR signaling is complicated by the fact that FGFR family members can form both homodimeric or heterodimeric pairs and that multiple alternative mRNA splicing events can occur for each receptor, greatly affecting FGF ligand specificity and sensitivity (4).

Disruption of FGFR signaling has been associated with several cancer processes. Mutations generated in the kinase domains of FGFRs 1, 3, and 4 have been shown to transform NIH3T3 cells, and to induce neurite outgrowth in PC12 cells (5). Additionally, activating mutations in FGFR2 and FGFR3 have been associated with bladder, cervix, and colorectal cancers (6,7). Although these and other activating mutations have been described, little is known about the role of specific FGFRs or the cellular mechanisms operating downstream of these receptors that can lead to a transformed phenotype. In addition to activating mutations, FGFRs have been shown to be abnormally expressed in a number of cancers including breast (8), thyroid (9), brain (10), and pancreas (11). FGFR mediated signaling has also been implicated in the progression of metastatic phenotypes (12,13) and has been shown to be at least partially involved in the development and persistence of antiestrogen resistance in human breast carcinomas (14,15). Our laboratory has previously shown that a dominant negative FGFR, containing only the extracellular, transmembrane, and juxtamembrane domains, is capable of inhibiting FGF1-dependent MCF-7 breast cancer cell growth in the presence of antiestrogens or in the absence of estrogen (16). Little is known, however, concerning the role of individual FGFRs in cancer-related signaling. For instance, it is not currently understood whether redundancy in the FGFR-signaling family will compensate for the loss of a specific

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FGFR, or whether specific FGFR homodimers or heterodimers are required for the development and/or progression of the specific neoplastic phenotypes. Therefore, it is important to determine whether targeting an individual receptor is sufficient to inhibit FGF-induced cancer proliferation or migration.

FGFRs have been shown to induce anchorage-independent growth of SW-13 adrenal carcinoma cells (17,18). In this study, we utilize FGF2 or FGF4, both of which have been shown to induce robust colony formation in soft agar, to stimulate SW-13 cells in order to elucidate the role of individual FGFRs in FGF-dependent growth conditions. The effects of knockdown of individual FGFRs using RNAi on FGF2- or FGF4-induced cell growth were determined by utilizing anchorage-independent colony formation assays. These assays are believed to closely mimic the malignant nature of cancer cells in humans (19). First, we determined that SW-13 adrenal cortex carcinoma cells express mRNA for FGFRs 1, 3, and 4 by quantitative RT-PCR. We then showed that transiently transfected siRNA duplexes targeting FGFR1 or FGFR3 can inhibit FGF2- or FGF4-induced growth of SW-13 cells and that this inhibition is independent of both MAPK and Akt activation. siRNA-mediated knockdown of FGFR4 has no significant effect on FGF2- or FGF4-induced colony formation. Furthermore, we show that cells transfected with a combination of siRNA targeting MEK1 and MEK2 inhibit both FGF2- and FGF4-induced soft agar colony formation. We also show that whereas FGFR knockdown has no effect on the growth of attached cells, the MEK1/2 siRNA duplexes are significantly inhibitory in this context as well.

Materials and methods

Chemicals, growth factors, and antibodies. Recombinant human FGF-2 and FGF-4 were obtained from Promega (Madison, WI), and recombinant human heregulin ß1 (HRGß1, residues 176-246 corresponding to the EGF-like domain) was from R&D Systems (Minneapolis, MN). Phenol red-free and phenol red containing improved modified Eagle's medium (IMEM) were obtained from Mediatech, Inc. (Herndon, VA), and fetal bovine serum was obtained from Gibco® (Invitrogen, Carlsbad, CA). Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-Akt and anti-Akt antibodies were also obtained from Cell Signaling Technology.

Small interfering RNA. Two siRNA duplexes targeting FGFR1 were obtained, 1a: 5'-GAAGUGCAUACACCGAGAC-3' and 1b: 5'-AUUCAAACCUGACCACAGA-3' from Xeragon Inc. (Valencia, CA) and Dharmacon, Inc. (Lafayette, CO) respectively. For siRNA targeting FGFR3 and FGFR4, four separate SmartPool® siRNA sequences (Dharmacon, Inc.) against each receptor were screened and the most efficient duplex was chosen for subsequent studies. For FGFR3, the target sequence was 5'-GGACGGCACACCCUACGUU-3'. The siRNA sequence used to develop the FGFR3 shRNA expression constructs (target sequence 5'-AGACGATGC CACTGACAAG-3') was chosen by placing the coding sequence for FGFR3 into the Oligoengine siRNA design tool available via the internet (www.oligoengine.com) and screened for efficacy prior to insertion into the pSuper vector. For FGFR4, the target sequence was 5'-AUACGGACAUCA UCCUGUA-3'. siCONTROL® duplexes (Dharmacon, Inc.) with no known homology to rat, mouse, or human genes were used as controls. After verifying siRNA sequences for efficiency versus specific FGFRs, siRNA duplexes modified for enhanced stability (siSTABLE™ siRNA) for FGFRs 1, 3, and 4 were obtained from Dharmacon, Inc. siRNA duplexes targeting both MEK1 and MEK2 have been described previously (20).

Cell lines and cell culture conditions. The SW-13 clonal cell line was derived from a human small cell adenocarcinoma of the adrenal cortex (21). Cells were maintained in IMEM containing 5-10% fetal bovine serum (FBS) and incubated in humidified conditions with 5% CO_2 at 37˚C. SWR3C is a polyclonal cell line derived from SW-13 stably transfected with pSuper-EGFP. SWR3.1 and SWR3.6 are independent clonally derived lines from SW-13 cells that were stably transfected with pSupR3-EGFP, an shRNA expression vector targeting FGFR3 (see below). The SWpsiRNAlacZ cell line is a polyclonal SW-13 derived cell line that stably expresses the psiRNAlacZ plasmid (Clontech, Palo Alto, CA) targeting ß-galactosidase.

Construction of pSuper-EGFP and pSupR3-EGFP plasmids and stable transfections. To construct the parent shRNA expression vector targeting FGFR3 (pSupR3), an siRNA sequence targeting FGFR3 and containing *Hin*dIII and NotI overhanging ends were ligated into the short hairpin expression pSuper vector (a gift from Dr Reuven Agami, The Netherlands Cancer Institute) that had been linearized by double restriction enzyme digest using *Hin*dIII and NotI enzymes. A 1.2-kb EGFP expression cassette excised from the pEGFP-C1 plasmid (Clontech) using AseI and MluI was filled-in with Klenow and then ligated into the pSupR3 vector linearized with NaeI restriction enzyme. The EGFP expression cassette was also ligated into pSuper to generate the pSupR-EGFP vector. This fragment includes a CMV promoter region followed by an EGFP expression region upstream of a polyadenylation region. To generate stable transfectants of SW-13 cells expressing shRNA targeting FGFR3, cells were cotransfected with pSupR3-EGFP and a blasticidin resistance vector (pEF6 Myc/His A) using Lipofectamine (Invitrogen). Blasticidin-resistant polyclonal and clonal populations of cells were isolated. Polyclonal populations of SW-13 cells stably expressing shRNA targeting ß-galactosidase (SWpsiRNAlacZ) served as a negative control cell line. To generate this line, the psiRNAlacZ plasmid (Clontech, Palo Alto, CA) was transfected into SW-13 cells as described above.

Real-time quantitative RT-PCR analysis. For QRT-PCR, mRNA was isolated using mRNACatcher™ 96-well plates (Invitrogen) according to the manufacturer's protocol for cells in monolayer. Briefly, cells were plated at $7.5x10^4$ cells per well in 24-well dishes in IMEM supplemented with 10% fetal bovine serum. Cells were transfected with siRNA duplexes 24 h after plating. Forty-eight hours after plating, transfection media was removed and cells were rinsed once in PBS before being lysed in 150-μl lysis buffer containing guanidine thiocyanate. Lysates were transferred to individual wells in the

mRNACatcher plates. Lysates were incubated for 90 min at room temperature and were then removed. The wells were then rinsed 3 times in a wash buffer. Elution buffer (50 μl) was then added to each well (65˚C, 5 min) and the eluate containing mRNA was transferred to microcentrifuge tubes for storage at -80˚C. First strand cDNA was synthesized using the SuperScript III kit (Invitrogen) according to the manufacturer's recommendations. Aliquots of the cDNA were combined in a 20 μl reaction mix containing 300 nM 5' forward primer, 300 nM 3' reverse primer, and 100 nM Fam-Tamra double fluorescently labeled MGB (minor groove binding) probe and subjected to quantitative PCR analysis on 384-well plates (Applied Biosystems, Foster City, CA). Human FGFR1, 3, and 4 sequences [GenBank accession no. X51803 (FGFR1), M58051 (FGFR3), X57205 (FGFR4)] were analyzed and gene-specific primers were determined using Assays-by-Design™ (Applied Biosystems). For FGFR1, the primers were 5'-CACAGAATTGGAGGCTACAAGGT-3' (forward) and GCACCACAGAGTCCATTATGATG (reverse). For FGFR3, the primers were 5'CTGAAGAACGGCAGGGAGTT-3' (forward) and 5'-CCAGGCTCCACTGCTGATG-3' (reverse). For FGFR4, the primers were 5'-GAGGAGGACCCCACAT GGA-3' (forward) and 5'-CGTACAGGATGATGTCCGTA TACCT-3' (reverse). Gene-specific probe sequences were 5'-CTCCAGGTGGCATAA-3' (FGFR1), 5'-CATTGGAG GCATCAAG-3' (FGFR3), and 5'-CAGCAGCGCCCGAG-3' (FGFR4). Reactions were carried out on an ABI Prism Taqman 7900 HT sequence detector (Applied Biosystems) according to the manufacturer's instructions. Reaction conditions were 50˚C for 2 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min.

The comparative CT method was used to represent the relative expression level of FGFRs in cells transfected with siRNA targeting individual FGFRs versus non-transfected or control transfected cells. ΔC_T values for each of the samples and the calibrator sample were obtained as the difference between CT values between the target transcript and human GAPDH for which the forward and reverse primer sets and probes were obtained from Applied Biosystems (part number 4326317E). The relative expression level is expressed as a unitless number and calculated as $2^{-\Delta\Delta T}$ as described (22).

Anchorage-independent colony formation assays. The ability of SW-13 cells transiently transfected with siRNA duplexes (Dharmacon, Inc.) targeting individual FGFRs to form colonies in soft agar was compared to control cell lines transiently transfected with non-targeting control siRNA (Dharmacon, Inc.) under growth factor-dependent conditions. Cells were plated at $2.5x10^5$ cells per well in 12-well dishes and transfected using DharmaFECT™ 2 reagent using 20 nM siRNA per well. For anchorage-independent colony formation assays using MEK1 and MEK2 siRNAs, cells were plated and transfected as above except that cells were cotransfected with MEK1 and MEK2 siRNAs at a concentration of 20 nM for each siRNA duplex for a final total siRNA concentration of 40 nM. Control cells transfected with non-targeting negative control siRNA were transfected to a final siRNA concentration of 40 nM. A mixture of 0.6% agar (1 μl) (Bacto-agar; Difco, Detroit, MI) containing 2X IMEM (BioSource, Int., Camarillo, CA) and 5% FBS supplemented phenol red-free IMEM was plated to form a bottom layer in 35-mm dishes (Corning, Acton, MA) and allowed to solidify at room temperature. Cells ($1x10^4$) per dish were suspended in 0.8 ml of a top agar solution of 0.36% agar in IMEM supplemented with either 5% FBS or 5% FBS plus either FGF2 or FGF4 at a final concentration of 20 ng/ml. All media contained heparin sulfate (Sigma-Aldrich) at a final concentration of 50 μg/ml. The top layer was allowed to solidify at room temperature and then all dishes were incubated for 12 to 14 days at 37˚C in a humidified 5% CO_2 atmosphere. Images of dishes were taken on a Spot camera (Diagnostic Instruments, Inc. Sterling Heights, MI) and colonies >60 μm were counted using ImagePro Plus software (Media Cybernetics, San Diego, CA). Statistical analysis was performed using the Student's t-test. Similarly, SW-13 clonal cell lines expressing shRNA targeting FGFR3 to form colonies in soft agar was compared to shRNA control cell lines under FGF-dependent conditions as described above.

Anchorage-dependent growth assays. In order to verify that the effects of FGFR1 and FGFR3 knockdown on FGF-induced anchorage-independent colony formation were in fact due to FGFR-specific knockdown, we tested the ability of these transfectants to grow under anchorage-dependent conditions using a 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxy-Phenyl)-2-(4-Sulfonyl)-2*H*-Tetrazolium (MTS) assay (Cell-Titer 96 AQueous one solution cell proliferation assay kit, Promega). SW-13 cells were plated at $1.5x10^5$ cells per well in 96-well plates. Following 24 h of plating, cells were transfected using DharmaFECT 2 transfection reagent (Dharmacon, Inc.) as described above. The following day, the transfection mix was removed and replaced with 5% FBS-IMEM. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for 7 days. Media were replaced after 4 days with fresh 5% FBS containing media. On Day 7, 40 μl of MTS assay reagent was added to each well and the plates were incubated at 37˚C for 2 h. Absorbance at 490 nm was read with a 96- well plate reader.

Immunoblot analyses. For the analysis of phospho or total ERK1/2, and Akt, Western blotting was performed on lysates prepared from SW-13 cells transiently transfected with siSTABLE siRNA (Dharmacon, Inc.) targeting FGFRs 1, 3, or 4, with a combination of a MEK1 and a MEK2 siRNA, or with non-targeting control siRNA (Dharmacon, Inc.). Cells were plated at $4x10^5$ cells per dish in 60-mm dishes in 10% FBS. Following 24 h of plating, cells were transfected with siRNA at a final concentration of 20 nM. As a positive control for these experiments, we included cells transfected with a combination of MEK1 and MEK2 siRNAs as described above at a concentration of 20 nM for each siRNA duplex for a final total siRNA concentration of 40 nM. For these assays, the non-targeting control siRNA was transfected to a final concentration of 40 nM to show that any effect of MEK siRNA on MAPK or Akt phosphorylation was not due to the higher concentration of MEK siRNA being used. The following morning, media was changed to plain IMEM for an additional 24 h. To treat the cells, appropriate volumes of FGF2, FGF4, or HRGß1 were added to plain IMEM. Volumes of plain IMEM (200 μl) plus additions were then added to each dish. Final concentrations were 20 ng/ml for FGF2 or

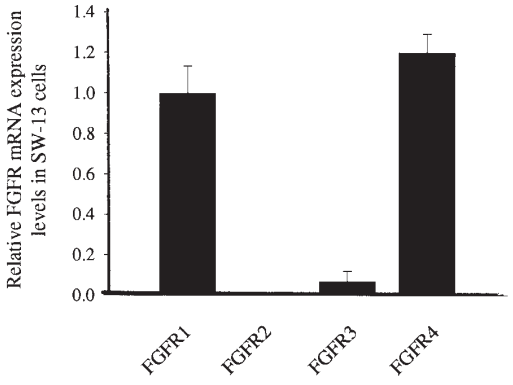

Figure 1. SW-13 human adrenal cortex carcinoma cells express mRNA for FGF receptors 1, 3, and 4. mRNA was isolated from untreated wild-type SW-13 cells and subjected to quantitative RT-PCR analysis using receptor-specific primers and probes. Amounts shown are relative to FGFR1. FGFR3 expression levels are ~10% of FGFR1 expression levels, and FGFR4 expression is ~110% of FGFR1. FGFR2 was not detectable in this cell line in this assay although the probes used were able to detect FGFR2 mRNA in a control cell line. QRT-PCR was performed on an ABI Prism 7900 HT. Error bars represent the standard error of the mean (SEM) from the values of triplicate wells.

FGF4 and 50 ng/ml for HRGß1. Cells were incubated in the conditions for 5 min and were then rinsed once in ice cold 1X PBS prior to lysis using 200 μl cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethylsulfonyl fluoride. Cell lysate (35 μg) per well was run on Criterion precast 10% SDS-PAGE gels (Bio-Rad, Hercules, CA). Proteins were transferred onto Protran filters (Bio-Rad) by electroblotting. The blots were then probed with specific antibodies against phospho-ERK1/2 p44/42 (Cell Signaling Technology). After probing with phospho-ERK1/2 p44/42 antibodies, blots were stripped with Restore stripping solution (Pierce, Rockford, IL) and reprobed with antibodies against total ERK1/2. Blots for phospho-Akt and total Akt were loaded and run as above using lysates from the same group of treated cells. Enhanced chemiluminescence with the Supersignal kit (Pierce) was used to detect bands. Western blots were also performed for analysis of phospho-ERK1/2 or total-ERK1/2 in SWR3C control cells, SWpsiRNAlacZ control cells, or SWR3 clonal cell lines stably expressing short hairpin RNA targeting FGFR3 as described above.

Results

SW-13 cells express fibroblast growth factor receptors. Previous studies have shown that untreated SW-13 cells will not form colonies in soft agar but will respond to certain exogenously supplemented growth factors, including pleiotrophin and fibroblast growth factors, by forming colonies in anchorage-independent colony formation assays (17,18). To determine the relative expression levels of individual FGFRs in this cell line, we performed QRT-PCR analysis on untreated SW-13 cells using receptor-specific primers. As shown in Fig. 1, SW-13 cells were found to express 3 of the 4 known FGFRs constitutively. In relative terms levels of FGFR4 mRNA were the highest, whereas FGFR3 mRNA was least expressed of the three receptors (Fig. 1). Probes for FGFR2 were not able to detect mRNA for this receptor in SW-13 cells

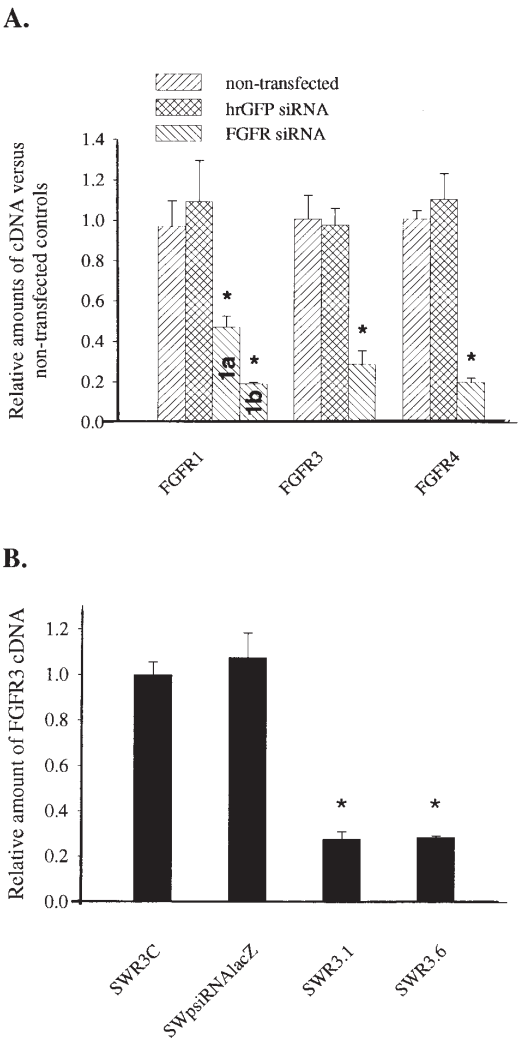

Figure 2. siRNA sequences targeting individual FGFRs (A) or shRNA targeting FGFR3 (B) are able to mediate knockdown of target mRNA in SW-13 cells. (A) SW-13 cells were transiently transfected with 20 nM final concentration of siSTABLE siRNA duplexes targeting individual receptors using DharmaFECT 2 transfection reagent (Dharmacon). Following 48 h of transfection, mRNA was isolated and cDNA was synthesized and subsequently subjected to quantitative RT-PCR analysis using receptor-specific primers and probes. Non-transfected cells or SW-13 cells transfected with siRNA targeting hrGFP served as controls. 1a and 1b represent siRNA duplexes targeting separate regions of FGFR1. Target FGFR mRNA levels were reduced by at least 70% for all three of the receptors expressed in this cell line. (B) An shRNA expression vector stably expressing an shRNA sequence targeting FGFR3 mediates target knockdown in SW-13 cells. SW-13 cells were stably transfected with an EGFP-shRNA expression vector as described in Materials and methods. mRNA was isolated, cDNA was synthesized, and subsequently subjected to quantitative RT-PCR analysis as described above. SW-13 cells stably expressing an empty control vector (SWR3C) or expressing shRNA targeting ß-galactosidase (SWpsiRNAlacZ) served as controls. Two clonal cell lines expressing the same shRNA vector targeting FGFR3, SWR3.1 and SWR3.6, both reduced FGFR3 levels by at least 70%. Statistics were performed using the Student's t-test. Error bars represent SEM of triplicate samples, $^{*}p<0.005$. Results are representative of 3 separate experiments.

in these assays (Fig. 1) although they were able to detect FGFR2 mRNA in MCF-7 breast carcinoma control cells (data not shown).

Selected FGFR siRNA sequences induced markedly decreased target mRNA levels in SW-13 cells. To determine the relative

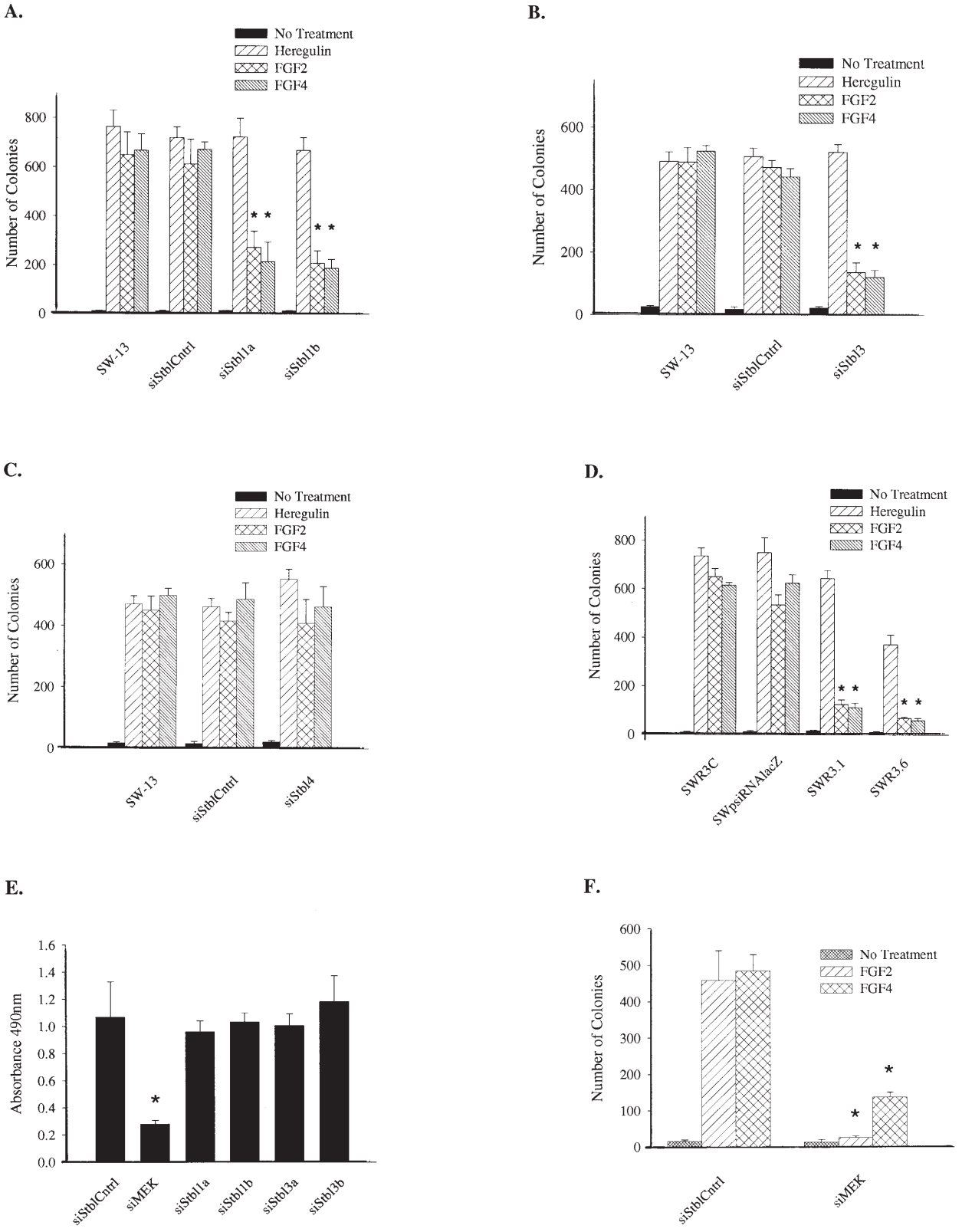

Figure 3. siRNA-mediated knockdown of either FGFR1 (A) or FGFR3 (B and D) is sufficient to inhibit FGF2- or FGF4-dependent anchorage-independent colony formation in SW-13 cells whereas siRNA mediated knockdown of FGFR4 has no effect on FGF2- or FGF4-dependent colony formation (C). SW-13 cells were transfected with siSTABLE siRNA (Dharmacon) targeting individual FGFRs or with non-targeting siSTABLE control siRNA (Dharmacon) using DharmaFECT 2 transfection reagent. Cells were lifted 24 h post-transfection and were plated in soft agar in triplicate in 35-mm dishes. Cells were incubated for 14 days prior to colony counts. For FGFR1, experiments were repeated using two separate siRNA sequences (siStbl1a and siStabl1b) targeting different regions of FGFR1 and similar results were obtained. (C) RNAi-mediated knockdown of FGFR4 had no significant effect on either FGF2- or FGF4-induced colony formation. SW-13 cells transfected with siRNA versus FGFR4 were capable of forming colonies in soft agar. (D) SW-13 clonal cell lines expressing a shRNA sequence targeting a separate region of mRNA relative to the duplex oligos resulted in similar inhibition of FGF-induced colony formation. (E) siRNA duplexes targeting FGFR1 and FGFR3 have no significant effect on growth of untreated SW-13 cells under anchorage-dependent conditions as determined using the MTS cell proliferation assay. (F) siRNA mediated knockdown of MEK1/2 using a cotransfection of siRNAs targeting MEK1 and MEK2 inhibited both FGF2- and FGF4-induced anchorage-independent colony formation of SW-13 cells. Statistics were performed using the Student's t-test. Error bars represent SEM of triplicate dishes, $^{*}p<0.005$. Results are representative of at least 2 separate experiments.

contribution of individual FGFRs to FGF-dependent growth in SW-13 cells, we downregulated each receptor using RNA interference (RNAi). To verify the ability of the selected siRNA sequences to knock down target mRNA levels, we transiently transfected these cells with siRNA duplexes targeting individual FGFRs and determined target mRNA expression levels via real-time quantitative RT-PCR analysis using receptor-specific primers and probes. Target knockdown was assayed 48 h post-transfection. Data shown in Fig. 2A indicate that siRNA targeted against FGFR1, FGFR3, and FGFR4 are able to effectively knock down target receptor mRNA levels by >70% (Fig. 2A). In addition, we also tested FGFR3 mRNA levels in two SW-13 derived clonal cell lines (SWR3.1 and SWR3.6) stably expressing short hairpin RNA (shRNA) targeting FGFR3. Results show that shRNA expression with sequences targeting FGFR3 is able to reduce target mRNA expression levels by at least 70% (Fig. 2B).

RNAi-mediated knockdown of individual FGFRs is sufficient to inhibit FGF2- or FGF4-dependent anchorage-independent colony formation. SW-13 cells are unable to form colonies in soft agar despite expressing high levels of endogenous FGF2, but will respond to supplementation with exogenous FGF2 by readily forming colonies (23). We have also found that SW-13 cells will respond to FGF4, FGF8, FGF 18, and heregulin ß1 by forming colonies under anchorage-independent conditions (data not shown). We utilized exogenous FGF2- or FGF4-induced colony formation as an end-point in order to determine the relative contribution of individual FGFRs to SW-13 anchorage-independent growth under FGF- dependent conditions. SW-13 cells were transiently transfected with stability-enhanced siRNA duplexes targeting either individual FGFRs or control duplexes (siCNTRL). Cells were then plated in soft agar with the addition of either FGF2, FGF4 or heregulin ß1, and incubated for 12-14 days prior to counting of colonies.

As shown in Fig. 3, FGF2, FGF4 and heregulin ß1 were all equally effective in inducing robust colony formation in soft agar. Knockdown of FGFR1 with siRNA duplexes targeting two distinct sequences (1a and 1b) reduced FGF2- and FGF4-dependent colony formation by 70-80% relative to controls, with no effect on heregulin ß1-induced colonies (Fig. 3A). Knockdown of FGFR3 also inhibited colony formation by ~70-80% (Fig. 3B). Knockdown of FGFR4 had no significant effect on FGF2- nor FGF4-induced colony formation (Fig. 3C). Time-course assays aimed at determining the duration of knock-down in these cells indicated that FGFR4 mRNA was still reduced by 50% seven days post-transfection (data not shown). SW-13 cells stably expressing shRNA targeting a separate region of FGFR3 were also plated in soft agar as described above. Similar to the results from assays using FGFR3 siRNA duplexes, colony formation was significantly decreased in these cell lines as compared to control cell lines expressing either empty shRNA expression cassettes (SWR3C) or in control cells expressing shRNA targeting ß-galactosidase (SWpsiRNAlacZ) (Fig. 3D).

In order to further demonstrate that the effects of FGFR1 or FGFR3 on FGF2- or FGF4-induced colony formation were due to target-specific knockdown and not off-target effects, we utilized an anchorage-dependent colony formation assay.

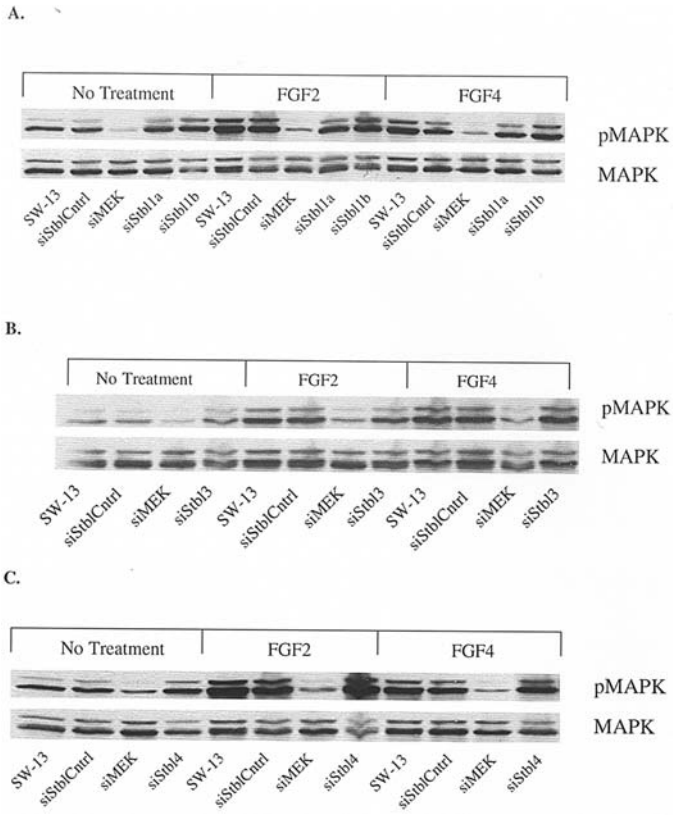

Figure 4. Knockdown of individual FGFRs has no significant effect on FGF2- or FGF4-induced MAPK phosphorylation in SW-13 cells. Western blot analysis of total and phosphorylated ERK1/2 in SW-13 cells transiently transfected with siSTABLE siRNA targeting individual FGFRs and treated with either FGF2 or FGF4 shows that RNAi-mediated knockdown of FGFR1 (A), FGFR3 (B), or FGFR4 (C) has no effect on FGF2- or FGF4-induced MAPK phosphorylation compared to either non-transfected control cells or cells transfected with non-targeting control siRNA. Cells transfected with siRNA duplexes targeting both MEK1 and MEK2 did show a significant reduction in either FGF2- or FGF4-induced MAPK phosphorylation. Cells were lysed, and lysate aliquots (30 μg) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total ERK1/2. Blots shown are representative of at least 3 separate experiments.

SW-13 cells are able to proliferate under anchorage-dependent conditions without the addition of exogenous growth factors. If the effects of FGFR1 or FGFR3 knockdown seen in anchorage-independent growth assays were due to off-target effects, then it is possible that inhibition of growth would also be seen in an anchorage-dependent assay. Transfection of SW-13 cells with FGFR1 or FGFR3 siRNA had no effect on the ability of untreated SW-13 cells to proliferate in an anchorage-dependent manner (Fig. 3E), further suggesting that the effects of FGFR1 and FGFR3 knockdown in FGF2- or FGF4-induced soft agar colony formation are specific for those targets.

RNAi knockdown of MEK1/2 inhibits both anchorage-independent colony formation and anchorage-dependent growth. FGFRs are known to signal through activation of the Ras-Raf-Mek signaling cascade (24). In order to determine if the effects of FGFR1 or FGFR3 knockdown on FGF-induced soft agar colony formation were due to effects on MEK-dependent signaling, we examined the effects of knocking down MEK1 and MEK2 on anchorage-independent colony

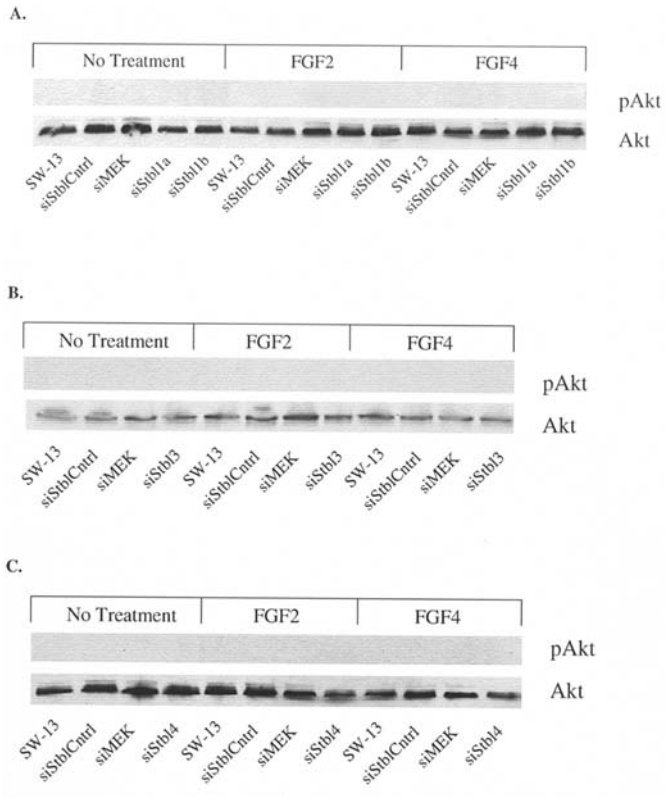

Figure 5. Treatment of SW-13 cells with either FGF2 or FGF4 does not induce Akt phosphorylation. SW-13 cells were transfected with siRNA sequences targeting FGFR1 (A), FGFR3 (B), or FGFR4 (C) and received either no treatment or treatment with either FGF2 or FGF4. Western blot analysis was performed to determine the effect of siRNA expression on FGF-induced Akt phosphorylation. Surprisingly, Akt was not phosphorylated in SW-13 cells when treated with either FGF2 or FGF4. Akt was activated when cells were treated with HRGß1 (data not shown). Cells were lysed, and lysate aliquots (30 μg) were run on SDS-PAGE gels and analyzed by Western immunoblotting for active (phospho) and total Akt. Blots shown are representative of at least 3 separate experiments.

formation in SW-13 cells. SW-13 cells were plated in soft agar 24 h after transfection with a combination of MEK1 and MEK2 siRNA in 12-well dishes as described in Materials and methods. Dishes were incubated for 14 days prior to counting of colonies. As shown in Fig. 3F, cells transfected with a combination of MEK1 and MEK2 siRNA showed significantly fewer FGF2- or FGF4-induced colonies than either non-transfected control cells or control cells transfected with a non-targeting control siRNA. These results suggest that MEK1/2 knockdown is sufficient to inhibit both FGF2- and FGF4-induced anchorage-independent colony formation in SW-13 cells.

Inhibition of FGF-dependent colony formation by FGFR knockdown is not dependent on inhibition of MAPK activation. Ligand mediated activation of FGFRs results in trans-autophosphorylation of juxtaposed intracellular kinase domains with subsequent recruitment of signaling molecules leading to phosphorylation and activation of components of the MAPK signaling pathway. In order to determine if knockdown of either FGFR1 or FGFR3 had effects on MAPK activation, we probed the phosphorylation status of ERK1/2 with phosphospecific antibodies by Western blot analysis in SW13 cells transiently transfected with siRNA duplexes targeting individual FGFRs or non-targeting control siRNA and treated with either FGF2 or FGF4. Cells were incubated in the transfection mix overnight and the following day, the transfection mix was removed and replaced with plain IMEM. Cells were incubated an additional 24 h in the plain IMEM to reduce background levels of MAPK phosphorylation. Cells were then either left untreated, or treated with either FGF2 or FGF4 for 5 min. Cells were lysed and whole cell lysates were subjected to immunoblot analysis. RNAi mediated knockdown of either FGFR1 or FGFR3 had no effect on FGF2- or FGF4-induced phospho ERK1/2 levels despite the fact that this knockdown significantly inhibited FGF2- or FGF4-induced anchorage-independent growth (Fig. 4A and B). siRNA-mediated knockdown of FGFR4 also had no effect on either FGF2- or FGF4-induced ERK1/2 phosphorylation (Fig. 4C). None of the transfected cells showed any effect on HRGß1-induced MAPK phosphorylation (Fig. 4A-C).

In addition to siRNA duplexes targeting FGFR3, we also determined the effects of shRNA-mediated knockdown of FGFR3 on FGF-induced MAPK phosphorylation using the SWR13 clonal cell lines. Cells were plated in 5% FBS-containing IMEM and incubated for 24 h at 37˚C. Media were then removed and replaced with plain IMEM as described above. Cells were treated with FGF2 or FGF4 for 5 min and then lysed, and lysates analyzed by immunoblotting as described above. We found no decrease in FGF-induced ERK1/2 phosphorylation in SW13 clones expressing FGFR3 shRNA (data not shown).

RNAi-mediated inhibition of FGF2- or FGF4-dependent SW-13 colony formation is independent of Akt activation. In addition to activation of ERK1 and ERK2, FGFs are also known to be able to induce activation of the PI3K/Akt pathway. In order to determine whether Akt was activated in SW-13 cells and whether this activation was inhibited by FGFR siRNA, we probed for Akt activation in SW-13 cells treated with either FGF2 or FGF4 via Western analysis. Surprisingly, although SW-13 cells do express Akt, it was not activated by either FGF2 or FGF4 (Fig. 5A-C). However, cells treated with HRGß1 did show an induction of Akt phosphorylation suggesting that this was not due to a general defect in Akt signaling in this line (data not shown). This suggests that Akt activation does not play a predominant role in FGF-specific signaling in these cells.

Discussion

We utilized the SW-13 cell line to explore the relative importance of individual FGFRs in mediating FGF2- or FGF4-induced anchorage-independent proliferation. SW-13 cells are derived from a human adrenal cortex adenocarcinoma and have been shown to respond to the addition of exogenous FGFs by forming colonies in soft agar (17,18). Wellstein *et al* (23) showed that SW-13 cells produce large amounts of endogenous FGF2, but that this FGF2 is not secreted and is not able to stimulate colony formation in soft agar. Pleiotrophin, another heparin-binding growth factor, has also been shown to induce SW-13 colony formation under anchorage-independent conditions (25), as has FGFs 1, 8, and 18, and

HRGß1 (Estes *et al*, unpublished data). On the other hand, SW-13 cells do not respond to other cancer derived growth factors such as TGF-α, TGF-ß, IGF, and PDGF (17,18). This could be explained by the fact that these cells do not express receptors for EGF, PDGF or IGF (26).

To gain a better understanding of the role that individual FGFRs play in FGF-dependent colony formation of SW-13 cells, we have utilized RNAi to selectively knock down individual FGFRs in this cell line. We show that transient transfection of siRNA duplexes targeting individual FGFRs can selectively knock down the expression levels of the FGFRs in these cells. Stable siRNA duplexes targeting FGFR1, 3 or 4 are able to reduce target mRNA levels by ≥70% (Fig. 2A). We also show that stable expression of shRNA targeting FGFR3 can selectively knock down its expression in SW-13 cells. Two separate SW-13 clonal cell lines stably expressing shRNA targeting the same sequence of FGFR3 are able to reduce target mRNA by 75% (Fig. 2B). The shRNA sequence used in these clonal cell lines, although distinct from the sequence used in the siRNA duplexes against this receptor in transient transfections, was equally effective in knocking down target mRNA and in inhibition of FGF2- and FGF4-induced soft agar colony formation. We were unable to obtain stable clones of cell lines expressing shRNA targeting receptors 1 or 4 that were consistently able to knock down target mRNA. Recent studies indicate that siRNA duplex efficacy does not always translate into effective knockdown when expressed as shRNA, possibly due to ineffective processing of the intermediate hairpin structures (27). This could account for the inability of these sequences to effectively knock down target FGFR mRNA when expressed as shRNA.

Given the redundancy in the FGF receptor family, in addition to the fact that family members are able to form heterodimers with other family members, it would not have been surprising had the knockdown of individual receptors not had significant effect on FGF2- or FGF4-induced cell growth. It is significant therefore that we find that knockdown of individual receptors, specifically FGFR1 or FGFR3, has a marked effect on either FGF2- or FGF4-induced colony formation in anchorage-independent assays (Fig. 3A, B and E) while knockdown of FGFR4 has no effect on this FGF-dependent growth (Fig. 3C). This suggests that FGFR1/FGFR3 heterodimers could be responsible for mediating the specific signaling cascade that results in FGF2- or FGF4-dependent growth under anchorage-independent conditions and that a decrease in the constitutive levels of either of these receptors is sufficient to abrogate the growth stimulating effects of these growth factors. Another possibility is that FGFR1 and FGFR3 homodimeric pairs could be acting individually to promote the FGF-induced effects on colony formation and that loss of either of these signaling dimers is sufficient to abrogate the growth effects of FGF2 and FGF4. Although knockdown of FGFR4 had no effect on anchorage-independent colony formation in these assays, it is possible that this receptor does play a role in FGF-induced growth but that the remaining amount of FGFR4 is sufficient to mediate the effects of FGF2 or FGF4 on colony formation.

In order to demonstrate the specificity of the effect of FGFR knockdown seen in soft agar assays, we determined the ability of SW-13 cells transfected with either control siRNA or with FGFR siRNA to grow under anchorage-dependent conditions. SW-13 cells will proliferate without the addition of exogenous growth factors in anchorage-dependent assays. If the siRNAs had a non-specific effect on cell growth it is possible that we would also see a reduction under anchorage-dependent conditions. Transfection with either of the two separate siRNA duplexes targeting FGFR1 or FGFR3 had no significant effect on the growth of attached SW-13 cells suggesting that the effects seen on anchorage-independent colony formation are specific for the individual FGFRs.

Activation of FGFRs is known to activate the Raf-MEK-ERK signaling cascade. After determining that knockdown of FGFR1 or FGFR3 inhibited FGF2- and FGF4-induced anchorage-independent colony formation we wanted to test the ability of siRNA targeting MEK1/2 to inhibit FGF2- and FGF4-induced anchorage-independent colony formation. If FGF2- or FGF4-stimulated FGFRs were signaling through the Raf-MEK-ERK in SW-13 cells, it would be possible that knockdown of MEK1/2 would also inhibit colony formation induced by these growth factors. Knockdown of MEK1/2 significantly inhibited both FGF2-and FGF4-induced colony formation, suggesting that this pathway plays an important role in mediating the growth effects of FGF2 and FGF4 on anchorage-independent colony formation.

Since knockdown of individual FGFRs was capable of inhibiting FGF2- or FGF4-induced colony formation, we next sought to determine if FGF-induced MAPK phosphorylation was also affected. In this study, we show that ERK1 and ERK2 are both activated by treatment with FGF2 or FGF4 in non-transfected parental SW-13 cell lines. Activation of the MAPK signaling cascade by FGFs leads to activation of downstream effector molecules that ultimately translocate to the nucleus and initiate transcription of a variety of genes including those responsible for cellular proliferation. Surprisingly, neither FGF2- nor FGF4-induced MAPK phosphorylation was affected by receptor knockdown (Fig. 4A and B). Similarly, siRNA-mediated knockdown of FGFR4 had no effect on FGF2 or FGF4 ERK1/2 activation (Fig. 4C). A double transfection using siRNA targeting MEK1 and MEK2 did result in a significant decrease in both FGF2- and FGF4-induced MAPK phosphorylation (Fig. 4A-C). Control cells transfected with stability enhanced non-targeting siRNA duplexes at the same concentration as the double transfected MEK siRNA also had no effect on MAPK phosphorylation (Fig. 4A-C). Given that knockdown of FGFR1, FGFR3, or MEK1/2 inhibit FGF2- and FGF4-induced colony formation but that knockdown of FGFR1 and FGFR3 have no effect on MAPK phosphorylation, we conclude that the MEK-ERK pathway is necessary but not sufficient for FGF-induced anchorage-independent growth.

It is possible that the growth signal initiated by FGF2 or FGF4 that allows for cellular proliferation and colony formation requires other signaling pathways that have been shown to be stimulated by FGFs. Studies have shown that pathways, including SAPK/JNK, STAT, and PI3K/Akt, can be activated in response to FGFs (28-30). One possible explanation for inhibition of colony formation could be an increase in the rate of apoptosis in SW-13 cells induced by FGFR-specific knockdown. Zhu *et al* (31) have shown that

shRNA-mediated inhibition of FGFR3 leads to apoptosis in three different multiple myeloma cell lines. PI3K/Akt is known to play a critical role in the apoptotic signaling pathway, with induction of Akt conferring protection against apoptosis (32). We sought to determine if Akt was activated by FGF2 or FGF4, and if this activation was affected by siRNA-mediated knockdown of FGFRs. Surprisingly, Akt was not phosphorylated when cells were treated with either FGF2 or FGF4 in either non-transfected control cells or in cells transfected with random control siRNA or siRNA targeting FGFRs. Probing for total Akt showed that Akt is present in these cells and SW-13 cells treated with HRGß1 did show activation of Akt, indicating that Akt is functional in these cells (data not shown). This suggests that Akt does not play a direct role in apoptosis in SW-13 cells, or that the inhibition of colony formation seen with knockdown of either FGFR1 or FGFR3 is not due to diminished cell survival as a result of abrogation of FGF pathways.

Another possibility for the lack of anchorage-independent growth could be the initiation of cell cycle arrest. Studies have shown that treatment with human pancreatic cancer cells with U0126 led to inhibition of ERK1/2 activity accompanied by cell cycle arrest but not apoptosis (33). Although ERK1/2 activity was not affected by FGFR knockdown in the SW-13 cell line, it is possible that knockdown of either FGFR1 or FGFR3 results in abrogation of alternative signaling pathways that can bypass the role of ERKs and lead to cell cycle arrest. Brown *et al* (34) have shown that specific marine sponge extracts can greatly decrease SW-13 cell survival by lowering cyclin B1 levels, leading to a G2/M cell cycle block and triggering apoptosis. They also showed that extracts from at least two marine sponges greatly inhibited MAPK phosphorylation but had no effect on SW-13 cell survival, while an extract from another sponge had little effect on MAPK phosphorylation but did inhibit cell growth (35). The present study further demonstrates the differential role that MAPK signaling can play in cell survival for a given cell lineage as it suggests that the proliferative effects of growth factors, specifically FGF2 or FGF4, on SW-13 cell anchorage-independent colony formation are independent of MAPK phosphorylation. In addition, it suggests that inhibition of colony formation is independent of effects on Akt activation, a signaling molecule whose activation has been shown to be involved in conferring protection against apoptosis.

Knockdown of FGFR1 or FGFR3 in SW-13 cells could negatively affect signaling pathways that provide resistance to suspension-induced apoptosis (anoikis) in such a manner that the cells lose the ability to proliferate in an anchorage-independent manner. Most transformed cells of human origin have lost the dependence on adhesion for survival and have acquired the ability to proliferate in an anchorage-independent manner (36-38). The exact mechanisms by which these cells acquire the ability to grow independent of adhesion are not fully understood. *In vitro* studies have suggested that activation of the MEK-ERK signaling pathway can play an important role in apoptosis. Zeng *et al* (39) showed that hepatocyte growth factor protected human head and neck squamous carcinoma cells against anoikis upon loss of matrix contact. Furthermore, they showed that this protection was both ERK and Akt dependent. These results are similar to studies which showed that oncogenic Ras inhibits anoikis of MDCK canine kidney epithelial cells and that both PI3K and Akt were activated and able to suppress anoikis in these cells (40). In contrast, McFall *et al* showed that although oncogenic Ras blocked anoikis in RIE-1 rat intestinal epithelial cells, it does not activate Akt in these cells (41). In addition, they showed that expression of constitutively active Akt did not provide protection from anoikis. In our studies using SW-13 cells, Akt was not activated by either FGF2 or FGF4. This suggests that FGF2 and FGF4 allow SW-13 cells to form colonies in soft agar by escaping anoikis and that this protection is independent of Akt activation.

In summary, our results suggest that the effects of siRNA-mediated knockdown of FGFR1 and FGFR3 on FGF2- or FGF4-induced anchorage-independent colony formation are independent of both ERK1/2 and Akt activation and that ERK1/2 activation alone is not sufficient to ensure cell survival in these assays.

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