ANTICANCER RESEARCH *24*: 3899-3906 (2004)

Functional Analysis of Focal Adhesion Kinase (FAK) Reduction by Small Inhibitory RNAs

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Abstract. *The focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to the points of cell contact with the extracellular matrix, called focal adhesions. Many factors induce tyrosine phosphorylation of FAK including growth factors, neuropeptides and integrin-dependent adhesion to the extracellular matrix. FAK has been implicated in several cellular processes such as invasion, motility, proliferation and apoptosis. In addition, FAK expression was shown to be elevated in a number of different human cancers, suggesting a role in the development of malignancy. We examined the biological functions of FAK using small inhibitory RNAs (siRNA) in cancer cells. Although FAK siRNA reduced the FAK protein levels by ~70% in several cancer cell lines, there was no clear evidence of apoptosis. However, in clonogenic and soft-agar assays in H1299, a lung cancer cell line, FAK siRNA treatment led to a 43% to 55% decrease in colony formation. Furthermore, FAK siRNA-treated cells displayed a decrease in migration when serum or EGF (epidermal growth factor) were used as chemo-attractants. Our results demonstrated that inhibition of FAK protein leads to alterations in cell growth and migration.*

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that phosphorylates a number of cell signaling molecules. FAK has been implicated in controlling integrin-mediated biological processes such as cell motility, migration, apoptosis and cell survival (1, 2). Extracellular matrix proteins and growth factors are known to activate FAK (1, 2). Following activation, FAK autophosphorylates on Tyr397, creating a binding site for Src homology 2 (SH2) domain-containing proteins. The SH2-containing proteins include phopholipase C-γ1, phosphoinositide 3-kinase, growth factor receptor bound protein 7 and Src. In addition to SH2-binding sites, FAK also contains proline-rich regions that serve as docking sites for SH3-containing proteins, including the crk-associated protein $p130^{cas}$ (1, 2).

FAK has been shown to be overexpressed in a number of human cancers including colon, breast, thyroid and prostate (3). The role of FAK in these cells and its relation to tumor growth is not understood. Several strategies for down-regulation of FAK gene expression have been employed in an effort to dissect the biological functions of this protein in cells. These include the use of antisense oligonucleotides (4), expression of the FAK-related non-kinase (FRNK) domain, an autonomously expressed non-catalytic carboxy terminal fragment of FAK (5-10), and use of FAK small interfering RNA (siRNA) (11). Ectopic overexpression of FRNK inhibits cell spreading, cell migration and growth factor signaling (5-10). Similarly, transient overexpression of the N-terminus of FAK in human breast cancer cells leads to cell detachment and apoptosis (28). Inhibition of FAK function by microinjection of either the carboxy terminal fragment of FAK or a monoclonal antibody against FAK causes cell cycle arrest and apoptosis (29, 30). A recent paper, using FAK siRNA, demonstrated that reduction in FAK protein levels potentiates gemcitabine-induced cytotoxicity both *in vitro* and *in vivo* (11).

In this report, we examined the role of FAK protein in regulating cellular growth and migration using siRNA technology. We demonstrated that reduction of FAK protein levels results in inhibition of both processes in cancer cells.

Materials and Methods

Cell culture. Human cancer cell lines that include human lung carcinoma H1299 and SV-40 transformed human embryonic kidney cell line HEK293T were obtained from the ATCC (Rockville, MD, USA) and were maintained in 5% CO_2 at 37˚C. H1299 and 293T cells were maintained in RPMI plus 10% fetal bovine serum and DMEM plus 10% fetal bovine serum, respectively.

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Key Words: Focal adhesion kinase, extracellular matrix, adhesion.

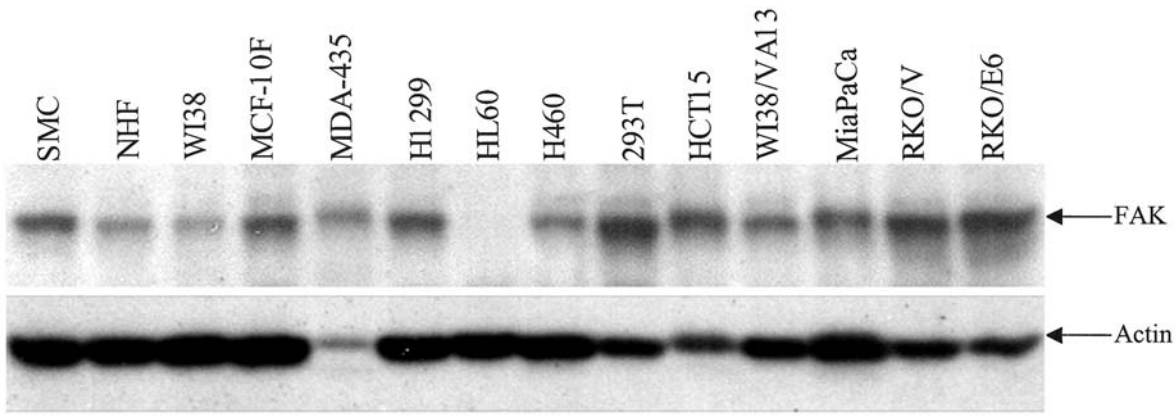

Figure 1. *Cell extracts from human normal and tumor cell lines were prepared and electrophoresed on SDS-PAGE followed by Western blot analyses as described in Materials and Methods.*

siRNA oligonucleotides and transfection. siRNA oligonucleotides against FAK were designed and synthesized by Dharmacon Research (Lafayette, CO, USA). FAK 379 (siRNA): AACCACCUG GGCCAGUAUUAU. FAK 379 (Scrambled control, Scr): AACCUGCACGGCCAGUAUUAU. FAK 560 (siRNA): AAGUAUGAGCUUGC UCACCCA. FAK 717 (siRNA): AAGUGGACCAGGAAAUUGCUU. FAK 717 (Scrambled control, Scr): AACGAUACCAGAAAUUGCUU. FAK 1013 (siRNA): AAUCCAGCUUGAACCAAGAG C. The siRNA oligonucleotides (30 to 100 nM) were transfected into several different cell lines using either Oligofectamine Reagent from Life Technologies (Rockville, MD, USA) or *Trans*IT-TKO reagent from Mirus Corp. (Madison, WI, USA) according to manufacturer's directions. Cells were harvested 48 h post-transfection and extracts were analyzed for FAK protein expression.

Protein extraction and Western blot analysis. Transfected cells were collected and the cell pellets were resuspended in lysis buffer [(20 mM Tris-HCl pH 7.4, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, 1% NP-40, 0.1% SDS and 10 mM NaF, plus the protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml) and PMSF (1 mM)]. This suspension was sonicated for 5 min at 4˚C using a sonicator (Misonix Inc, Farmingdale, NY, USA). Cells were spun briefly and supernatants were collected for determination of protein concentration by the Bio-Rad assay (Bio-Rad, San Diego, CA, USA). For Western blotting, 20-40 μg of protein from the total cell lysate was fractionated by SDS-PAGE. The proteins on these gels were then transferred to immobilon-P membranes (Millipore, Bedford, MA, USA), using transfer buffer (25 mM Tris, 190-mM glycine and 10% methanol). Membranes were blocked with blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Tween 20, 5% non-fat dry milk), and the membranes were then incubated with the indicated antibodies. Human FAK and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used at 1:1000 dilutions. After treatment with blocking buffer without 5% non-fat dry milk (washing buffer), a dilute solution (1:1000) of horseradish peroxidase linked anti-rabbit donkey serum (Amersham, Arlington Heights, IL, USA) was added. Membranes were then washed with washing buffer and immune detection was performed using the ECL Western blotting detection system (Amersham).

Soft-agar assay. One milliliter of complete media (with 10% fetal bovine serum) containing 0.5% agar was layered on the bottom of each well of a 6-well plate and allowed to cool until it solidified (~30 min). Cells were transfected and allowed to incubate for 48h. After treatment with trypsin–EDTA solution, cells were collected, counted, and resuspended in complete medium containing 0.3% agar at 5 X 10^3 per ml. Two ml of the cell suspension was layered on top of the bottom agar and allowed to solidify. Plates were incubated for 10-14 days and colonies were stained with P-iodonitrotetrazolium violet (Sigma-Aldrich, St.Louis, MO, USA). Colonies (> 200 μm) were scored and counted using image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

Clonogenic assay. One thousand FAK siRNA transfected cells were plated in a 10-cm dish and medium was changed every 2 to 3 days until the colonies were formed (7-10 days). Colonies were fixed with 100% methanol for 10 min then stained with Geimsa staining solution (Sigma-Aldrich) for 30 min. Plates were washed with water and air-dried. Stained colonies were scored with the aid of image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

Migration assay. Exponentially growing H1299 cells were trypsinized and counted. 4X10^6 cells per ml were incubated with 50nM of the fluorescent dye Calcein-AM (Molecular Probes, Eugene, OR, USA) and incubated for 30 min at room temperature in the dark. Migration assay of the fluorescent cells (50000 cells) was performed using the ChemoTX system (Neuro Probe, Inc., Gaithersburg, MD, USA) in the presence or absence of either 10% serum or 10 nM epidermal growth factors as chemoattractants. Fluorescent cells that move through the filter were measured from the bottom of the plate in a Tecan SpectraFluor plate reader (Research Triangle Park, NC, USA) using 485-nm excitation and 530-nm emission filters.

Results

FAK is expressed ubiquitously in normal and cancer cell lines. To determine the status of FAK protein expression in human cells, we surveyed a number of cancer as well as normal cells.

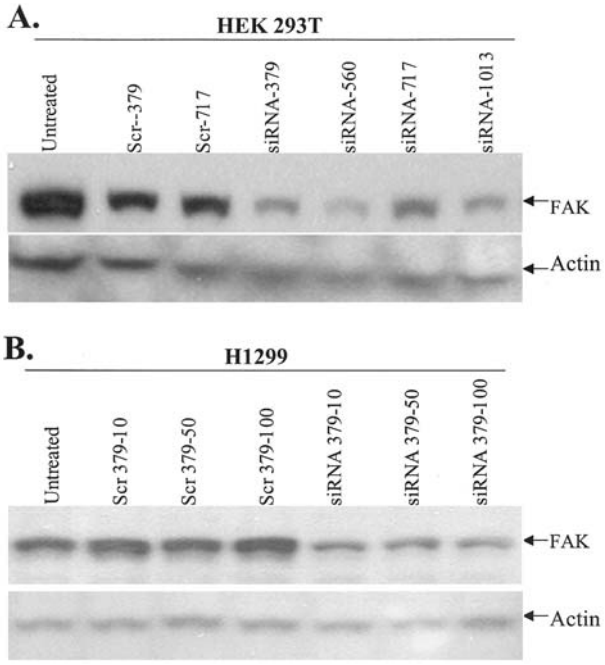

Figure 2. *Cell extracts from scrambled control (Scr) and FAK siRNA oligonucleotides transfection were prepared and electrophoresed on SDS-PAGE followed by Western blot analyses as described in Materials and Methods. Panel (A) shows HEK 293 cells. Panel (B) shows H1299.*

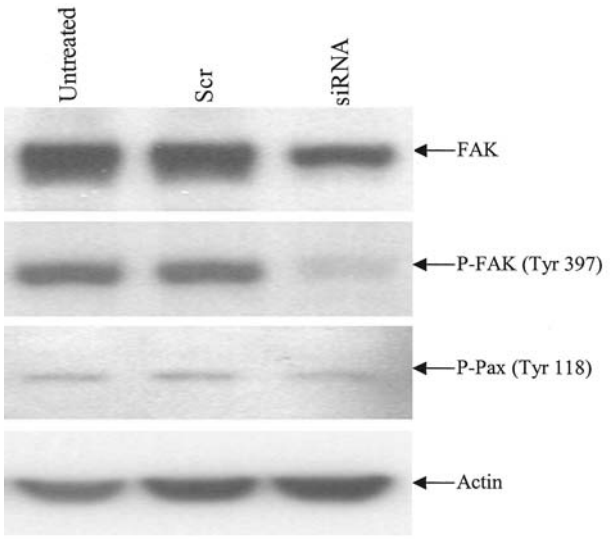

Figure 3. *H1299 cell extracts from scrambled (Scr) and FAK siRNA oligonucleotides treated (100 nM each) were prepared and electrophoresed on SDS-PAGE followed by Western blot analyses as described in Materials and Methods.*

FAK protein expression was analyzed by Western blotting using a FAK-specific antibody. Normal cell lines included in the experiment were smooth muscle cells (SMC), normal human skin fibroblasts (NHF), normal human lung fibroblast (WI38), and a normal breast cell line (MCF-10F). Cancer cell lines consisted of breast (MDA-MB-435), lung (H1299, H460, WI38/VA13), colon (HCT15, RKO/V, RKO/E6), pancreas (MiaPaCa) and leukemia (HL60). As shown in Figure 1, FAK protein was expressed in both normal and cancer cell lines except HL-60. It was shown previously that FAK was not detected in the HL60 cell line (12) when HL60 cells were grown in suspension. When HL60 cells were attached following phorbol ester treatment, FAK was expressed in this cell line. It is well documented that FAK is required for anchorage-independent growth (1, 2).

FAK protein is reduced by FAK siRNA in HEK 293T and H1299 cells. We chose H1299 and HEK 293T cells for our transfection experiment using FAK siRNAs as these two cell lines displayed a greater than 90% transfection efficiency as measured by a fluorescent-labelled siRNA (data not shown). We designed a number of FAK siRNAs and tested them in HEK 293T cells. As shown in Figure 2A, all four FAK siRNAs (30 nM) reduced the protein level of FAK after 48h by at least 70%. Two scrambled controls (Scr 379/Scr 717) showed minor effects in reducing the FAK protein. We chose FAK siRNA-379 for further study. The FAK siRNA-379 was titrated in H1299 cells from 10 nM to 100 nM. As shown in Figure 2B, FAK protein was reduced by more than 80% by the siRNA. In this particular cell line, the FAK scrambled control had no effect on the FAK protein level.

FAK phosphorylation (tyrosine 397) was reduced by FAK siRNA treatment. We examined the phosphorylation at tyrosine 397 that has been shown to be important for FAK activity (1,2). As shown in Figure 3A, FAK protein reduction resulted in a >90% inhibition of FAK phosphorylation. Furthermore, paxilin, a downstream target of FAK (14,15), showed a slight but consistent reduction of paxilin phosphorylation at tyrosine 118. This result shows that FAK protein reduction leads to inactivation of FAK function and its downstream signal transduction pathway.

FAK protein reduction resulted in decreased colony formation. To further examine the effect of FAK depletion by FAK siRNA, we assessed colony formation of transfected cells in both clonogenic and soft-agar assays. As shown in Figure 4, there was a 47% decrease in colony formation in the soft-agar assay (4347 colonies *vs.* 2266 colonies) when compared to the scrambled control. Anchorage-independent growth is one of the hallmarks of cancer cells. In a similar but anchorage-dependent clonogenic assay (Figure 5), there was also a decrease of 31% (777 colonies *vs.* 536 colonies) in colony formation.

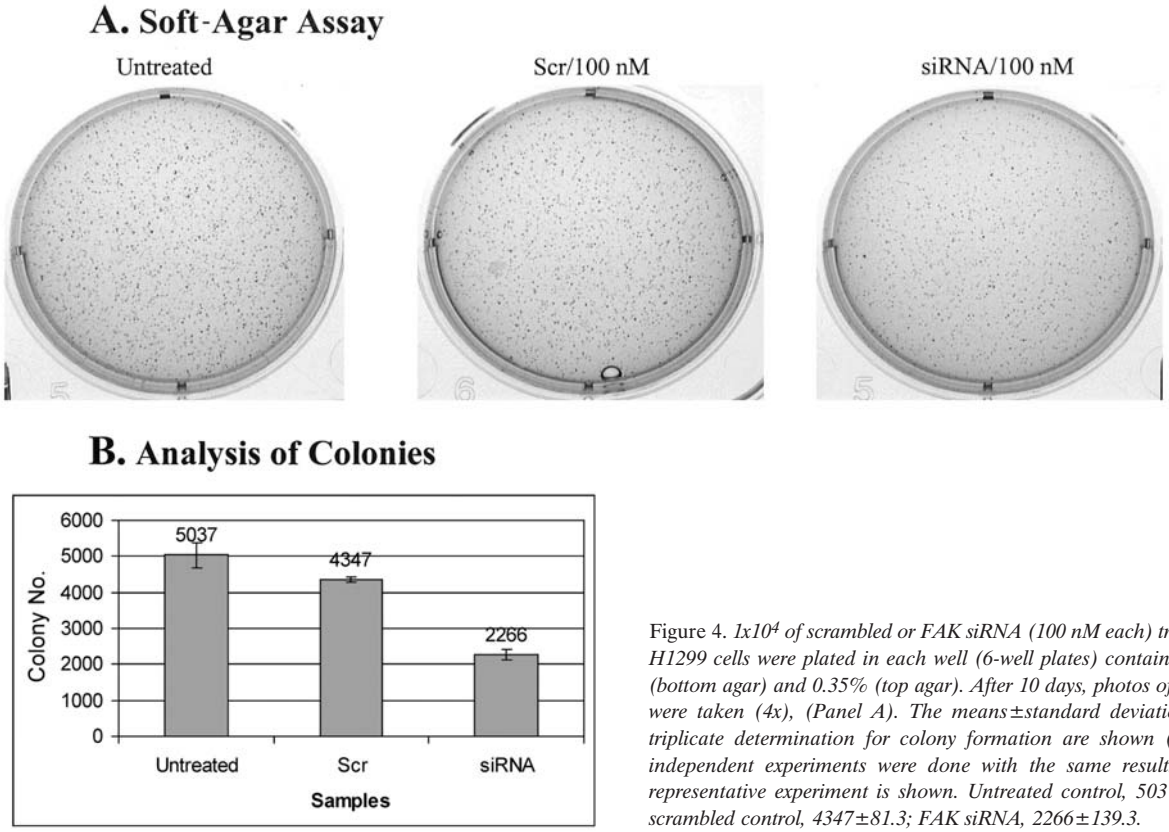

Figure 4. *1x10⁴ of scrambled or FAK siRNA (100 nM each) transfected H1299 cells were plated in each well (6-well plates) containing 0.5% (bottom agar) and 0.35% (top agar). After 10 days, photos of colonies were taken (4x), (Panel A). The means±standard deviations from triplicate determination for colony formation are shown (B). Two independent experiments were done with the same results, and a representative experiment is shown. Untreated control, 5037±342.7; scrambled control, 4347±81.3; FAK siRNA, 2266±139.3.*

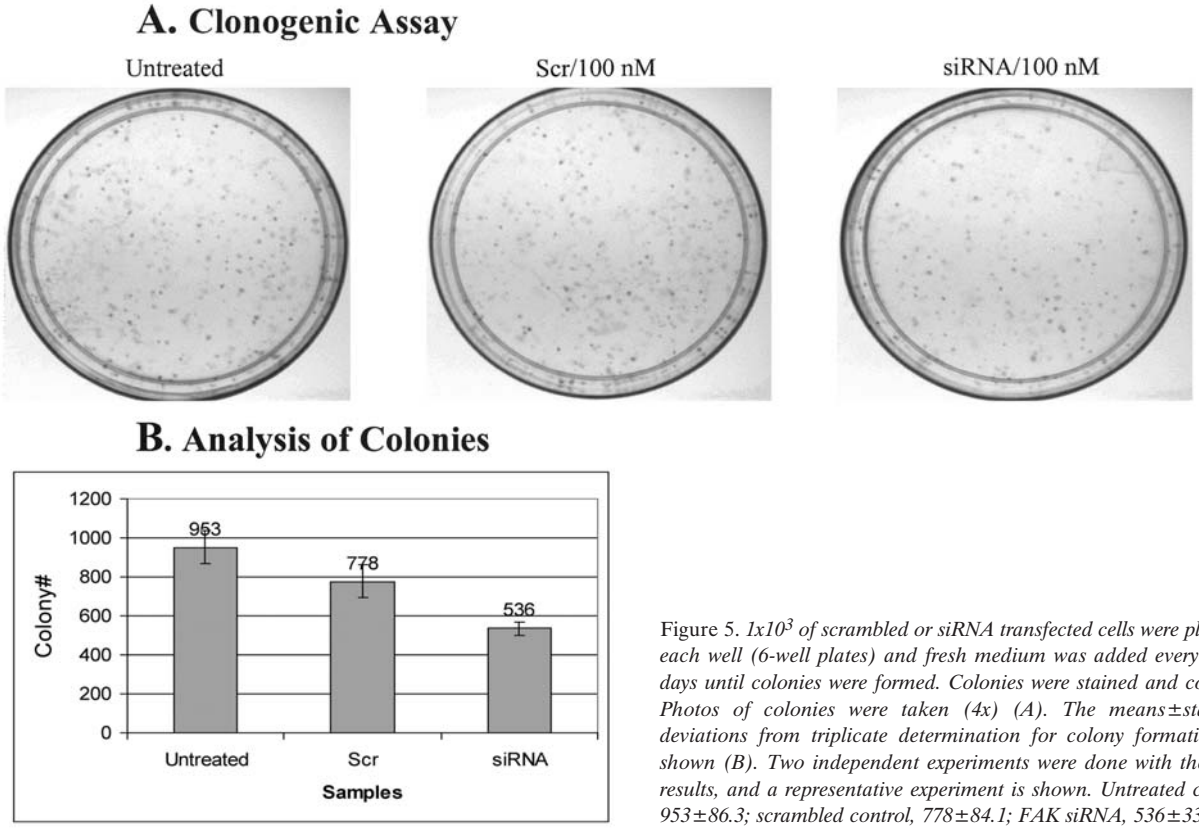

Figure 5. *1x10³ of scrambled or siRNA transfected cells were plated in each well (6-well plates) and fresh medium was added every 2 to 3 days until colonies were formed. Colonies were stained and counted. Photos of colonies were taken (4x) (A). The means±standard deviations from triplicate determination for colony formation are shown (B). Two independent experiments were done with the same results, and a representative experiment is shown. Untreated control, 953±86.3; scrambled control, 778±84.1; FAK siRNA, 536±33.2.*

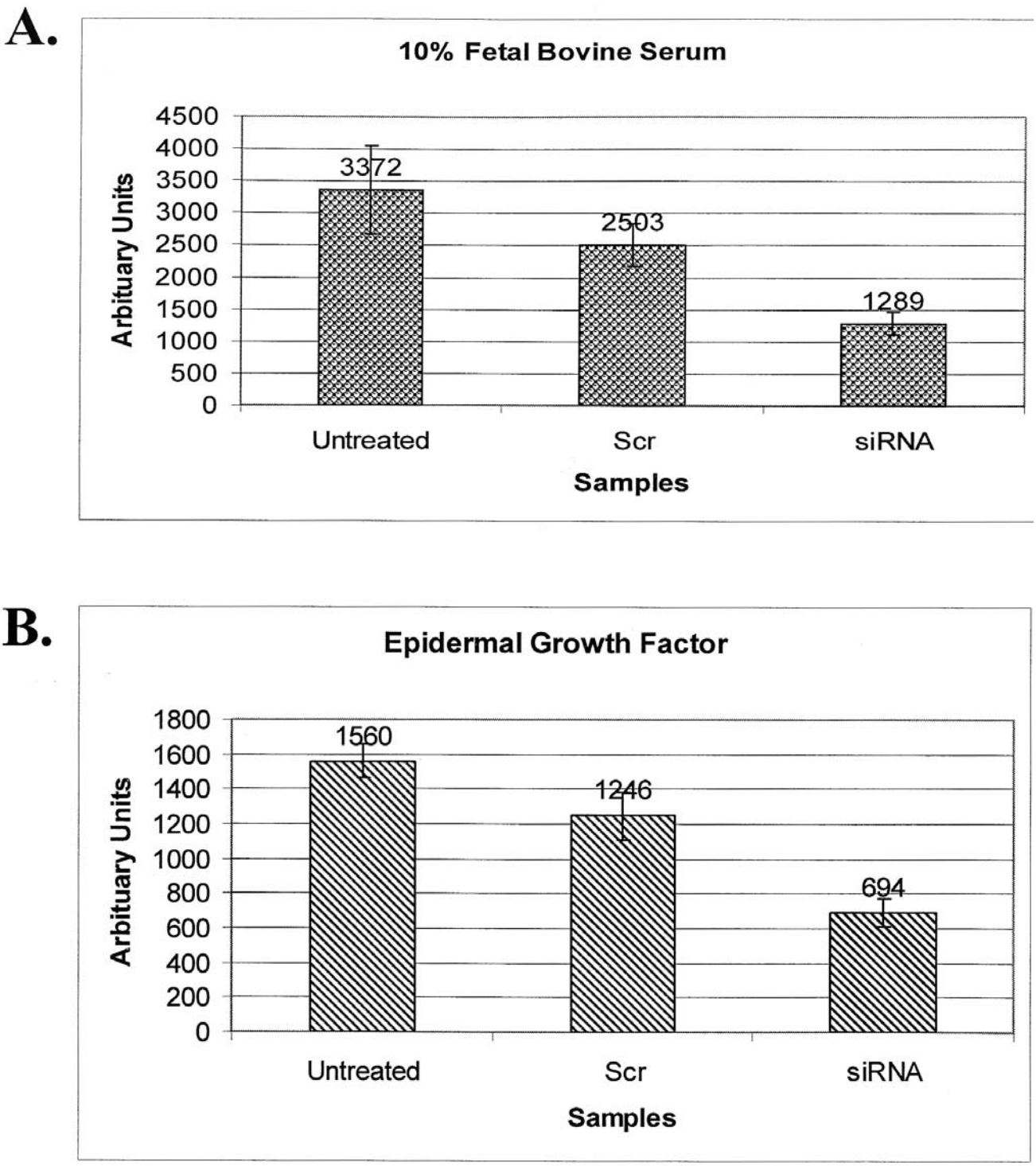

Figure 6. *H1299 cells transfected with scrambled (Scr) or FAK siRNA were examined in the migration assay in the presence of either 10% fetal bovine serum (A) or 10 nM epidermal growth factor (B) as described in Materials and Methods. The means±standard deviations from triplicate determination for colony formation are shown (B). Two independent experiments were done with the same results, and a representative experiment is shown. For 10% fetal bovine serum, untreated control, 3372±688.5; scrambled control, 2503±330.0; FAK siRNA, 1289±173.8 were obtained (A). For epidermal growth factor, untreated control, 1560±93.3; scrambled control, 1246±136.0; FAK siRNA 694±79.8 were obtained (B).*

We have not seen any evidence of apoptosis by FAK siRNA treatment in either H1299 or HEK 293T cells (data not shown). In addition, cells were collected and counted at 48 h and 72 h post-transfection with siRNA oligonucleotides. There was no discernable difference in number of detached cells, or morphology between siFAK- and control-treated cells (data not shown).

FAK protein reduction led to cell migration against serum and epidermal growth factor. It is well known that FAK is involved in cell migration and invasion (1-3). To examine this property, FAK siRNA-treated H1299 cells were tested for cell migration potential in the presence of either 10% fetal bovine serum or epidermal growth factor. As shown in Figure 6A, FAK siRNA-treated cells showed a 50% decrease in cell migration in the presence of 10% fetal bovine serum. Similarly, there was a 42% decrease in cell migration when EGF was use as a chemoattractant. This data confirms that FAK is plays an important role in cell migration.

Discussion

In this paper, we demonstrated that (a) FAK siRNAs reduced the FAK protein in H1299 and 293T cells; (b) FAK siRNA inhibited colony formation in both soft-agar and clonogenic assays, and, finally, (c) FAK siRNA suppressed cell migration when challenged with fetal bovine serum or epidermal growth factor. In previous studies with FAK antisense oligonucleotides (4) or FAK-related non-kinase (FRNK, the dominant negative FAK carboxy-terminal domain), there was an induction of apoptosis and detachment of cells (5-11). It has been shown very recently that use of FAK siRNA caused no significant induction of apoptosis in pancreatic cancer cells (12). In support of this work, we did not see any evidence of apoptosis induction by FAK siRNA.

Several lines of evidence implicate FAK in the regulation of cell migration and motility. First, FAK- deficient cells migrate poorly in response to chemotactic signals (2, 19). Introduction of FAK antisense oligonucleotides, as well as FRNK into a human adenocarcinoma cell line (A549), inhibited EGF-stimulated cell motility (8). Second, overexpression of FRNK blocked cell spreading as well as chemotaxis and migration (16, 17). FAK siRNA or FAK antisense oligonucleotides decreased cell adhesion to collagen and fibronectin in human colon cancer cells (SW620) (18). Third, overexpression of FAK in Chinese hamster ovary (CHO) cells enhanced cell migration (19). Our work, analyzing the effect of FAK siRNA on cell migration (Figure 7), supports the previous reports indicating FAK plays an important role in cell migration and motility.

FAK is overexpressed in a variety of human tumors such as prostate (20), breast (21), colon (22-24), ovarian (26) and thyroid (27). Considering the roles that FAK plays in a human cancer including cell survival, apoptosis and migration, FAK is an attractive target for cancer therapy. It will be interesting to develop small molecules that can inhibit the kinase function of FAK *in vitro* as well as *in vivo*. Furthermore, it will be interesting to see if small molecule inhibitors of FAK kinase used in combination with other chemotherapeutic drugs have synergistic effects *in vivo*. This may lead to better efficacy of FAK kinase inhibitors for treating cancer patients.

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Received July 28, 2004
Accepted October 20, 2004