

Focal Adhesion Kinase Promotes the Aggressive Melanoma Phenotype

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Abstract

Malignant melanoma continues to remain a significant health threat, with death often occurring as a result of metastasis. The metastatic phenotype typically is characterized by augmented tumor cell invasion and migration in addition to tumor cell plasticity as shown by vasculogenic mimicry. Therefore, understanding the molecular mechanisms that promote an aggressive phenotype is essential to predicting the likelihood of metastasis at a stage when intervention may be possible. This study focuses on the role of focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase important for many cellular processes, including cell survival, invasion, and migration. We found FAK to be phosphorylated on its key tyrosine residues, Tyr³⁹⁷ and Tyr⁵⁷⁶, in only aggressive uveal and cutaneous melanoma cells, which correlates with their increased invasion, migration, and vasculogenic mimicry plasticity. Additionally, we confirmed the presence of FAK phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶ in both cutaneous and uveal melanoma tumors *in situ*. Examination of a functional role for FAK in aggressive melanoma revealed that disruption of FAK-mediated signal transduction pathways, through the expression of FAK-related nonkinase (FRNK), results in a decrease in melanoma cell invasion, migration, and inhibition of vasculogenic mimicry. Moreover, we found that FRNK expression resulted in a down-regulation of Erk1/2 phosphorylation resulting in a decrease in urokinase activity. Collectively, these data suggest a new mechanism involved in promoting the aggressive melanoma phenotype through FAK-mediated signal transduction pathways, thus providing new insights into possible therapeutic intervention strategies. (Cancer Res 2005; 65(21): 9851-60)

Introduction

The incidence of malignant melanoma has been steadily increasing. Although cutaneous melanoma remains more common, uveal melanoma is the primary intraocular tumor occurring in adults with significant morbidity (reviewed in ref. 1). The major health threat arising from malignant melanoma, both cutaneous and uveal, is death from metastatic disease, involving invasion and migration of tumor cells from the primary tumor to a distant site(s) within the body. Therefore, identification of prognostic markers useful for

predicting an aggressive melanoma phenotype and the likelihood of metastasis could potentially increase the probability of a successful intervention resulting in a more favorable clinical outcome.

Melanoma metastasis is a multistep process involving the movement of an invading melanoma cell through its basement membrane, thus exposing it to different extracellular matrix (ECM) components. Melanoma cells acquire the ability to recognize these ECM components by ectopically expressing different ECM receptors, including integrins (reviewed in ref. 2). The recognition of these new ECM components by receptors (such as integrins) results in the activation of signal transduction cascades within the melanoma cell to further promote migration and invasion. Signal transduction pathways activated by integrin signaling primarily involve focal adhesion kinase (FAK), a 125-kDa cytoplasmic tyrosine kinase responsible for mediating many cellular processes, including cell survival, migration, and invasion. Integrin clustering as triggered by binding to ECM components results in FAK phosphorylation. Initially, phosphorylation of FAK occurs on its major autophosphorylation site, Tyr³⁹⁷. Phosphorylation of this tyrosine initiates a cascade of signal transduction events that results in the phosphorylation of subsequent tyrosine residues, including Tyr⁵⁷⁶, which is located within the kinase domain of FAK and renders the molecule a fully active kinase (reviewed in ref. 3). Unregulated increases in cell survival mechanisms, migration, and invasion can contribute to a tumor's ability to metastasize to distant sites within the body. Therefore, FAK is often found to be overexpressed and/or constitutively active in numerous cancers, including melanoma, prostate, thyroid, colorectal, ovarian, and oral tumors (4–9).

Our laboratory has been investigating the molecular mechanisms that promote an aggressive melanoma phenotype resulting in an increased metastatic potential. Studies have primarily focused on understanding the migratory and invasive potential of aggressive melanoma cells as well as tumor cell plasticity as shown by vasculogenic mimicry (reviewed in ref. 10). To date, we have identified numerous signal transduction components that seem to play significant roles in mediating the aggressive properties of melanoma cells (reviewed in ref. 10). Although we are beginning to understand the involvement of some of the signaling pathways that regulate cell invasion, migration, and vasculogenic mimicry, the complexity of the coordinated molecular interactions underlying these processes remains to be elucidated. In this study, we focused on the role of FAK as a significant mediator of the aggressive melanoma phenotype.

This study tests the hypothesis that increased FAK activity in aggressive melanoma tumor cells promotes cellular invasion, migration, and ultimately vasculogenic mimicry, all involved in melanoma metastasis. To test this premise, we analyzed the levels of FAK expression and phosphorylation in a panel of aggressive and poorly aggressive human uveal and cutaneous melanoma cell lines. Although we found little difference in the levels of FAK protein among

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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the various cell lines tested, we observed a marked increase in FAK phosphorylation, specifically on Tyr³⁹⁷ and Tyr⁵⁷⁶, in the aggressive melanoma cells, which correlated with an increase in invasive behavior and vasculogenic mimicry. Additionally, we confirmed the presence of FAK phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶ in both cutaneous and uveal melanoma tumors *in situ*. Furthermore, expression of the FAK-related nonkinase (FRNK) in aggressive melanoma cells, which acts to disrupt FAK signaling, resulted in an inhibition of melanoma vasculogenic mimicry concomitant with a decrease in melanoma cell invasion and migration. We found this biological effect to be mediated in part through an Erk1/2 signaling pathway that resulted in a down-regulation of urokinase and matrix metalloproteinase (MMP)-2/membrane type 1-MMP (MT1-MMP) activity. These results indicated that FAK seems to be a key mediator of the aggressive melanoma phenotype as characterized by increased invasion, migration, and vasculogenic mimicry, suggesting that FAK may serve as a new target for therapeutic intervention in treating aggressive melanomas or preventing emergence of melanoma clones with enhanced metastatic capabilities.

Materials and Methods

Cell culture. The human cutaneous (C8161 and A375P) and human uveal (MUM-2C, MUM-2B, and C918) melanoma cell lines have been described previously (11, 12) and were maintained in RPMI 1640 (Invitrogen Life Technologies, Inc. Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). The human cutaneous melanoma cell lines 1205Lu and WM278 (a generous gift from Dr. Meenhard Herlyn, The Wistar Institute, Philadelphia, PA) were maintained on Melanoma Tu 2% medium (MCDB 153, Sigma Chemical Co., St. Louis, MO) supplemented with L-15 (Invitrogen Life Technologies), 5 µg/mL bovine insulin (Sigma Chemical), 2% FBS, and 1.68 mmol/L calcium chloride. Human epidermal melanocytes (MC0018) derived from neonatal foreskins were cultured in Medium 254 with human melanocytes growth supplement (Cascade Biologics, Portland, OR). Cell cultures were determined to be free of *Mycoplasma* contamination using a PCR-based detection system (Roche, Indianapolis, IN).

Three-dimensional cultures. Three-dimensional type I collagen matrices were used to evaluate the vasculogenic mimicry potential of the tumor cells and were prepared as described previously (13). C8161 cells either untransfected or transfected with FRNK were seeded onto the three-dimensional type I collagen matrices and cultured for 6 days. C8161, MUM-2B, and C918 cells were either left untreated (DMSO; control) or treated with the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated

kinase (ERK) kinase (MEK) 1/2 inhibitor, U0126 (1 or 10 µmol/L final concentration in DMSO; Cell Signaling Technologies, Beverly, MA), and seeded onto the three-dimensional type I collagen matrices and cultured for a period of 6 days (C8161) or 4 days (MUM-2B and C918). Fresh medium containing inhibitor was added every 48 hours.

Electrophoresis and immunoblotting. Whole-cell lysates were collected from cells cultured on three-dimensional type I collagen matrices as described previously (13). Protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Corp., Rockford, IL). Whole-cell lysates (20 µg) were separated by 10% or 12% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Blots were blocked with either 5% nonfat milk in TBS-TB [0.05% Tween 20, 0.05% bovine serum albumin (BSA)] or 5% nonfat milk in TBS-B (0.05% BSA; for anti-FRNK analysis) for 1 hour at room temperature. Blots were incubated with anti-FAK[pY397] (1:1,000; BioSource, Camarillo, CA), anti-FAK[pY576] (1:1,000; BioSource), anti-FAK (clone 77; 1:1,000; BD Biosciences, Lexington, KY), anti-FAK/FRNK (1:500; BC3; Upstate Biotechnology, Lake Placid, NY), anti-Erk1/2[pTpY185/187] (1:1,000; BioSource), or anti-Erk1/2 pan (1:1,000; BioSource) followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary (1:5,000; Bio-Rad, Hercules, CA). Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunohistochemistry. Formalin-fixed, paraffin-embedded archival tissue was obtained from patients classified as having radial growth-phase cutaneous melanoma or vertical growth-phase cutaneous melanoma (Loyola University, Chicago, IL) or aggressive uveal melanoma xenografts obtained by injecting 1×10^6 C918 tumor cells into the subretinal space of nude mice as described previously (14) and grown for 4 weeks. Following deparaffinization and antigen retrieval using citrate buffer (Richard-Allan Scientific, Kalamazoo, MI), slides were washed in TBS with Tween 20 (Richard-Allan Scientific), and three blocking steps were applied: 0.03% hydrogen peroxide followed by avidin and biotin blocks (avidin/biotin blocking kit, Vector Laboratories, Inc. Burlingame, CA). Lastly, serum-free protein block (Richard-Allan Scientific) was applied, after which slides were incubated in primary antibodies, anti-FAK[pY397] (1:100) or anti-FAK[pY576] (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), for 1 hour, and staining was detected using the Multispecies HRP/3,3'-Diaminobenzidine (DAB) Detection System kit (Richard-Allan Scientific). Slides were counterstained using Meyer's hematoxylin. Negative control slides were prepared by incubating the tissue with an appropriate concentration of rabbit IgG (DAKO Cytomation, Inc., Carpinteria, CA). Images were captured using an Axioskop 2 (Carl Zeiss, Inc., Thornwood, NY) and Spot 2 camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using the Zeiss Axiovision 2.0.5 software (Carl Zeiss).

Stable transfections. To express the FRNK (pcDNA3.1-FRNK; a generous gift provided by Dr. Michael Schaller, University of North Carolina, Chapel Hill, NC) in C8161 cells, the cells were transfected with 350 ng DNA using LipofectAMINE Plus reagent (Invitrogen Life Technologies) following

Table 1. Biological properties of human melanoma cells

Culture designation	Invasive potential (%)*	Vasculogenic mimicry [†]	Reference
C8161 (cutaneous, metastasis)	High, (14.6-17.1) ± 0.7	+	34
A375P (cutaneous, primary)	Poor, 1.5 ± 0.01	-	35
1205Lu (cutaneous, experimental metastasis)	Moderate, (7.1-8.6) ± 0.5	+	36
WM278 (cutaneous, VGP)	High, (8.7-14.5) ± 1.8	+	37
C918 (uveal, primary)	High, (12.5-14.0) ± 0.5	+	12
MUM-2B (uveal, metastasis)	High, (13.3-15.1) ± 0.6	+	12
MUM-2C (uveal, metastasis)	Poor, (2.0-2.4) ± 0.06	-	12

*Invasiveness was calculated as the percentage ± SE of cells capable of invading a basement membrane-coated polycarbonate membrane over 24 hours within the MICS chamber compared with the total number of cells seeded ($n = 6$ wells per variable and run in duplicate experiments).

[†]Vasculogenic mimicry was assessed by the ability of tumor cells seeded onto a three-dimensional type I collagen matrix to form tubular networks in a 6-day assay.

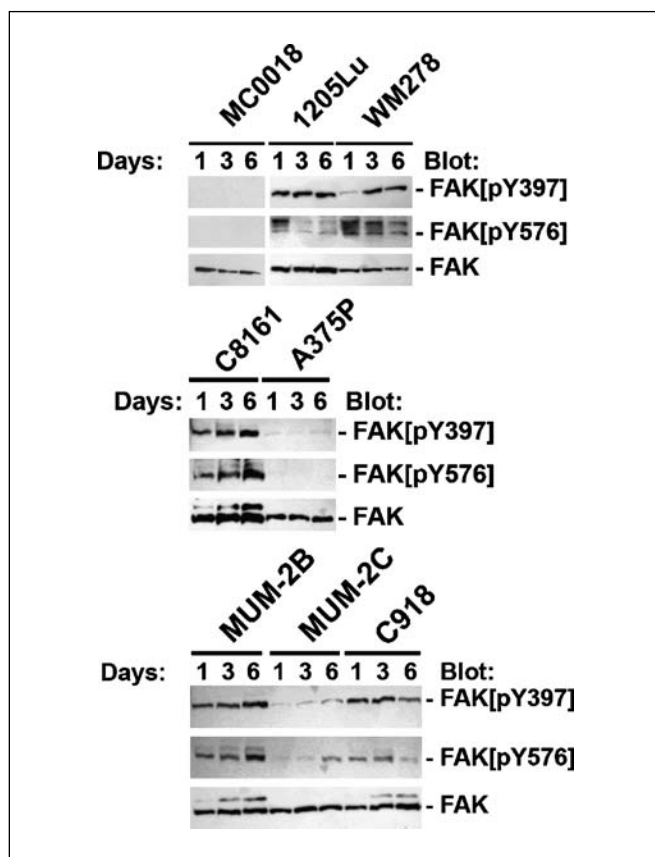


Figure 1. A Western blot analysis of FAK in aggressive melanoma cells shows phosphorylation on Tyr³⁹⁷ and Tyr⁵⁷⁶. Normal melanocytes (MC0018), aggressive melanoma cells (1205Lu, WM278, C8161, MUM-2B, and C918), and poorly aggressive melanoma cells (A375P and MUM-2C) were cultured on three-dimensional type I collagen matrices. At 1, 3, and 6 days after seeding cells onto matrix, cells plus matrix were recovered by scraping in the presence of lysis buffer (13) and proteins from whole-cell lysates were separated using 10% SDS-PAGE followed by electroblotting onto nitrocellulose membranes. The resulting Western blots were probed with either anti-FAK[pY397] or anti-FAK[pY576] to detect FAK phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶, respectively, followed by stripping the blot and reprobing with anti-FAK antibodies.

the manufacturer's protocol. Twenty-four hours after transfection, the cells were replated in normal growth medium. Twenty-four hours after plating, G418 (600 µg/mL) was added to the growth medium. Colonies were selected and screened by Western blot analysis to identify FRNK-expressing cells. As a control, cells were transfected with empty pcDNA3.1 vector.

Invasion and migration assays. The invasive potential of the cells was analyzed using the membrane invasion culture system (MICS) as described previously (15). For invasion, an equal number of cells were seeded into the upper wells of the MICS chamber, which is separated from the lower wells by 10-µm polycarbonate membrane (Osmonics Co., Livermore, CA) coated with a defined matrix consisting of 50 µg/mL each of human laminin and human collagen IV (Sigma Chemical) in 2 mg/mL gelatin-10 mmol/L acetic acid/PBS base or coated with gelatin for migration assays. Chambers were incubated for 24 hours (invasion) or 6 hours (migration) at 37°C, 5% CO₂, after which cells in the lower chamber wells were collected and counted. Samples were analyzed in triplicate and the statistical significance of the observed differences was determined using the Student's *t* test (Sigma Plot for Windows, SPSS, Inc., Chicago, IL). For invasion or migration assays where the MEK inhibitor U0126 was used, cells were either left untreated or treated with 1 or 10 µmol/L U0126 for 24 hours before seeding into the chamber in the presence of inhibitor.

Zymography and membrane type I-matrix metalloproteinase activity assays. Conditioned medium was collected from C8161, MUM-2B,

or C918 cells left untreated (DMSO; control) or treated with 1 or 10 µmol/L U0126 or from C8161 cells untransfected, transfected with empty vector, or transfected with FRNK cultured on three-dimensional type I collagen matrices for 24 and 48 hours. Cell debris was removed from the samples by centrifugation at 4,000 rpm for 5 minutes. Active urokinase was detected using casein/plasminogen zymography as follows: conditioned medium (10 µL) was loaded onto a 10% polyacrylamide gel containing 1 mg/mL casein and 5 units/mL plasminogen (American Diagnostica, Stamford, CT); after electrophoresis, the gel was developed as described previously (16). Gelatin zymography was used to detect the levels of active MMP-2 as follows: serum-free conditioned medium was collected and subjected to SDS-PAGE using 0.1% (w/v) gelatin containing 10% polyacrylamide gels as described previously (17). For MT1-MMP activity assays, C8161, MUM-2B, and C918 cells were cultured on three-dimensional type I collagen matrices and either left untreated (DMSO; control) or treated with 1 or 10 µmol/L U0126 for 48 hours. The level of MT1-MMP activity was determined in the samples using an immunoadsorbed Biotrak assay kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol and as described previously (17). The experiments were done in triplicate, and the samples were analyzed in duplicate. The values are reported as a percentage of control and statistical significance of the observed differences was determined using the Student's *t* test (Sigma Plot for Windows).

Results

Focal adhesion kinase is phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶ in aggressive human cutaneous and uveal melanoma *in vitro* and *in situ*. The work presented here examined the signal transduction events that regulate the aggressive melanoma phenotype as characterized by increased invasive and migratory potential as well as an ability to engage in vasculogenic mimicry as an example of tumor cell plasticity. In particular, we focused on the levels of FAK phosphorylation in various melanoma tumor cell lines derived from both human uveal and cutaneous melanoma tumors. FAK is phosphorylated on numerous tyrosine residues, including Tyr³⁹⁷, its major autophosphorylation site, and Tyr⁵⁷⁶, which is located in the kinase domain. Phosphorylation on these two domains indicates that FAK is a fully active kinase and can promote certain cellular responses, including an increase in cell migration and invasion (reviewed in ref. 3). The cell lines used for this study have been characterized previously and are summarized in Table 1. To better mimic the microenvironment that invading melanoma cells may encounter, we cultured normal melanocytes and aggressive or poorly aggressive melanoma tumor cells on a three-dimensional type I collagen matrix for 6 days and then assessed their ability to form vasculogenic-like networks (vasculogenic mimicry) as described previously (13). Cells cultured on these three-dimensional matrices were also harvested and analyzed by Western blot analysis for the presence of FAK protein after days 1, 3, and 6. In all cases, FAK protein levels were relatively equal among the normal melanocytes and aggressive or poorly aggressive melanoma tumor cells (Fig. 1); equal loading was verified by Coomassie stain (Supplementary Fig. S1). Next, the levels of phosphorylated FAK in the same cells were determined. Western blot analysis revealed that FAK is phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶ in the aggressive melanoma cells but not in normal melanocytes or poorly aggressive melanoma cells during the formation of vasculogenic-like networks (vasculogenic mimicry) in three-dimensional culture (Fig. 1). Furthermore, differences in the ability of melanoma cells to invade *in vitro* correlated with the increased levels of FAK phosphorylation on Tyr³⁹⁷ and Tyr⁵⁷⁶ (Table 1).

To determine if FAK phosphorylation could be validated in aggressive cutaneous melanoma or uveal melanoma *in situ*, we examined histologic sections of patient tissues for the presence

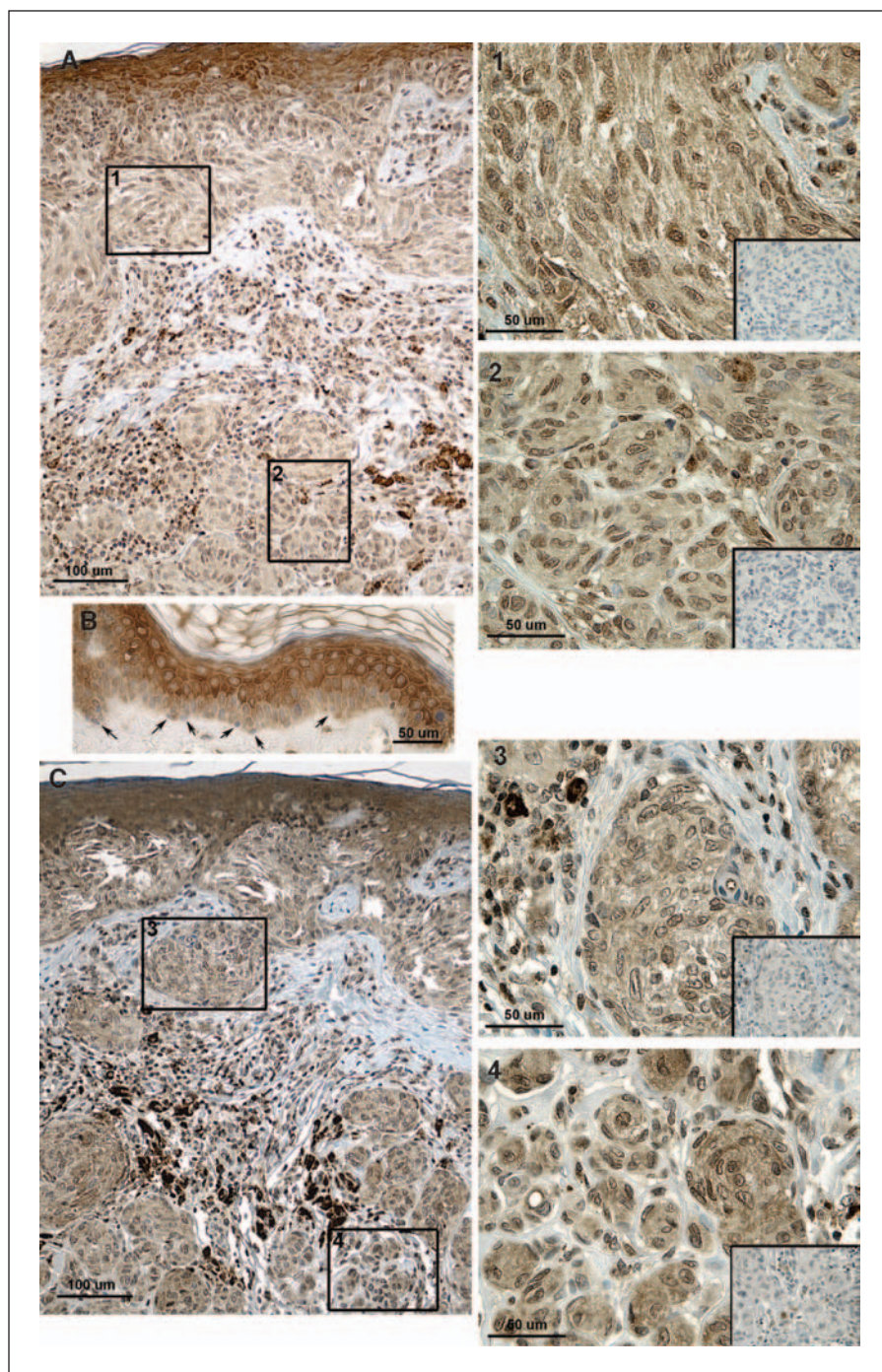


Figure 2. Immunohistochemical analyses of FAK phosphorylated in Tyr³⁹⁷ and Tyr⁵⁷⁶ in late-stage cutaneous and uveal melanoma. A-C, histologic sections of patient tissues with cutaneous melanoma were incubated in the presence of anti-FAK[pY397] (A and B; arrows delineate normal melanocytes) or anti-FAK[pY576] (C) antibodies followed by exposure to DAB brown chromagen and counterstained with Meyer's hematoxylin. 1 and 2 are shown at high magnification and represent both radial (1) and vertical (2) growth phases stained with anti-FAK[pY397] antibodies; inset, same area incubated with an isotype control antibody. 3 and 4 are shown at high magnification and represent both radial (3) and vertical (4) growth phases stained with anti-FAK[pY576] antibodies; inset, same area incubated with an isotype control antibody.

of FAK phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶. Fourteen different paraffin-embedded tissues obtained from patients classified as having radial growth-phase or vertical growth-phase cutaneous melanomas were analyzed. In all tissues, there was evidence of positive staining for both FAK[pY397] and FAK[pY576] [$\geq 50\%$ of the tumor staining positive for either antibody as determined by a pathologist (B.J.N.)]. A representative slide is shown in Fig. 2, which shows an aggressive cutaneous melanoma stained with anti-FAK[pY397] (Fig. 2A) or anti-FAK[pY576] (Fig. 2C) antibodies. Closer examination of the radial (Fig. 2A-1 and C-3) and vertical (Fig. 2A-2 and C-4) growth phases at higher magnification revealed strong cytoplasmic as well as some nuclear

staining of FAK phosphorylated on both Tyr³⁹⁷ and Tyr⁵⁷⁶. These primary cutaneous melanomas included numerous cases in which a wide basal incision was done such that normal-appearing skin was included in the specimen. This normal skin allowed us to evaluate the immunoreactivity of normal, non-neoplastic melanocytes. As noted in Fig. 2B, the entire basal epidermal layer was negative for FAK[pY397] or FAK[pY576] (data not shown), including numerous normal melanocytes (arrows). A table summarizing the degree of immunoreactivity of both anti-FAK[pY397] and anti-FAK[pY576] for all 14 cases analyzed is presented in Supplementary Table S2. Likewise, Fig. 2D and E shows aggressive C918 primary uveal melanoma

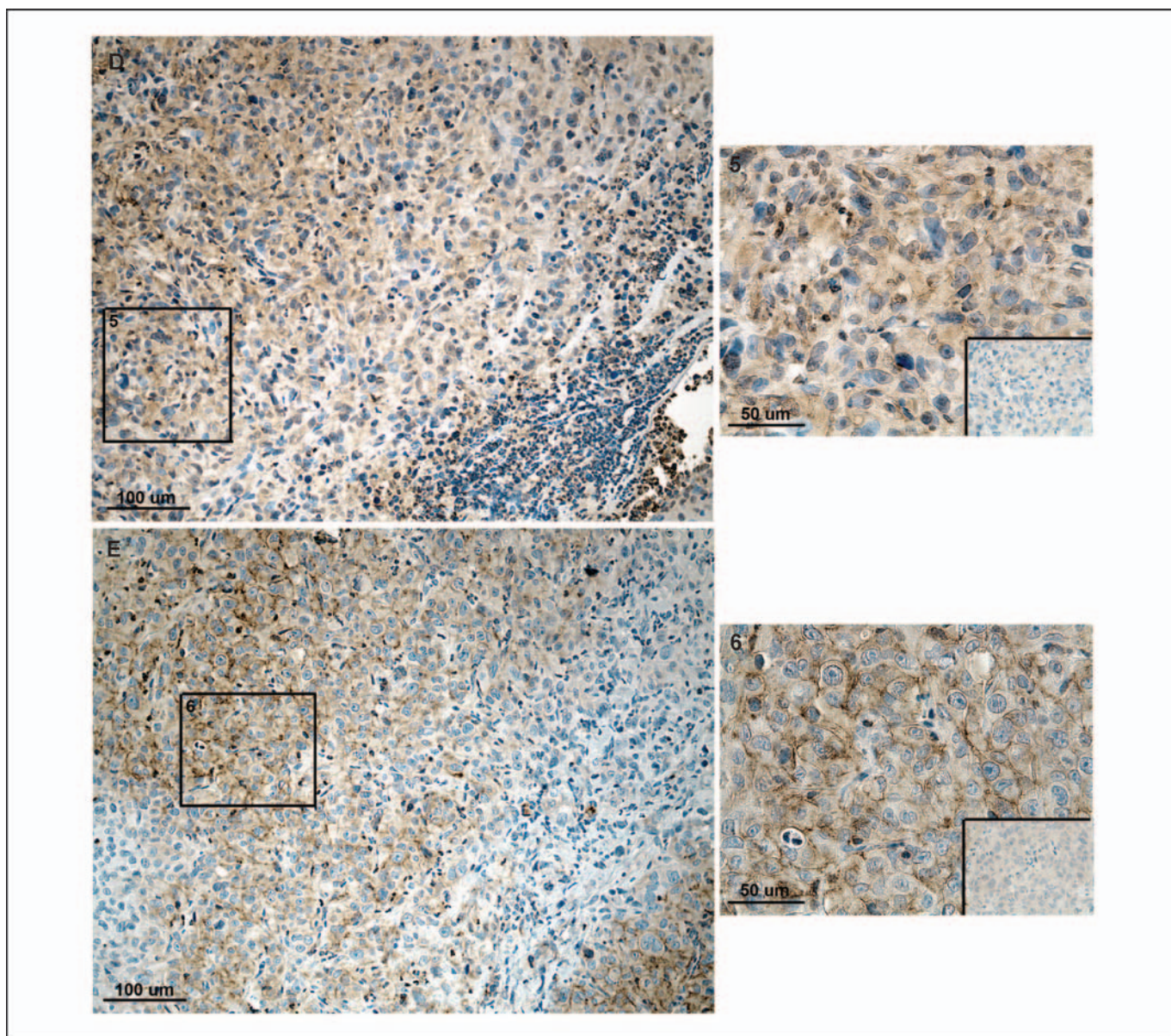


Figure 2. Continued. *D* and *E*, uveal melanoma xenograft taken from an intraocular injection of C918 into nude mice incubated in the presence of anti-FAK[pY397] (*D*) or anti-FAK[pY576] (*E*) antibodies followed by exposure to DAB brown chromagen and counterstained with Meyer's hematoxylin. 5 and 6 represent high magnification stained with anti-FAK[pY397] (5) or anti-FAK[pY576] (6); insets, same area incubated with an isotype control antibody.

xenograft stained with anti-FAK[pY397] (Fig. 2*D*) or anti-FAK[pY576] (Fig. 2*E*) antibodies. Analyses of the tissue samples at higher magnification (Fig. 2*D-5* and *E-6*) also revealed strong cytoplasmic and focal membrane staining of FAK phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶. It is noteworthy that we did not observe the same kind of nuclear staining for phosphorylated FAK in the aggressive uveal melanoma xenograft as we did with the aggressive cutaneous melanoma tissues. These immunohistochemical staining results confirmed the finding of phosphorylated FAK in melanoma tumor tissues similar to that seen in aggressive melanoma tumor cells *in vitro*.

Expression of focal adhesion kinase-related nonkinase disrupts melanoma invasion, migration, and vasculogenic mimicry. To investigate the role FAK plays in mediating melanoma cell invasion and vasculogenic mimicry, the aggressive

melanoma cells were transfected with FRNK. FRNK can interact with focal adhesion proteins in the same manner as FAK; however, because it lacks a kinase domain, it is unable to potentiate downstream signal transduction events, thereby acting as a dominant-negative FAK protein (18, 19). Attempts to transfect C8161, MUM-2B, and C918 aggressive melanoma tumor cells resulted in only two positive clones from C8161 designated as C8161-FRNK-1 and C8161-FRNK-2. Analyses by Western blot indicated that these two clones expressed varying amounts of FRNK protein compared with untransfected C8161 or two different empty vector C8161-neo clones (Fig. 3*A*). When these transfected cells were analyzed for their invasive and migratory potential compared with two different C8161 cells transfected with an empty neo vector, we found a 60% decrease in their invasiveness (Fig. 3*B*; Supplementary Fig. S2*A*) and a 70%

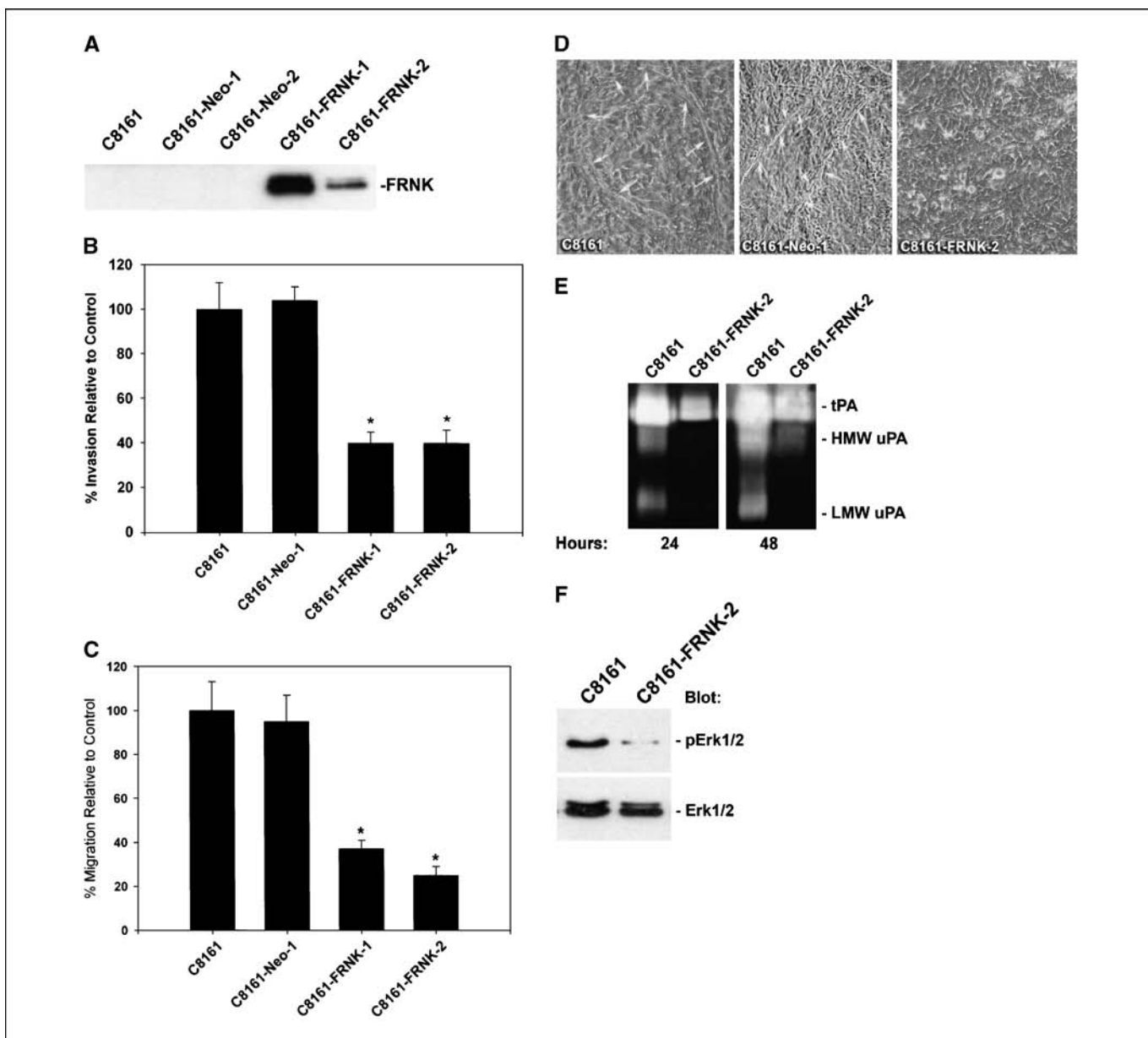


Figure 3. FRNK expression down-regulates Erk1/2 phosphorylation as analyzed by Western blot as well as urokinase activity as shown with casein/plasminogen zymography resulting in an inhibition of melanoma vasculogenic mimicry and a decrease in invasion and migration as evaluated using the MICS. **A**, Western blot confirmation of FRNK expression in transfected C8161 cells. Whole-cell lysates obtained from parental C8161 cells, two different empty vector neo-transfected C8161 clones (*C8161-Neo-1* and *C8161-Neo-2*), and two different C8161-FRNK clones (*C8161-FRNK-1* and *C8161-FRNK-2*) were separated using 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. The resulting Western blots were probed with anti-FRNK antibodies. **B**, invasion for untransfected C8161 cells (*C8161*), neo-transfected C8161 cells (*C8161-Neo-1*), or FRNK-transfected C8161 cells (*C8161-FRNK-1* and *C8161-FRNK-2*) was calculated as a percentage of cells able to invade through a matrix (collagen IV, laminin, and gelatin)-coated polycarbonate membrane within a 24-hour period using the MICS assay compared with the total number of cells seeded and normalized to control. The 60% decrease in invasion of both FRNK-transfected C8161 cells compared with control and neo-transfected cells was statistically significant (*, $P < 0.001$). **C**, migration for untransfected C8161 cells (*C8161*), neo-transfected C8161 cells (*C8161-Neo-1*), or FRNK-transfected C8161 cells (*C8161-FRNK-1* and *C8161-FRNK-2*) was calculated as a percentage of cells that migrated through a gelatin-coated polycarbonate membrane containing 10- μ m pores within a 6-hour period using the MICS assay compared with the total number of cells seeded normalized to the control. The 75% decrease in migration of the FRNK-transfected cells compared with the control or neo-transfected cells was statistically significant (*, $P < 0.001$). **D**, bright-field microscopy of C8161, C8161-neo (*C8161-Neo-1*), or C8161 transfected with FRNK (*C8161-FRNK-2*) cells seeded onto three-dimensional type I collagen matrices for 6 days. Arrows delineate tube formation. Original magnification, $\times 20$. **E**, casein/plasminogen zymography was used to analyze the levels of active urokinase in conditioned medium collected from C8161 and FRNK-transfected C8161 (*C8161-FRNK-2*) cultured on three-dimensional type I collagen matrices at 24 and 48 hours. **F**, respective Western blot analysis of phosphorylated Erk1/2 in C8161 and FRNK-transfected C8161 (*C8161-FRNK-2*). Proteins from whole-cell lysates collected from C8161 and C8161-FRNK-2 cells were separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes. The resulting Western blot was probed with anti-Erk1/2[pTpY185/187] antibodies, stripped, and reprobed with anti-Erk1/2 antibodies.

decrease in their ability to migrate (Fig. 3C; Supplementary Fig. S2B). Because both clones showed the same level of reduction in invasion and migration, only the C8161-FRNK-2 clone was used for future experiments. It was subsequently found that C8161-

FRNK-2 cells were unable to form vasculogenic-like networks compared with the untransfected C8161 or C8161-neo-1 control (Fig. 3D). These results suggest that FAK may play a role in mediating melanoma vasculogenic mimicry possibly through

signaling events associated with the invasive and migratory potential of these cells.

Expression of focal adhesion kinase-related nonkinase down-regulates urokinase activity concomitant with a decrease in Erk1/2 phosphorylation. To ascertain a potential mechanism by which FAK signaling may mediate melanoma invasion and vasculogenic mimicry, we examined specific proteolytic enzymes known to participate in the remodeling of the ECM. Given that the urokinase-type plasminogen activator (uPA) receptor (uPAR)/urokinase signaling pathway has been linked to both FAK and Erk1/2 phosphorylation in many different tumor types (20–22), we examined conditioned medium obtained from C8161, two different empty vector C8161-neo clones, and C8161-FRNK-2 cells cultured on three-dimensional type I collagen matrices for 24 and 48 hours for urokinase activity. Figure 3E shows a reduction in activity of both high molecular weight (55-kDa proform; Supplementary Fig. S2C) and low molecular weight (35-kDa active form) urokinase. Western blot analysis of whole-cell lysates of C8161, two different empty vector C8161-neo clones, and C8161-FRNK-2 cells using phosphospecific Erk1/2 antibodies further indicated that there is a significant reduction in the levels of phosphorylated Erk1/2 in the C8161-FRNK-2 cells versus the C8161 cells (Fig. 3F; Supplementary Fig. S2D). Together, these results suggest that FAK signaling leads to Erk1/2 phosphorylation resulting in an increase in the levels of urokinase secreted by these cells.

Erk1/2 regulates urokinase and matrix metalloproteinase-2/membrane type 1-matrix metalloproteinase activity, thus promoting melanoma invasion and vasculogenic mimicry. To determine if urokinase activity was regulated by the Erk1/2 signaling pathway in the melanoma cells, we used the MEK1/2 inhibitor U0126, which functions to decrease the levels of phosphorylated Erk1/2. For these studies, conditioned media from C8161, MUM-2B, and C918 cells cultured on three-dimensional type I collagen matrices and left untreated (DMSO; control) or treated with 1 or 10 $\mu\text{mol/L}$ U0126 (Fig. 4A) were analyzed for urokinase activity. Figure 4A shows that treatment of all three cell lines with 10 $\mu\text{mol/L}$ U0126 diminishes the level of both the 55-kDa proform and the 35-kDa active form of urokinase, which correlates with the reduction of urokinase activity in the FRNK-expressing C8161 cells (compare Fig. 3E with Fig. 4A, top). Western blot analyses of whole-cell lysates showed that treatment with 10 $\mu\text{mol/L}$ U0126 also inhibited Erk1/2 phosphorylation to the same extent as expression of FRNK protein in the C8161 cells (compare Fig. 3F with Fig. 4A, bottom). Furthermore, treatment with the MEK inhibitor U0126 decreased the levels of active MMP-2 (Fig. 4B) and MT1-MMP (Fig. 4C) in the C8161, MUM-2B, and C918 cells.

Lastly, we tested the role of the Erk1/2 pathway in promoting melanoma invasion, migration, and vasculogenic mimicry. For these experiments, we also used a second MEK1/2 inhibitor, PD98059. For invasion, C8161, MUM-2B, or C918 cells were left untreated or treated with 1 $\mu\text{mol/L}$ PD98059 (Supplementary Fig. S3A) or with 1 or 10 $\mu\text{mol/L}$ U0126 and then analyzed for changes in their invasiveness compared with control (untreated) cells. Figure 4D shows that treatment with 1 $\mu\text{mol/L}$ U0126 did not significantly change the ability of the cells to invade; however, treatment with 10 $\mu\text{mol/L}$ U0126 resulted in ~40% to 60% reduction in invasion, which is similar to the decreases seen with the C8161-FRNK cells. Furthermore, a complete inhibition of vasculogenic mimicry occurred after culturing the C8161 cells on

three-dimensional type I collagen for 6 days or the MUM-2B and C918 cells on three-dimensional type I collagen for 4 days in the presence of 10 $\mu\text{mol/L}$ U0126 (Fig. 4E). Similarly, inhibition of vasculogenic mimicry was seen when C8161 cells were treated for 6 days with 1 $\mu\text{mol/L}$ PD98059 as shown in Supplementary Fig. S3B. Contrary to what we observed in the C8161 cells expressing FRNK protein, there was no change in the ability of the cells to migrate after treatment with either 1 or 10 $\mu\text{mol/L}$ U0126 (Supplementary Fig. S4). These results suggest that FAK may promote an aggressive melanoma phenotype by up-regulating invasion and vasculogenic mimicry through an Erk1/2-mediated signal transduction pathway, although migration seems to be regulated through a separate FAK-mediated signal transduction pathway.

Discussion

The major health threat for malignant melanoma is death from metastasis. Understanding the signal transduction events that promote the aggressive melanoma phenotype is essential to deciphering the mechanisms that regulate tumor metastasis. Invasion, migration, and vasculogenic mimicry are all characteristics of an aggressive melanoma phenotype. This study addressed the role of FAK in mediating these processes. Collectively, FAK was found to regulate all three components of the aggressive melanoma phenotype in part by regulating the secretion of proteolytic enzymes, such as urokinase, as well as the ability of the tumor cells to migrate. Although it seems that FAK signals through Erk1/2 to promote urokinase secretion in aggressive melanoma cells, the signaling mechanism(s) that regulates migration of these cells remains to be determined. The model presented in Fig. 5 summarizes the proposed signal transduction pathways activated downstream of FAK and is responsible, at least in part, for promoting an aggressive melanoma phenotype.

Metastasis is a multistep process requiring the ability of a melanoma cell to escape control of its surrounding microenvironment and invade the basement membrane. Once through the basement membrane, the melanoma cell comes into contact with an interstitial microenvironment, which may be more conducive to promoting increased invasion and migration. One key feature of the new microenvironment is the composition of various ECM components and the acquisition of a new integrin profiles enabling the melanoma cells to recognize these newly encountered ECM components (reviewed in ref. 2). The integrins on the cell surface bind to the ECM components, thereby initiating signal transduction events within the cell that promote cell survival, migration, and invasion. One such cytoplasmic kinase responsible for potentiating these signal transduction events is FAK, which is recruited to the site of cell-ECM attachment during focal adhesion formation. Upon integrin clustering as a result of binding to the ECM, FAK is recruited to and becomes phosphorylated on its major autophosphorylation site, Tyr³⁹⁷, as the focal adhesion site begins to take shape. Autophosphorylation of FAK initiates a cascade of signal transduction events that results in the phosphorylation of subsequent tyrosine residues, including Tyr⁵⁷⁶, which is located within the kinase domain of FAK and renders the molecule a fully active kinase (reviewed in ref. 3). FAK-mediated signal transduction pathways promote cell survival mechanisms, migration, and invasion, which if left unregulated can contribute to a tumor's ability to metastasize to distant sites within the body.

Deciphering the molecular signature of an aggressive melanoma phenotype is key to understanding and predicting the metastatic

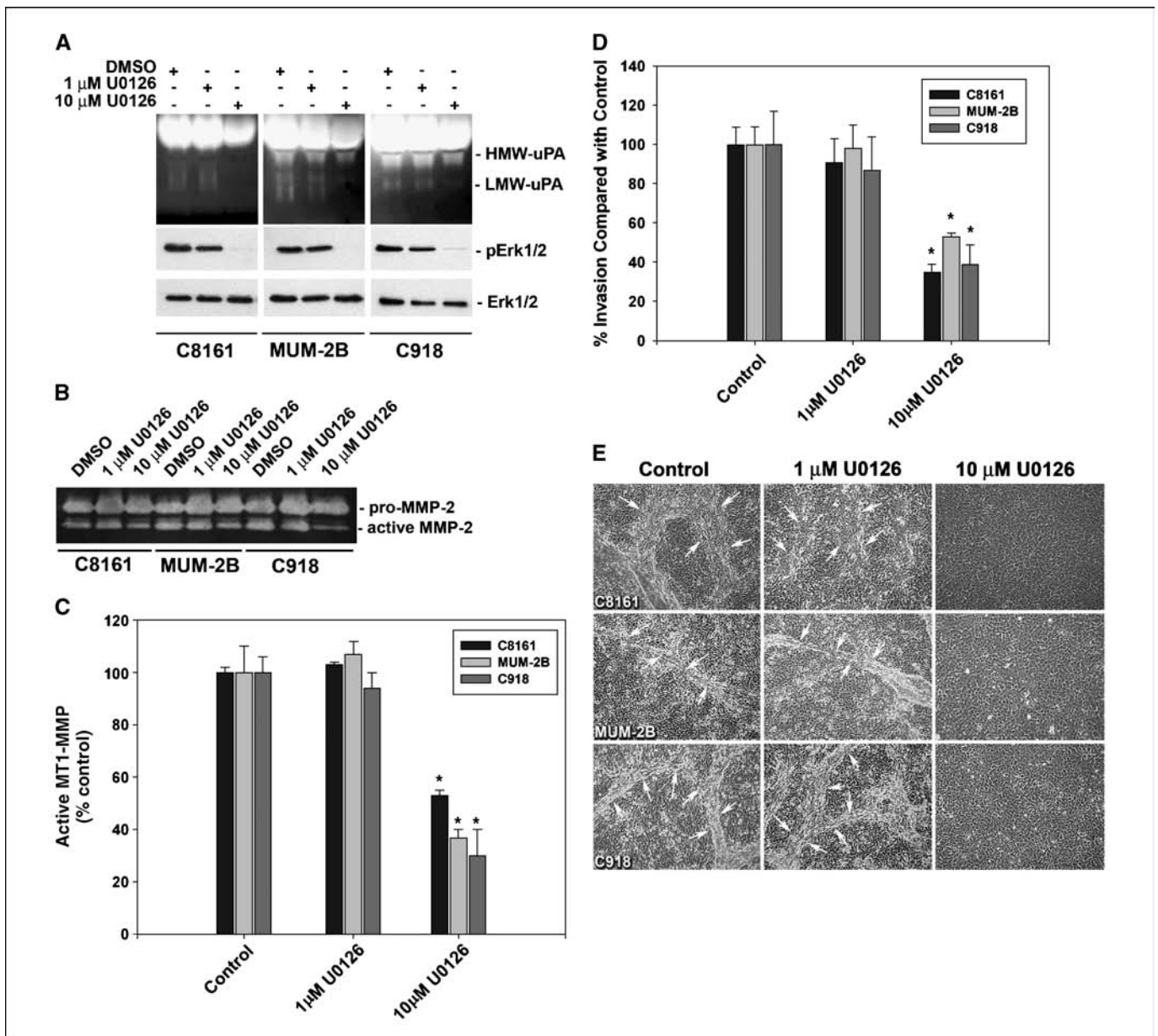


Figure 4. Treatment of aggressive melanoma cells with U0126 decreases Erk1/2 phosphorylation as shown by Western blot analysis concomitant with a decrease in urokinase and MMP-2 as shown by zymography as well as a decrease in MT1-MMP activity measured using an activity assay, resulting in an inhibition of melanoma vasculogenic mimicry and a decrease in invasion as evaluated using the MICS. *A*, casein/plasminogen zymography was used to analyze the levels of active urokinase in conditioned medium from C8161, MUM-2B, and C918 cells cultured on three-dimensional type I collagen matrices and either left untreated (DMSO; control) or treated with 1 or 10 μ M U0126 for 48 hours. Respective Western blot analysis of phosphorylated Erk1/2 from C8161, MUM-2B, and C918 cells left untreated (DMSO; control) or treated with 1 or 10 μ M U0126 for 48 hours. Proteins from whole-cell lysates were separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes. The resulting Western blots were probed with anti-Erk1/2[pTpY185/187] antibodies, stripped, and re probed with anti-Erk1/2 antibodies. *B*, gelatin zymography was used to analyze the levels of active MMP-2 in conditioned medium from C8161, MUM-2B, and C918 cells cultured on three-dimensional type I collagen matrices and either left untreated (DMSO; control) or treated with 1 or 10 μ M U0126 for 48 hours. *C*, MT1-MMP activity was measured using an ELISA assay for C8161, MUM-2B, or C918 cells left untreated (DMSO; control) or treated with 1 or 10 μ M U0126 for 48 hours. Relative levels of MT1-MMP activity are represented as a percentage of control. The 55% to 75% decrease in the levels of active MT1-MMP observed in the 10 μ M U0126-treated cells compared with untreated controls was statistically significant (*, $P < 0.001$). *D*, invasion for C8161, MUM-2B, and C918 cells either left untreated (DMSO; control) or treated with 1 or 10 μ M U0126 was calculated as a percentage of cells able to invade through a matrix (collagen IV, laminin, and gelatin)-coated polycarbonate membrane within a 24-hour period using the MICS assay compared with the total number of cells seeded and normalized to control. The 40% to 70% decrease in invasion for C8161, MUM-2B, and C918 cells observed after treatment with 10 μ M U0126 compared with untreated control cells was statistically significant (*, $P < 0.001$). *E*, bright-field microscopy of C8161, MUM-2B, and C918 cells left untreated (DMSO; control) or treated with 1 or 10 μ M U0126 and cultured on three-dimensional type I collagen matrices for 6 days (C8161) or 4 days (MUM-2B and C918). Arrows delineate tube formation.

potential within the tumor. Therefore, the purpose of this study was to further identify key signal transduction events that underlie the aggressive melanoma phenotype as characterized by increased invasion, migration, and vasculogenic mimicry potential. We

hypothesized that FAK could play a key role in promoting the aggressive melanoma phenotype because its increased expression has already been linked to tumor cell aggressiveness in other tumors (4–9). The data presented throughout this study indicate that,

although FAK expression is relatively equal among normal melanocytes, poorly aggressive and aggressive melanoma cells, consistent with other reports for melanoma (23), FAK is phosphorylated on its key tyrosine residues, Tyr³⁹⁷ and Tyr⁵⁷⁶, in only the most aggressive melanoma cells, which correlates with increased invasion, migration, and vasculogenic mimicry. Furthermore, we showed that FAK is phosphorylated on Tyr³⁹⁷ and Tyr⁵⁷⁶ in aggressive cutaneous and uveal melanoma tissues *in situ*. Interestingly, although we observed some nuclear staining for phosphorylated FAK in the cutaneous melanoma tissue samples analyzed, we failed to observe the same phenomenon in the uveal melanoma xenografts. This disparity could be attributed to the different location of these melanomas; uveal melanomas arise in the choroid, iris, and ciliary body of the eye, whereas cutaneous melanomas arise in the skin. It is noteworthy that phosphorylated FAK associated with the nucleus (in our study) has not been reported previously and may represent a significant new finding that should be pursued. Collectively, these results suggest that FAK-mediated signal transduction pathways are important for promoting the aggressive melanoma phenotype. As proof of principle, disruption of FAK-mediated signaling pathways by overexpressing FRNK, which acts as a dominant-negative protein, resulted in a decrease in all three of these cellular events associated with a more aggressive phenotype.

Signaling events downstream of FAK are complex and result in the activation of many cellular pathways affecting cell survival, cell growth, angiogenesis, cellular invasion, and cellular migration (3, 24). Our findings indicate that disruption of FAK signaling by overexpression of FRNK has a significant effect on invasion, migration, and vasculogenic mimicry by aggressive melanoma cells used in this study. To better understand a mechanism for how these processes could be regulated by FAK, we first examined the proteolytic enzymes known to be important for invasion in several tumor cell types, including melanoma. In particular, there have been

reports linking both uPA/uPAR and MMP with FAK signaling (21, 25–27). We found urokinase activity to be greatly reduced by FRNK expression in the aggressive melanoma cells. However, we did not see an effect on MMP-2 activity or MT1-MMP activity in the FRNK-transfected C8161 melanoma cells (data not shown). To identify the signaling pathways downstream of FAK that were responsible for urokinase secretion in the aggressive melanoma cells, we turned our attention to the MAPK pathways, specifically the MEK1/2-Erk1/2 pathway, because it has been linked to urokinase activity in aggressive breast cancer (20, 21). We found Erk1/2 phosphorylation to be significantly decreased in the FRNK-expressing C8161 cells compared with the untransfected C8161 cells. Moreover, we found that treatment with two different MEK inhibitors, both of which reduced Erk1/2 phosphorylation to the level seen with FRNK expression, decreased urokinase activity concomitant with a decrease in invasion and vasculogenic mimicry in the aggressive melanoma cells. These results coincide with those found with overexpression of the FRNK protein in the C8161 cells and suggest that FAK signals through an Erk1/2-mediated pathway to promote urokinase secretion.

In order for aggressive melanoma cells to engage in vasculogenic mimicry, they must have the ability to migrate and invade the ECM to form vasculogenic-like networks (see video at <http://www.childrensmrc.org/hendrix/supplemental/quicktime/>). Although blocking the phosphorylation of Erk1/2 using specific inhibitors diminished invasion and vasculogenic mimicry in the aggressive melanoma cells, we saw no effect on migration in these cells. Subsequently, we explored specific proteolytic enzymes that are important for invasion and vasculogenic mimicry. Using the MEK1/2 inhibitor U0126 to down-regulate Erk1/2 phosphorylation, we found decreases in both MMP-2 and MT1-MMP activity, which could result in the decreases seen in invasion and vasculogenic mimicry. Given our previous observations concerning the role of MMP-2 and MT1-MMP in melanoma vasculogenic mimicry (17, 28), we suggest that Erk1/2 signaling may regulate the proteolytic enzymes secreted by the aggressive melanoma cells, which results in the observed decreases in invasion and vasculogenic mimicry and a result of significant decrease in urokinase, MMP-2, and MT1-MMP activities—all necessary components enabling these tumor cells to remodel the ECM and invade and engage in vasculogenic mimicry.

Although we found a decrease in urokinase activity mediated in part through an Erk1/2 signal transduction pathway, FRNK expression in the C8161 cells resulted in no effect on MMP-2 and MT1-MMP activity. These results suggest that there could be another signal transduction pathway independent of FAK signaling, which is responsible for Erk1/2 phosphorylation in the aggressive melanoma cells. One possibility for such a signaling pathway is the RAS-RAF-MEK-ERK-MAPK pathway. Mutations in N-Ras or B-Raf are very common in cutaneous melanoma and often result in increased phosphorylation of Erk1/2 (reviewed in ref. 29). Sumimoto et al. showed that disruption of the B-Raf signaling pathway in melanoma cell lines using RNA interference technology resulted in a down-regulation of invasion concomitant with a decrease in Erk1/2 phosphorylation and MMP-2 activity (30). In spite of a large body of evidence supporting a role for N-Ras and/or B-Raf mutations in cutaneous melanoma, these mutations are not typically associated with uveal melanoma, although these tumors also have constitutive activation of the MAPK pathway (31).

A second potential role for FAK signaling in the aggressive melanoma cells is by promoting migration. In this regard, Haskell et al. investigated the role of FAK in mediating angiogenesis in

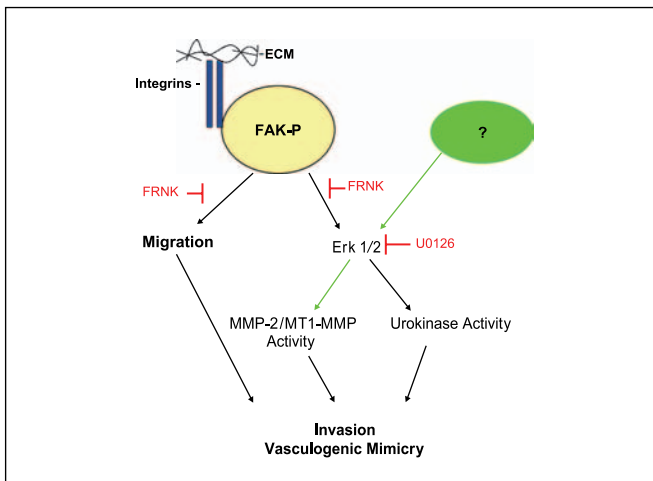


Figure 5. Hypothetical model for the signal transduction pathways promoting the aggressive melanoma phenotype. In this model, FAK signaling regulates invasion, migration, and vasculogenic mimicry through two different signaling pathways. FAK signals through Erk1/2 to increase the levels of urokinase activity, thus regulating invasion of the aggressive melanoma cells. Additionally, FAK seems to signal through unknown downstream effectors to promote migration in the aggressive melanoma cells that may contribute to an increase in invasion and vasculogenic mimicry potential. Lastly, a second signal transduction pathway, which lies upstream of Erk1/2 but not downstream of FAK signaling, may promote an increase in MMP-2 and MT1-MMP activity that can function together to increase invasion and vasculogenic mimicry in the aggressive melanoma cells.

malignant astrocytic tumors (24). These investigators found that FAK is phosphorylated on Tyr³⁹⁷ in endothelial cells of the highest-grade tumors. Furthermore, they reported that expression of FRNK in brain microvascular endothelial cells inhibited tube formation in three-dimensional collagen gels and haptotactic migration toward collagen and fibronectin ECMs. These results suggested that FAK promotes endothelial angiogenesis in part through the regulation of endothelial cell migration.

Cellular migration is a complex process that requires the precise cooperation of various signal transduction pathways, which facilitate the formation of new focal adhesions, lamellipodia, and filopodia at the leading edge of the cell in conjunction with retraction of the cell's posterior processes. FAK has been found to be a key regulator of cellular migration by initiating many of the signal transduction pathways necessary for this process to occur. In melanoma specifically, there has been a correlation between expression of FAK and increased migration potential (32, 33). Using the K1735 murine mouse model for melanoma, Li et al. found that expressing FRNK in these cells resulted in a decrease in FAK phosphorylation on Tyr³⁹⁷ concomitant with a 90% decrease in the

migration capability of these cells (33). Together, these results support our observations that signal transduction pathways initiated by FAK play an important role in mediating melanoma cell migration and offer a new potential mechanism for the effects shown on melanoma invasion and vasculogenic mimicry.

These studies have added yet another important signaling component, FAK, as a key mediator of the aggressive behavior of melanoma cells. As we develop a better understanding of the signal transduction pathways at work in regulating melanoma invasion, migration, and vasculogenic mimicry, we may identify new avenues for the therapeutic intervention of aggressive melanoma.

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