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The Chemopreventive Bioflavonoid Apigenin Inhibits Prostate Cancer Cell Motility through the Focal Adhesion Kinase/Src Signaling Mechanism

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Abstract Prostate cancer mortality is primarily attributed to metastatic rather than primary, organ-confined disease. Acquiring a motile and invasive phenotype is an important step in development of tumors and ultimately metastasis. This step involves remodeling of the extracellular matrix and of cell-matrix interactions, cell movement mediated by the actin cytoskeleton, and activation of focal adhesion kinase (FAK)/Src signaling. Epidemiologic studies suggest that the metastatic behavior of prostate cancer may be an ideal target for chemoprevention. The natural flavone apigenin is known to have chemopreventive properties against many cancers, including prostate cancer. Here, we study the effect of apigenin on motility, invasion, and its mechanism of action in metastatic prostate carcinoma cells (PC3-M). We found that apigenin inhibits PC3-M cell motility in a scratch-wound assay. Live cell imaging studies show that apigenin diminishes the speed and affects directionality of cell motion. Alterations in the cytoskeleton are consistent with impaired cell movement in apigenin-treated cells. Apigenin treatment leads to formation of "exaggerated filopodia," which show accumulation of focal adhesion proteins at their tips. Furthermore, apigenin-treated cells adhere more strongly to the extracellular matrix. Additionally, apigenin decreases activation of FAK and Src, and phosphorylation of Src substrates FAK Y576/577 and Y925. Expression of constitutively active Src blunts the effect of apigenin on cell motility and cytoskeleton remodeling. These results show that apigenin inhibits motility and invasion of prostate carcinoma cells, disrupts actin cytoskeleton organization, and inhibits FAK/Src signaling. These studies provide mechanistic insight into developing novel strategies for inhibiting prostate cancer cell motility and invasiveness.

Prostate cancer is the most common noncutaneous malignancy in American males (over 186,000 cases diagnosed yearly), and the second leading cause of cancer-related deaths in American men (28,660 estimated deaths in 2008; ref. 1). In prostate cancer, most deaths are attributed to metastatic disease rather than primary, organ-confined prostate cancer (2–5). Numerous epidemiologic studies (6) suggest that the metastatic behavior of prostate cancer may be an ideal target for pharmacologic intervention with chemopreventive agents. Because prostate cancer is typically diagnosed in older men, chemopreventive and/or chemotherapeutic strategies to delay its progression may have a substantial impact on clinical outcome. Epidemiologic studies have shown that Asian men experience a lower incidence of metastatic prostate cancer than Western men, which is attributed to dietary and/or life-style factors (3, 4). Interestingly, some studies suggest that the incidence of primary cancer may be similar in Asian and Western populations, and that the higher rate of prostate cancer-related deaths in the United States is due to a higher rate of prostate cancer metastasis (3, 5).

Apigenin (4′, 5, 7-trihydroxyflavone) is a nontoxic and nonmutagenic flavone present in fruits and leafy vegetables. Its major sources include tea, onions, parsley, thyme, celery, and sweet red pepper (7). Epidemiologic studies have shown an

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inverse association between consumption of vegetables and fruits and risk of human cancers at many sites (8–10).

Apigenin was shown to be nonmutagenic in the Ames assay and in Chinese hamster V79 cells (11). Studies in numerous laboratories show that it displays a wide variety of anticarcinogenic effects in skin (12, 13), breast (12), colon (12, 14), ovarian (15), and prostate (16–18) cancer cells. Apigenin fed by gavage inhibits prostate tumor xenograft growth in mice through up-regulation of insulin-like growth factor-3 (12). Apigenin's chemopreventive activity in multiple organ sites is likely tied to its ability to modulate pathways involved in cell cycle control (12, 19, 20), apoptosis (12, 16, 21, 22), and signal transduction (12, 19, 23–25). Apigenin inhibits the activation of the phosphoinositide 3-kinase/Akt pathway in numerous cell types including prostate cancer cell lines (12, 19), potentially by blocking the ATP binding site of phosphoinositide 3-kinase (26). Other signal transduction pathways modulated by apigenin include epidermal growth factor receptor and Src tyrosine kinase pathways in colon carcinoma and breast carcinoma cells (12, 24, 25). A study in bovine thymocytes found that among 22 flavonoids and related compounds, apigenin was the only compound that inhibited the activity of Src (24). Apigenin inhibits migration and invasion in breast cancer and melanoma cells (12) and matrix metalloproteinase (MMP)-9 activity in breast cancer cells (12). Apigenin can target key pathways involved in adhesion, motility, and invasion, showing a potential role for apigenin in chemoprevention of metastasis.

Actin polymerization and filament elongation at the leading edge of the cell, coupled to actin/myosin filament contraction at the rear, are the driving forces for cell migration (27). Integrins are heterodimeric transmembrane receptors that link the extracellular matrix (ECM) to the actin cytoskeleton at points of substrate interaction termed focal adhesions. Signals initiated by ECM-integrin interactions are transduced into cells through the activation of integrin-associated focal adhesion kinase (FAK) and Src (28). Signaling from FAK/Src leads to actin cytoskeleton remodeling, which is instrumental for migration and invasion through the ECM. Cell motility is an attractive therapeutic target of advanced and aggressive prostate cancers, especially because it is a critical requirement for tumor invasion (29).

In this study, we investigated the effect of apigenin on prostate cancer cell motility and invasion. We found that apigenin inhibited PC3-M cell motility in a scratch wound assay. Using a novel micropatterning line assay, we found that apigenin disrupted the persistence of motion and diminished the velocity of the prostate cancer cells. Apigenin treatment leads to formation of "exaggerated filopodia," which show accumulation of focal adhesion proteins at their tips. Furthermore, apigenin-treated cells adhere more strongly to the ECM. Apigenin treatment resulted in actin cytoskeleton remodeling and decreased phosphorylation of FAK and Src. Additionally, we showed that phosphorylation of Src is important for PC3-M cell motility and cytoskeleton remodeling, because the effect of apigenin on motility and cytoskeleton remodeling was blunted by constitutively active Src (caSrc). We also showed that apigenin inhibited the formation of actin-rich structures during invasion. These studies provide mechanistic insight into the role of apigenin in inhibition of prostate cancer cell motility and invasiveness.

Materials and Methods

Cell culture

PC3-M cells (a gift from Dr. R.C. Bergan, Northwestern University, Chicago, IL), C4-2B cells (a generous gift from Dr. R.S. Taichman, University of Michigan, Ann Arbor, MI), and DU145 cells (ATCC) were cultured in RPMI 1640 (Mediatech) containing 10% heat-inactivated fetal bovine serum (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (Mediatech).

Reagents and antibodies

Apigenin and DMSO were from Sigma. FAK monoclonal antibody, pFAK (Y576), pFAK (Y925), Src, and cortactin polyclonal antibodies were from Santa Cruz Biotechnology. Fibronectin, pFAK (Y397) monoclonal antibody, and VASP monoclonal antibody were from BD Biosciences. pFAK (Y397), FAK, pSrc (Y416), and Src polyclonal antibodies, Src, and p-Tyrosine monoclonal antibodies were from Cell Signaling Technologies. Rhodamine and Alexa-633 Phalloidin were from Molecular Probes/Invitrogen. Lipofectamine was from Invitrogen. Horseradish peroxidase–conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were from Bio-Rad Laboratories. Conditioned 804G medium enriched for Laminin 5 protein was prepared as described previously (30).

Scratch wound assay and live cell imaging

PC3-M cells were plated in 24-well plates (120,000 cells per well) in medium containing 25 μmol/L apigenin or solvent control for 16 h, scratch wound was made by scratching the confluent monolayer with a 20-μL pipette tip. Cells were washed with PBS and media containing fresh apigenin or solvent control was added. Cells were imaged immediately after wounding. Twenty-four and 48 h after wounding, cells were washed with PBS, and fresh media containing apigenin or solvent control was added before imaging. The percentage wound closure was determined using ImageJ software. For live cell imaging, PC3-M cells were plated in Lab-Tek (Nunc) four-well chambered cover glass (175,000 cells per well) with apigenin or solvent control for 16 h. A scratch wound was made using a 26-gauge needle, and cells were placed into the Application Solution Multidimensional Workstation (ASMDW; Leica) for 17 h, with phase time-lapse recordings taken at 30-min intervals using a ×20 objective. Quantitations were made using MetaMorph 6.1 imaging software (Universal Imaging).

Line assay

The substrates for line assays were fabricated using the Anisotropic Solid Microetching technique as previously described (31). Briefly, ultrathin (~30-50 nm) gold layers supported on Ti (5 nm) thermally evaporated on glass. Etching was done using micropatterned hydrogel "stamps" delivering (at the rate of 28 Å/s) solution of gold etchant to the glass/Au interface while simultaneously removing etching products into the stamp's bulk. This process yielded arrays of transparent lines (2 μm edge resolution) surrounded by opaque regions of gold. Subsequent derivatization of the unetched gold with self-assembled monolayers of oligo (ethylene glycol) alkane thiols [$HS(CH2)_{11}(OCH2CH2)_6OH$; ProChimia] rendered it resistant to cell adsorption. Cells were plated onto the substrates, and they localized and migrated only on the Laminin 5–coated etched lines. The transparency of these "tracks" for cell locomotion enabled live-cell imaging of cell motions and quantification of cell motility parameters (31).

To monitor the effects of apigenin, cells were adhered and spread on micropatterned substrates overnight, then treated with medium containing 25 μmol/L apigenin or solvent control for 16 h. Movie was taken for an additional 16 h.

Time lapse spreading and motility assays

Spreading. PC3-M cells were treated with media containing apigenin or solvent control for 16 h. Cells were then detached and plated on FN-coated two-well chambered cover glass in media containing apigenin or solvent control and allowed to attach for 1 h. Live cell

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imaging was then done in the ASMDW for 8 h, with phase time-lapse recordings taken at 5-min intervals using a ×20 objective.

Motility. PC3-M cells were treated with media containing apigenin or solvent control for 16 h, then detached and plated on FN-coated two-well chambered cover glass in media containing apigenin or solvent control. Cells were allowed to attach and spread for 5 h, and then they were placed in the ASMDW for 8 h, with phase time-lapse recordings taken at 5-min intervals using a ×20 objective. Quantitations were made using Metamorph 6.1 imaging software.

Cell detachment assay

Cell detachment assays were carried out as previously described, with some modifications (32). Briefly, PC3-M cells were plated in 96-well plates (40,000 cells per well). Cells were then treated with apigenin or solvent control for 16 h. The media was then removed and the cells were incubated with trypsin (1:50 dilution) for 10 min. The trypsin solution was removed, and the remaining cells were washed with PBS and then fixed with formalin, followed by staining with methylene blue.

Immunofluorescence

PC3-M cells were treated with serum-free media containing apigenin or solvent control for 16 h, then detached by calcium sequestration and plated on FN-coated coverslips in 24-well plates (10 μg/mL FN; 40,000 cells per well). After 1 or 2 h at 37°C, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked in 10% goat serum in PBS. Cells were incubated with primary antibody (VASP; Cortactin) in blocking solution, washed and incubated with secondary antibody (conjugated to Alexa-488), then counter-stained with rhodamine-phalloidin. Coverslips were mounted, visualized with LSM confocal microscopy (×40 magnification), and analyzed using LSM Image Examiner software.

For Src and pFAKY397 immunofluorescence, cells were plated on FN-coated coverslips. After 1 h, coverslips were incubated in 3.7% formal saline solution for 5 min at room temperature, and fixed with acetone at −20°C for 2 min. Coverslips were incubated with primary antibody washed and incubated with secondary antibody (conjugated to Alexa-488). Coverslips were mounted, visualized with a Leica upright microscope (model DMR) at ×40 magnification, and images were captured using Hamamatsu Orca digital camera (model C4742-95) and MetaMorph 6.1 imaging software.

Immunoblotting

PC3-M cells were treated with serum-free media containing apigenin or DMSO for 16 h, detached and replated onto FN-coated wells (10 μg/mL FN; 40,000 cells per well). After 2 h at 37°C, cells were lysed in Triton lysis buffer containing protease and phosphatase inhibitors. Lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, followed by immunoblotting with antibodies against pFAKY397, pFAKY576, pFAKY925, FAK, pSrcY416, Src, cortactin, VASP, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Invadopodia assay

Coverslips for invadopodia assay were prepared as previously described (33). PC3-M cells were treated with apigenin or solvent control for 16 h, detached and replated on oregon green–conjugated gelatin coated coverslips in 24-well plates (10 μg/mL gelatin; 80,000 cells per well). After 2 and 9.5 h at 37°C, cells were fixed with 3.7% paraformaldehyde in 5% sucrose, permeabilized with 0.5% filtered Triton X-100, and blocked in 10% goat serum in PBS. Cells were incubated with cortactin antibody in blocking solution, washed, incubated with secondary antibody, and counter-stained with phalloidin-alexa-633. Coverslips were mounted and visualized with confocal microscopy (×40 magnification).

Transient transfections and scratch wound assays

Retroviral vector pLXSH containing caSrc was a gift from Dr. Jonathan Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA). Cells were transfected according to Lipofectamine packaging instructions. Briefly, cells were plated at 95% confluency and transfected with either empty vector (pLXSH) or caSrc (Y527). The next day, cells were treated with apigenin or solvent control for 16 h. A scratch wound was made as described above. After 22 h, cells were imaged and lysed to analyze expression of p-Src and total Src.

MTS assay

PC3-M cells were plated in 96-well plates (8000 cells per well) and treated with 25 μmol/L apigenin or DMSO for 24 and 48 h. Cell survival was determined using the MTS titer assay according to manufacturer's instructions (Promega). Formazan (proportional to the number of living cells) was quantitated by measuring absorbance at 490 nm using the spectrophotometer.

Results

Apigenin inhibits PC3-M motility in a scratch wound assay

It has been reported that apigenin inhibits migration in melanoma (12), breast cancer (12), and ovarian cancer cells (34). However, the effect of apigenin on cell motility has not been shown in prostate cells, and little is known about the mechanism by which apigenin inhibits cell migration. Therefore, we determined the effect of apigenin on cell motility in the prostate carcinoma cell line PC3-M using a scratch-wound assay as a model system. Our results showed that apigenin treatment significantly inhibited the motility of PC3-M as well as other prostate cancer cell lines C4-2B, DU145, and PC3-LN4 in the scratch-wound assay (Supplementary Fig. S1; Fig. 1A and B). This effect was not due to decreased cell viability as shown by MTS assay (Supplementary Fig. S2). To better assess how apigenin inhibits cell motility, we did live cell imaging studies of scratch wound closure. Our data further show that apigenin affected cell motility, with apigenin-treated cells showing 66% decreased movement from the origin compared with control cells (Supplementary Movie S1; Fig. 1C and D).

Apigenin inhibits persistence length and velocity of PC3-M cells in a scratch wound assay and on a line assay

Cell migration is regulated by both speed and directionality of cell motility (27, 29, 35, 36). Directional migration (i.e., cell motility in one direction) can involve either externally directed migration during chemotaxis or the intrinsic propensity of cells to continue migrating in the same direction without turning (i.e., intrinsic persistence of migration). Many migratory processes in development and tissue remodeling use intrinsic cell migration properties (37).

To determine if apigenin affects the directionality of cell movement, we analyzed individual cells by live cell imaging. We measured distance and trajectory for each track to determine cell persistence. Apigenin-treated cells had a 45% decreased distance/trajectory ratio, demonstrating decreased cell persistence in those cells (Fig. 2A and B).

We used a micropatterning technique that uses a combination of small-scale etching and surface chemistry based on self-assembled monolayers to study directional persistence length and cell velocity. With this approach, we can fabricate substrata constraining motions of motile cells to transparent

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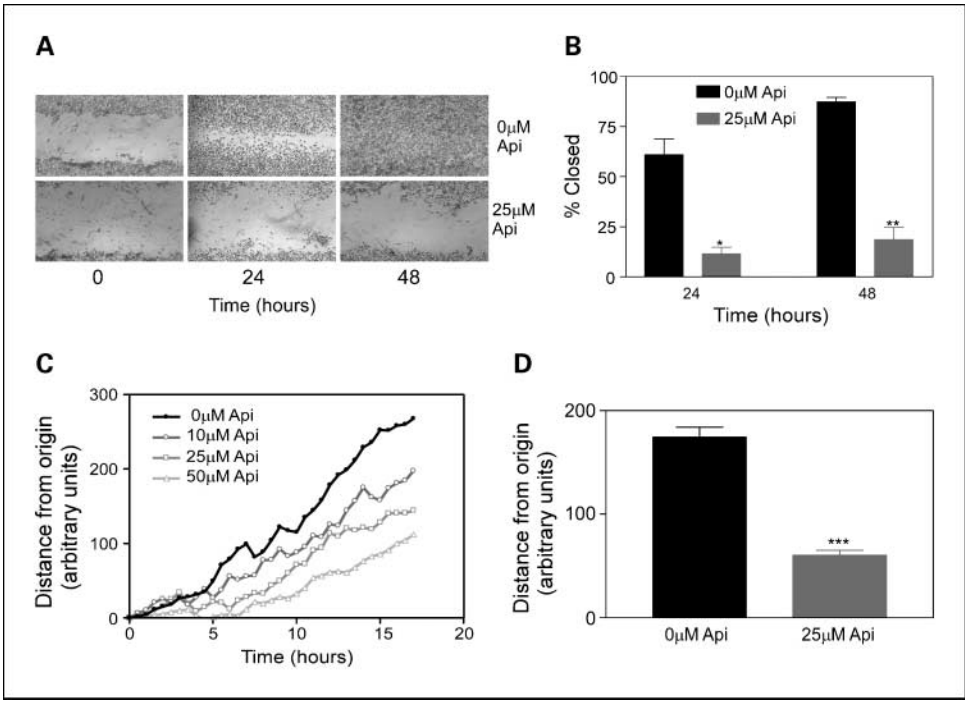

Fig. 1. Apigenin inhibits PC3-M motility. *A* and *B*, PC3-M cells were plated for a scratch wound assay and pretreated with apigenin or solvent control for 16 h. Photographs were taken at 0, 24, and 48 h after wounding. The percent wound closure was calculated using Image J software. Each time point was compared with 0 time point for each treatment condition. *A*, a representative image; *B*, shows the quantitation of one experiment done in triplicate. At 24 h, the wounds in the control cells were 61 ± 4.6% closed and 11.6 ± 1.4% closed for the apigenin-treated cells. At 48 h, the wounds in the control cells closed 87.5 ± 1.3% compared with 18.6 ± 6.7% for the apigenin-treated cells. *, difference between control and apigenin-treated cells at 24 h ($P < 0.02$); **, difference between control and treated cells at 48 h ($P < 0.01$). *C* and *D*, PC3-M cells were plated for live cell imaging of wound closure and pretreated with apigenin or DMSO control for 16 h, with fresh media containing apigenin or solvent control added at the start of the experiment. Distance from origin was calculated using MetaMorph 6.1 imaging software. *C*, the path of representative cells plated in 0, 10, 25, or 50 μmol/L apigenin. *D*, the average distance from the origin over 17 h for 40 cells treated with 25 μmol/L apigenin versus control. The distance traveled by the control cells was 174.4 ± 9.7 compared with 60.4 ± 4.7 for the apigenin-treated cells. ***, difference between control and treated cells ($P < 0.001$).

microtracks surrounded by nonadhesive, opaque regions, and can analyze distributions of persistence times and lengths. The key features of these assays are that they constrain cell motions to only one dimension and, owing to high-optical contrast between cell-adhesive and cell-resistant regions, allow for analysis of cell trajectories. This is especially advantageous in establishing "persistence times" and "lengths"—that is, times and distances the cells move one direction before reversing the direction of motion (31).

These movies of "cells on lines" provided us a means of comparing the persistence and velocity of cells through tracking the displacement of each cell with time. A representative image of control and apigenin-treated PC3-M cells on lines is shown in Fig. 2C. Plots of these frequency distributions are shown in Fig. 2C. Results show that persistence length decreased after treatment with apigenin (Supplementary Movie S2; Fig. 2C). The characteristic persistence length of 21 control and 19 apigenin-treated cells was found to be 17.6 ± 0.8 μm and 6.7 ± 0.4 μm, respectively. Velocities of the PC3-M cells also decreased (Supplementary Movie S2; Fig. 2D). The characteristic velocity of 31 control cells was found to be 19.4 ± 0.57 μm/hour, compared with 6.1 ± 0.18 μm/hour in 51 apigenin-treated PC3-M cells.

Fewer total cells treated with apigenin (37%) moved compared with the control cells (68%). Therefore, the data again support the conclusion that apigenin hinders cell movement and affects persistence (or directionality) of cell movement. Because directed cell migration is an essential component of cancer cell invasion during metastasis (27), these results suggest that apigenin could be useful in interfering with the invasiveness of prostate cancer cells.

Apigenin-treated cells display "exaggerated filopodia" and stronger attachment to the matrix

Cell migration depends in part on the strength of transient cell-substratum attachments. On weakly adhesive surfaces, cell-substratum interactions cannot provide traction, so the cells spread poorly and motility is not possible. On strongly adhesive surfaces, the cells are well-spread and immobilized, and regular dynamic disruption of cell-substratum attachments is difficult so locomotion cannot occur. However, cell motility is possible with an intermediate strength of cell-substratum interactions. The terms weak and strong adhesion are relative to the level of motile force generated within the cell and transmitted to the cell-substratum attachments (38). We wanted to better understand how apigenin is affecting PC3-M cell motility, so we did single-cell motility assays of cells on FN using time-lapse video microscopy. Figure 3A shows still images from the time-lapse microscopy studies showing that apigenin treated cells have very long "sticky" protrusions, known as exaggerated filopodia (39). Supplementary Movie S3 shows that the exaggerated filopodia present on the apigenin-treated cells seem to be strongly attached to the surface, requiring substantial force to detach and snap back to the cell body.

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Using wind-rose plots, Fig. 3B shows the change in individual PC3-M cell tracks when cells are treated with medium containing solvent control or apigenin. The apigenin-treated cells did not move far from the origin and changed directions frequently as opposed to the more motile and persistent control cells. Additionally, we found that apigenin-treated cells have fewer protrusions than control cells and move more slowly than control cells (Supplementary Fig. S3)

Figure 3C shows the quantitation of the protrusions exhibited by the control cells and apigenin-treated cells. Apigenin-treated cells have longer protrusions with a longer duration than the ones in the control cells. There is an accumulation of focal adhesion proteins as shown by increased staining with anti-pFAKY397 antibody (Fig. 3D) and paxillin (data not shown) at the tips of the exaggerated filopodia on the apigenin-treated cells.

Based on these results, we wanted to test if apigenin treatment could strengthen cell-matrix adhesion. We did a cell detachment assay and observed that cells treated with apigenin adhere more strongly to the ECM than the control cells (Fig. 3D).

Apigenin induces remodeling of the actin cytoskeleton and inhibits formation of actin structures during cell motility

Actin polymerization and filament elongation at the leading edge of the cell, coupled to actin/myosin filament contraction at the rear, are thought to be the driving forces for cell migration (27). Lamellipodia represent filament elongation at the front of a moving cell and filopodia sense the extracellular environment and help determine the direction of cell movement. Both lamellipodia and filopodia are actin-rich protrusions driven by actin polymerization. Stress fibers are actin structures connected to focal complexes and are responsible for the contractile tension needed for cell translocation.

Because apigenin not only affects movement of the cell, but persistence (or directionality) of movement, and actin structures are intricately connected to cell motility and directionality of cell motility, we next determined whether apigenin treatment resulted in actin cytoskeleton remodeling. PC3-M cells plated on FN for 1 hour formed membrane ruffles, or lamellipodia, which stain positively for Cortactin. Cortactin activates Arp2/3 when bound to filamentous actin and inhibits debranching of Arp2/3 complexes *in vitro*. Therefore, it has been theorized that it serves as a stabilizer of the putative actin filament branches in the lamellipodium (40). However, our results shown in Fig. 4A show that apigenin-treated cells failed to form lamellipodia. These cells also looked morphologically different than control cells, and displayed long actin-rich protrusions (Fig. 4A). Similar results were obtained with Arp2 (data not shown).

PC3-M cells plated on FN form numerous filopodia that stain positively for VASP. Ena/VASP binds to free barbed ends of actin filaments in the distal tips of lamellipodia/filopodia, and antagonizes capping proteins that inhibit filament elongation. Apigenin-treated cells, however, formed far less VASP-positive filopodia (Fig. 4B). The effect of apigenin on filopodia formation may help to explain the effect on the directionality of cell movement, because inhibition of filopodia formation leads to impaired motility (41).

Interestingly, Western blot analysis showed that apigenin-treated PC3-M cells also had lower levels of Cortactin and VASP proteins than control cells, suggesting that apigenin may be affecting their mRNA levels or protein stability. It has previously been shown that apigenin can affect mRNA levels and/or protein stability of FAK, Cox-2, p53, and HIF-1α (34, 42–44).

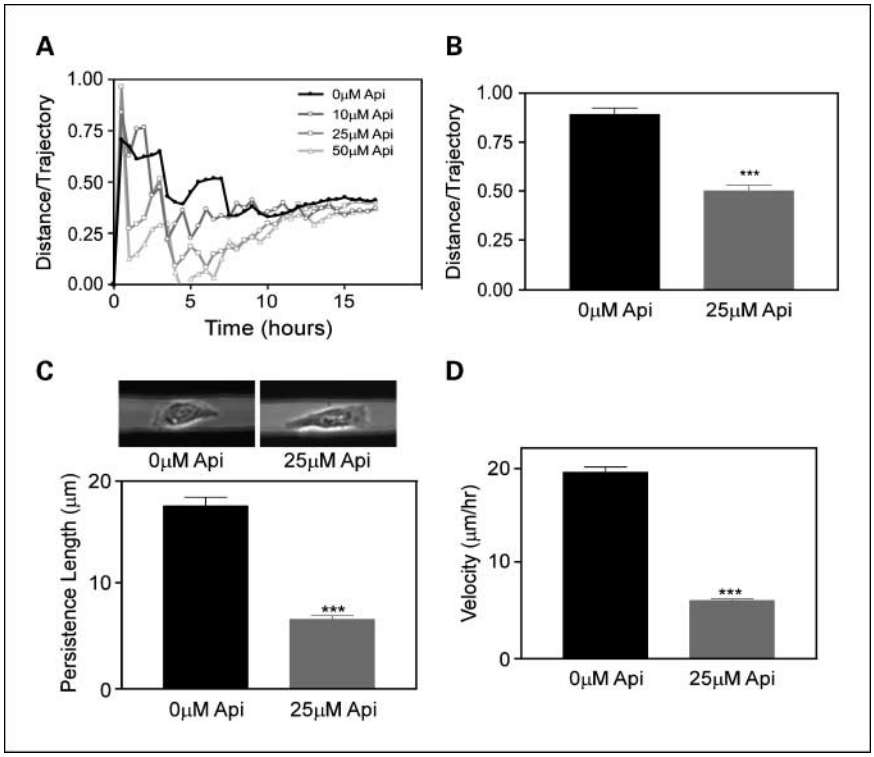

Fig. 2. Apigenin inhibits persistence length and velocity in PC3-M cells. *A* and *B*, apigenin reduces directional motility of PC3-M cells: PC3-M cells were plated and pretreated with apigenin or DMSO control. Fresh medium containing apigenin or solvent control was added and live cell imaging of wound closure was done. Distance/trajectory (d/t) was measured using MetaMorph 6.1 imaging software. *A*, d/t of a representative cell; *B*, the quantitation of 40 cells. The average d/t for control cells was 0.89 ± 0.03, and the average d/t for apigenin-treated cells was 0.5 ± 0.03. *C* and *D*, apigenin inhibits persistence length and velocity in PC3-M cells on a line assay: PC3-M cells (apigenin-treated or solvent control) were imaged on line assays. *C* and *D*, a representative image of cells on lines with cell persistence and velocity as a quantitation of 31 control cells and 51 apigenin-treated cells, respectively. *B*, *C*, and *D*, ***, difference between control and treated cells ($P < 0.001$).

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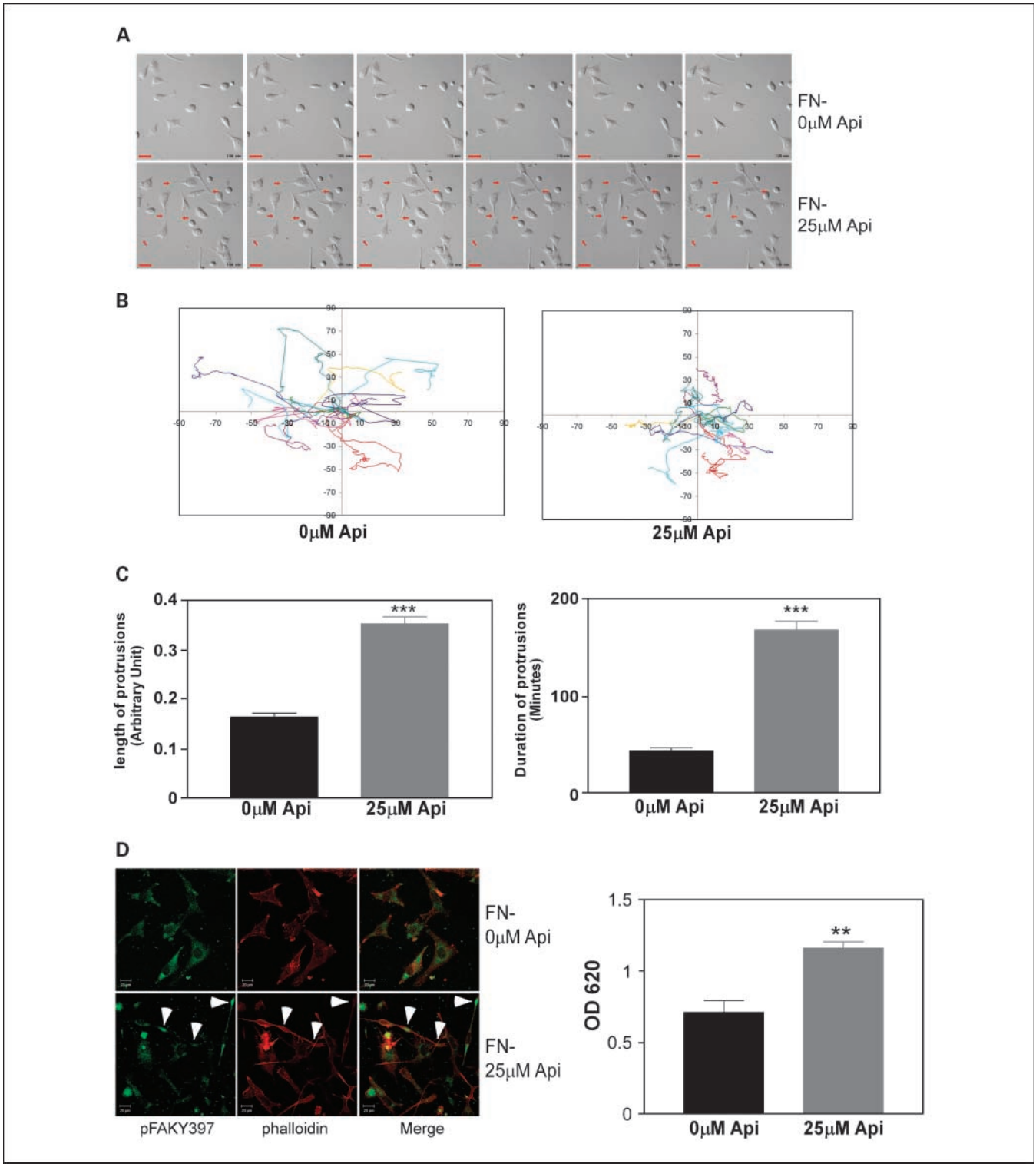

Fig. 3. Apigenin-treated cells have exaggerated filopodia and stronger attachment to the matrix. *A,* stills from phase time-lapse microscopy. PC3-M cells were treated with apigenin or solvent control for 16 h, then detached and plated on FN-coated two-well chambered cover glass and allowed to attach and spread for 5 h before being placed in the ASMDW for live cell imaging. The stills are from 100 to 125 min. *Arrows,* exaggerated filopodia. *Scale bar,* 20 μm. *B,* wind-rose plots of cell tracks from time-lapse microscopy. Each wind-rose plot shows centroid tracks from 10 representative tracks, with the initial position of each track superimposed at 0,0 for clarity. *C,* length and duration of protrusions from time-lapse microscopy. Two hundred forty-one control cells were analyzed with an average length of 0.16 ± 0.01 and average duration of 43.6 ± 2.8 min. One hundred seventy-six apigenin-treated cells were analyzed with an average length of 0.35 ± 0.01 and an average duration of 167.4 ± 9 min. ***, difference between control and treated cells ($P < 0.001$). *D, left,* PC3-M cells were treated with apigenin or solvent control for 16 h, then detached and plated on FN-coated coverslips. After 9.5 h, cells were fixed and stained for pFAKY397. *Arrows,* accumulation of pFAKY397 at the tips of the exaggerated filopodia. *Right,* PC3-M cells were plated in 96-well plates, treated with medium containing apigenin or solvent control for 16 h, then trypsin was added for the cell detachment assay. The cells remaining on the plate were fixed and stained with methylene blue and absorbance was measured at 620 nm. Average absorbance for control cells was 0.7 ± 0.08, and for apigenin-treated cells, it was 1.16 ± 0.04. **, difference between control and treated cells ($P < 0.003$).

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Apigenin-treated PC3-M cells plated on FN for 2 hours also showed decreased stress fiber formation compared with control cells, as shown by phalloidin staining (Supplementary Fig. S4A). Apigenin-treated cells also exhibited an altered morphology compared with control cells. Because stress fibers are connected to focal complexes and are responsible for the contractile tension needed for cell translocation, the inability of apigenin-treated cells to form stress fibers may explain the impaired motility. Additionally, these results suggest that focal complexes may be defective in these cells (see below).

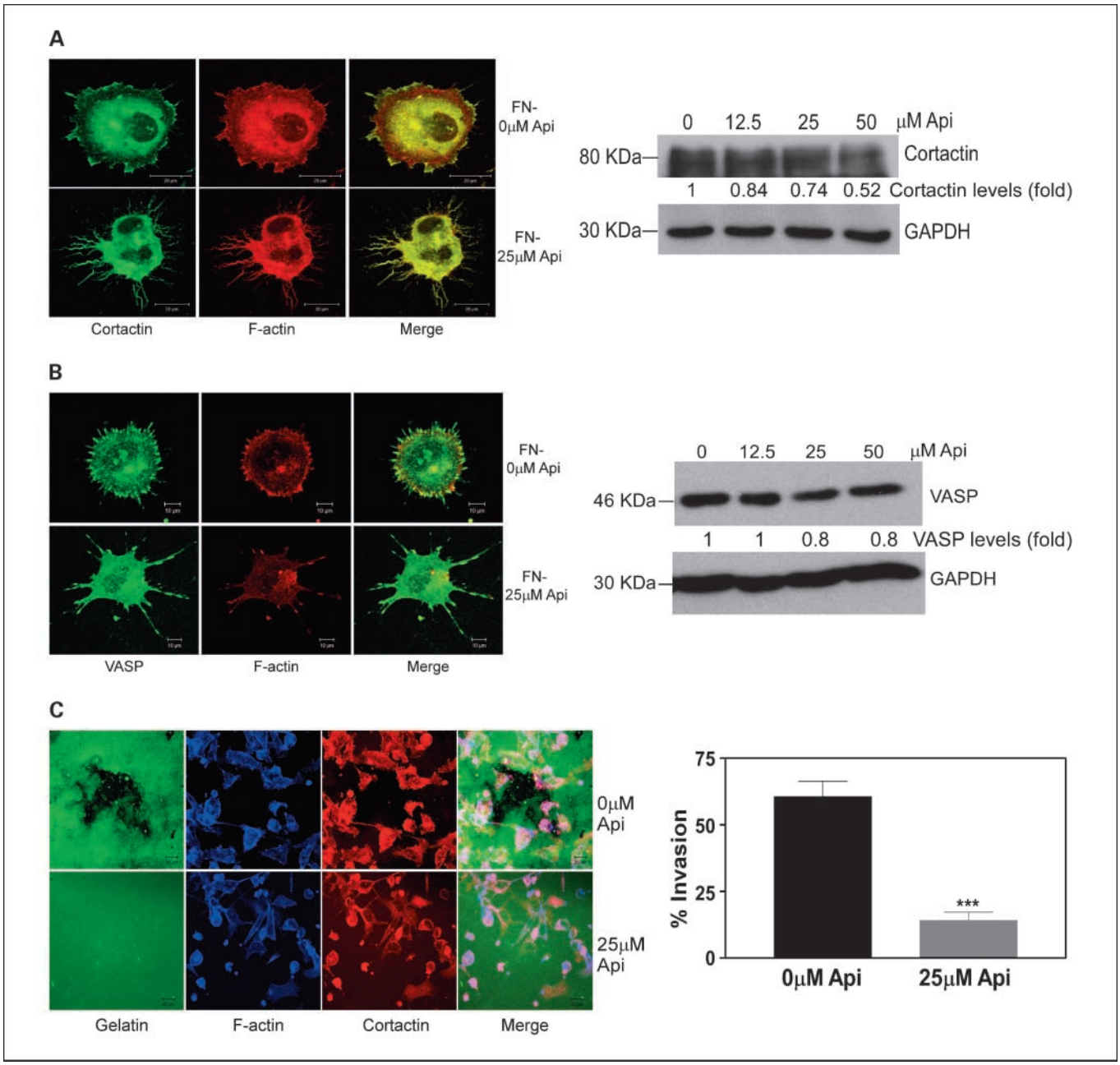

Fig. 4. Apigenin disrupts actin cytoskeleton structures involved in cell motility and cell invasion. *A* and *B,* for immunofluorescence, PC3-M cells were treated with medium containing apigenin or solvent control for 16 h, detached and plated on FN-coated coverslips, and after 1 h, cells were stained for Cortactin (*A*) or VASP (*B*). For Western blot, PC3-M cells were treated with apigenin or solvent control for 16 h, detached, plated on FN-coated wells, and, after 1 h, lysed and subjected to SDS-PAGE, followed by immunoblotting with antibodies against Cortactin and GAPDH (*A*) or VASP and GAPDH (*B*). Cortactin and VASP levels were determined by normalizing to GAPDH levels for each treatment. Average decrease in cortactin expression from three experiments with 25 μmol/L apigenin was 0.79 ± 0.07. Average decrease in VASP expression from three experiments with 25 μmol/L apigenin was 0.85 ± 0.02. *C,* PC3-M cells were treated with medium containing apigenin or solvent control for 16 h, then detached and plated on gelatin-coated coverslips, and invadopodia were visualized by confocal microscopy after 9.5 h; *green,* gelatin; *red,* cortactin; *blue,* phalloidin. *Arrows,* colocalization of cortactin and phalloidin with degraded matrix (invadopodia). Quantitations of invadopodia formation (% invasion measured as the number of cells localized over areas of degraded matrix relative to the total number of cells). Experiment was done in duplicate, with five fields imaged per coverslip, with at least 140 cells being counted to determine the percent invasion. The % invasion for the control cells was 60.6 ± 5.72% compared with 14.1 ± 2.43% for apigenin-treated cells. ***, difference between control and treated cells ($P < 0.001$).

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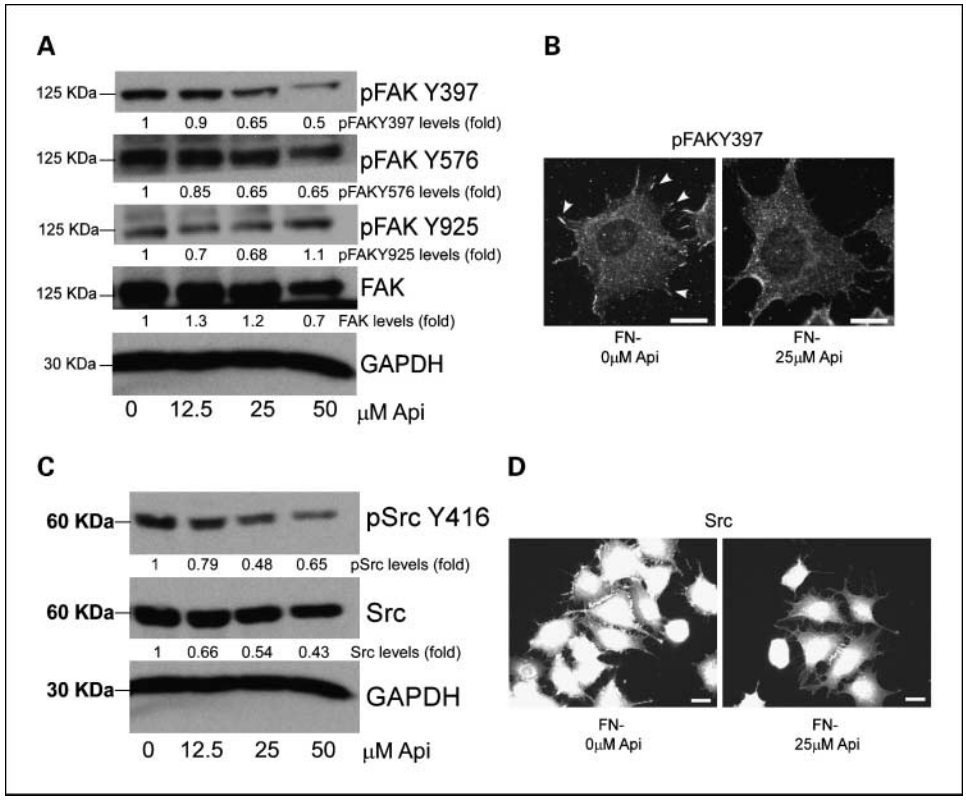

Fig. 5. Apigenin inhibits FAK/Src activation. *A,* PC3-M cells were treated with apigenin or solvent control for 16 h, plated on FN for 2 h, followed by immunoblotting with antibodies against pFAKY397, Y576/577, Y925, total FAK, and GAPDH. Levels of pFAK were determined by normalizing to FAK levels for each treatment. Total FAK levels were determined by normalizing to GAPDH levels for each treatment. Average decrease in pFAKY397 expression with 25 μmol/L apigenin was 0.875 ± 0.2. The average decrease in pFAKY576 expression with 25 μmol/L apigenin was 0.79 ± 0.12. The average decrease in pFAKY925 expression with 25 μmol/L apigenin was 0.865 ± 0.1. The average decrease in FAK expression with 25 μmol/L apigenin was 0.8 ± 0.1. *B,* PC3-M cells were treated with apigenin or solvent control for 16 h, then detached and plated on FN-coated coverslips. After 1 h on FN, PC3-M cells were fixed, followed by immunofluorescence with anti-FAK (Y397) antibody. *Arrows,* focal adhesions staining positively for pFAK. *Scale bar,* 20 μm *C*. PC3-M cells were treated with apigenin or solvent control for 16 h, detached and plated on FN-coated coverslips for 1 h, followed by immunoblotting with antibodies against p-SrcY416, total Src, and GAPDH. Levels of pSrc were determined by normalizing to Src levels for each treatment. Total Src levels were determined by normalizing to GAPDH levels for each treatment. The average decrease in pSrc expression with 25 μmol/L apigenin was 0.53 ± 0.02, and the average decrease in Src expression with 25 μmol/L apigenin was 0.78 ± 0.14. *D,* PC3-M cells were treated with apigenin or solvent control for 16 h, then detached and plated on FN-coated coverslips. After 1 h on FN, PC3-M cells were fixed, followed by immunofluorescence with antibodies against Src. *Scale bar,* 20μm.

To better assess the role of apigenin on cell spreading, we did a spreading time course on FN. Supplementary Fig. S4B shows still images from the time-lapse microscopy from 1, 2, and 3 hours. This experiment showed that apigenin-treated cells do not spread as well or as fast as the control cells. Even at 3 hours after plating, the apigenin-treated cells remain very round and lack lamellipodia.

Apigenin induces remodeling of the actin cytoskeleton and inhibits formation of actin structures during cell invasion

Recent research has reinforced the concept that tumor invasion is the net result of dysregulated cell motility (29). Induced cell motility has come to be recognized as the dominant regulator of tumor invasion (27), particularly as matrix degradation and remodeling is now understood to be limited rather than extensive, and a partner to motility (27). Apigenin inhibits migration and invasion in breast cancer and melanoma cells and MMP-9 activity in breast cancer cells (12). We have previously shown that apigenin inhibits invasion (in a Boyden chamber invasion assay; ref. 44), affects the speed and direction of cell motility, and alters the actin cytoskeleton. Thus, we next determined whether apigenin treatment affected actin structures during invasion by conducting an invadopodia assay. ECM degradation by invadopodia mediated by released and exposed proteases, including MMPs (45), can be visualized *in vitro*.

The functional definition of invadopodia is colocalization of cortactin and actin with degraded matrix (45). Control cells stained positively for cortactin and actin, which colocalized with degraded matrix. This occurred as early as 2 hours, and became more pronounced at 9.5 hours. However, cells treated with apigenin showed decreased staining for cortactin and actin, as well as fewer areas of degraded matrix at both 2 and 9.5 hours (Supplementary Fig. S4; Fig. 4C). We expressed the amount of invadopodia being formed as "percent invasion" by calculating the ratio of the number of cells localized over degraded matrix (black areas) to the total number of cells. Invadopodia formation was inhibited by 77% in apigenin-treated cells compared with the control cells (Fig. 4C).

Apigenin inhibits FAK/Src activation

Signals initiated by ECM-integrin interactions are transduced into cells through activation of integrin-associated

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proteins, such as FAK and Src (28). Recent studies in FAK- and Src-deficient cells implicate these two molecules as critical mediators of integrin adhesion turnover that promote cell migration (46). Src-mediated phosphorylation of FAK is required for actin stress fiber formation and focal adhesion assembly associated with cell attachment and spreading on ECM, as well as for focal adhesion turnover (47). Integrin-stimulated FAK phosphorylation at Y397 creates a high-affinity binding site for Src family tyrosine kinases (46). The binding of Src with FAK leads to tyrosine phosphorylation of the remaining sites in FAK and changes its interactions with components of various signaling pathways (46). In many signaling contexts, FAK-Src complex acts to control cell shape and focal contact turnover during cell motility (46).

High levels of FAK expression and signaling are correlated with higher invasive and migratory capacity of prostate cancer (29). Signaling from FAK/Src leads to migration, invasion,

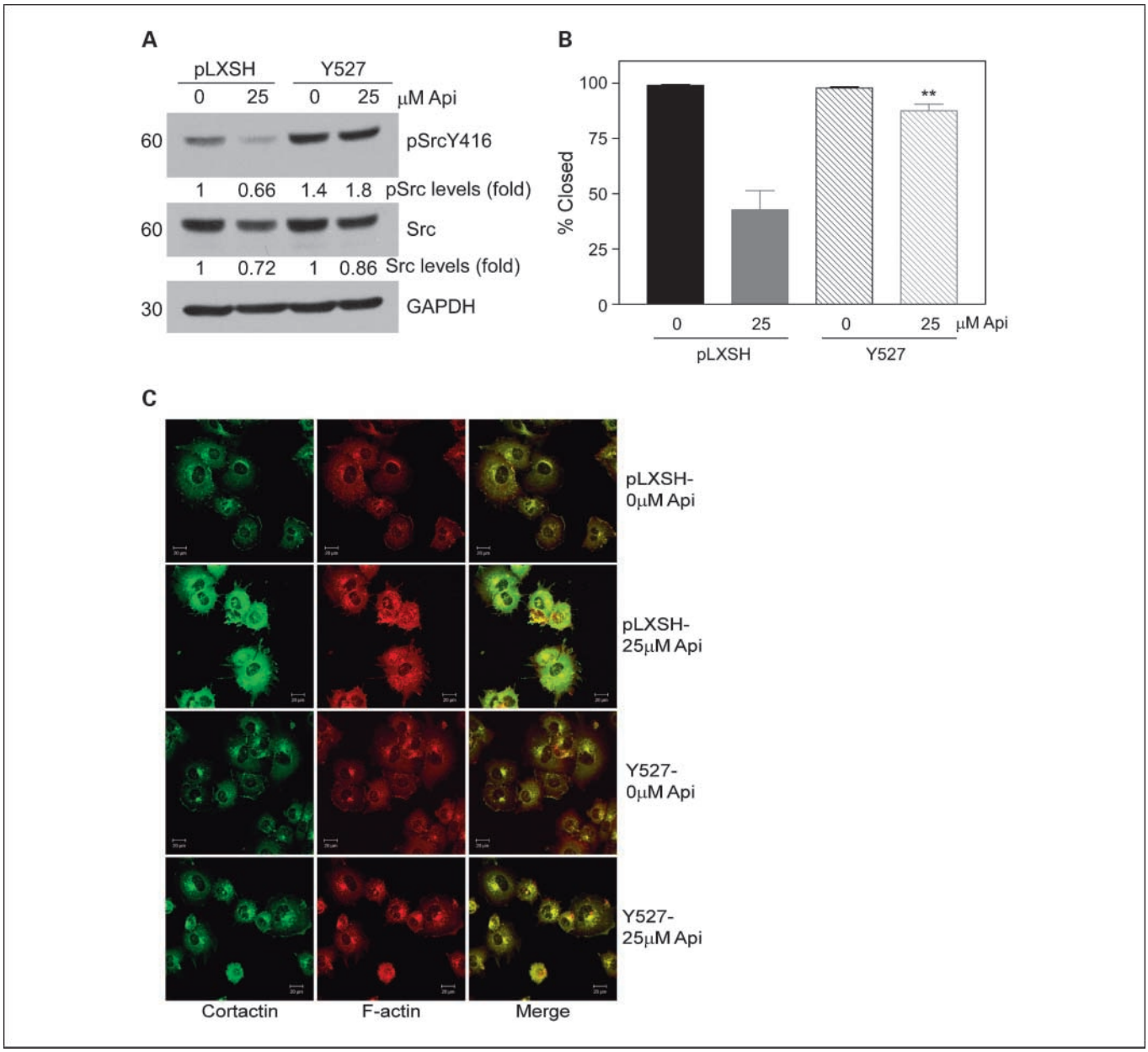

Fig. 6. The expression of caSrc blunts the effect of apigenin on cell motility. *A,* PC3-M cells were plated in 24-well plates and transfected with either empty vector (*pLXSH*) or vector containing caSrc (*Y527*). Twenty-four hours after transfection, cells were treated with apigenin or solvent control for 16 h and then subjected to a scratch wound assay. Pictures were taken after 24 h, then cells were lysed, and a Western blot was done with antibodies against pSrcY416, total Src, and GAPDH. Levels of pSrc were determined by normalizing to Src levels for each treatment. Total Src levels were determined by normalizing to GAPDH levels for each treatment. *B,* scratch wound closure was quantitated by comparing the size of the wound at 24 h to the 0-h time point for each condition using Image J software. Quantitations are from one experiment done in triplicate. **, difference between pLXSH plus 25 μmol/L apigenin and Y527 plus 25 μmol/L apigenin cells ($P < 0.02$). *C,* PC3-M cells were plated in 10-cm dishes and transfected with either empty vector or caSrc vector. Twenty-four hours after transfection, cells were treated with media containing apigenin or solvent control for 16 h, then detached and plated on FN-coated coverslips. After 1 h, cells were fixed and stained for cortactin.

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and alterations in the actin cytoskeleton during both motility and invasion. Src kinase signaling has been shown to be both necessary and sufficient for invadopodia and podosome formation (48–50). Moreover, FAK has been found in actively degrading invadopodia (50, 51).

Therefore, we determined if the effects of apigenin on motility and actin remodeling are mediated through FAK/Src signaling. Apigenin-treated PC3-M cells showed decreased phosphorylation of FAK in a dose-dependent manner (Fig. 5A). FAK phosphorylation was decreased by 35% (Y397), 35% (Y576), and 32% (Y925) in apigenin-treated cells (25 μmol/L) compared with control cells. In addition, apigenin-treated cells also had decreased levels of total FAK protein (30% less than control cells at 50 μmol/L).

Figure 5B shows the effect of apigenin on p-FAK by immunofluorescence. In concordance with Western blot data and our previous observations of the effect of apigenin on the cytoskeleton, we observed that apigenin-treated cells showed decreased staining for p-FAK at focal adhesions, as well as cytoskeletal remodeling. Because apigenin modestly decreased p-FAK at Src substrates Y576 and Y925, we next determined if apigenin affects activation (phosphorylation at tyrosine 416) of Src. Apigenin-treated cells showed a decreased activation of Src in a dose-dependent manner compared with control cells. Apigenin treatment (25 μmol/L) led to a 52% reduction in Src phosphorylation. Additionally, we observed that apigenin-treated cells expressed 46% less Src protein than control cells (Fig. 5C).

Figure 5D shows effect of apigenin on Src by immunofluorescence. We again observed that apigenin-treated cells looked morphologically different than control cells, with several long protrusions. Moreover, levels of Src staining were decreased in apigenin-treated cells, consistent with Western blot data showing decreased levels of Src protein.

Src is important for the effect of apigenin on motility and cytoskeleton remodeling

To determine if the effect of apigenin on motility was through decreased activation of Src, we used a caSrc construct. PC3-M cells transfected with empty vector and treated with apigenin had decreases in both p-Src and total Src protein, compared with cells treated with medium containing solvent control. caSrc cells treated with apigenin displayed decreased levels of total Src protein; however, the amount of p-Src relative to total Src was the same as control cells transfected with empty vector (Fig. 6A). When PC3-M cells were transfected with caSrc, apigenin was no longer able to inhibit cell motility in a scratch wound assay (Fig. 6B). These data indicate that apigenin's inhibition of PC3-M cell motility is mediated through Src signaling.

We next determined if the effect of apigenin on cytoskeleton remodeling was through decreased activation of Src. We did immunofluorescence studies on cells transfected with the caSrc construct. As shown in Fig. 6C, expression of caSrc blunts the effect of apigenin on cell morphology and cytoskeleton remodeling. Specifically, caSrc cells treated with 25 μmol/L apigenin showed both a reduced level of lamellipodia formation compared with the solvent control–treated cells transfected with empty vector, and a reduced number of cells with long actin-rich protrusions compared with the apigenin-treated empty vector transfected cells. This suggests that, although activation of Src is important for cytoskeleton remodeling in these cells, it by itself is not sufficient for completely reversing the effect of apigenin.

Discussion

As a critical requirement for tumor invasion, cell motility is an attractive therapeutic target for inhibiting advanced and aggressive prostate cancers (29). Using a scratch-wound assay and looking at invadopodia formation, we showed here that apigenin inhibited prostate cancer cell motility and invasion. Actin reorganization is considered to be a driving force for cell migration, which is regulated by both the speed and directionality of cell motility (40). In this study, we showed that apigenin treatment led to actin cytoskeleton remodeling concomitant with decreased cell motility. We found that apigenin-treated cells have exaggerated filopodia and stronger attachment to the matrix, which may explain their decreased motility. Furthermore, FAK and Src (major regulators of actin cytoskeleton remodeling, cell motility, and invasion) are inhibited by apigenin. Our Western blot analysis and immunofluorescence experiments showed that apigenin inhibited the phosphorylation of FAK at tyrosines 397, 576, and 925 and phosphorylation of Src at tyrosine 416. Furthermore, expression of a caSrc blunted the effect of apigenin on cell motility and cytoskeleton remodeling. Taken together, these findings point to a unique chemopreventive mechanism of apigenin activity in prostate cancer.

We used a novel micropatterning line assay, which constrains cell motions to only one dimension and allows for highly qualitative analysis of "persistence times" and "lengths," as well as cell velocities (31). Using line assays, we found that apigenin inhibited cell migration, consistent with the notion that apigenin treatment results in decreasing the distance a cell moves in one direction before reversing direction. Future studies using line assays will distinguish between the regulatory roles of apigenin on cell velocity and persistence length by using fluorescence to monitor the dynamics of both actin (velocity) and microtubule (persistence) cytoskeletal components (52).

Because the cytoskeleton is critical for cell motility and cell invasion, we investigated the effect of apigenin on cytoskeleton remodeling. We describe here a profound effect of apigenin on actin structures, as shown by immunofluorescence staining for Cortactin, VASP, and filamentous actin. We also observed that apigenin-treated cells formed exaggerated filopodia that strongly adhered to the ECM. This finding is intriguing because there are no extensive studies on the role of bioflavonoids on the cytoskeleton. Future studies are warranted to investigate whether actin cytoskeleton remodeling is a general property of bioflavonoids or a specific attribute of apigenin. Additional studies are needed to further delineate the mechanism of actin cytoskeleton remodeling by apigenin. Because cell motility is a critical requirement for tumor invasion (29), understanding how apigenin inhibits prostate cancer cell motility through actin remodeling can provide insight into the mechanism of apigenin-mediated chemoprevention.

FAK/Src signaling has previously been shown to play a role in cell motility and invasion in large part through orchestrating the cytoskeletal remodeling needed for cell movement (53). In prostate cancer, FAK is known primarily for its role in cell

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motility and cytoskeletal rearrangement, as supported by *in vivo* and *in vitro* evidence (53). Additionally, a large body of evidence shows that increased expression and activation of FAK correlates with more aggressive prostate cancer (53). Src has been shown to be involved in many aspects of prostate cancer biology, such as adhesion and cell migration through its interactions with FAK, and invasion through Src regulation of MMPs (53) Our studies presented herein show not only that apigenin inhibits FAK and Src but also that caSrc overrides apigenin's inhibition of cell motility and cytoskeleton remodeling. These findings are consistent with the hypothesis that the FAK/Src signaling axis lies at the root of apigenin's inhibition of cell motility, cell invasion, and actin cytoskeleton remodeling.

Because FAK and Src also depend on ECM remodeling, integrin receptors, and their numerous downstream effectors (46), future studies are warranted to elucidate the role of these factors in apigenin-mediated effects on motility and the cytoskeleton. Additionally, because expression of caSrc did not completely reverse the effect of apigenin on cytoskeleton remodeling, this indicates that other signaling molecules are involved in the process. It will be especially interesting to determine whether apigenin can alter the expression of integrin receptors shown to be important for cell motility in prostate cancer. For example, $\alpha_v\beta_3$ is important for prostate cancer cell migration (53) and $\alpha_{6p}\beta_1$ gives tumor cells selective advantages for metastases (29). α_{6p} is a novel form of the α_6 subunit found in prostate cancer cells, which is paired with β_1.

In conclusion, considering apigenin is found in many fruits and vegetables and has been shown to be nontoxic when fed *in vivo* to mice as a dietary additive (54), our results are further evidence of apigenin's potential as an effective chemopreventive agent in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Chemopreventive Bioflavonoid Apigenin Inhibits Prostate Cancer Cell Motility through the Focal Adhesion Kinase/Src Signaling Mechanism

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