Cyclin-Dependent Kinase 5 Activity Controls Cell Motility and Metastatic Potential of Prostate Cancer Cells

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Abstract

We show here that cyclin-dependent kinase 5 (CDK5), a known regulator of migration in neuronal development, plays an important role in prostate cancer motility and metastasis. P35, an activator of CDK5 that is indicative of its activity, is expressed in a panel of human and rat prostate cancer cell lines, and is also expressed in 87.5% of the human metastatic prostate cancers we examined. Blocking of CDK5 activity with a dominant-negative CDK5 construct, small interfering RNA, or roscovitine resulted in changes in the microtubule cytoskeleton, loss of cellular polarity, and loss of motility. Expression of a dominant-negative CDK5 in the highly metastatic Dunning AT6.3 prostate cancer cell line also greatly impaired invasive capacity. CDK5 activity was important for spontaneous metastasis *in vivo*; xenografts of AT6.3 cells expressing dominant-negative CDK5 had less than one-fourth the number of lung metastases exhibited by AT6.3 cells expressing the empty vector. These results show that CDK5 activity controls cell motility and metastatic potential in prostate cancer. (Cancer Res 2006; 66(15): 7509-15)

Introduction

Prostate cancer is the most commonly diagnosed cancer in American men. Although there have been significant advances in the early detection and treatment of localized prostate cancer, metastasis, the key determinant of lethality in the disease, still represents a major clinical challenge (1, 2). Therefore, the identification of target proteins responsible for the control of metastasis in prostate cancer may be important for treating this disease (3).

The control of cell migration, one of the critical processes of metastasis, has been extensively studied not only in cancer cells, but also in normal central nervous system development. We hypothesized that these studies in neural development may provide clues to the control of cell migration in metastasis. In normal cerebral cortical development, successive layers of nascent postmitotic neurons migrate radially from the ventricular zone, through previously deposited layers of neurons (4). In mice, this process is complete by embryonic day 18, with six layers of neurons. Because the nascent neurons migrate through previously deposited layers, the most recently born neurons are deposited in the successive outermost layers. Neuronal migration has been shown to involve loss of cadherin-dependent cell-cell adhesion, to leave the ventricular zone, and processes of cell motility and invasion, for migration through the already deposited layers of neurons.

At both the cellular and molecular levels, the processes of metastasis are strikingly similar to parallel processes in neuronal migration. In both systems, cell motility requires the development of cell polarity, protrusion of lamellipodia, translocation of the cell body, and retraction of the cell tail. In both systems, these processes involve reorganization of the actin and tubulin cytoskeletal elements, and spatially specific activation of the small GTPases Rho, Rac, and Cdc42 (4–9). Many proteins have been shown to be associated with increased cell motility in metastasis (5, 10, 11), and it is notable that many of these same proteins also control cell motility in other systems, including neuronal migration.

In this context, we considered that an upstream "central control" element for neuronal migration, cyclin-dependent kinase 5 (CDK5), might also be involved in the control of metastasis. CDK5 was originally identified for its homology to the CDK family of proteins, although it has no cell cycle function (12). Its kinase activity is strictly dependent on the association of CDK5 with its specific activator proteins, p35, and the somewhat less well-characterized p39 (13, 14); expression of either of these activators is indicative of CDK5 activity. Although CDK5 is expressed ubiquitously in cells, the restricted expression of p35 and p39 limit CDK5 activity mainly to postmitotic cells of neural origin (15, 16). Homozygous deletions of CDK5, p35, or p35/p39 have shown that CDK5 activity is necessary for neuronal migration (16–19). These mice display a defect in cortical development, resulting in an inversion of the layers of the cortex due to a failure of neuronal migration. The effects of CDK5 on neuronal migration are mediated through cytoskeletal changes and release of negative regulators of migration such as cellular adhesion molecules. Consistent with this role in neuronal cells, CDK5 has been implicated in changes in actin dynamics, microtubule stability, and neurite outgrowth through the phosphorylation of molecules including Pak1, Fak1, and Ezrin (20–25). It has also been implicated in the dissociation of β-catenin and N-cadherin, resulting in a loss in homotypic cellular adhesion (26).

In this present study, we show that CDK5 is active in prostate cancer. In prostate cancer cells, CDK5 seems to control cytoskeletal remodeling, cell polarity, and cell motility. In addition, the inhibition of CDK5 dramatically inhibited spontaneous metastasis in the Dunning prostate cancer model. Thus, CDK5 activity seems to control cell motility and metastasis in prostate cancer.

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

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Materials and Methods

Cell culture. LnCaP, PC3, and DU145 cell lines were obtained from American Type Culture Collection (Manassas, VA). The Dunning rat prostate cancer cell lines: AT6.3, AT3.1, MatLu, and MatLyLu cells were described previously (27, 28). The human prostate cancer cells, DU145 and PC3, were maintained in RPMI 1640 supplemented with 10% bovine calf serum (Hyclone, Logan, UT) and penicillin (50 IU/mL)/streptomycin (50 μg/mL; Life Technologies, Gaithersburg, MD). Medium for LnCaP cells was the same as above, except that fetal bovine serum was substituted for bovine calf serum. AT6.3, AT3.1, MatLu, and MatLyLu cells were maintained in the same media as the DU145 and PC3 cells, with the addition of 0.1 μg/mL of dexamethasone. All cells were seeded on laminin-coated plates (Sigma, St. Louis, MO) for all immunohistochemistry and cell motility experiments.

Western blotting. Cells were washed with PBS and harvested by scraping the cells with 1× SDS lysis buffer [2% SDS and 62.5 mmol/L Tris (pH 6.8)] containing protease inhibitor cocktail (P8340; Sigma) and briefly sonicated. Protein concentration was determined using bicinchoninic acid protein assay reagents (Pierce, Rockford, IL) and 20 to 50 μg of protein lysates were loaded per well. Lysates were electrophoresed on SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Blots were probed at 4°C overnight with primary antibodies in 5% milk/TBST. The antibodies for Western blotting were p35 (C19; Santa Cruz, Santa Cruz, CA), CDK5 (C8, Santa Cruz), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD).

Reverse transcription-PCR. Cells were grown to 90% confluence and RNA was harvested using Trizol (Invitrogen, Carlsbad, CA). The RNA was treated with RNase-free DNase to remove any contaminating genomic DNA. cDNA was synthesized using Superscript II reverse transcriptase and PCR for p35 was done. A control was also done in the absence of reverse transcriptase to further eliminate the possibility of contaminating genomic DNA. The forward primer for the human p35 was 5′ CCTGAAGAAGTCGCTGTCGTG 3′ and the reverse primer was 5′ TGTGAAGTAGTGTGGGTCGGC 3′. The forward primer for the rat p35 was 5′ GACCAGGGTTTCATCACACC 3′ and the reverse primer was 5′ AGCATCTTGGAGCTCATGAG 3′.

DNA constructs and stable cell lines. The dominant-negative *CDK5* construct was kindly provided by Dr. Li-Huei Tsai (Department of Pathology, Harvard Medical School, Boston, MA) and contains a D144N mutation (21). This CDK5dn construct was subcloned into a bidirectional Tet vector, pBI-EGFP, which also expresses enhanced green fluorescent protein (EGFP) under the control of a Tet-responsive element (BD Biosciences, Bedford, MA). The pBI-EGFP vector was modified by adding a zeocin resistance gene for selection (kindly provided by Dr. Kornel Schuebel, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD). Stable AT6.3 clones were made by transfecting the Tet-Off promoter construct, pTTa (BD Biosciences), into the cells and selecting with G418. Clones were selected for their ability to highly express a transgene in the absence of doxycycline whereas being tightly regulated in the presence of it. The pBI-EGFP *CDK5dn* and pBI-EGFP empty vectors were transfected into a stable Tet-Off clone, and clones with high level expression under tight control of doxycycline were selected.

Kinase assay. CDK5 kinase activity was measured using the SignaTECT protein kinase assay system (Promega, Madison, WI; ref. 29). The biotinylated peptide substrate was PKTPKKAKKL. Cells were lysed in a buffer containing 50 mmol/L of Tris-HCl (pH 7.5), 250 mmol/L of NaCl, 1 mmol/L of EDTA (pH 8.0), 10 mmol/L of NaF, and 1× protease inhibitor cocktail (Sigma). Cell lysates were then immunoprecipitated with either the p35 antibody (C-19, Santa Cruz), CDK5 antibody (C-8, Santa Cruz) or normal rabbit IgG as a control. The kinase assay was done according to the SignaTECT protocol. As a control for CDK5 specificity, the assay was also done in the presence of 1 μmol/L of the CDK inhibitor, roscovitine. Kinase assays were done at least twice.

***In vitro* invasion assay.** The invasion assay was done using BD Biocoat invasion chambers with growth factor reduced Matrigel in 24-well format (BD Biosciences). AT6.3 empty vector and AT6.3 *CDK5dn* cells were suspended in serum-free RPMI 1640 at a concentration of 1×10^5 cells/mL, and 0.5 mL of each was added to the invasion chambers in quadruplicate. RPMI 1640 (0.75 mL) supplemented with 10% fetal bovine serum was added to each well of the plate to act as a chemoattractant and the plates were placed in an incubator for 18 hours. Cells that invaded through the insert were stained with crystal violet and eight high-power fields were counted per insert.

Immunohistochemistry and immunofluorescence. 3,3′-Diaminobenzidine immunostaining was done using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) on tissue microarray samples. An epitope retrieval step with boiling in 9 mmol/L of sodium citrate (pH 6.0) was used for the p35 antibody (C19, Santa Cruz). For fluorescent staining of cells, cells seeded on laminin-coated plates were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were probed for mouse monoclonal α-tubulin (Molecular Probes, Eugene OR). The secondary antibody was antimouse Alexa 568 (Molecular Probes). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma) to stain the nuclei. Expression of the dominant-negative CDK5 or empty vector in the AT6.3 was monitored by the expression of GFP.

Small interfering RNA-mediated knockdown of CDK5. The SMARTpool small interfering RNAs (siRNA) targeted to human CDK5 (M-003239-01-0005) and the siCONTROL nontargeting siRNA #1 (D-001210-01-20) were synthesized by Dharmacon (Lafayette, CO). DU145 cells were seeded at 300,000 cells per well in a laminin-coated six-well plate 1 day prior to transfection. The cells were transfected using Dharmafect siRNA transfection reagent 1 (T-2005-01, Dharmacon), according to the manufacturer's protocol. Successful knockdown of CDK5 was shown by Western blotting for CDK5.

Motility experiments. Motility experiments were done on laminin-coated plates. *In vitro* wound healing assays were done on confluent DU145 cells, PC3 cells, and AT6.3 empty and *CDK5dn* clones. The media on the confluent cells was replaced with RPMI 1640 with 0.5% fetal bovine serum media and an area of cells was scraped off using a rubber-tipped cell scraper. Fluorescent or light microscopic images were taken at time 0 and at 48 hours for the AT6.3 cells, and at 24 hours for the DU145 and PC3 cells. Roscovitine was used at 10 μmol/L for the inhibition of CDK5. Control cells were treated with 0.2% DMSO as a vehicle control.

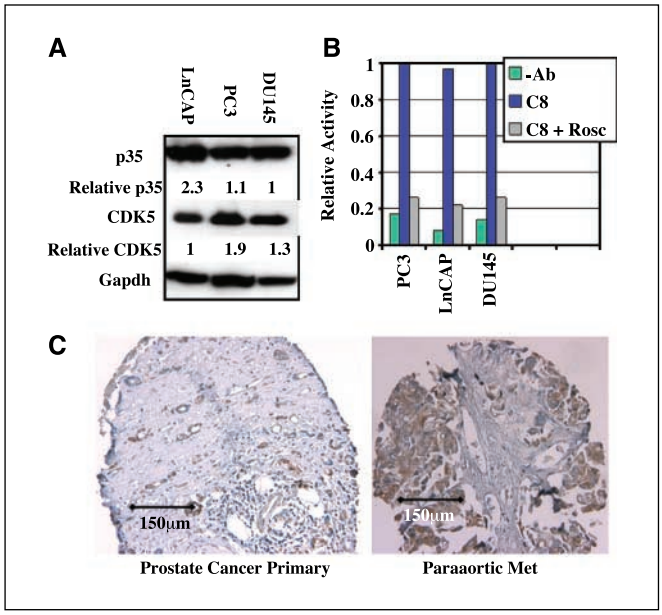

Figure 1. P35 expression and CDK5 activity in prostate cancer. *A,* Western blot of human prostate cancer cells for p35, CDK5, and GAPDH loading control. Relative levels of p35 and CDK5 were determined using the Typhoon 8600 Phosphorimager (Molecular Dynamics, Piscataway, NJ) and normalized for GAPDH loading. *B,* kinase assays of immunoprecipitated CDK5 from human prostate cancer cell lysates. All results were normalized to the DU145 cell line, designated as 1. *C,* immunohistochemical staining of p35 in representative samples of resected prostate cancer and a paraaortic prostate cancer metastasis. Phase photomicrographs were taken with a Nikon DXM camera.

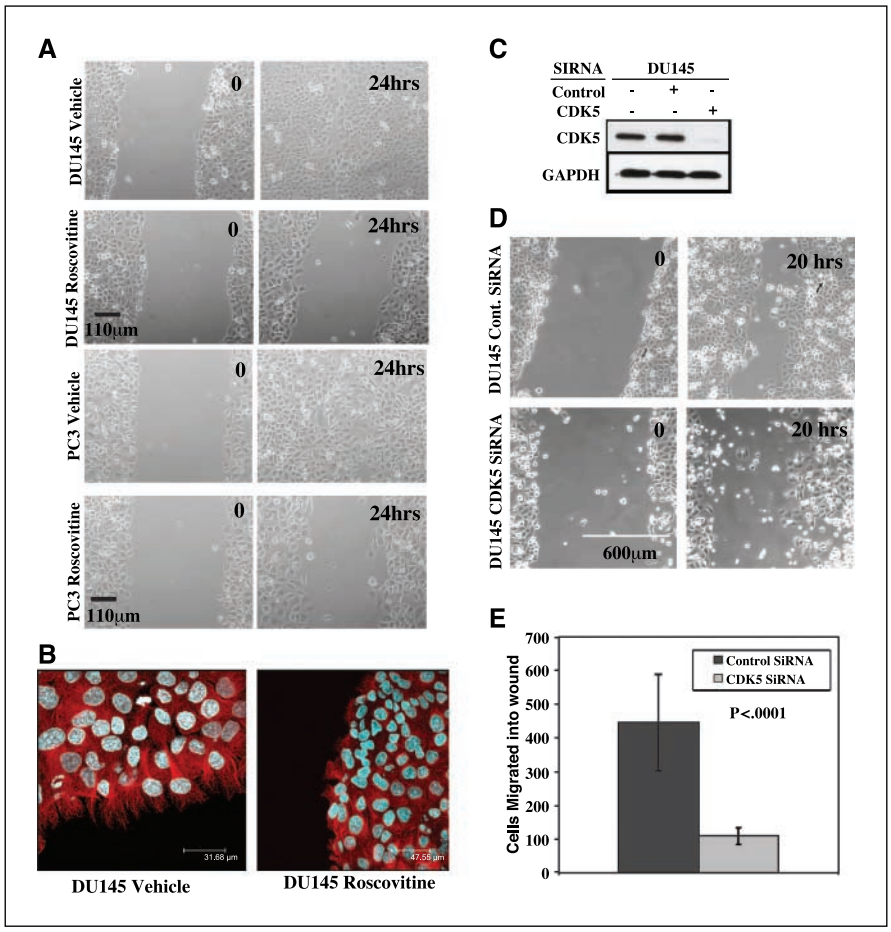

Figure 2. CDK5 activity is necessary for motility in human prostate cancer cells. *A, in vitro* wound assay of DU145 cells and PC3 cells at 0 and 24 hours after clearing the region on the plate with a rubber scraper. Phase photomicrographs were taken with a Nikon DXM camera. *Bottom,* cells were treated with 10 μmol/L of roscovitine to block CDK5 activity. Untreated cells were treated with 0.2% DMSO as a vehicle control. *B,* α-tubulin immunofluorescence of DU145 cells 1 hour after initiating wound on the plate in the presence or absence of 10 μmol/L of roscovitine. Untreated cells were treated with 0.2% DMSO as a vehicle control. DAPI was used to counterstain the nucleus. Images were taken using a Leica TCS confocal microscope. *C,* Western blot of CDK5 in DU145 cells treated with a control siRNA (Dharmacon) and the CDK5 SMARTpool siRNA (Dharmacon). Relative levels of CDK5 were determined using the Typhoon 8600 Phosphorimager (Molecular Dynamics) and corrected to GAPDH loading. *D, in vitro* wound assay of DU145 cells treated with siRNA. Images were taken with a Nikon DXM camera at 0 and 22 hours. *E,* quantitation of migration of siRNA-treated cells in the cleared region on the plate. The images were corrected for the wound size at the 0 time point and divided into grids. The cells which were in this region were then counted and reported as cells migrating into the wound.

For time-lapse experiments, induced cells were seeded sparsely on six-well plates. After 6 hours, time-lapse photomicroscopy was done at 10-minute intervals for 18 to 24 hours using Metamorph imaging software (Universal Imaging, Downingtown, PA). Phase and fluorescent images were taken at each time point.

***In vivo* spontaneous metastasis assay.** The *in vivo* spontaneous metastasis assay was done as described previously, using AT6.3 empty vector and AT6.3 *CDK5dn* clones (27, 28). Cells (5×10^5) were injected subcutaneously into the right flank of 10 NCR nude mice (Taconic, Germantown, NY) per clone. Xenografts were grown for 5 weeks with weekly measurements taken to assess growth rates *in vivo*. Lungs were harvested, inflated with 4% paraformaldehyde, fixed in Bouin's solution, and external lung metastases were counted. Results were reported as metastases per gram of primary tumor (30, 31).

Statistical analysis. Data were expressed as mean ± SD, with significance determined by Student's two tailed *t* test.

Results

CDK5 and its activator, p35, are expressed in prostate cancer samples. The functions shown for CDK5 in neuronal cell motility and adhesion are strikingly similar to processes shown to be involved in metastasis. This led us to hypothesize a role for CDK5 in the metastatic potential of prostate cancer. A previous report has shown that the prostate cancer cell lines, DU145 and LnCaP, express the CDK5 activator, p35, as well as its cleavage product, p25 (32). We confirmed that CDK5 is expressed in a panel of prostate cancer cell lines: LnCaP, DU145, and PC3 (Fig. 1*A*). In addition, these cell lines express p35, the activator of CDK5, as shown by Western blot (Fig. 1*A*). Kinase assays done on the prostate cancer cell lines showed CDK5 activity, which was inhibited by the CDK5 inhibitor, roscovitine (Fig. 1*B*).

P35 is also expressed in primary human prostate cancers. We evaluated p35 expression in a metastatic prostate cancer tissue microarray, representing 32 individual patient prostate cancer samples and numerous correlated metastases for each sample. Twenty-eight of the 32 individual prostate cancers (87.5%) and their correlated metastases were positive by immunohistochemistry for p35. Figure 1*C* shows representative slides of a primary prostate cancer and a prostate cancer metastasis. Our results show that p35 is expressed not only in advanced prostate cancer, but also in 10 out of 17 normal prostate samples and one out of two prostatic intraepithelial neoplasia lesions, although relative differences of p35 expression cannot be completely ruled out.

Roscovitine, an inhibitor of CDK5, and CDK5 siRNA block the motility of prostate cancer cells in a wound healing assay. The expression and activity of CDK5 in prostate cancer suggested that CDK5 might function in a manner similar to its role in migration in neural development. A wound healing assay for cell migration was done on the human prostate cancer cell lines, DU145 and PC3, to determine whether the motility of the cells was dependent on CDK5 activity. Both cell lines were capable of migrating into and repopulating the cleared region of the laminin-coated plates in 24 hours (Fig. 2*A*). When the cells were treated with the CDK5 inhibitor, roscovitine, the cells could no longer migrate into the cleared region (Fig. 2*A*). DU145 and PC3 cells treated with

roscovitine migrated into the wound at a rate of 9% and 18.5% of vehicle treated cells, respectively. This suggested a critical role for CDK5 in the motility of these human prostate cancer cell lines.

CDK5 activity has been shown to affect actin and tubulin polymerization in remodeling of the cellular cytoskeleton (20–25). After 1 hour, immunofluorescent staining of α-tubulin in DU145 cells in a wound healing assay showed that the cells reoriented themselves toward the cleared region (Fig. 2*B*). They projected lamellipodia extending into the open area on the plate to allow for motility into this area (Fig. 2*B*). The cells behind the first row of cells also projected their lamellopodia in between the cells in the first row to the wound. In the roscovitine-treated cells, the cells no longer reoriented themselves toward the clearing and did not project the prominent lamellopodia that the untreated cells did (Fig. 2*B*). The random orientation of the roscovitine-treated cells at the edge of the wound, as well as reduced prominence of the tubulin structures, suggests an inability of the cells to establish polarity for efficient motility in the absence of CDK5 activity (Fig. 2*B*).

SiRNA knockdown of CDK5 expression was also effective in blocking cell motility in DU145 cells. CDK5 siRNA was effective at reducing the expression of CDK5 protein to <5% of levels in untreated cells whereas the control siRNA had no effect (Fig. 2*C*). In a wound assay, the cells treated with CDK5 siRNA exhibited significantly impaired repopulation of the wound at 20 hours (Fig. 2*D*); the CDK5 knocked down cells had ~20% of the number of cells in the wound as the control siRNA cells (Fig. 2*E*). This difference was significant as determined by Student's *t* test ($P < 0.0001$). These results with roscovitine and siRNA validate the importance of CDK5 for motility in these cells.

Expression of a dominant-negative CDK5 construct in AT6.3 cells. The importance of CDK5 in the motility of the human prostate cells suggested a possible role for CDK5 in prostate cancer metastasis. To examine this role, it was necessary to use a prostate cancer model that was capable of spontaneous metastasis *in vivo*. The Dunning rat prostate cell lines are capable of efficiently metastasizing to the lungs from a s.c. xenograft in a nude mouse model (27, 28, 33). This *in vivo* model has been used for the identification of several metastasis suppressor genes in prostate cancer, including KAI1, CD44, and MKK4 (33–35). We showed that the Dunning rat prostate cancer cell lines express p35 by reverse transcription-PCR (RT-PCR) and Western blot, whereas CDK5 expression was shown by Western blotting (Fig. 3*A*). We also showed that the Dunning cell lines have CDK5 kinase activity, and that this activity could be inhibited by roscovitine (Fig. 3*B*). We stably expressed a dominant-negative *CDK5* construct (D144N; *CDK5dn*) to block CDK5 activity in AT6.3 cells (21). The dominant-negative expression construct was subcloned into a bidirectional vector expressing GFP in a Tet-Off system, and the cells were characterized for effects on cell motility, polarity, and invasion. Quantification of total CDK5, endogenous and dominant-negative, was done by Western blot for each of the dominant-negative *CDK5* AT6.3 clones and empty vector clones. There was at least a 10-fold increase in the clones expressing the dominant-negative *CDK5* construct as compared with empty vector–expressing cells, indicating efficient expression of the dominant-negative

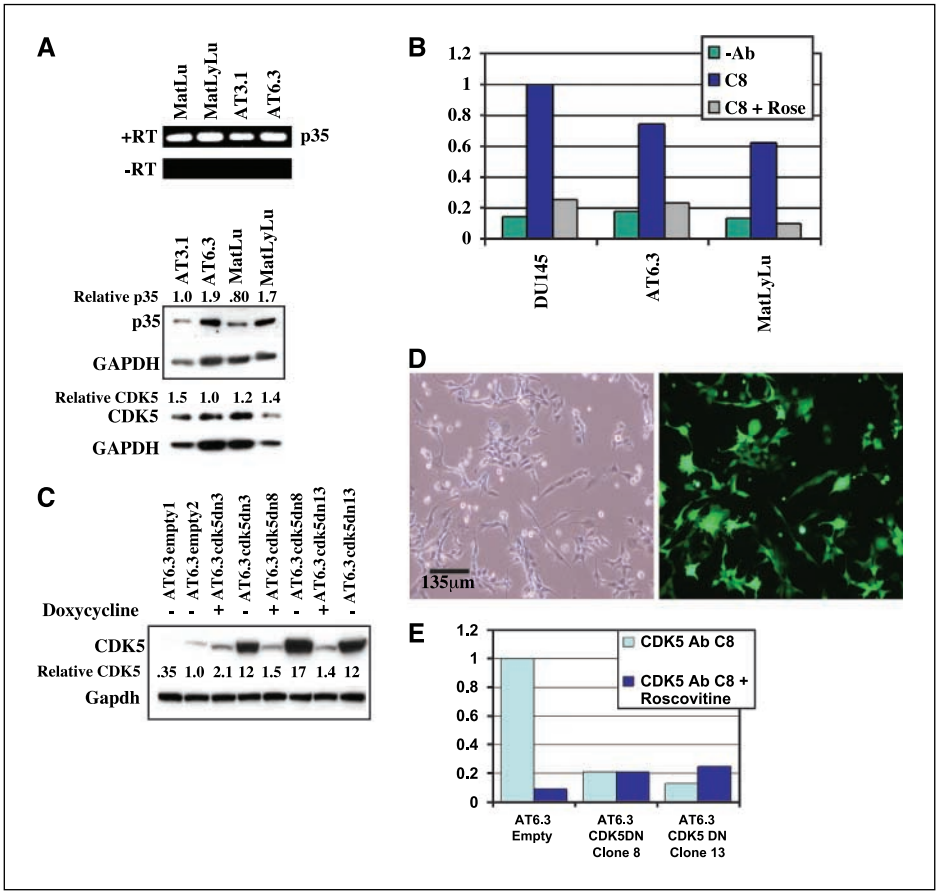

Figure 3. Blocking CDK5 activity in Dunning prostate cancer cell lines with expression of a dominant-negative CDK5. *A,* p35 expression in Dunning prostate cancer cell lines as determined by RT-PCR and Western blot. Controls for RT-PCR were done by using samples without added reverse transcriptase to control for genomic DNA. GAPDH was used as a loading control for Western blotting. Relative levels were calculated as described in Fig. 2. *B,* kinase assays of immunoprecipitated CDK5 from Dunning prostate cancer cell lysates. All results were normalized to the DU145 cell line, designated as 1. *C,* CDK5 Western blot of stable AT6.3 clones expressing empty vector or three different *CDK5* dominant-negative clones. Expression of CDK5 was induced by the removal of doxycycline for 48 hours. *D,* phase and GFP fluorescent photomicrographs of stable AT6.3 cells expressing bidirectional pBI-EGFP with *CDK5dn*. Almost 100% of the cells express the transgene. *E,* kinase assay of the AT6.3 cells expressing the empty vector and the CDK5 dominant-negative clones.

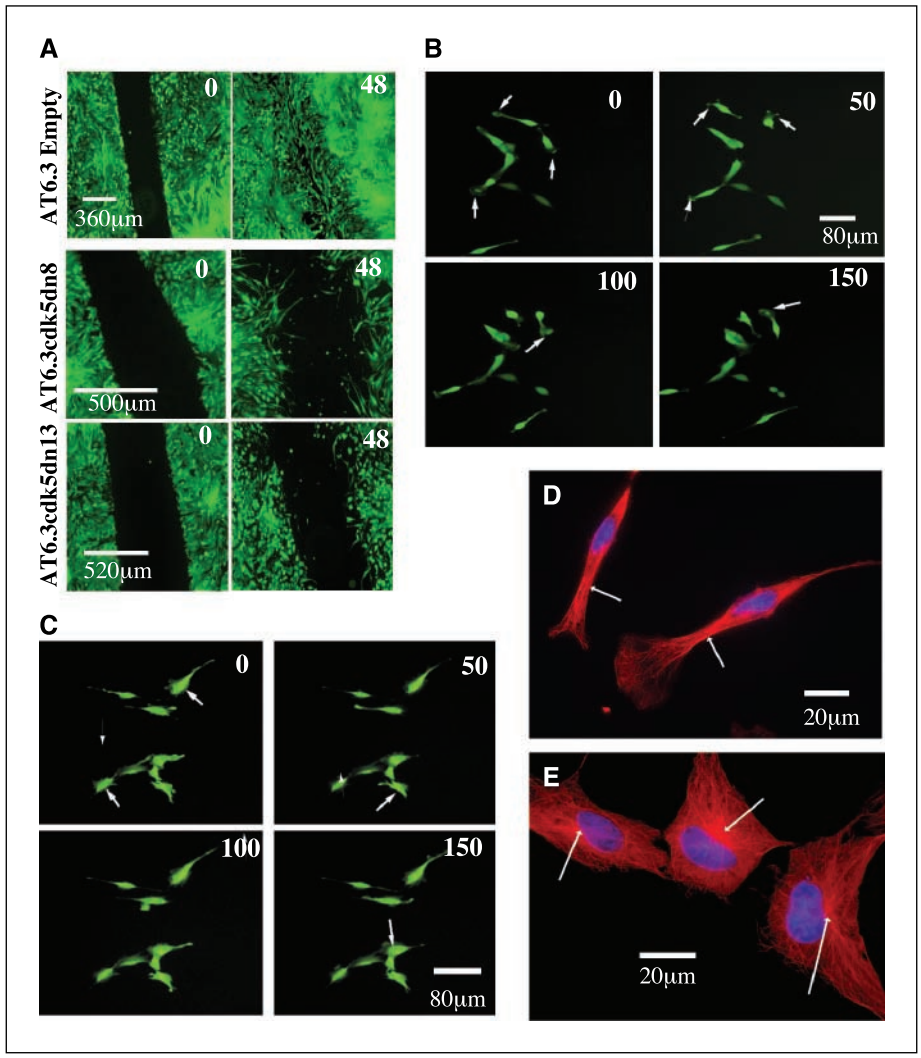

Figure 4. CDK5 activity is necessary for the motility and polarity of AT6.3 cells. *A,* wound assay on AT6.3 empty clone 1, AT6.3 *CDK5dn* clone 8, and AT6.3 *CDK5dn* clone 13 cells. Assays were done in low-serum medium on laminin-coated plates. Photomicrographs were taken at 0 and 48 hours after clearing cells. *B,* time-lapse photomicrographs of AT6.3 empty cells clone 2. *Arrows,* the leading edge of individual cells. Although movement is random due to a lack of chemoattractant, the presence of polarity is evident, marked by creation of a leading edge and movement of the cells. This is further illustrated in Supplemental Movie 1. *C,* time-lapse photomicrographs of AT6.3 *CDK5dn* c8 cells. *Arrows,* individual cells, showing a flatter morphology with an apparent increase in the number of lamellipodia. Although the cells are elongated, there does not seem any leading edge in these cells. This is further illustrated in Supplemental Movie 2. Time-lapse photomicrography was done using Metamorph software. GFP fluorescence was used to assure visualization of only cells expressing the vector in both assays. *D,* immunofluorescent staining of α-tubulin in AT6.3 empty vector stable clone. DAPI was used to counterstain the nuclei in these cells. There is an obvious presence of polarity in these cells. *Arrows,* MTOCs, which are located between the nucleus and the leading edge. *E,* immunofluorescent staining of α-tubulin in AT6.3 *CDK5dn* clone 8 cells. DAPI was used as a counterstain. Cells have lost polarity and have a more disorganized tubulin. *Arrows,* the MTOC in these cells, which is shifted back toward the nucleus, and unlike the empty vector AT6.3 cells, there is no obvious leading edge, although lamellipodia remain.

construct (Fig. 3*C*). Monitoring of GFP expression in the minus doxycycline-induced cells showed expression in 100% of the cells in the stable clones (Fig. 3*D*). We also showed that the AT6.3 cells expressing the dominant-negative CDK5 also had no CDK5 kinase activity when the Tet-responsive CDK5 dominant-negative clones were induced. There was no obvious effect on the growth or survival of these cells when CDK5 activity was blocked by the expression of the dominant-negative constructs (data not shown).

Blocking of CDK5 activity with a dominant-negative construct in AT6.3 cells results in a loss of motility of the cells through effects on the cytoskeleton. Expression of CDK5dn in AT6.3 cells resulted in a loss of motility in an *in vitro* wound assay on laminin-coated plates. Cells in the two empty vector clones migrated efficiently into the scraped region, and in 48 hours, had repopulated the cleared region of the plate (Fig. 4*A*). In contrast, in the AT6.3 *CDK5dn* clones, the cells no longer migrated into the cleared region, and even after 48 hours, the cleared region still remained mostly unpopulated (Fig. 4*A*).

The results of the *in vitro* wound assay showed that CDK5 activity is important for the ability of AT6.3 cells to migrate from a confluent region to an opening on the plate. However, the assay did not allow discrimination between effects on cell motility versus homotypic adhesion. To further implicate CDK5 in cell motility, we used time-lapse photography to follow thinly seeded individual cells. Fluorescence was used to show expression of the vector containing the dominant-negative construct with GFP or the empty vector with GFP alone. This was done on laminin-coated plates in normal media with no chemoattractant, so that there was no directed movement toward a gradient. AT6.3 cells with the empty vector expressing GFP moved efficiently, with obvious remodeling of the cytoskeleton necessary for movement (Fig. 4*B*). These cells showed obvious polarity, with a distinct cellular front and back. These cells were completely capable of random movement around the plate (Supplemental Movie 1). In contrast, AT6.3 *CDK5dn* cells showed an almost complete loss in their ability to migrate around the plate (Fig. 4*C*; Supplemental Movie 2). Even at 16 hours, there was only minimal movement in the cells, as shown in Supplemental Movie 2, suggesting greatly impaired cell motility. The cells lacked the ability to establish polarity and seemed to randomly reorganize their cytoskeleton. They also seemed to adhere tightly to the laminin substratum, possibly contributing further to their lack of motility (36). These results suggest that CDK5 activity is necessary for the motility of these metastatic prostate cells on extracellular matrix, similar to the effects we had observed in roscovitine-treated DU145 human prostate cancer cells.

Expression of the dominant-negative *CDK5* construct in AT6.3 cells resulted in extensive changes in the cytoskeleton, especially evident in microtubules. Immunohistochemical staining for α-tubulin showed that when AT6.3 clones with empty vector (AT6.3 empty) were seeded on laminin-coated plates, tubulin was organized in a front-to-back fashion with evident cell polarity typical for migrating cells (Fig. 4*D*; ref. 9). There also was an apparent microtubule-organizing center (MTOC) present in front of the nucleus toward the leading edge, as is often seen in motile cells (9, 37). When AT6.3 clones expressing the dominant-negative *CDK5* (AT6.3 *CDK5dn*) were seeded on laminin-coated plates, the cells seemed to be flatter and larger, with few lamellipodia, and a loss of cell polarity (Fig. 4*E*). The MTOC was also abnormally localized near the nucleus in these cells, with a loss of microtubule organization, further suggesting a loss of polarity (9). These results illustrate the importance of CDK5 activity in the microtubule organization necessary for motility of the AT6.3 prostate cancer cells, similar to that which was seen for the human prostate cancer cells.

CDK5 activity is necessary for invasion and metastasis in AT6.3 cells. The decreased cell motility in AT6.3 *CDK5dn* cells correlated with dramatically lower invasive potential. We assayed the invasive potential of these cells using Matrigel-coated invasion chambers. The AT6.3 *CDK5* dominant-negative clones only invaded at ~5% the efficiency of the AT6.3 empty vector cells (Fig. 5*A*). Each high-power field in the empty vector had an average of 86 cells invaded, whereas the two dominant-negative clones had an average of four and five cells invaded per high-power field. Figure 5*B* shows an example of invasion of the empty vector AT6.3 cells and the CDK5dn AT6.3 cells.

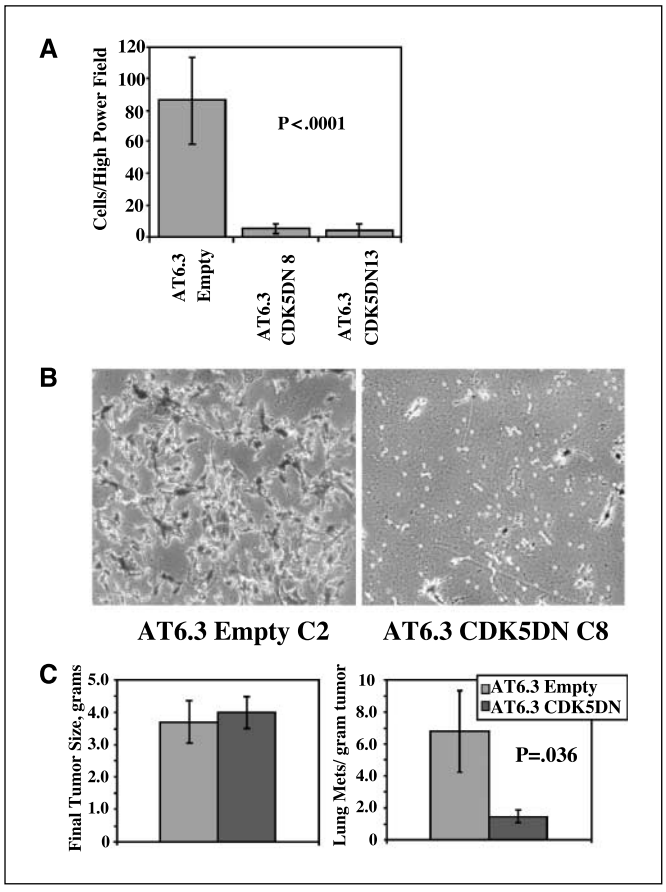

Figure 5. CDK5 activity is important for invasion and metastasis of AT6.3 cells. *A,* matrigel invasion assay was done on AT6.3 empty vector versus 2 AT6.3 *CDK5dn* clones. Assay was done using four wells each. All assays were stopped at 18 hours and eight high-power fields were counted per well. Levels of invasion of the cells were designated as cells invaded per high-power field. AT6.3 empty vector versus either dominant-negative clones ($P < 0.0001$). *B,* images of representative fields in the invasion assay for the empty vector AT6.3 cells and the CDK5DN expressing AT6.3 cells. Cells were stained with crystal violet. *C,* a spontaneous *in vivo* metastasis assay was done with AT6.3 empty and *CDK5dn* clones injected s.c. in nude mice. At 5 weeks, the mice were sacrificed and lung surface metastases were counted. Average size of primary tumor and number of surface pulmonary metastases per gram of primary tumor.

Because our data implicated CDK5 in motility and invasion in prostate cancer cells, it seemed likely that blocking CDK5 activity would interfere with metastasis *in vivo*. To examine the role of CDK5 activity in metastasis, we did *in vivo* spontaneous metastasis experiments using these stable AT6.3 cell lines. Subcutaneous xenografts were allowed to grow for 5 weeks, at which time mice were sacrificed and lung surface metastases were counted. Although the average size of the primary xenograft tumors for the AT6.3 empty vector cells and the AT6.3 *CDK5dn* cells were approximately equivalent (Fig. 5*C*), blocking of CDK5 activity resulted in a 79% reduction in the number of lung metastases per milligram of primary xenograft tumor (Fig. 5*C*). These results show that CDK5 activity is integral to the metastatic capacity of AT6.3 cells.

Discussion

Metastasis bears a substantial resemblance to the normal process of neural migration in development of the cerebral cortex, in which the postmitotic neurons invade preexisting layers of neurons to migrate to their appropriate positions in the cerebral cortex (4). The control of cell motility, polarity, adhesion, and invasion are necessary components of these processes. In both neuronal development and prostate cancer, CDK5 activity is necessary for these cellular functions to occur. CDK5 activity may have a role in metastasis in other cancers as well, because we have found that p35 is expressed in several other cancer types, including lung, pancreatic, thyroid, and melanoma.[5] Our data do not necessarily suggest that CDK5 is aberrantly active in metastatic cancer; rather, because we observed p35 expression in some apparently normal prostate epithelia, we suggest that CDK5 activity may serve a normal function in these cells. Therefore, whereas CDK5 activity is necessary for metastasis in prostate cancer, it may not be sufficient by itself to cause metastasis.

One may speculate that the pathways controlled by CDK5 in prostate cancer may be similar to those in neuronal migration, and may define mechanisms generally involved in metastasis. Numerous substrate proteins for CDK5 have been identified in neurons, and a remarkable fraction of these CDK5 targets are effectors of cytoskeletal structure, motility, and adhesion. These CDK5 targets include Pak1 (actin polymerization), doublecortin and NUDEL (microtubule remodeling), src, FAK, PIPKI (integrin activation and cytoskeletal coupling), β-catenin and presenilin (cellular adhesion), and Dab1 (cell positioning; refs. 20, 24, 38–44). Many of these proteins have already been implicated in the control of motility and invasion in nonneuronal cells, including cancer cells (5, 10, 11).

Metastasis is a multistage process. These stages have been defined as local invasion, intravasation into the circulation, survival and transport in the circulation, extravasation from the

[5] C.J. Strock, et al., in preparation.

bloodstream, and growth in the metastatic site (1–3, 45–47). Progression through these stages requires changes in cellular phenotype, such as cellular motility, antiapoptotic capability, adhesion molecule expression, expression of matrix metalloproteinases and other proteases, and expression of angiogenic factors and other paracrine or autocrine factors. Several authors have emphasized that if even one requisite step of the multistep metastatic process could be blocked, it would result in the abrogation of clinically relevant metastasis (1–3, 45). This is an important point that has led to intensive and promising preclinical and clinical efforts, especially for interventions targeting angiogenesis or protease expression (48, 49). In this context, we emphasize that we do not envision that CDK5 activity alone is sufficient to activate all steps of metastasis; we suggest that in a subset of cancers, CDK5 may control some of the necessary functions in metastasis, including motility. Therefore, blocking CDK5 activity in these cancers may provide a therapeutic window to inhibit metastasis.

In summary, we have identified CDK5 activity as an integral component of motility and metastatic potential in prostate cancer cells. In these cells, CDK5 seems to control cytoskeletal remodeling, cell polarity, and cell motility, and inhibition of CDK5 dramatically inhibited spontaneous metastasis. Because metastasis is one of the most clinically relevant events in prostate cancer progression, CDK5 may be a potentially important therapeutic target to limit metastasis in prostate cancer.

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