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Calpain-2 as a Target for Limiting Prostate Cancer Invasion[1]

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ABSTRACT

Mortality and morbidity of prostate cancer result from extracapsular invasion and metastasis. This tumor progression depends on active cell motility. Previous studies have shown that calpain-regulated rear detachment enabling forward locomotion is required for cell migration initiated by growth factor and adhesion receptors. Therefore, we asked whether calpain would be a target for limiting tumor progression, using as our model the PA DU-145 human prostate carcinoma cell line and a highly invasive subline, wild-type DU-145, derived from it. *In vitro*, the calpain-specific inhibitor CI-I (ALLN) and the preferential-but-less-specific inhibitor leupeptin decreased transmigration of both cell lines across a Matrigel barrier. These calpain inhibitors limited epidermal growth factor-induced motility but did not alter the growth rate of the tumor cells, as expected. Antisense down-regulation of the growth factor-activated calpain-2 (m-calpain) isoform also reduced transmigration and cell motility. These *in vitro* findings were then buttressed by *in vivo* studies, in which i.p. DU-145 tumor xenografts were treated with leupeptin. Tumor invasion into the diaphragm was reduced by leupeptin treatment for both the PA and wild-type DU-145 cells (from 1.7 to 0.78 for the parental line and 2.3 to 1.2 for the invasive derivative, respectively). Tumor cells of both types engineered to express calpain-2 antisense constructs also demonstrated a similar 50% reduced invasiveness *in vivo*. Finally, we found by gene expression survey of 53 human prostate tumors and 23 normal prostates that calpain was not up-regulated in relationship to invasiveness or metastatic activity, consistent with expectation from the biological role of this effector. Taken together, these results strongly suggest that epigenetic activation of calpain plays an important role in the invasion of human prostate cancer and that it can be targeted to reduce tumor progression.

INTRODUCTION

Prostate cancer is among the most frequent tumors in men (1), with the vast majority of morbidity and mortality resulting from tumor spread beyond the prostate (2, 3). Thus, work has focused on molecular changes that invasive and metastic tumors acquire to enable them to breach the barrier matrices and extend beyond the prostate capsule. Whereas there are a number of cell properties and their controlling signaling pathways, we have focused on cell migration as a critical rate-limiting step in tumor invasion (4–7). Extravasating and metastatic cells have been observed as displaying active motility during these actions (8–10). Therefore, inhibition of tumor cell motility should provide a novel therapeutic approach.

Cell motility is a highly orchestrated process that requires cell protrusion of leading lamellipodia with subsequent new adhesions, contraction through the cell body, and release from the substratum at the trailing edge (11). Each of these biophysical processes is controlled coordinately by biochemical signaling cascades (12). Such cascades can be initiated by adhesion receptors, notably integrins (13), or by growth factor receptors, although the specific elements in signaling chains may vary dependent on the initiating signal (12). The rear detachment step appears to be regulated by convergent signaling from growth factors and integrin (14, 15). Calpains are required for deadhesion of the tail during both haptokinesis (16) and chemokinesis (17, 18), at least on moderately to highly adhesive surfaces (19). However, it appears that integrins activate the calpain-1 (μ-calpain) isoform, whereas growth factor receptors trigger calpain-2 (m-calpain). As these two ubiquitously coexpressed proteins are highly homologous and appear to cleave the same targets, this convergence is likely because of differential regulation of the calpain isoforms (14, 20). Inhibition of calpain does block the motility of fibroblasts and myofibroblasts (16, 17), as well as keratinocytes (21). In the one study to date examining calpain-dependency of motility in carcinoma cells, inhibition of calpain in bladder carcinoma cells limited both motility and transmigration of a Matrigel barrier *in vitro* (22). The effects of inhibiting calpain were similar to when other motility-related signals are blocked, such as peritoneal lymphocyte γ-mediated cytoskeleton reorganization (22–24). Thus, there is promise that calpain may be a target for limited tumor invasiveness. However, this has yet to be determined in animal models.

Calpains are a family of >12 known mammalian intracellular limited proteases that share a similar catalytic structure (25). The two ubiquitous isoforms, calpain-1 and -2, are the best characterized and defined by their calcium requirements for *in vitro* activation. Whereas the biochemistry and structural biology of the ubiquitous calpains is highly advanced (25–28), the cell biology of these enzymes is lagging because of questions of mode of activation *in vivo* (14, 15). Calpains contribute not only to cell motility, as noted above, but also are likely involved in cell proliferation and apoptosis (15, 20, 29). Still less is known about the role of calpains in carcinogenesis and tumor progression. There is a report in a subset of 21 clear cell renal carcinomas of calpain-1, being up-regulated at the mRNA level in metastatic tumors compared with node-negative tumors (30). The gastric-specific calpain-9 is down-regulated in carcinomas from that tissue, although whether it is related to differentiation status or tumorigenesis is still open to question (31, 32). On the other hand, the decrease of muscle-specific calpain-3, and reciprocal increase in calpain-2 and ubiquitin-dependent proteolysis in muscles during cancer cachexia is almost assuredly a secondary organismal effect unrelated to tumor growth and progression (33). However, because calpain is regulated in an epigenetic manner and detection of changes in calpains are not expected, either calpain activity has to be determined directly or challenged in experimental systems to substantiated potential roles in tumor biology.

To investigate the role of calpain in prostate cancer invasion, we used the androgen-independent cell line DU 145 (PA; Ref. 34) and its derivative, WT,[3] which overexpresses the full length of EGFR and which has been shown to be more invasive (35, 36). Because the signature of activated calpain within cells is not known, we could not survey *de novo* tumors for activation status. Rather, we used an interventional strategy to establish proof of concept that calpains contribute to tumor invasion. Both ubiquitous calpains were inhibited

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[3] The abbreviations used are: WT, wild-type; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; CI, calpain inhibitor; AS, antisense; MAP, microtubule-associated protein; V, vector; ECM, extracellular matrix.

pharmacologically by the calpain-specific inhibitor CI-I (ALLN) or the calpain-preferential but broad-spectrum cysteine-serine protease inhibitor, leupeptin. This latter agent was chosen because it has been used in mice and even, on the basis of compassionate release, in humans with little toxicity evident (37, 38). To confirm calpain targeting and identify the key isoform, AS down-regulation of calpain-2 was performed in these cells. Our findings indicate that calpain may represent a key molecular switch that regulates a rate-limiting step in tumor invasion.

MATERIALS AND METHODS

Cell Lines and Reagents. Human DU 145 prostate carcinoma cell line and its derivative WT DU145 (35, 36) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum supplemented with L-glutamine (2 mM), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and antibiotics; 350 mg/ml of G418 was added to the medium for the WT cells. Medium was purchased from Life Technologies, Inc. (Gaithersburg, MD). The parental DU145 cells are referred to as PA DU145, whereas those cells overexpressing full length, WT EGFR are referred to as WT DU145. Human recombinant EGF was purchased from BD Biosciences, CI-I (ALLN) from Biomol (Plymouth Meeting, PA), and leupetin and all of the other reagents were purchased from Sigma (St. Louis, MO).

Plasmids and DNA Constructs. To generate a minigene complementary to human calpain-2, we chose a sequence that spanned the translation initiating ATG, as AS to this sequence was productive (17). Human cDNA coding for 80 pb (C2AS) minigene was generated by RT-PCR using the following primers: 5′ oligo sequence 5′ACCGCAGCATGGCGGGCA; and 3′ reverse oligo sequence 5′TGGCCCTCTCGTGGGAGC. The cDNA was cloned into pBluescript II KS vector, digested with *Xho*I and *Bam*HI, and inserted into the *Xho*I and *Bam*HI sites of the mammalian pCEP4 expression vector. cDNA was sequenced to verify correct orientation and sequence. Expression was obtained by electroporation into DU145 cells. Stable transfectant cells were selected by supplementing the medium with 100 μg/ml hygmomycin. These cells are referred to as C2AS WT or PA DU145, whereas the vector only controls are named V WT or PA DU145.

AS Oligonucleotides. Phosphorothioate AS oligodeoxynucleotides were synthesized by DNA synthesis facility (University of Pittsburgh). The sequences of calpain-2 AS have been described previously, 5′CGCGATGCCCGCCCGCCATGCT (39). A scrambled (SCR: 5′TCGTACCGCCCGCCCGTAGCGC) phosphothiorated oligonucleotide was used as a control. These sequences and their complementary sequences presented no similarity with other target mRNA, as best we could determine using the BLASTN program.

Quiescent cells were transfected using the superfectin reagent according to the manufactured protocol. Briefly, cells plated in 12-well plates were incubated with 20 μM of oligonucleotide with 7.5 μl of superfect in a final volume 500 μl for 3 h, then washed twice with PBS and incubated with or without 1 nM EGF for 24 h. For invasion assay, cells were counted and transferred into the transwell chambers. Otherwise, cells were kept in the same plate and used for MAP2 assay or wounded (0 h) for the migration assay.

Migration Assay. An *in vitro* "wound healing" assay was used to assess cell motility in two dimensions (40). Cells (10^5) were plated on a six-well plate and grown to confluence in their regular medium. To minimize the autocrine signaling, confluent cells were kept in 1% dialyzed FBS, then wounded using a rubber policeman (0 h). Cells were washed twice with PBS and treated with or without specific effectors for 24 h. Photographs were taken at 0 and 24 h, and the distance traveled was determined by subtracting the values obtained at 0 from 24 h. Mitomycin C (0.5 μg/ml) was used to limit proliferation (41).

Calpain Activity Assays. Calpain activity was detected in living cells or in the whole cell lysates using BOC or MAP2 assays, respectively, as described previously (17). Briefly, for BOC, cells were plated on glass coverslips at between 50 and 70% confluence in their regular media. Quiescent cells were treated with or without 1 nM EGF, CI-I, or leupetin for 24 h. t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (0.5 μM; Molecular Probes, Eugene, OR) is added to the cells for 20 min followed by 1 nM EGF for 10 min. The activity of calpain was detected by the increase of fluorescence noted on the cleavage of the substrate BOC using an Olympus fluorescent microscope (model BX40 with an Olympus M-NUA filter), and representative images were captured using a spot CDD camera. The exposure and time settings were fixed within each experimental series.

To determine calpain activity in cell lysates, MAP2 (Cytoskeleton, Denver, CO) was labeled with DTAF by incubation of MAP2 and dichlorotriazinylaminofluorescein in (pH 8.5) PIPES buffer for 30 min at 4°C. Labeled MAP2 was then isolated by size exclusion column chromatography and dialyzed against (pH 7.5) HEPES buffer overnight. Cells were grown to confluence in six-well plates, quiesced for 24 h, and treated or not with 1 nM EGF. Cells were washed twice with ice-cold PBS and lysed with cell lysis buffer [20 nM HEPES (pH 7.4), 10% glycerol, 0.1% Triton X-100, 500 mM sodium chloride, and 1 mM sodium vanadate]. After removing the cell debris by centrifugation, 0.9 μg of DTAF-labeled MAP2 was added to the samples with 20 μM free Ca^{2+} concentration. Fluorescence was immediately measured by an Aminco-Bowman Series II spectrofluorimeter (Spectronic Instruments Inc., Rochester, NY) at excitation and emission wavelength of 470 and 520 nm, respectively, for 3 min at room temperature

To detect the total potential calpain activity in a cell, we used casein zymography. Twenty μg of cell lysate were resolved under nonreducing conditions by PAGE in HEPES-imidazole buffer with 5 mM EDTA that separates calpain-1 and -2 isoforms. After washing, gels were incubated for 20 h in a calpain activation buffer (20 mM 4-morpholinepropanesulfonic acid 2 plus 5 mM beta-mercaptoethanol) containing 5 mM $CaCl_2$ or in 4-morpholinepropanesulfonic acid buffer without $CaCl_2$ and with EDTA as a control. The gels were stained for protein content with transparent bands identified by comparison to calpain standards. The density of the bands was measured using NIH image.

Immunoblotting. Protein expression was determined as described previously (17). Briefly, cells were washed in PBS and lysed in SDS lysis buffer before analysis by reducing SDS-PAGE. Primary antibodies included anticalpain-2 (clone N-19 and C-19; Santa Cruz Biologics, Santa Cruz, CA), anticalpain-1 (Biomol), and antiactin (Sigma). Bands were visualized using alkaline-phosphatase-coupled secondary antibody (Promega, Madison, WI).

Cell Proliferation Assay. Mitogenic stimulus was determined by direct cell counting. Cells were plated in 24-well plates and cultured for 24 or 48 h in their regular medium, with or without leupeptin or CI-I. The number of cells was determined using a Coulter Counter model Z2 (Miami, FL).

Invasion Assays. Invasive potential was determined *in vitro* by transmigration of an ECM (5). Matrigel invasion chamber plates were obtained from Becton Dickinson/Biocoat (Bedford, MA). The upper surface of the matrix was challenged with 20,000 cells, a number derived from empirical experimentation (22, 23, 35). Cells were kept in serum-free medium containing 1% BSA for the first 24 h and then replaced with only serum-free medium for the remaining 24 h; the lower chamber contained medium containing 10% serum for the entire assay. Enumeration of the cells that invaded through the matrix over a 48-h period was accomplished by visually counting cells on the bottom of the filter, as per routine procedures, after any uninvaded cells were removed from the top of the filter with a cotton swab. In all of the cases, individual experiments were performed in duplicate chambers.

True invasiveness of the cells was determined *in vivo* using the diaphragm invasion model (5, 24, 36). For the first experimental series, 14 male 6-week-old Balb/*c nu/nu* athymic mice (day 0) were inoculated i.p. with 2×10^6 PA or WT DU145 cells and randomly separated into two groups at day 10. After 10 days, the mice received daily i.p. injections of 12 mg/kg of leupeptin or diluent only for 30 days. In the second experimental series, mice were inoculated with either PA or WT DU145 expressing C2AS minigene or V alone to assess AS down-regulation of calpain-2 on tumor invasion after 60 days. In all of the cases, invasion was determined as follows. Mice were sacrificed, and the diaphragm and any tumors were removed, fixed in 10% paraformaldehyde, and stained with H&E. Invasiveness was scored semiquantitatively on a four point scale measuring the greatest extent of invasion into the diaphragm muscle, with 0 being no invasion and 4 being complete transmigration of the diaphragm. Mice without evident diaphragmatic tumors were not included in the invasion scoring. Each experiment was repeated and the data collated for the two experiments. The number of mice challenged was determined *a priori* for a 95% confidence level of determining a difference ($P < 0.05$) using the assumptions of 80% diaphragmatic tumors with a 30% difference in invasiveness between the comparison groups; this yielded a minimum mouse number of 12 mice per test set. These assumptions were based on prior experimentation

of altered EGFR or peritoneal lymphocyte signaling (24, 36). All of the animal experiments were certified by the University of Pittsburgh and Pittsburgh VA Medical Center Institutional Animal Care and Use Committees.

Microarray Gene Expression Analysis. We queried the gene expression profile of 53 prostate cancers and 23 normal donors using the Affymetrix (Santa Clara, CA) system. These human tumor queries were determined as exemption 4 under pre-existing data and excess pathological specimens by the University of Pittsburgh Institutional Review Board; specimens were provided by an "honest broker," and the investigators were blinded as to patient identity. Designation of invasive (aggressive; $n = 29$) and localized (organ-confined; $n = 24$) was per pathology report for clinical use. In addition, 23 normal human prostates from organ donors were run in parallel.

Samples of prostate tissues obtained from prostatectomy were dissected and trimmed to obtain pure tumor (completely free of normal prostate acinar cells) or normal prostate tissues. Sandwich-frozen sections were performed by board-certified genito-urinary pathologists to examine the purity of the tumors. These tissues were then homogenized. Total RNA was extracted and purified with Qiagen RNeasy kit (Qiagen, San Diego, CA). Five μg of total RNA were used in the first strand cDNA synthesis with T7-d(T)$_{24}$ primer [GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)$_{24}$] by Superscript II (Life Technologies, Inc., Rockville, MD). The second-strand cDNA synthesis was performed at 16°C by adding *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RnaseH in the reaction. This was followed by the addition of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phenol-chloroform and ethanol precipitation. The purified cDNA was then incubated at 37°C for 4 h in an *in vitro* transcription reaction to produce cRNA labeled with biotin using MEGAscript system (Ambion, Inc., Austin, TX).

Hybridization was as follows. Fifteen to 20 μg of cRNA were fragmented by incubating in a buffer containing 200 mM Tris-acetate (pH 8.1), 500 mM KOAc, and 150 mM MgOAc at 95°C for 35 min. The fragmented cRNA were then hybridized with a pre-equilibrated Affymetrix chip at 45°C for 14–16 h.

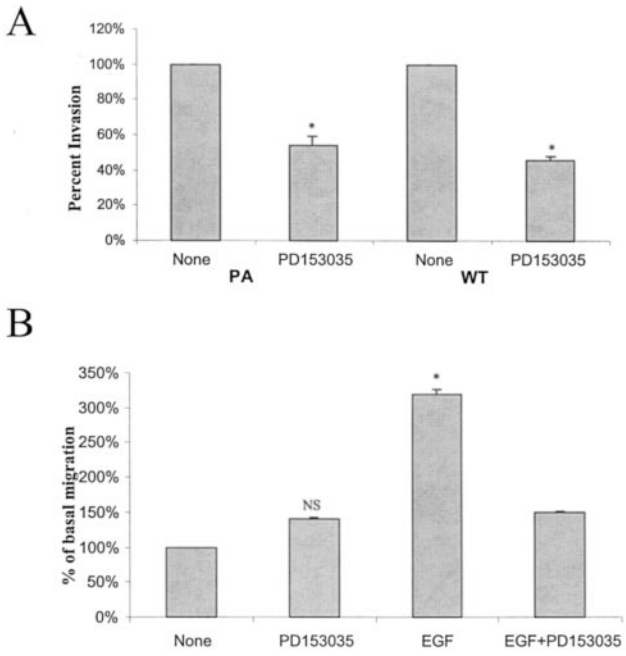

Fig. 1. DU145 invasion and migration are dependent on EGFR signaling. *A,* PA or WT DU145 cells (20,000) were plated in the upper chamber of the transwell plate in medium containing 1% BSA and 500 ng/ml EGFR inhibitor (PD153035). After 24 h, the medium in the upper chamber was replaced with serum-free medium containing PD153035 for another 24 h. The bottom chamber contained complete medium with 10% FCS and PD153035. Cells in the upper compartment were removed by wiping with a cotton swab, and invasive cells were stained according to the manufacture's protocol. *B,* PA DU145 cells were plated in six-well plates and quiesced for 24 h before an *in vitro* wound-healing assay. Cell movement into the denuded space was assessed in the presence of EGF (1 nM) and/or PD153035 (500 ng/ml) or diluent. All of the experiments were performed in triplicate and repeated at least twice. Effects are normalized to diluent alone for the respective cell line; in *A* absolute invasiveness was 2.00 ± 0.24 in WT over PA DU145 cells. $**P < 0.05$ as compared with no treatment, NS, not significant; *bars,* ±SD.

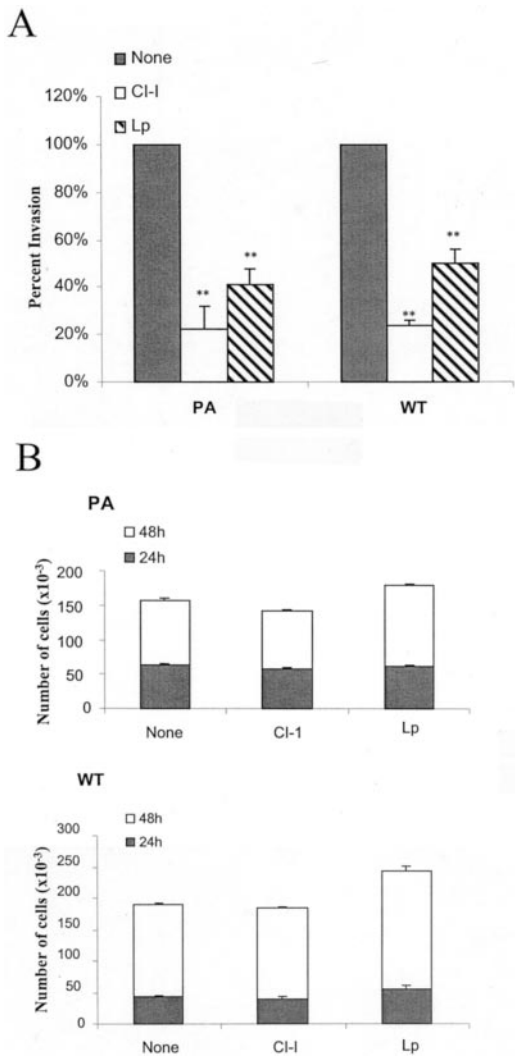

Fig. 2. Calpain inhibition reduces invasiveness of PA and WT DU145 cells *in vitro*. *A,* cells (20,000) were plated in the upper chamber of the transwell plate in medium containing 1% BSA and 2 μg/ml CI-1 or 100 μM of leupeptin (*Lp*). After 24 h, the medium in the upper chamber was replaced with serum-free medium containing CI-1 or Lp for another 24 h. The bottom chamber contained complete medium with 10% FCS and CI-I or Lp. Cells in the upper compartment were removed by wiping with a cotton swab, and invasive cells were stained according to the manufacture's protocol. *B,* cells (20,000 PA or 10,000 WT) were plated in 24-well plates and cultured for 24 or 48 h in CI-I or leupeptin (*Lp*). Cell numbers were enumerated directly. All of the experiments were performed in triplicate and repeated at least twice. $**P < 0.01$; *bars,* ±SD.

After the hybridization mixtures were removed, the chips were then washed in a fluidic station with low-stringency buffer (6× saline-sodium phosphate-EDTA, 0.01% Tween 20, and 0.005% antifoam) for 10 cycles (2 mixes/cycle) and stringent buffer (100 mM 4-morpholinepropanesulfonic acid, 0.1 M NaCl and 0.01% Tween 20) for 4 cycles (15 mixes/cycle), and stained with streptoavidin phycoerythrin. This was followed by incubation with biotinylated mouse antiavidin antibody, and restained with strepto-avidin phycoerythrin. The chips were scanned in a HP ChipScanner (Affymetrix Inc.) to detect hybridization signals.

Data were analyzed by importing the hybridization data from text files into an Microsoft excel spreadsheet. GeneSpring 4.2 along with Michael Eisen's cluster and tree view software were the primary analysis tools. Principle component analysis and logistic regression were performed using S-Plus (Statistical Sciences, Inc.) statistical software.

RESULTS

Invasiveness of DU-145 Prostate Cell Lines Is EGFR-dependent. EGFR overexpression correlates with tumor progression and invasion (5, 7). We tested the hypothesis that motility induced by

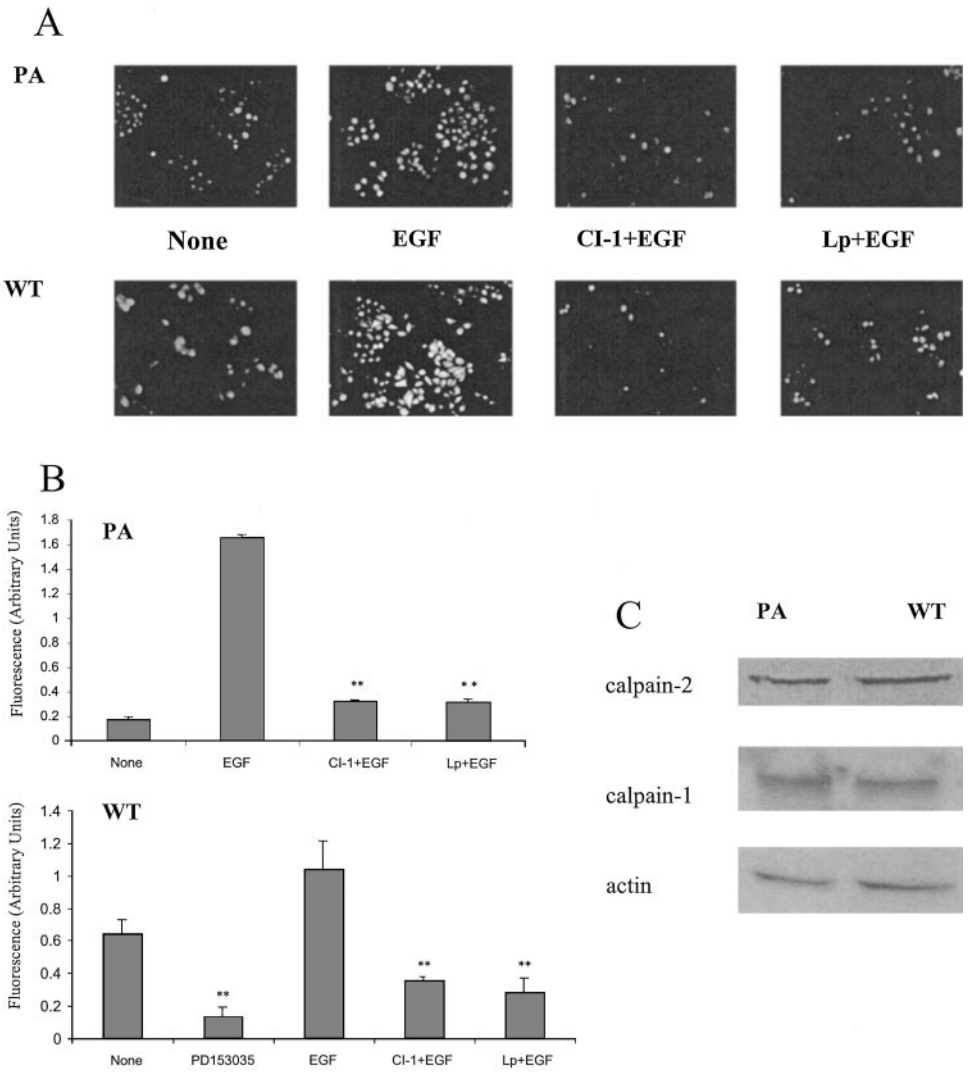

Fig. 3. Leupeptin and CI-I block EGF-induced calpain activation. *A*, quiescent WT and PA DU145 cells were treated with CI-I (ALLN; 2 μg/ml) or leupeptin (*Lp*; 100 μM) for 24 h before loading with BOC-Leu-Met-CMAC for 20 min. Cells were then stimulated with EGF (1 nM) for 10 min before visualizing with a preset CCD camera. The shown exposures are set so only cells with activated calpain are seen; in all of the frames similar numbers of cells were present as determined by phase contrast performed in parallel. *B*, cells were quiesced for 24 h before exposure to EGF (1 nM). Cells were treated with CI-I, leupeptin (as in *A*), or PD153035 (500 nM). Cells were lysed and cleared cytosolic lysates evaluated for their ability to cleave DTAF-labeled MAP2 as described. *C*, cells were grown in complete medium in six-well plates, washed, lysed, and proteins separated by SDS-PAGE. Equal protein loads were immunoblotted for calpain-2, calpain-1, or actin as a loading control. All of the experiments were repeated at least twice with the calpain assays performed in duplicate. $**P < 0.01$, as compared with diluent alone for PD153035 and compared with EGF treatment for CI-I and leupeptin.

autocrine EGFR signaling is a rate-limiting step in the invasion using our model of variously invasive syngeneic DU145 prostate cancer cell lines. Exposure of the moderately invasive PA DU145 or the highly invasive WT DU145 cells to the EGFR kinase inhibitor PD153035 decreased significantly the invasiveness through Matrigel even in the absence of exogenously added EGFR ligand (Fig 1*A*). The data are normalized to the respective mock-treated controls; WT DU145 cells are 1.7 (35) to 2.0 (data herein; $P < 0.05$) times more invasive than PA DU145 cells. EGFR-signaled cell motility was examined under conditions that minimize autocrine EGFR signaling (Fig. 1*B*). As shown in PA DU145 cells, EGF increased motility, which was abrogated by PD153035. These data support the previous literature (22, 35, 36) and demonstrate that the invasiveness of these cells is driven by EGFR signaling.

CIs Reduce DU 145 Cell Invasiveness *in Vitro*. The initial question we asked was whether calpain signaling was required for transmigration of an ECM. Transmigration of Matrigel by the moderately invasive PA DU145 and highly invasive WT DU145 lines was determined in the presence of CI-1 (ALLN; 2 μg/ml) or leupeptin (100 μM; Fig. 2*A*). The number of cells that reached the lower chamber within 48 h was significantly decreased by both inhibitors in both cell lines; the absolute invasiveness of WT DU-145 cells was 2.0-fold that of PA DU-145 cells. This agent-related decrement in cells transmigrated was not secondary to decreased proliferation (15, 20, 29, 39), as the concentrations of CI-1 and leupeptin used in this assay did not block cell proliferation (Fig. 2*B*).

Calpain activation was inhibited by both CI-1 and leupeptin (Fig. 3). First, we ascertained calpain activity *in vivo* by visualizing the bright blue fluorescence after the proteolysis of BOC-LM-CMAC, a calpain-selective substrate (42). Induced calpain activity was inhibited by both CI-1 and leupeptin in both PA and WT cells lines (Fig. 3*A*). In addition, we quantitated calpain activation using cleavage of the prefluorescence substrate DTAF from MAP2; again, both inhibitors limited or eliminated EGF-induced activation of calpain (Fig. 3*B*). WT DU145 demonstrated a somewhat higher basal activity as expected because of increased autocrine EGFR signaling (35), as it was inhibited by the pharmacologic agent PD153035 (Fig. 3*B*). Induced calpain activity was inhibited by both CI-1 and leupeptin to a level similar to that seen in the presence of PD153035 (Fig. 3*B*). The higher basal activity observed in WT compared with PA cells is not attributable to a higher amount of calpain-1 or -2 expression (Fig. 2*C*). Thus, we had evidence for calpain inhibition limiting tumor cell invasion.

Down-Regulation of Calpain-2 Limits PA DU145 Invasion. Molecular targeting of calpain was required, as leupeptin, in particular, and possibly even CI-1 inhibit proteases in addition to calpain. We used AS approaches to calpain-2 to abrogate signaling through this molecule (17). Oligonucleotides against calpain-2 in PA DU145 cells

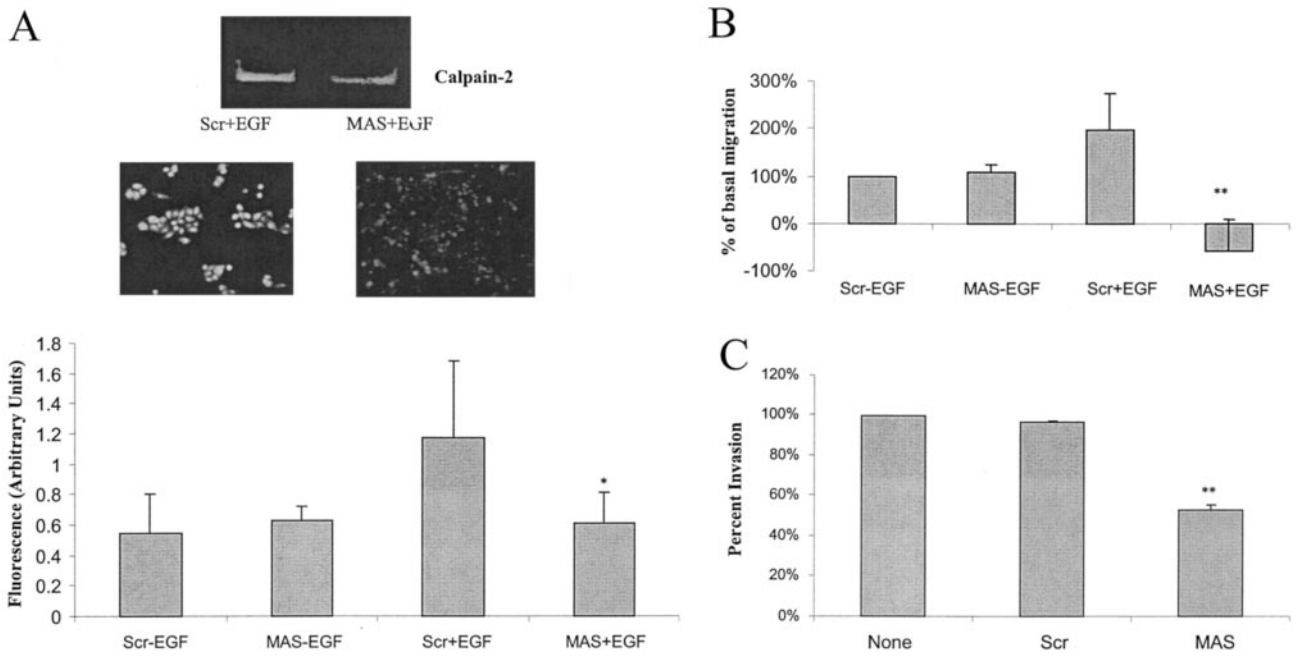

Fig. 4. AS oligonucleotides to calpain-2 decrease PA DU145 cell invasiveness and migration. Phosphothiorated oligonucleotides (20 μM) specific for calpain-2 (*MAS*; Ref. 17) were added to cells; a scrambled oligonucleotide (*Scr*) of similar composition served as a control. *A*, calpain activation was assessed by zymography (*top panel*), BOC-Leu-Met-CMAC (*middle panel*), or MAP2 (*bottom panel*) as described. *B*, the effect of MAS or Scr oligonucleotides on PA DU145 cell migration across a two-dimensional surface were calculated as a percentage of the values obtained with the Scr oligonucleotide alone. *C*, the invasion through Matrigel was evaluated as previously described and the number of cells that transmigrated through the Matrigel were normalized to no treated cells. All of the experiments were repeated at least twice, with the assays performed in duplicate. $**P < 0.01$; NS, not significant; *bars*, $\pm$SD.

limited EGF-induced calpain activation cell migration and transmigration of the Matrigel barrier (Fig. 4). A control scrambled oligonucleotide did not effect these parameters.

We generated a stable PA DU145 derivative in which an 80-bp minigene around the calpain-2 translation initiation site was expressed in the AS direction from the cytomegalovirus promoter. In these cells, calpain-2 levels were reduced by >30% as quantified using an NIH program (Fig. 5); such partial down-regulation was expected because calpain-2 is required for cell viability and growth (15, 20, 29, 39); importantly, a similar level of calpain-2 down-regulation eliminates EGF-induced calpain activity and motility in fibroblasts (17). These cells were significantly less invasive than a PA DU145 derivative expressing the vector alone as a control. This decrement in invasiveness was not because of decreased cell numbers, whether reduced proliferation or survival, because the two derivative polyclonal lines grew at the same rate. In sum, these data strongly suggest that calpain-2 activation is required for increased tumor cell motility and subsequent invasiveness *in vitro*.

Leupeptin and Down-Regulation of Calpain-2 Decreases DU145 Invasiveness *in Vivo*. Our data *in vitro* show that calpain activity is required for cell transmigration throughout a "defined" layer of ECM. To investigate the role of calpain in an *in vivo* environment where complex and various interactions occur, we used the murine tumor xenograft model of diaphragm invasion (24, 36). This assay was used because it is more easily quantitated than invasiveness of orthotopic tumor growth for both technical and biological reasons; however, the semiquantitative scores of diaphragm invasion correlate well with the qualitative assessment of invasiveness of orthotopic tumors (24, 36). The pharmacological agent chosen was leupeptin because this has been used in both mice and humans with minimal toxicity (37, 38). Either PA or WT DU145 cells were inoculated i.p. into athymic mice and allowed to establish for 10 days before treatment with leupeptin or diluent alone. The WT DU145 tumors demonstrated increased invasion *in vivo* ($P < 0.05$ compared with PA DU145 tumors), similarly to the increment *in vitro* transmigration of Matrigel; this finding is consistent with our previous reports (24, 36). For both cell lines, leupeptin treatment significantly reduced the extent of invasion into and through the diaphragm (Table 1). Invasion into other soft organs was not scored because of difficulty in quantitation but qualitatively reflected this difference. The reduction of invasiveness seen with leupeptin was not attributable to decreased tumor growth, because tumors in the diaphragm with the same size from treated or not treated mice showed different level of invasiveness (Fig. 6). This is expected, because leupeptin did not affect cell proliferation (Fig. 2*B*).

Verifying that this invasiveness was because of calpain inhibition required a second approach because leupeptin inhibits other proteases, both intracellularly as well as extracellularly. We repeated the diaphragm invasion assay using the PA and WT DU145 cells expressing the calpain-2 AS minigene or vector alone (Table 2). Mice inoculated with the calpain-2 AS showed 50% less invasiveness compared with the mice carrying the vector alone (Fig. 7). The PA DU145 cells exhibited high significance in themselves, whereas the WT DU145 were marginally inhibited; this affect is likely because of the few mice challenged in this second series, which was curtailed because of the outcome of the P DU145 cells. Again the tumor take rates and size of the diaphragm tumors were indistinguishable between the sublines expressing C2AS or V constructs. This degree of inhibition of invasiveness by slightly more than half was in line with the extent of inhibition shown by leupeptin.

Calpain Levels Are Not Altered in Human Prostate Tumors. The above data strongly suggest an epigenetic role for calpain in enabling tumor cell motility and subsequent invasion. To address whether this is also altered gene expression levels of calpain-2 in prostate tumors, we analyzed 29 aggressive/invasive or metastatic tumors and 24 organ-confined tumors (Table 3). In addition, 23 normal prostates were queried on the same chip set. We also examined the expression of calpain-1 and calpastatin, because these might alter

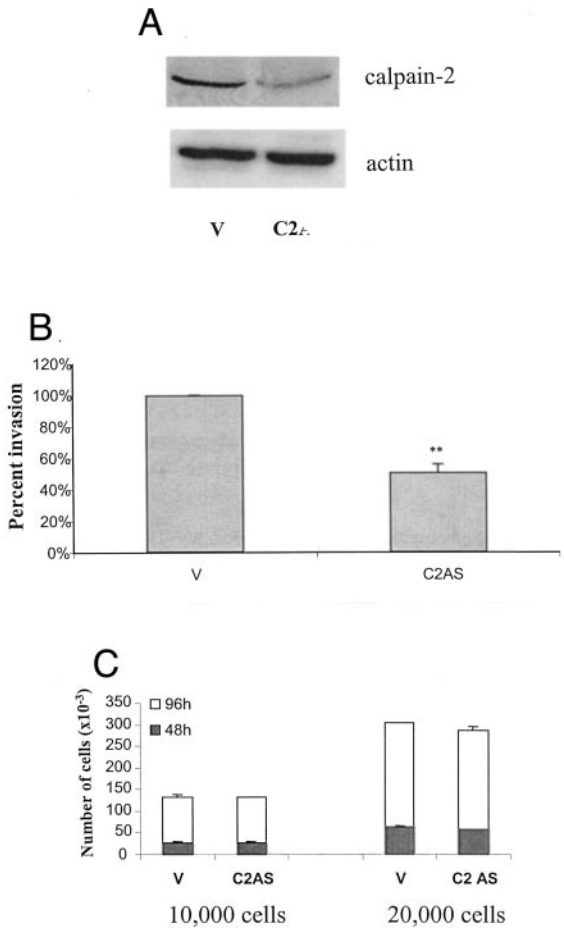

Fig. 5. AS minigene down-regulation of calpain-2 reduces PA DU145 invasiveness. PA DU145 cells were stably transfected with a minigene that expressed AS across the translation initiation site of calpain-2 or an empty vector. *A*, calpain-2 levels were ascertained by immunoblotting. Invasiveness through Matrigel (*B*) and cell proliferation (*C*) were evaluated as described. All of the experiments were repeated at least twice, with the invasiveness assay performed in duplicate and the cell counting in triplicate. $^{**}P < 0.01$; *bars*, ±SD.

the calpain activity balance. Analysis of the expression levels of all five of the calpain-related hybridization spots using the Welch *t* test indicated minimum variation of gene expression across all of the samples. Calpain-2 and calpain-1 expression were characterized as being at moderate levels, whereas expression of calpastatin was minimum. No significant expression change was identified when aggressive prostate cancer was compared with normal donor or organ-confined prostate cancers.

DISCUSSION

Tumor invasion is a complex process that involves cellular migration, interaction with the microenvironment, and survival at the ectopic site. We and others have shown that cell migration is a rate-limiting step in this process (5). Thus, key molecular switches required for functional migration may be successfully targeted to limit tumor spread. Previous studies have shown that the calpain proteases are required for rear deadhesion during productive motility whether initiated by adhesion-related signals or growth factors (14, 16–18). Synthesizing these finding for tumor invasiveness, an initial report demonstrated that blockade of calpain limited both the motility and invasiveness *in vitro* of bladder carcinoma cells (22). As local invasion generates a great part of the morbidity of prostate cancer, we asked whether blockade of calpain signaling would limit this spread. Herein, we report that pharmacological and molecular inhibitors of calpain-2 significantly reduce the motility and invasiveness of DU145 human prostate carcinoma cells both *in vitro* and *in vivo*. These data suggest that calpain may be rationally targeted to limit prostate cancer spread.

Our data strongly implicate calpain-2 control of cell motility as the operative target. However, this assignment is compromised by the lack of selectivity of the pharmacological agents for the calpain-2 isoform; this is especially true for the broad spectrum inhibitor leupeptin. Despite this uncertainty of inhibition, leupeptin was chosen, because it has been used in both animals and humans with minimal reported toxicity (37, 38). Still, a strong case for calpain-2 being the critical element is made by the fact that AS approaches to calpain-2 mimic the findings with leupeptin and CI-I. Whereas leupeptin inhibits both intracellular and extracellular proteases, and ECM remodeling might be hindered (43), the expression of the AS calpain-2 minigene should not alter the myriad of extracellular proteases. Thus, a confluence of data support targeting calpain-2.

A second point of contention may rest on which cell behavior is limited by calpain inhibition. In many settings calpain activity is required for cell proliferation or apoptosis in addition to motility (20). Our *in vitro* data suggest that in this setting our level of calpain inhibition does not affect cell proliferation (Fig. 2*B;* Fig. 5*C*). However, the *in vivo* experiments are not readily amenable to such analyses; although the fact that the tumor take rate (Tables 1 and 2) and size of the tumors were indistinguishable between the calpain-inhibited and control tumors is reassuring that overall cell number is not the main target of calpain inhibition.

It is possible that the increased motility and invasion may be indirectly related to calpain activation because the broad spectrum of calpain targets may also involve regulation of secreted proteases. We feel that this is an unlikely mechanism, because our earlier works failed to demonstrate differences in protease production between PA and WT DU145 (35). Furthermore, because motility over a two-dimensional surface is also affected, the need for extracellular proteases to modify a "barrier" matrix is limited, although others have suggested that matrix metalloprotease 9 is required for dispersion of cohesive keratinocytes even over a matrix surface (44). However, in our earlier survey of cellular proteases produced by DU145 sublines, matrix metalloprotease 9 was secreted at equivalent levels by the three syngeneic lines (35). Lastly, EGF only activates calpain-2 in the immediate subplasma membrane locale (45), and, thus, protease maturation is not likely globally affected by such localized signaling. However, until the identification of the specific target of calpain during induced motility (14), both indirect as well as direct molecular mechanisms must be considered.

Prostate cancer motility and invasion likely uses both ubiquitous calpain isoforms, calpain-2 and calpain-1, for cell movement. This is because prostate carcinoma cells present both integrins capable of promoting haptokinesis and EGFR-mediate autocrine signaling loops that induce chemokinesis (46). Calpain-1 (μ-calpain) has a calcium-dependency that can be attained in living fibroblasts and epithelial

Table 1 *Leupeptin decreases tumor invasiveness in mice*

Control mice inoculated with PA or WT cells were injected with diluent HBSS, and the experimental mice received 12 mg/kg of leupeptin. Mice were sacrified and diaphragm scored for diaphragm invasiveness from 0 to 4. Diaphragm tumors represent the total number of mice with tumors in the diaphragm.

	PA + HBSS	PA + leupeptin	WT + HBSS	WT + leupeptin
Diaphragm tumors	14/14	13/14	14/14	13/14
Diaphragm invasiveness	1.71	0.7[a]	2.35	1.25[b]

[a] $P < 0.01$.
[b] $P < 0.05$.

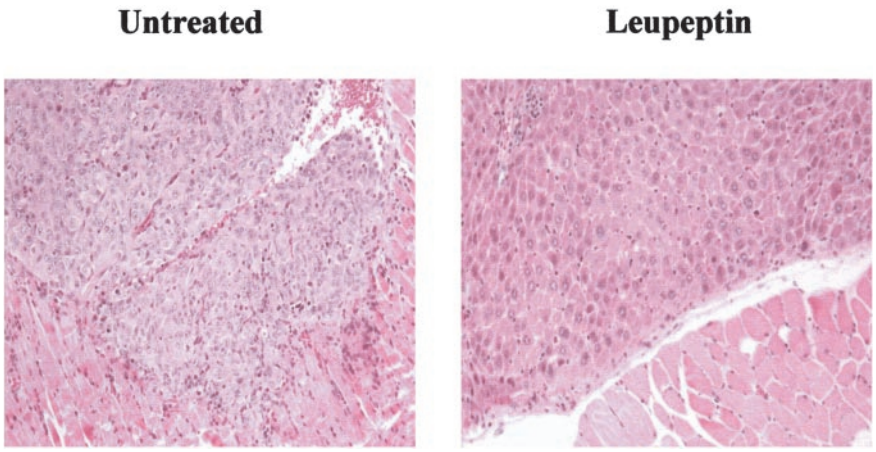

Fig. 6. Leupeptin reduces diaphragmatic invasion *in vivo*. Six-week-old male *BALB/c nu/nu* mice were injected i.p. with 2 million PA DU-145 cells. Ten days later, mice were separated into two groups, one was daily given i.m. injection of 12 mg/kg of leupeptin for 30 days, and the other control group was injected with a similar volume of HBSS. Diaphragms were isolated and evaluated by histopathology. Shown are representative invasion values of 2+ (diluent) and 0 (*Leupeptin*).

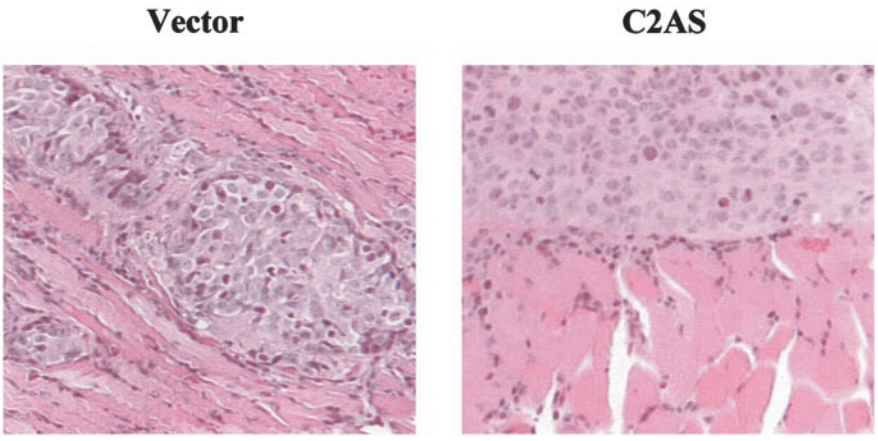

Fig. 7. AS down-regulation of calpain-2 reduces diaphragmatic invasion *in vivo*. Six-week-old male *BALB/c nu/nu* mice were injected i.p. with 2 million PA DU-145 cells expressing the minigene or vector alone. Sixty days later the diaphragms were isolated and evaluated by histopathology. Shown are representative invasion values of 3+ (*vector*) and 1 (*C2AS*).

cells (47). The autocrine EGFR-mediated signaling would activate calpain-2 preferentially via an ERK mitogen-activated protein kinase pathway at the inner face of the plasma membrane (17, 45). Thus, there appears to be a convergent signaling through the two ubiquitous calpain isoforms to regulate cell deadhesion (14). Whereas this might suggest that the best target for intervention is the end target, there are reasons to focus on calpains. First, the presumably common end target(s) might be individually sufficient, but none are actually required, making specific intervention ineffectual. Second, it is likely that the end target of calpain is a structural component and, thus, not readily "inhibitable," although the activation of the rho-GTPase may suggest sensitive points for intervention (48). Third, the ability to inhibit only one isoform may limit toxicity, because homeostatic mechanisms that require low level motility, such as colonic or skin epithelial replacement, would use one of the isoforms and not the other in the absence of injury repair needs (49). Unfortunately, the commonly available inhibitors such as leupeptin and CI-I do not distinguish between the isoforms, making molecular approaches the only viable option at present to determine whether inhibition of a single isoform can accomplish blockade of tumor invasiveness. Obviously, new, isoform-specific small molecule inhibitors would greatly advance our understanding of the physiology of calpain activation.

The question remains of whether these findings in model systems translate to the human clinical situation. We surveyed 53 specimens from human prostate tumors and normal prostate tissue. Segregated by tumor stage, invasiveness, and metastases, we found no significant differences in mRNA levels of these tissues. This is in contradistinc-

Table 2 *Antisense down-regulation of calpain-2 decreases prostate tumor invasiveness in mice*

Mice injected with PA or WT DU-145 cells expressing calpain-2 minigene (C2AS) were compared with mice receiving cells transfected with the vector alone (V). Results are the average of diaphragm score of 9 mice *versus* 8 for PA cells and 4 *versus* 3 for WT cells.

	V PA DU145	C2AS PA DU145	V WT DU145	C2AS WT DU145
Diaphragm tumors	9/10	8/10	4/5	3/5
Diaphragm invasiveness	2.33	1.13[a]	3.50	1.67[b]

[a] $P < 0.05$.
[b] $P < 0.10$.

Table 3 *Calpain expression does not differ in human prostate cancers based on tumor invasiveness*

Probe set	Description	Average of AC[a]	Average of OCC	Average of ND	AC/ND	*P*	AC/OCC	*P*
33384	Calpastatin	64.228	68.457	67.957	0.9451	0.6445	0.9382	0.8002
33385_g	Calpastatin	288.345	298.095	324.826	0.8877	0.1824	0.9673	0.4202
33908_	Calpain-1	2,485.08	2,451.91	2,356.19	1.0547	0.785	1.0135	0.9962
37001_	Calpain-2	1,560.52	1,507.79	1,751.10	0.8912	0.1428	1.035	0.7373
47510_r	CAST Calpastatin	293.507	288.238	256.426	1.1446	0.3642	1.0183	0.9132

[a] AC, aggressive prostate cancer ($n = 29$); OCC, organ-confined prostate cancer ($n = 24$); ND, normal prostate donor ($n = 23$).

tion to a recent report in which calpain-2 mRNA was found to be mildly (1.4 times) up-regulated in prostate carcinomas in conjunction with cadherin cleavage (50). We did not note this increased transcript level in our series of tumors, although the reasons for this discrepancy are not evident at present. However, in a different tissue, calpain-2 levels were not increased over that in normal skin in either squamous or basal cell carcinomas (51). Another calpain isoform reported altered in tumors, calpain-9 (31) is not reliably detectable in our prostate tissues: neither normal donor nor tumor (data not shown). According to accepted models of calpain activation (14, 15, 25), the lack of transcriptional change is not unexpected. Calpains appear to be activated at a post-translational level with calcium or other mechanisms, such as coactivators or phosphorylation (52–54),[4] being the operative event. In fact, in studies that attempt to exogenously express calpains, one usually fails to attain even a doubling of calpain levels, as higher activity leads to apoptosis (55). Thus, to demonstrate increased calpain activation in invasive tumors would require a way to assess *in situ* activation. For live cells, this can be accomplished by fluorescent substrates (Fig. 2). However, in nonliving cells we need to develop reagents to detect either the post-translational modifications that mark activation or colocalization of the activator cofactors.

In summary, we found that targeting calpain can limit prostate cancer cell invasiveness both *in vitro* and *in vivo*. This was likely because of the inhibition of rear deadhesion during growth factor-induced motility. In fact, CI-I limits EGFR-mediated deadhesion of DU145 cells (data not shown) similar to the calpain-dependent detachment of fibroblasts (17) and epithelial keratinocytes (21). Our operative model of calpain function during tumor invasion posits an epigenetic or post-translational activation of calpain-2 rather than significant changes in protein levels. A survey of mRNA profiles of human prostate carcinoma specimens supports this by failing to demonstrate calpain gene expression differences between invasive and noninvasive carcinomas. However, to fully demonstrate the validity of this model will require a knowledge of how calpain-2 is activated and development of tools to detect such changes in activation. Additionally, the targeting of calpain-2 as a rational therapeutic intervention strategy will also necessitate new reagents, isoform-specific inhibitors. Because of the high degree of homology at the amino acid and structure levels (25) molecular agents offer the greatest hope of discriminatory agents. Thus, the full exposition of this potential novel target to limit tumor progression will rely as much on technical developments as on biological insights.

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[4] Unpublished observations.

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