The American Journal of Pathology, Vol. 175, No. 2, August 2009

DOI: 10.2353/ajpath.2009.080417

Tumorigenesis and Neoplastic Progression

Suppressive Roles of Calreticulin in Prostate Cancer Growth and Metastasis

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Calreticulin is an essential, multifunctional Ca^{2+}-binding protein that participates in the regulation of intracellular Ca^{2+} homeostasis, cell adhesion, and chaperoning. Calreticulin is abundantly expressed and regulated by androgens in prostate epithelial cells. Given the importance of both calreticulin in multiple essential cellular activities and androgens in prostate cancer, we investigated the possibility of a role for calreticulin in prostate cancer progression. Immunohistochemistry revealed the down-regulation of calreticulin in a subset of human prostate cancer specimens. Prostate cancer cells overexpressing exogenous calreticulin produced fewer colonies in both monolayer culture and soft agar. Furthermore, calreticulin overexpression also inhibited tumor growth in the orthotopic PC3 xenograft tumor model and macroscopic lung metastasis in the rat Dunning AT3.1 prostate tumor model. To address the potential mechanism of calreticulin suppression of prostate cancer, we generated calreticulin mutants with different functional domains deleted. The calreticulin mutants containing the P-domain, which binds to other endoplasmic reticulum chaperone proteins, were sufficient for the suppression of PC3 growth in colony formation assays. Overall, our data support the hypothesis that calreticulin inhibits growth and/or metastasis of prostate cancer cells and that this suppression requires the P-domain. ***(Am J Pathol 2009, 175:882–890; DOI: 10.2353/ajpath.2009.080417)***

Prostate cancer was the most frequently diagnosed non-skin cancer and the third leading cause of cancer death among American men in 2008.[1] Androgens are intimately associated with prostate cancer progression and as such, androgen ablation remains the standard therapy for patients with metastatic prostate cancer.[2] However, hormone therapy is not curative and the vast majority of treated patients eventually experience disease progression. Elucidating the mechanism of androgen influence on prostate cancer is important as it may facilitate the development of more effective therapies and methods of disease prevention.

Androgen action is mediated through the androgen receptor, which controls the expression of androgen-responsive genes.[3] As androgen-responsive genes likely play important roles in prostate cancer progression, the characterization of their expression patterns and functions should provide insight into the roles of androgen in disease development. *Calreticulin* is one of the androgen-responsive genes in the prostate.[4,5] Androgen ablation by castration rapidly down-regulates calreticulin at both

Supported by grants from the National Institute of Health, R01 DK51193, Prostate Cancer Specialized Program Of Research Excellence (SPORE), CA90386, Department of Defense Prostate Cancer Research Program, DAMD17-01-1-0088. M.A. and F.J. were supported by Chicago Baseball Charity Pre-doctoral Fellowship. S.E.E. was funded by a Ruth L. Kirschstein National Research Service Award from the National Institute of Health Training Grant.

Accepted for publication May 14, 2009.

Supplemental material for this article can be found on *http://ajp.amjpathol.org.*

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the mRNA and protein levels by more than tenfold. In contrast, androgen replacement rapidly restores the expression of calreticulin in the regrowth of the castrated prostate. Northern blot analysis of the tissue-specificity of calreticulin expression in the rat model shows that the most abundant levels occur in the prostate, as compared with the liver, kidney, brain, heart, muscle, and seminal vesicles. *In situ* hybridization and immunohistochemistry studies demonstrate that prostatic epithelial cells specifically express calreticulin.[5,6] The expression profile and androgen-responsiveness of calreticulin in the prostate indicate that calreticulin may play a key role in androgen action in prostate epithelial cells.

Calreticulin performs a variety of functions within the cell. This evolutionarily conserved protein localizes to the endoplasmic reticulum (ER),[7–9] but possibly may also be found at the cell surface.[10,11] Within the ER, calreticulin serves as a molecular chaperone to ensure proper folding of glycoproteins.[12–15] In addition, calreticulin modulates intracellular Ca^{2+} homeostasis by its ability to bind Ca^{2+} with high affinity.[6,16–18] Other possible activities include integrin α-binding and cell adhesion,[19–22] major histocompatibility class I assembly,[23] steroid-mediated gene regulation,[24–26] as well as Zn^{2+} binding and storage.[27] Gene knockout experiments further underscore the importance of this protein, as loss of calreticulin results in embryonic death from defective cardiac development.[28] Calreticulin consists of three distinctive domains. The N-domain (residues 1 to 180) is thought to bind heavy metals (Zn^{2+}) and interact with other ER chaperones, nuclear receptors, and nucleic acids.[29] The P-domain (residues 181 to 290) contains a proline-rich region that forms an extended arm structure and interacts with other chaperones in the lumen of the ER. Lastly, the C-domain (residues 291 to 400) is a highly acidic region that binds Ca^{2+} and is involved in Ca^{2+} storage.[30]

Although the role of calreticulin in normal cellular functions and during embryogenesis is well-established, its role in human carcinogenesis remains poorly understood.[31] In the current study, we demonstrate the down-regulation of calreticulin protein in a subset of human prostate cancer specimens. Furthermore, we show that calreticulin overexpression in prostate cancer cells inhibits prostate tumor growth and metastasis and that its growth inhibitory role requires the P-domain. Overall, our present study provides evidence, for the first time, that calreticulin is capable of suppressing prostate cancer progression.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed as described previously.[32] We used 21 paraffin-embedded archival blocks obtained from human prostatectomy specimens. In 11 of the specimens was found high grade prostate intraepithelial neoplasia; in 10 specimens, cancer with Gleason grade 3 + 3; and in 3 specimens, cancer with Gleason grade 4 + 4. All of the specimens also contained benign glandular epithelial cells. For the immunohistochemistry, we used unstained sections, 4-μm thick. The immunostaining was performed using a polyclonal anti-calreticulin antibody as described previously.[5] Sections immunostained in the absence of primary antibody were included as controls for nonspecific staining. The slides were scored under a $\times$10 microscopic field by two pathologists (S.S.D. and M.P.) and the results were recorded as either no down-regulation (dash), mild down-regulation (arrow), or significant down-regulation (two arrows) of the calreticulin level in cancer cells relative to the benign glandular cells in the same slide.

We also evaluated calreticulin expression in prostate cancer tissue microarrays. Tissue microarray blocks were prepared by transferring paraffin-block tumor cylindrical cores from 'donor' blocks to one 'recipient' block.[33] Each tissue core was characterized by a genitourinary pathologist (M.P.). Immunostaining of the tissue cores was performed with anti-calreticulin antibodies. Two observers (S.E.E. and S.K.) then independently isolated the area of characterized tissues within the tissue core and scored the intensity of immunostaining using a subjective score of 0 (no staining) to 3 (intense staining). If the scoring discrepancy between the two observers was 1.5 or greater, the tissue core was discarded from analysis. Average immunostaining score for each type of prostatic histology was then determined.

Specimens in tissue microarrays were grouped according to their pathology and histological Gleason grade. Statistical analysis was performed using SPSS statistical software (SPSS Inc, Chicago, IL). The analysis of calreticulin expression levels using the immunostaining score among individual histological groups was performed by the Games-Howell post hoc test and overall evaluation using an analysis of variance test. Evaluation of immunostaining scores greater or less than 1 was done using the Pearson χ^2 test. Values of $P < 0.05$ were considered statistically significant.

Constructs

The vectors pcDNA3.1 (Hygro) and pcDNA3.1 (Neo) were obtained from Invitrogen (Carlsbad, CA). Through PCR, HindIII and XhoI sites were added to the 5′ and 3′ ends, respectively, of rat calreticulin (rtCrt), using the following primers: 5′RtCrt-HindIII, 5′-GGG*AAGCTT*ATGCTCCTTTCGGTGCCGCTCC-3′ and RtCrtREV-XhoI, 5′-GGG*CTCGAG*GCCAGTGGCATCCTCCTCATCTTC-3′. The resultant PCR product was cloned into a pcDNA3.1 (N) vector containing the hemaglutinnin (HA) epitope, followed by the KDEL sequence that is found at the carboxyl-terminus of calreticulin. Domain mutant constructs were created similarly. All sequences were confirmed either by the Northwestern University Sequencing Core Facility (Chicago, IL) or Macrogen (Seoul, S. Korea). To clone green fluorescent protein (GFP)-tagged calreticulin expression vectors, GFP was PCR-amplified from pEGFP-C1 (Invitrogen, Carlsbad, CA), using the following primers: GFPCRTFOR.2, 5′-GATATC*CTCGAG*ATGGTGAGCAAGGGCGAGGAG-3′ and GFPREV.1, 5′-GGG*TCTAGA*CTACAGCTCGTCCTTGGCCTGCTTGTACAGCTCGTCCATGCCGAG-3′. We then replaced the HA tag with the GFP cDNA in the expression constructs.

Cell Culture, Colony Formation Assay, and Transfection

The human androgen receptor-negative prostate cancer line PC3 and the human androgen receptor-positive androgen-sensitive prostate cancer lines LNCaP were acquired from ATCC (Manassas, VA). The rat Dunning AT3.1 tumor cell line was kindly provided by Dr. Allen Gao (University of California, Davis, CA). PC3 and LNCaP cells were maintained in RPMI media supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine (complete media without drug) (Mediatech, Inc., Manassas, VA). AT3.1 cells were grown in RPMI-complete media that contained 250 nmol/L dexamethasone. (VWR, West Chester, PA).

In colony formation assays, PC3 cells, at approximately 50% confluence in 6 well plates, were transfected in duplicate using Fugene 6.0 (Roche, Indianapolis, IN). The following day, the duplicate transfectants were combined and then diluted 1:50 and 1:100 in media containing either hygromycin (150 μg/ml) (VWR, West Chester, PA) or G418 (500 μg/ml) (Gemini Bio-Products, West Sacramento, CA). After 2 weeks in the drug-containing media, the stable clones were counted. Similarly, LNCaP cells, at approximately 60% to 70% confluence, were transfected in 6-well plates using Lipfectamine 2000 (Invitrogen, Carlsbad, CA). After 2 days, cells were resuspended in media containing either hygromycin (150 μg/ml) or G418 (500 μg/ml) and then cultured for approximately 3 weeks, at which time the colonies were counted. To control for transfection efficiency in colony formation assay, cells cultured in 6-well plates were transfected with 2.0 μg of pcDNA3 empty vector, pcDNA-Calreticulin, or pcDNA-HA-Calreticulin expression vectors in the presence of 0.25 μg of the pEGFP-C1 plasmid via Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in OPTI-MEM medium (Invitrogen, Carlsbad, CA) and after 4 hours the medium was replaced with RPMI 1640 medium. Twenty hours after transfection, cells were lysed in modified radioimmunoprecipitation buffer [50 mmol/L Tris (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO)]. GFP and HA-Calreticulin expression were determined by Western analysis using mouse anti-GFP polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse anti-HA monoclonal (Covance, Denver, PA) antibodies as described previously.[34]

Stable transfection was done similarly as above. When colonies were approximately 3 mm in diameter, they were isolated either by pipette tip or through the use of a cloning ring. Western blots were performed to determine relative levels of calreticulin, as described previously.[6]

Intracellular Localization of GFP-Tagged Constructs

PC3 cells were plated on coverslips in 6-well plates. At approximately 30% to 50% confluence, cells were transfected with the GFP-tagged constructs as described above. After 1 to 2 days, the coverslips were fixed in 4% paraformaldehyde for 15 minutes, permeabilized with 1% Triton X-100 (VWR, West Chester, PA) for 5 minutes, and then stained with calnexin (Cnx) antibody (Stressgen, Victoria, BC, Canada). Following extensive washing, coverslips were incubated in tetramethylrhodamine B isothiocyanate-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour. Cells were observed and imaged using a Nikon TE2000U microscope attached to a digital camera. Images were captured and edited using Metamorph software 6.0 (Downingtown, PA) and Adobe Photoshop 7.0 (San Jose, CA).

Soft Agar Assay

Soft agar assays were performed as described previously.[35] Briefly, 2 ml of bottom agar in complete media (0.6%) was poured into 6-well plates. After solidification and drying, 1 ml of top agar (0.4%) containing 30,000 stably-transfected cells was applied to the bottom layer. After cooling, plates were placed in a 37°C CO_2 incubator for 15 days and then the number of colonies was determined in five random fields per well in two different wells.

Orthotopic Injection Model

Athymic nude mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) and maintained in the Northwestern Animal Care Facility, according to approved animal care and use protocols. Orthotopic injections were performed similarly as described previously.[36,37] Briefly, PC3 cells (1×10^6 cells in 20 μl) overexpressing calreticulin were injected orthotopically into the prostate of anesthetized nude mice using a 30-gauge needle along with a Hamilton syringe. Proper injection was demonstrated by the production of a bubble within the prostate capsule. The mice were sutured using Ethicon sutures (Johnson & Johnson, Somerville, NJ) and stapled. Mice were euthanized approximately 30 days after the injection of tumor cells. The urogenital organs (bladder, prostate, and seminal vesicles) were excised and weighed immediately.

AT3.1 Subcutaneous Injection Model

This experiment was performed as described previously.[38,39] Briefly, 1×10^5 AT3.1 cells in 500 μl were injected subcutaneously into the right flank of 4- to 6-week-old male nude mice (Charles River Laboratories, Frederick, MD). Ten mice were used for each cell line. After 20 to 25 days, when the tumor burden became excessive, the mice were euthanized. Primary tumors at the site of injection were excised and weighed. The lungs were also removed, fixed in Bouin's solution and the number of macrometastases recorded.

Results

Calreticulin Is Down-Regulated in Prostate Cancer

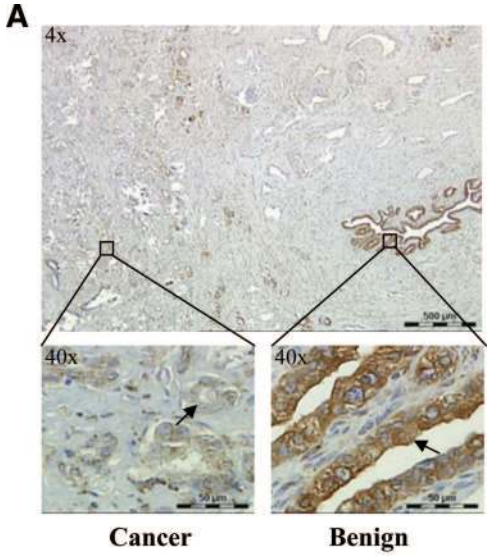
To investigate a possible role for calreticulin in prostate cancer progression, we performed immunohistochemical analysis of human prostate cancer specimens and demonstrated a decrease in calreticulin expression, as compared with benign tissue adjacent to the tumor (Figure 1A). After surveying benign tissue, high grade prostatic intraepithelial neoplasia lesions and tumors with a Gleason score of 6 or 8, we also found that the frequency and magnitude of calreticulin down-regulation appear to increase with Gleason score (Figure 1B).

To further evaluate calreticulin expression in prostate cancer progression, we conducted immunohistochemical analysis on prostate tissue microarrays containing a total of 341 tissue cores, of which 277 (81.2%) met the criteria that the scoring discrepancy between the two observers was less than 1.5 and were further analyzed (Figure 1C). The average immunostaining score for benign prostate tissue (1.21) was higher than that of intermediate (Gleason 7: 0.91, $P = 0.007$) and high grade prostate cancers (Gleason 8 or 9: 0.77, $P = 0.006$). Low grade prostate cancer stained more intensely for calreticulin when compared with high grade disease (1.10 vs. 0.77, $P = 0.048$). Calreticulin expression in nodal metastases, while significantly different from benign tissue (0.85 vs. 1.21, $P = 0.023$), was most similar to intermediate grade prostate cancer.

We then extended our analysis to evaluate if down-regulation of calreticulin expression was more common in prostate cancer than in benign tissue. We defined the immunostaining score of 1.0 or less as calreticulin down-regulation, and a score greater than 1.0 as normal or increased calreticulin expression. Calreticulin down-regulation was more common in cancerous versus benign tissue (Figure 1D). Benign tissue exhibited an immunostaining score of 1.0 or less in 45% of the cores compared with Gleason 5 or 6 (49%), Gleason 7 (63%), and Gleason 8 or 9 (77%) cancers. The incidence of down-regulation also increased along with histological aggressiveness (Pearson χ^2, $P = 0.004$), as Gleason 8 or 9 cores had immunostaining scores of 1.0 or less much more frequently than Gleason 5 or 6 cores (77% vs 49%). While nodal metastases were the most frequent tissue type to demonstrate calreticulin down-regulation, the low number of tissue cores ($n = 17$) prevented any meaningful analysis.

Calreticulin Inhibits Colony Formation of Prostate Cancer Cells in Monolayer Cultures

The ability to form colonies *in vitro* represents one characteristic of the transformed phenotype in prostate



B

Calreticulin staining in human CaP specimens

Down-regulation	HGPIN (n=11)	Gleason 6 (n=10)	Gleason 8 (n=3)
↓↓	0	10%	33.3%
↓	36.4%	30%	33.3%
-	63.6%	60%	33.3%

C

Calreticulin staining in CaP tissue microarray

	No. of Cores	Staining Score	S.D.
Benign	82	1.21	0.53
Gleason 5 or 6	89	1.10	0.44
Gleason 7	67	0.91*	0.55
Gleason 8 or 9	22	0.77*	0.48
Metastasis	17	0.85*	0.38

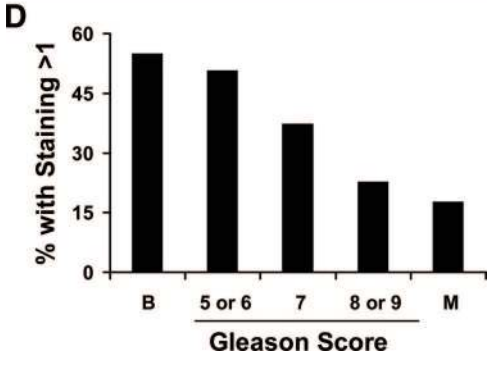


Figure 1. Calreticulin is down-regulated in prostate cancer. **A:** Representative immunostaining of calreticulin expression in a human prostate cancer specimen with a Gleasone score of 4 + 3. **Arrows** indicate either benign or cancerous prostatic epithelial cells. The size bars are equal to 500 μm and 50 μm in pictures taken with ×4 and ×40 objectives, respectively. **B:** The summary of immunostaining results for 21 human prostate cancer specimens. The dash indicates no down-regulation, one **arrow** means mild down-regulation, and two **arrows** indicate significant down-regulation of calreticulin expression in cancer cells relative to the benign glandular epithelial cells on the same slide. **C:** Summary of calreticulin staining intensity in prostate cancer tissue microarray. The staining scores for Gleason 7 ($P = 0.007$), Gleason 8 or 9 ($P = 0.006$), and nodal metastasis ($P = 0.023$) specimens are statistically different from that of the benign tissue staining and are indicated by an **asterisk**. **D:** Immunostaining score stratification based on prostate histology in tissue microarray.

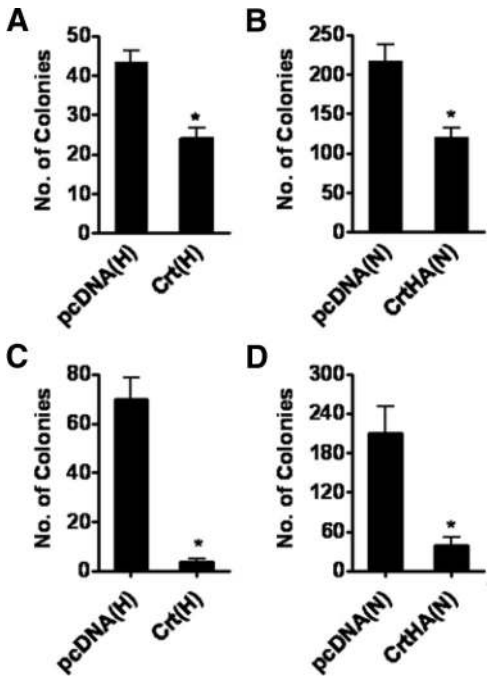


Figure 2. Inhibition of monolayer colony formation in PC3 and LNCaP cells stably transfected with calreticulin-expression vectors. **A** and **B:** PC3 cells and (**C** and **D**) LNCaP cells were stably transfected with (**A** and **C**) either a vector encoding untagged calreticulin and a hygromycin resistance cassette [Crt(H)] or the corresponding empty vector [pcDNA(H)]. Cells were also transfected with (**B** and **D**) either a vector encoding HA-tagged calreticulin and a neomycin resistance cassette [CrtHA(N)] or the corresponding empty vector [pcDNA(N)]. The number of colonies in each column represents the mean of three independent experiments. Error bars represent SEM. Statistical significance (*$P < 0.05$) was determined by Student's *t*-test (Graphpad Prism).

cancer.[40] To investigate whether calreticulin expression modulates *in vitro* growth, we performed monolayer colony formation assays using PC3 and LNCaP cells stably transfected with either calreticulin-expression or control vectors. Experiments were done with both HA-tagged and untagged exogenous calreticulin. In PC3 cells, the untagged and HA-tagged calreticulin constructs both caused a twofold decrease in colony formation as compared with the empty control vectors ($P < 0.05$) (Figure 2, A and B). In LNCaP cells, calreticulin expression caused an even greater fold inhibition of colony formation ($P < 0.05$) (Figure 2, C and D). The reason for the decrease in colony formation was not due to a decrease in the transfection efficiency of the cultures that received the calreticulin construct, because the calreticulin constructs used in our experiments did not affect the co-transfection efficiency of a GFP expression vector (data not shown). These observations suggest a growth inhibitory role for calreticulin.

Calreticulin Overexpression Inhibits Anchorage-Independent Growth in Soft Agar

To further investigate the growth inhibitory activity of calreticulin in prostate cancer cells, we tested the effect of calreticulin overexpression on prostate cancer cell growth in soft agar. Figure 3, B and C show the results from two representative PC3 clones that stably overexpress HA-tagged calreticulin, CrtHA.6 and CrtHA.10. Both clones expressed approximately twofold more calreticulin than either the parental PC3 cells alone or cells transfected with the control vector, as determined by Western blot (Figure 3A). When placed in soft agar in multiple independent experiments, CrtHA.6 and CrtHA.10 exhibited statistically-significant reductions in anchorage-independent growth as compared with control cells ($P < 0.05$, Figure 3C). As shown in Figure 3B, the diameter of the largest CrtHA.6 and CrtHA.10 colonies was approximately half that of the largest parental or the control vector-transfected PC3 colonies, which is also consistent with a growth suppressive function of calreticulin. Transfection with the control vector had no effect on growth. Anchorage-independent growth, considered an indication of cellular transformation, often corresponds with tumor growth in nude mice. Therefore, our result in soft agar assay suggests that calreticulin may possess tumor suppressive activity and so its down-regulation in prostate cancer specimens may contribute to malignant growth *in vivo*.

Calreticulin Overexpression Inhibits the Growth of Orthotopic PC3 Xenograft Tumors

To test whether calreticulin has tumor suppressive function *in vivo*, we examined the effect of calreticulin overexpression in a PC3 orthotopic xenograft tumor model in nude mice. We had initially isolated a group of six PC3 clones that overexpress calreticulin. Based on expression levels and growth properties in soft agar, we chose CrtHA.10 as the most representative clone and thus used it for subsequent *in vivo* experiments. When injected into the dorsal prostate of nude mice, the CrtHA.10 clone produced significantly less growth in the GU tract than injection with the mock control ($P < 0.05$, Figure 3D). Notably, in all cases, mice injected with the mock control cells had enlarged iliac lymph nodes, suggesting metastasis to the lymph nodes. In contrast, we observed no change in the iliac lymph nodes in the mice injected with CrtHA.10 (data not shown). However, the inhibition of the potential lymph node metastasis by calreticulin overexpression in this model could be secondary to the growth inhibition by calreticulin. The *in vivo* growth inhibition by calreticulin is consistent with the observation that calreticulin overexpression clone CrtHA.10 displayed a reduced growth rate relative to the empty vector-transfected PC3 cells (Figure 3E).

The P Domain Plays a Critical Role in the Inhibition of Colony Formation in PC3 Cells

To explore the potential mechanism of calreticulin action in prostate cancer, we mapped the domains essential for prostate cancer suppression. Calreticulin consists of three distinct domains: N, P, and C (see supplemental Figure 1A at *http://ajp.amjpathol.org*). Calreticulin is localized to ER and consists of three distinct domains: N, P, and C (supplemental Figure 1A at *http://ajp.amjpathol.org*). We

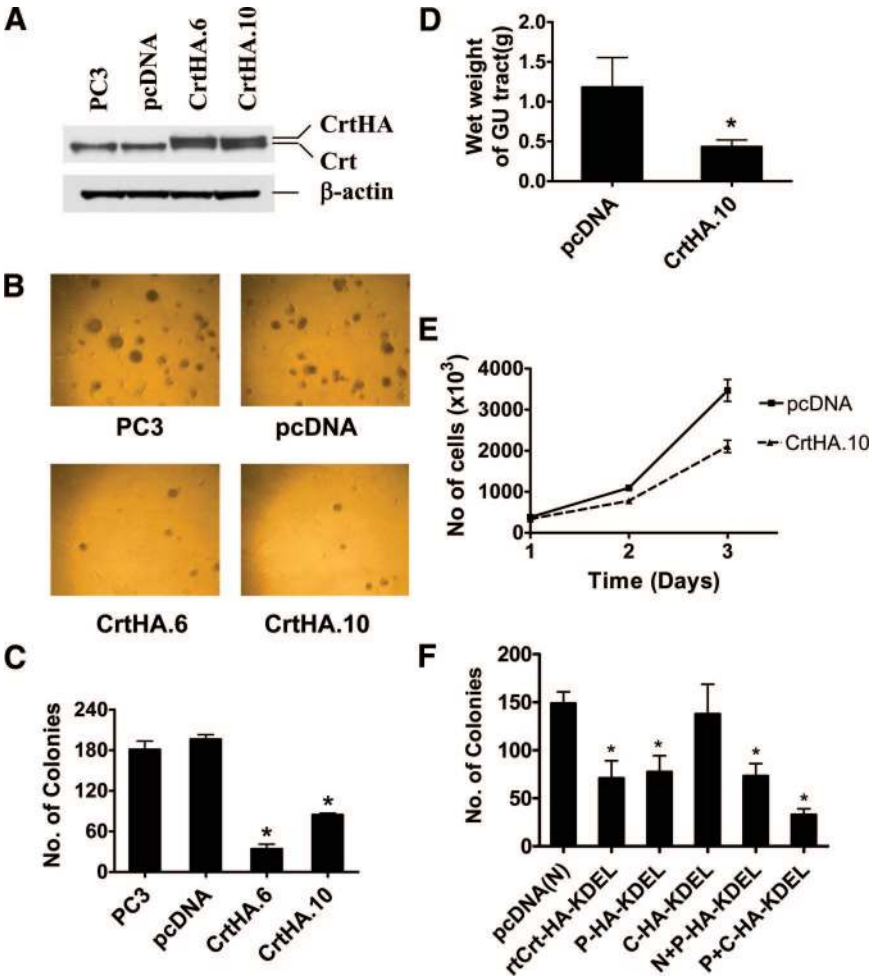


Figure 3. Overexpression of calreticulin inhibited PC3 growth both *in vitro* and *in vivo*. **A:** Western blot analysis of calreticulin expression in parental PC3 cells and PC3 sublines stably transfected with a HA-tagged calreticulin expression vector (CrtHA.6 and CrtHA.10) or empty vector (pcDNA). β-actin served as a loading control. **B:** Colony formation of the parental PC3 cells and the indicated stable sublines in soft agar. Representative pictures were taken 15 days after seeding the cells in soft agar. **C:** Quantitative analysis of colony formation of the parental PC3 cells and the indicated stable sublines in soft agar. Clones were seeded in duplicate. Clones were grown in soft agar for 15 days and then the colonies counted in five random fields per well, in both wells. The values represent the mean of three independent experiments. Error bars represent SEM. **D:** Effect of calreticulin overexpression on orthotopic PC3 xenograft tumor growth. Wet weights of the genitourinary (GU) tracts of the mice injected with the indicated PC3 sublines were determined. Each group consisted of 10 mice. Error bars represent SEM. Statistical significance ($^*P < 0.05$) was determined by Student's *t*-test (Graphpad Prism). **E:** Effect of calreticulin overexpression on PC3 cell growth *in vitro*. Error bars represent SEM. Cell numbers were counted using Coulter Counter at indicated time after plating. **F:** Effect of wild-type and mutant calreticulin constructs on PC3 growth in colony formation assays. All of the constructs were tagged with HA at the N-terminus of the KDEL signal. The number of colonies formed after transfection with the indicated vectors was determined as previously described. Values are shown as the mean of eight independent experiments with error bars representing SEM. Statistical significance ($^*P < 0.05$) was determined by Student's *t*-test (Graphpad Prism).

created mutants that expressed these individual domains or combinations of them, along with GFP and the ER-retention sequence KDEL found at the carboxyl-terminus of calreticulin. Before studying the functionality of the mutants, we assessed their subcellular localization since phenotypes arising from mislocalized protein would not be related to endogenous function. After transfection with the various expression constructs, cells were stained with an antibody that detects calnexin, an insoluble ER membrane protein that binds to calreticulin to help regulate glycoprotein folding.[41] Cells were then examined by fluorescent and phase-contrast microscopy. As expected, we found that the full-length calreticulin construct, Crt-GFP-KDEL, co-localized with calnexin in the ER (see supplemental Figure 1B at *http://ajp.amjpathol.org*). GFP-KDEL served as a control and localized evenly between the nucleus and cytoplasm in live cells (data not shown). However, fixed cells had a slightly lower fluorescent signal in the cytoplasm as compared with the nucleus (see supplemental Figure 1C at *http://ajp.amjpathol.org*), a possible result of the fixation or permeabilization process. In the case of the N-domain GFP fusion (N-GFP-KDEL), all cells exhibiting GFP fluorescence had a rounded phenotype consistent with cell death (see supplemental Figure 1C at *http://ajp.amjpathol.org*). Not surprisingly, very few cells exhibited green fluorescence because virtually all of the N-GFP-KDEL transfected cells were unable to survive (data not shown). Since N-GFP-KDEL appeared to have toxic effects, further analyses with this construct were not undertaken. With the remaining constructs, the majority of fluorescing cells produced a pattern consistent with ER localization. The calnexin signal and the GFP signal from the calreticulin-domain mutants co-localized in the merged image (see supplemental Figure 1C at *http://ajp.amjpathol.org*). Supplemental Figure 1D (at *http://ajp.amjpathol.org*) displays a graphical representation of calreticulin localization in over 100 fluorescent cells per transfected construct. All of the domain mutants, except the N-GFP-KDEL, displayed predominant ER localization and were used in further studies described in the manuscript.

We next investigated the ability of the domain mutants to affect growth using a colony formation assay. For this experiment we used the smaller HA tag instead of the GFP tag to minimize the possibility of functional interference. The P-HA-KDEL was able to inhibit colony formation at a level similar to that seen with the full-length protein (Figure 3F), suggesting that the P domain plays a key role in calreticulin suppression of PC3 colony formation. Transfection with N+P-HA-KDEL resulted in a similar number of colonies as transfection with P-HA-KDEL. No-

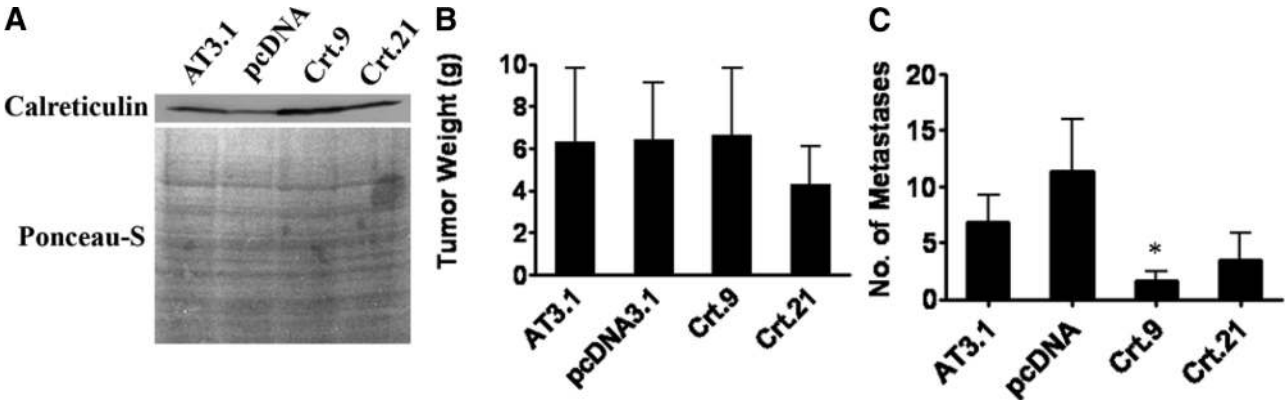


Figure 4. Calreticulin overexpression inhibits lung macrometastasis in the rat Dunning AT3.1 subcutaneous xenograft tumor model. **A:** Expression of calreticulin in the transfected rat Dunning AT3.1 prostate cancer cell line. Western analysis was performed to assess the expression of calreticulin in parental AT3.1 cells, as well as in AT3.1 cells transfected with empty vector (pcDNA), and calreticulin-expression vectors (Crt.9 and Crt.21). The loading of whole protein extract was evaluated by Ponceau-S staining. Densiometric analysis allowed for the determination of relative expression levels. **B:** Effect of calreticulin expression on primary tumor wet weight in the AT3.1 xenograft tumor model. The primary tumors were excised and weighed after sacrifice. **C:** Effect of calreticulin overexpression on AT3.1 xenograft tumor lung macrometastasis in nude mice. The parental Dunning AT3.1 and indicated sublines were injected subcutaneously into 4- to 6-week-old male athymic nude mice. Ten mice were used for each indicated cell lines. Twenty days after injection, the tumor-bearing mice were sacrificed according to a procedure approved by the institutional animal care and use committee. The lungs were fixed in Bouin's solution and lung macrometastases were scored. Error bars represent SEM. Statistical significance ($^*P < 0.05$) was determined by Student's *t*-test (Graphpad Prism).

tably, transfection with P+C-HA-KDEL produced the fewest colonies (Figure 3F).

Calreticulin Overexpression Reduces Lung Macrometastases in the Rat Dunning AT3.1 Xenograft Tumor Model

Calreticulin influences cell adhesion to the substratum, possibly through its ability to bind to integrin or to modulate expression of cytoskeleton proteins.[20,21,42,43] The fundamental relationship between cell adhesion and metastasis[44] suggests a possible role for calreticulin in metastasis. To examine a potential role for calreticulin in metastasis, we used a rat Dunning subcutaneous xenograft tumor model that produces countable lung macrometastases in nude mice.[45–47] We established two AT3.1 stable clones that overexpress calreticulin, Crt.9 and Crt.21, as well as a mock-transfected clone. Western analysis demonstrated that AT3.1 cells transfected with the control vector expressed approximately 50% less calreticulin than the parental AT3.1 cells (data not shown). At the same time, Crt.9 expressed approximately 1.5-fold more calreticulin than the parental line and threefold more than the mock line (Figure 4A). Crt.21 expressed calreticulin at a level slightly higher than that in the parental line. Interestingly, increased calreticulin expression did not inhibit the growth of AT3.1 cells in culture (data not shown) or the growth of primary tumors in nude mice (Figure 4B). However, mice injected with Crt.9 had a statistically significant decrease in the number of lung metastases ($P < 0.05$, Figure 4C). These observations argue for a metastasis-suppressive role of calreticulin, independent of growth, in prostate cancer.

Discussion

Although many studies have shown that calreticulin is a multifunctional protein essential for animal development, whether this protein plays a role in human cancer pathology is not entirely clear. Based on the evidence presented here, we propose that calreticulin contributes to the suppression of prostate cancer. Immunostaining of human prostate cancer specimens from hormone-manipulation–naïve patients showed the down-regulation of calreticulin in a subset of prostate cancer specimens. Moreover, the introduction of exogenous calreticulin inhibited colony formation of LNCaP and PC3 cells in independent experiments. Calreticulin overexpression also inhibited tumor growth in an *in vivo*, orthotopic PC3 xenograft tumor model, and decreased PC3 anchorage-independent growth in soft agar. Calreticulin was reported to increase cell-to-cell and cell-to-substratum adhesion, suggesting a potential role for calreticulin in suppressing metastasis. To test the potential role of calreticulin in prostate cancer metastasis, we used the AT3.1 rat Dunning model.[45] While overexpression of calreticulin suppressed the growth of primary orthotopic PC3 tumors in nude mice, it had no effect on the growth of primary subcutaneous AT3.1 tumors. Further studies will be needed to determine whether calreticulin function in tumor xenografts is influenced by implantation site or is cell line-specific. Regardless, the lack of growth inhibition by calreticulin in the subcutaneous AT3.1 model made it an excellent choice for testing the effects of calreticulin on metastasis. As expected, calreticulin overexpression inhibited macroscopic lung metastasis from the primary Dunning AT3.1 tumor. The ability of calreticulin to suppress tumor metastasis is consistent with its ability to enhance cell adhesion.[20,42,43] Increased expression of calreticulin resulted in the increased cell–substratum and cell–cell adhesiveness and was likely a result of an increase in vinculin and N-cadherin,[21,43] proteins essential for cell–cell adhesion. Subsequent studies have implicated that calreticulin's role as a modular of Ca^{2+} homeostasis may regulate cell adhesion by affecting kinase-dependent adhesion to fibronectin via c-Src and CaMKII activity.[48–50] The existence of different mechanisms for calreticulin to modulate cell adhesion suggests that calreticulin suppression of tu-

mor metastasis may involve multiple pathways. In the AT3.1 xenograft tumor model, calreticulin may inhibit the release of cancer cells from the primary tumor, the growth of tumor metastasis, and/or additional steps involved in metastasis. Further studies will be needed to clarify the mechanism by which calreticulin inhibits metastasis.

Based on structure/functional studies, calreticulin consists of three domains: N, P, and C.[31] Before investigating the potential contributions of the individual domains to prostate cancer suppression, we first evaluated their subcellular localization using domain mutants tagged with GFP (see supplemental Figure 1 at *http://ajp.amjpathol.org*). Our studies showed that the individual P and C domains, like the full length protein, localized to the ER of PC3 cells. The N domain, however, induced cell death. Interestingly, N + P fusion proteins were more likely to localize to the ER than those with P domain alone. Thus, the possibility exists that the N domain may modulate P domain localization in the ER. We also observed that P + C domain-containing fusion proteins were more likely to localize to the ER than fusion proteins containing C or P domain alone. This may reflect an enhancement in ER localization due to the combination of two ER localization signals. The construct GFP-KDEL served as a control for these experiments. When at the carboxyl-terminus, KDEL is thought to act as an ER retention signal for calreticulin.[51] However, KDEL was not sufficient for the ER localization of GFP as we found GFP-KDEL in both the nucleus and cytoplasm, but not specifically in the ER (supplemental Figure 1C at *http://ajp.amjpathol.org*). This also indicates that other regions in calreticulin are important for the ER localization of the protein, supporting a previous report that multiple mechanisms maintain calreticulin in the ER.[51]

Colony formation assays suggested that constructs containing the P domain inhibited the growth of PC3 cells. The P domain alone produced similar inhibitory effects as the full-length protein whereas the C domain alone did not inhibit colony formation, suggesting that the functions associated with C may not be critical in the suppression of metastasis. While we were not able to assess the function of the N domain alone due to its cytotoxicity, the addition of the N domain to the P domain did not significantly enhance the inhibitory activity of the P domain. In contrast, a combination of the C + P domains displayed a greater ability to inhibit colony formation than both the P domain alone and full-length calreticulin. These observations suggest that the individual domains may modulate each other's activity as well as location.

As the P-domain is sufficient to inhibit colony formation in PC3 cells, the functions associated with this domain are potentially important in the suppression of prostate cancer growth. The proline-rich P-domain (aa 180 to 303) contains two sets of sequence repeats. Studies indicate that the P domain, which displays a high degree of homology with calnexin, is a site of chaperone activity and oligosaccharide binding and, together with the N domain, is responsible for chaperone activity.[31] The P domain forms an extended-arm structure and can bind to other chaperones, such as ERp57, in the lumen of the ER. Although the P domain contributes little to calcium storage, this high affinity-low capacity calcium binding domain is responsible for the calreticulin inhibition of repetitive intracellular calcium waves.[52] However, other studies have shown that calcium and zinc responsive domains are restricted to the N and C regions of calreticulin.[53] This suggests that the effects of calreticulin on prostate cancer growth are mediated through the protein's chaperone activity, interaction with its binding partners, or some other, as yet undefined, function. Since the P domain can modulate multiple cellular activities, further studies will be required to determine which of these activities is responsible for the growth inhibition of prostate cancer cells. It is important to recognize that the other domains of calreticulin, N and C, may also modulate tumor suppression.

The finding that calreticulin suppresses growth of prostate cancer cells suggests that the protein may also contribute to the homeostasis of the normal prostate. The normal prostate is constantly exposed to androgens. Androgens significantly induce calreticulin expression in the luminal epithelial cells of the prostate. Given the ability of calreticulin to suppress growth, the androgen-induced expression of the protein should restrict the growth of the prostate. The down-regulation of calreticulin in prostate cancer cells, in turn, may disrupt the androgen-dependent homeostasis of the prostate, contributing to the continuous proliferation of the cancerous cells in the prostate.

In conclusion, our studies argue for a suppressive role of calreticulin in prostate cancer. We present evidence that calreticulin suppresses tumor growth and metastasis both *in vitro* and *in vivo*. Results presented here suggest that the growth suppression is primarily mediated through the P domain. The suppressive role of calreticulin is consistent with its down-regulation in a subset of human prostate cancer specimens. Our findings establish a link between calreticulin and a specific human disease, prostate cancer.

Acknowledgments

We thank Moira Hitchens and Laura Pascal for critical reading and editing and Marie Acquafondata, Katherine O'Malley, and Junkui Ai for technical assistance. We also are grateful to an anonymous reviewer for insightful suggestions.

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