

Paracrine Calcitonin in Prostate Cancer is Linked to CD44 Variant Expression and Invasion

KENNETH A. ICZKOWSKI^{1,2}, A. LEVI OMARA-OPYENE¹,
TRUPTI R. KULKARNI³, MEGHA PANSARA¹ and GIRISH V. SHAH³

¹Department of Pathology, Immunology, and Laboratory Medicine of The University of Florida and

²Veterans Administration Medical Center, 1601 S.W. Archer Road, Gainesville, FL 32608-1197;

³Department of Pharmaceutical Sciences, School of Pharmacy, 700 University Avenue,
University of Louisiana, Monroe, LA 71209-0470, U.S.A.

Abstract. *Background:* Calcitonin (CT) exerts an autocrine/paracrine influence on prostatic tumor invasion through coupling to transduction protein $G_s\alpha$. Cell adhesion glycoprotein CD44 variant v7-v10 also facilitates invasion, but its modulation by the CT- $G_s\alpha$ system was unexplored. *Materials and Methods:* LnCaP, PC-3 and metastasis-derived PC-3M cell lines were studied, including cells modified therefrom: $G_s\alpha$ -QL, expressing mutant constitutively active $G_s\alpha$ protein, and CT+, overexpressing CT. CD44 variant expression was evaluated *in vivo* after orthotopic implantation into nude mice, and *in vitro* by real-time RT-PCR and Western blotting. *Results:* Both mRNA and protein levels of the CD44 variant were minimal in PC-3M tumor implants, but elevated in $G_s\alpha$ -QL. Exogenous CT stimulated invasion into Matrigel strongly in LnCaP and CT+, and less in PC-3 and $G_s\alpha$ -QL. By Western blot analysis, untreated $G_s\alpha$ -QL and CT+ cells overexpressed CD44 variant compared with LnCaP or PC-3. By quantitative RT-PCR, exogenous CT dose-dependently increased CD44 variant mRNA to seven-fold. Pharmacologic agents that stimulated or inhibited $G_s\alpha$ activity or stimulated adenylyl cyclase produced proportionate dose-dependent effects on both CD44 variant expression and Matrigel invasion. *Conclusion:* This paracrine factor, acting through cyclic AMP, regulates the expression of CD44v7-10, which modulates the tumor phenotype.

Correspondence to: Kenneth A. Iczkowski, M.D., Pathology and Laboratory Medicine Service (113), VA Medical Center, 1601 S.W. Archer Road, Gainesville, FL 32608-1197. Phone: (352) 376-1611 x 4522; FAX: (352) 379-4023; e-mail: iczkoka@pathology.ufl.edu

Key Words: Calcitonin, CD44, G-protein, invasion, paracrine, prostate cancer, signal transduction.

Normal prostatic epithelium contains receptors for a variety of neuroendocrine factors that exert a mitogenic influence. One of them is the 32-amino acid peptide calcitonin (CT). We have demonstrated, by both *in situ* hybridization and immunohistochemistry in prostate cancer, that CT expression is increased in proportion to tumor grade (1). Exogenously added CT stimulates the proliferation of normally slow-growing LnCaP prostate cancer cells by increasing cAMP concentration and cytoplasmic Ca^{2+} transients, and increases their chemotaxis by currently unknown mechanisms (2, 3). Moreover, both primary prostatic tumor and cultured prostate cancer cells express high-affinity CT receptors (2), establishing an autocrine/paracrine role for CT. The receptor site for CT has been demonstrated to be coupled with the membrane-bound stimulatory GTP-binding protein, $G_s\alpha$ (4, 5). Ligand-induced and/or constitutive activation of $G_s\alpha$ in prostate cancer augments tumor growth (6, 7) and invasion (8). We (G.V.S.) have constructed PC-3M cell variants called CT+ and $G_s\alpha$ -QL, which stably express CT or constitutively active $G_s\alpha$, respectively. $G_s\alpha$ -QL cells show increased cAMP accumulation, increased DNA synthesis (7), faster proliferation, greater invasiveness *in vitro*, and more metastasis formation *in vivo* (8).

Cell adhesion molecules such as transmembrane glycoprotein CD44, by forming complexes with extracellular matrix protein, facilitate tumor cell dyshesion and migration through a matrix. The *standard* isoform of CD44 (CD44s) results from expression of its exons 1-5 and 16-20 with intervening exons spliced out; longer splice *variant* isoforms (CD44v1-10) result from altered mRNA processing to include sequences from one or more of exons 6-15. CD44s expression is ubiquitous, whereas CD44v are restricted to epithelia. Carcinomas express novel and increased variant isoforms, reflecting dysregulated mRNA processing. In

several studies from this laboratory, we have shown that a variant isoform of CD44 is characteristically present in prostate cancer, but not benign prostate (9-12). In this paper, we show that exogenous or endogenous CT stimulates invasion to varying extents in four prostate cancer cell lines that represent the spectrum of invasiveness. Given that the CD44v7-10 isoform is characteristically overexpressed in primary (9-11) and metastatic (11) prostate cancer and facilitates cell invasion (11), we sought to determine whether its expression was altered by endogenous or exogenous stimulation of CT- $G_s\alpha$ signaling. We verified that this interaction existed and that $G_s\alpha$ and CT+ cells also overexpressed CD44v7-10. Moreover, we discovered that pharmacologic manipulation of $G_s\alpha$ or adenylyl cyclase significantly modulated CD44v7-10 expression and invasion.

Materials and Methods

Cell lines and culture. LnCaP cells were purchased (American Type Culture Collection (ATCC), Manassas, VA, USA) and incubated in RPMI 1640 with L-glutamine, 10% fetal calf serum, and antibiotics at 37°C in a 5% CO₂ incubator. PC-3 cells (also from ATCC) were incubated in Ham's F12-K medium plus the above supplementary ingredients. The CT+ cell line was prepared by stably transfecting PC-3 metastatic (PC-3M) tumor cells with mammalian expression vector pcDNA3.1, containing cDNA to express the CT peptide constitutively. The second line, $G_s\alpha$ -QL, was prepared by transfection with this same vector containing cDNA for $G_s\alpha$ -QL, a mutant, constitutively active $G_s\alpha$ protein that lacks GTPase activity (6). PC-3M, $G_s\alpha$ -QL and CT+ cells were maintained in complete medium (RPMI 1640 supplemented with L-glutamine, 5% fetal calf serum, 12% horse serum, 50 U/mL penicillin, 50 µg/mL streptomycin and 20 µg/mL fungizone).

Prostatic tumor formation in vivo. Metastasis-derived PC-3M parental cells, or those stably expressing plasmids for either wild-type $G_s\alpha$ or $G_s\alpha$ -QL (8), were orthotopically injected into dorsolateral lobes of the prostates of nude mice. The protocol used 1x10⁶ cells in 20 µl Hank's Balanced Salt Solution, as described previously (13). Each treatment group included five to eight mice. The mice were monitored for tumor growth and distant metastases in lymph nodes by palpation. Necropsy was performed 5 weeks after the implantation and tumor tissue was harvested from the prostates (8). The specimens were formalin-fixed and paraffin-embedded.

Effect of calcitonin on cell invasion. Studies were performed in six-well two-tier invasion chambers (Collaborative Biomedical Products, Bedford, MA, USA). 2.5x10⁵ LnCaP, PC-3, $G_s\alpha$ -QL, or CT+ cells per well were seeded in the upper insert in 2 mL serum-free basal medium (RPMI 1640 medium containing 0.1% BSA, 150 mg/ml of G418, 4 mM L-glutamine, 100 µg/ml penicillin G and 100 µg/ml streptomycin). The lower chamber contained 2 mL of chemoattractant medium: 70% complete medium, 10% fetal bovine serum, plus 20% conditioned medium obtained from subconfluent cultures.

CT was obtained (Peninsula Laboratories, San Carlos, CA, USA) and added to medium at a 50 nM final concentration, more

than sufficient to induce DNA synthesis (7) for test groups of each of these four cell lines. CT was omitted from control groups of each of the four cell lines. The incubations were carried out for 36 h. After this period, upper inserts were removed, and uninvaded cells removed from the upper Matrigel surface using cotton swabs. The invasive cells should penetrate through the Matrigel layer and be situated on the outside bottom of the upper insert. Gel inserts were fixed, stained using Diff Quik staining (Dade Diagnostics, Aguilar, PR, USA), and mounted on glass slides. The total number of cells on the entire gel was counted. Based on three experimental trials, the absolute numbers of treated invaded cells were normalized to those of control cells (11), and the results were reported as mean % ± standard error.

In situ hybridization for CD44v9. CD44v9 mRNA was evaluated in tumors grown in nude mice. An antisense DNA probe for CD44v9 was designed (Invitrogen, Carlsbad, CA, USA) as follows: Biotin-AAAGGACCCAGTCTTAGCTGAGGTCAGTGGGA-Biotin. A sense probe was designed in the reverse sequence. Sections from paraffin-embedded tissue were floated in DEPC-treated water baths for slide preparation. *In situ* hybridization was performed as previously (10). Briefly, after deparaffinization, tissue was digested with Proteinase K (DAKO, Carpinteria, CA, USA). The probe was diluted to a final concentration of 100 nM in CEP Hybridization buffer (Vysis, Downers Grove, IL, USA) delivering 20 ng/slide, heated to 80°C to prevent secondary structure formation, coverslipped, and hybridized at 37°C overnight. Two stringency washes were done using 2X SSC with 0.1% Nonidet P-40 at 73°C for 5 min. Slides were transferred to PBS pH=7.4 and peroxide block was performed for 10 min with 3% H₂O₂ in methanol. Detection was accomplished using the tyramide signal amplification method of the GenPoint kit (DAKO).

Immunohistochemical staining. CD44v7-10 reactivity was evaluated in tumors grown in nude mice. Five µm sections were deparaffinized in xylene and alcohols and soaked for 30 min in Tris-buffered saline, pH=7.5 with 0.1% Tween-20 (TBST). Slides were subjected to steam heat antigen retrieval in citrate buffer, pH=6 for 2 x 30 min. Slides were quenched in 3% H₂O₂ in methanol for 10 min and rinsed well in distilled water, then in TBST. An "Inhibitor solution" (Ventana) was applied for 4 min followed by blocking antibodies for 10 min (20% normal swine serum in Tris-HCl, pH=7.6). Specific monoclonal anti-CD44v7/8 antibody (SeroTec, Raleigh, NC, USA) was used at 1:100 or anti-CD44v9 antibody from the supernatant of mouse hybridoma cells HB-258 (American Type Culture Collection, Manassas, VA, USA) was used neat. The specificity of these antibodies was confirmed by Western blot using colon cancer as a positive control. Antibody was applied at 4°C, overnight. Biotin block (Ventana) was applied for 3 min. A secondary antibody (Dako LSAB Kit mouse/goat/rabbit) was applied for 25 min. Slides were rinsed in TBST, covered with avidin-biotin complex for 25 min, and rinsed in TBST. Diaminobenzidine served as the chromogen, and slides were counterstained with hematoxylin. Negative controls consisted of application of non-immune whole rabbit serum at 1:300 dilution.

SDS-polyacrylamide gel electrophoresis and Western blot analysis for CD44v9. A pellet from treated or untreated cultured cells was lysed in RIPA lysis buffer (Upstate Biologicals, Lake Placid, NY, USA) freshly supplemented with the protease inhibitors: 2 µg/mL Pepstatin A, 1.5 µg/mL Leupeptin and 1 mM PMSF. The cell lysate

was treated with an equal volume of 2% SDS and the protein concentration was estimated by the Lowry method.

One part of 5x sample buffer (formulated as described by Bio-Rad, Hercules, CA, USA) was added to four parts of the solubilized proteins containing 40 µg of sample/lane. The proteins were then denatured for 5 min in 100°C boiling water bath and loaded into wells of a 4% stacking gel (10). Five µL of protein marker (RPN 756V Amersham Pharmacia, Piscataway, NJ, USA) were also loaded on each gel. SDS-PAGE was performed according to the Laemmli method (15) using 12% polyacrylamide gels. After electrophoresis, the protein was blotted onto nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad). The membrane was hybridized overnight to primary antibody. CD44v7-10 expression was assessed using neat HB-258 supernatant, as detailed in the immunostaining section above. Goat anti-mouse IgG labeled with horseradish peroxidase (Pierce, Rockford, IL, USA) was used as a secondary antibody at 1:10,000 dilution. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce, Rockford IL, USA), or a colorimetric system (Opti-4CN kit, Bio-Rad). To verify equal protein loading, all membranes were next re-hybridized with mouse monoclonal anti- α -tubulin (clone DM1A, Sigma, St. Louis, MO, USA) at 1:2000 dilution and developed as above. A 50-kD band was expected.

Real-time RT-PCR for CD44 variant. A pellet was obtained from PC-3M cells that had been plated at 20,000/well in two 6-well plates. At 70% confluency, cells were serum-starved for 8 h, then four wells were treated for 12 h with each of the following: 0, 1.0, 10, or 100 nM CT. Total RNA was extracted in Trizol (Invitrogen), resuspended in 20 µl of diethylpyrocarbonate-treated water, and its concentration was measured. Complementary DNA (cDNA) was synthesized from 4 µg total RNA in a 20 µl reaction mixture, with reverse transcriptase buffer: (Tris 500 mmol/L, pH 8.3, potassium chloride 500 mmol/L, magnesium chloride 80 mmol/L, dithiothreitol 100 mmol/L), 10 units RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), oligo(dT)₁₅ primer 25 µmol/L (Promega), 2 mmol/L each of dNTP and 150 units avian myeloblastosis reverse transcriptase (Invitrogen). PCR reaction used 4 µg cDNA plus the manufacturer's master mix (Applied Biosystems, Foster City, CA, USA) in a volume of 25 µL. Samples were run in triplicate with primers and probe for CD44v; samples were also amplified with 18S ribosomal RNA primers and detected with an 18S probe (all Applied Biosystems). The amplification protocol was as follows: hold 50°C 2 min, 95°C 10 min, then 40 cycles of (95°C for 0:15 and 60°C for 1:00) using the iCycler iQ system (Bio-Rad). Data were analyzed by the 2- $\Delta\Delta C_T$ method (14) to determine the fold change in gene expression. Taking ΔC_T to represent the difference between the CD44v C_T and the 18S ribosomal RNA C_T , the $\Delta\Delta C_T$ (Mean C_T of treated – mean C_T of calibrator) was computed for each sample using the mean ΔC_T of untreated cells as the calibrator. Two raised to – (this power) represented the fold mRNA expression.

Pharmacologic manipulation of $G_s\alpha$ protein and adenylyl cyclase. To stimulate the $G_s\alpha$ protein in PC-3 cells, the non-hydrolyzable GTP analog Gpp(NH)p was obtained (Sigma). Inhibition of $G_s\alpha$ protein activity in $G_s\alpha$ -QL cells was accomplished using a non-hydrolyzable GDP analog, GDP- β S (Sigma), for which the effective dose was expected to be up to 200 µM (16). Cultured cells were permeabilized with saponin at a 0.01% concentration for 7 min,

then the saponin was removed and G-protein drugs (or for controls, no drug) were added. To compare the effect of stimulating adenylyl cyclase in PC-3 and $G_s\alpha$ -QL cells, forskolin was obtained (Sigma). Cells were treated with 5-100 µM forskolin dissolved in DMSO and untreated cells received DMSO alone. All stimulatory or inhibitory drugs were added to 4 mL cell culture at graded concentrations, for 36 h, after which cells were harvested for protein preparations and immunoblotting as above. In separate experiments, 250,000 cells/well were treated with these same pharmacologic agents while Matrigel invasion assays were carried out as detailed in the second paragraph of Materials and Methods.

Results

$G_s\alpha$ activity correlates with CD44v9 expression in vivo. *In situ* hybridization and immunostaining for CD44v9 were performed on sections of tumor grown in nude mice, consisting of PC-3M cells, $G_s\alpha$ -wild-type and $G_s\alpha$ -QL cells. The PC-3M cells showed virtually no mRNA signal (Figure 1A), the $G_s\alpha$ -wild-type cells showed slight cytoplasmic reactivity (Figure 1B), and the $G_s\alpha$ -QL cells showed intense reactivity (Figure 1C). Immunostaining demonstrated similar trends for CD44v9 protein (Figures 1D-F) and CD44v7/8 (data not shown).

Effect of exogenous or endogenous CT or constitutively active $G_s\alpha$ on invasion. The effect of CT on Matrigel invasion was tested in the normally noninvasive LnCaP cells and invasive PC-3, $G_s\alpha$ -QL and CT+ cells (Figure 2). The number of invaded LnCaP cells per insert was the smallest with or without added CT, but the addition of CT more than doubled the number of invaded cells compared to that of controls. An increase of similar magnitude was seen in CT+ cells. Lesser increases in invasion were noted in PC-3 cells. $G_s\alpha$ -QL cells exhibited the highest invasiveness among the cell lines tested in this study, and exogenous CT did not significantly increase invasiveness.

Relationship between CT and/or $G_s\alpha$ and CD44v9 expression in vitro. Since CD44v9 expression facilitates invasion, and CT increased the invasiveness of cell lines, we tested the effect of CT on CD44 variant expression. Triplicate Western blot analyses for CD44v9 were performed in four cell lines spanning the spectrum of invasiveness. The highest CD44v9 expression was seen in unstimulated $G_s\alpha$ -QL and CT+ cells, by comparison to the less invasive PC-3 and LnCaP. Addition of CT caused an increased CD44v9 protein expression in all four cell lines tested (Figures 3A-B). The increases in $G_s\alpha$ -QL and CT+ cells were not as marked as in LnCaP and PC-3 cells and this probably reflects the high baseline expression of CD44 variant in unstimulated $G_s\alpha$ -QL and CT+ cells. At the mRNA level, exogenous CT at doses of 1 or 10 nM doubled expression in PC-3M cells, and 100 nM resulted in 7-fold higher mRNA levels (Figure 4).

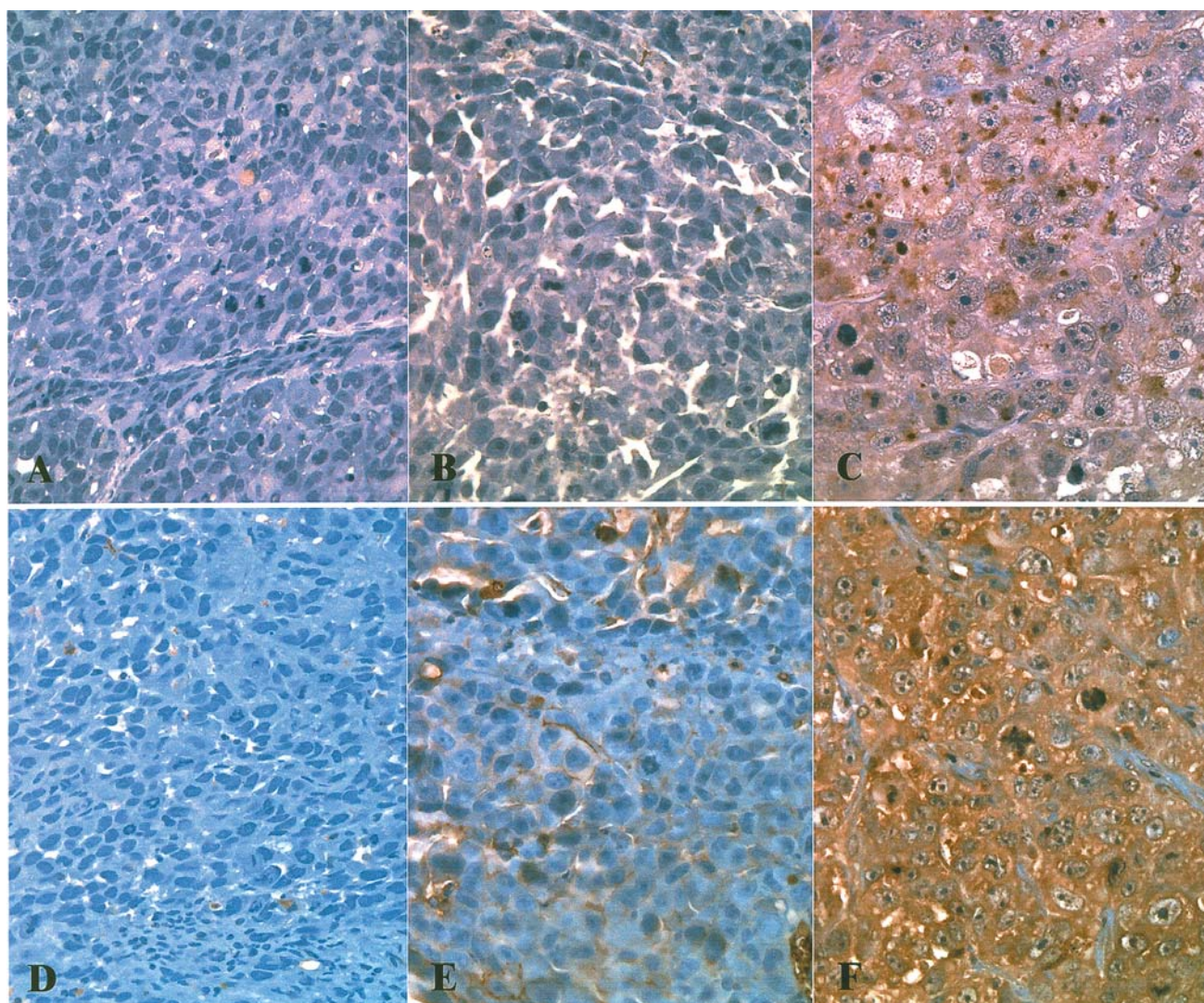


Figure 1. *CD44v9* messenger RNA and protein in tumor resulting from orthotopic implantation in nude mouse prostates (all 600x). A-C. *In situ* hybridization. Parental PC-3M cells have no signal (A), but minimal signal is seen in PC-3M cells transfected with a wild-type $G_s\alpha$ plasmid (B), and more abundant signal is noted in $G_s\alpha$ -QL cells, expressing mutant, constitutively active $G_s\alpha$ (C). D-F. Immunohistochemistry. Parental PC-3M cells have no reactivity (D). Cytoplasmic reactivity is seen in PC-3M cells transfected with a wild-type $G_s\alpha$ (E). $G_s\alpha$ -QL cells demonstrate the strongest reactivity (F). The expression is proportional to the differential $G_s\alpha$ protein expression among these cell lines. Therefore, this hormonal signaling system activates *CD44v7-10* transcription.

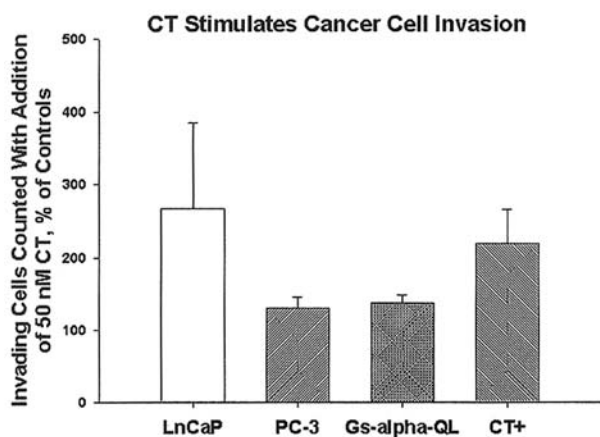


Figure 2. Comparative counts of invasive cells after addition of 50 nM exogenous CT peptide to the upper chamber of Matrigel assays. Invasion was stimulated in four prostate cancer cell lines. Bar graph represents the mean number of invaded cells, normalized to untreated controls, based on triplicate trials \pm SD. Increases in invasion by cell line (with unstimulated and stimulated counts in parentheses) were LnCaP, 167% (20-80 and 100-170); PC-3, 30% (626-8604 and 865-8779); $G_s\alpha$ -QL, 38% (969-5820 and 1540-7354); CT+, 119% (829-1425 and 1446-4418). LnCaP results were based on small numbers, precluding estimation of significance. Results may reflect that PC-3 does not express detectable CT receptor mRNA, whereas the PC-3M derived $G_s\alpha$ -QL and CT+ cells and LnCaP do (7).

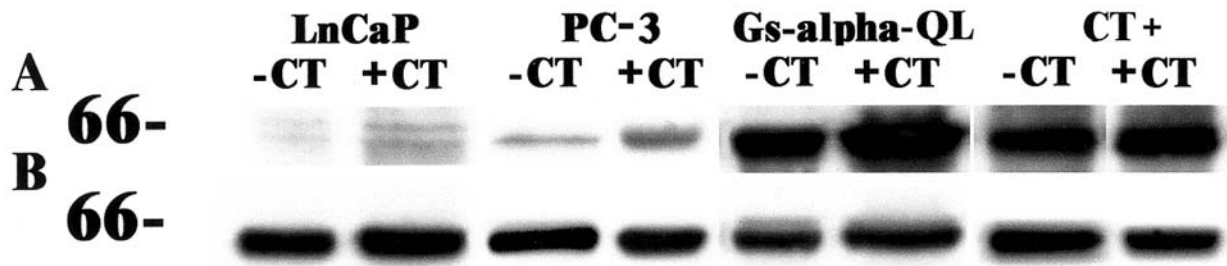


Figure 3. Western blot analyses for CD44v9 in prostate cancer cells. A. Untreated samples of $G_s\alpha$ -QL and CT+ consistently demonstrate higher expression of CD44v9 cleavage product at 66 kD than do LnCaP or PC-3 cells. After exposure of 250,000 cells to no agent or 50 nM CT, increased expression was seen in LnCaP and PC-3 cells which had lower baseline expression, while increases were marginal in $G_s\alpha$ -QL and CT+ cells in which baseline expression was higher. We postulate that these alterations in CD44 variant expression are mediated through the linkage of the CT receptor to $G_s\alpha$ protein. B. α -tubulin loading control.

Pharmacologic activation of $G_s\alpha$ or stimulation of adenylyl cyclase stimulates CD44v9 expression. To examine the role of $G_s\alpha$ in the CT-induced increase in CD44v9 expression, we acutely activated $G_s\alpha$ with graded concentrations of stimulatory drug Gpp(NH)p, as described in Materials and Methods. The cell lysates were then processed for Western blot analysis. Dose-dependent increases in CD44v9 were observed. At the highest doses, the PC-3 cells expressed CD44v9 strongly, similar to untreated $G_s\alpha$ -QL cells (Figures 5A-B). Functionally, the result was 50% increased Matrigel invasion. To examine the role of adenylyl cyclase induction of CD44v9 by CT, we tested the effect of forskolin, a direct activator of adenylyl cyclase. Forskolin dose-dependently increased CD44v9 expression in the LnCaP and PC-3 cells (Figures 5C-D). Treatment of all cell lines with the maximally effective forskolin dose caused the greatest increases in Matrigel invasion in LnCaP and PC-3 (Figure 5E). Conversely, the $G_s\alpha$ protein inhibitor GDP- β S reduced CD44v9 expression in $G_s\alpha$ -QL cells to the level of the PC-3 cells, and this effect was dose-dependent up to 200 μ M (Figures 5F-G). The drug inhibited Matrigel invasion of $G_s\alpha$ -QL by 32%.

Discussion

Our study produced three related findings: (i) Calcitonin (CT), like CD44 variant v7-10 (11), has functional significance in prostate cancer since it stimulates invasion. Invasion *in vitro* was increased by endogenous or exogenous CT in four cell lines tested. (ii) Endogenous or exogenous stimulation of the CT- $G_s\alpha$ protein system in prostate cancer cells correlated with expression *in vitro* and *in vivo* (in xenografts) of the CD44v7-10 sequence that is characteristically overexpressed in prostate cancer and facilitates invasion (10, 11). (iii) The influence of CT- $G_s\alpha$ on CD44v7-10 expression and invasion could be modulated by applying drugs that stimulate or inhibit $G_s\alpha$ activity or that stimulate adenylyl cyclase.

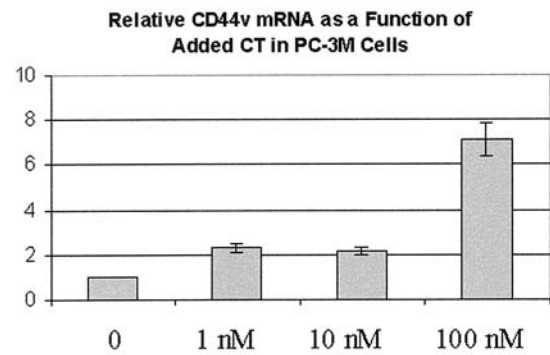


Figure 4. Real-Time RT-PCR. In PC-3M cells treated with 0, 1, 10, or 100 nM CT, the ΔC_T values for CD44 variant mRNA were 5.97, 4.76, 4.87 and 3.14, respectively. Using a normalization method (14), these values translate to relative mRNA expression of 1 (control), 2.30, 2.14 and 7.10, respectively.

CT is a 32-amino acid peptide described in the prostate (17) that is secreted by primary prostatic epithelium and at several-fold higher levels in cancer than benign epithelium (18). CT can bind in a saturable fashion to plasma membrane fractions of prostate cancer cells (2). Furthermore, its relevance to *in vitro* growth was studied in explant-derived primary prostate cancer; exogenous CT induced a two-fold increase in DNA synthesis, and anti-CT serum caused a 56% decline (7). CT is also of special interest because its messenger RNA (1, 7) and protein (1) localize to neoplastic secretory cells, whereas expression of other neuroendocrine markers is restricted to the smaller population of neuroendocrine cells that accompany prostatic neoplasia (17). Indeed, we have shown that CT is increased in proportion to tumor Gleason grade and tumor stage (1). CT, thus, may be a determinant of prostate cancer aggressiveness, helping to discriminate the approximate 10% of cases that show the poor prognostic findings of extraprostatic spread or

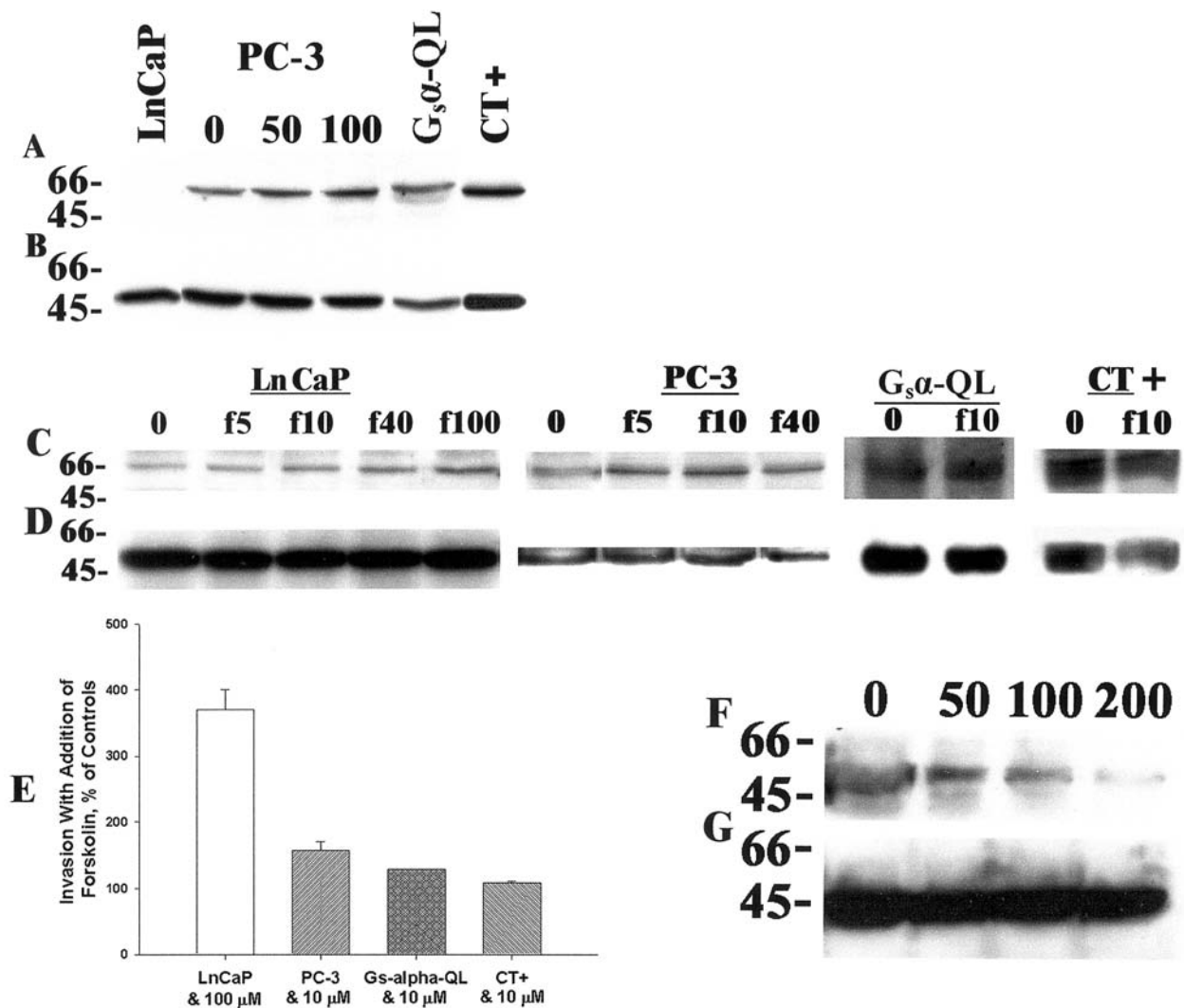


Figure 5. Western blots for CD44v9 showing influence of the $G_s\alpha$ protein and adenylyl cyclase in 250,000 treated or untreated cells. A. PC-3 prostate cancer cells have been exposed to 50 or 100 μ M of G-protein stimulatory drug Gpp(NH)p. After 36 hours, the treated PC-3 cells (lanes 2-3) express CD44v9 at a higher level, similar to untreated $G_s\alpha$ -QL cells (lane 4). Untreated LnCaP cells are also shown (lane 5). B. α -tubulin loading control. Functionally, Matrigel invasion of 250,000 PC-3 cells exposed to 100 μ M Gpp(NH)p is increased to $150.0\% \pm 19.5\%$ of that of controls. C. Stimulation with 5-10 μ M forskolin (f), an adenylyl cyclase agonist that raises cAMP, dose-dependently increased CD44v9 expression in LnCaP and PC-3 cells. In PC-3 cells, maximal effect is observed at 10 μ M, whereas in LnCaP cells, a response is noted up to 100 μ M. With $G_s\alpha$ -QL or CT+ cells, negligible effect of forskolin was noted. Intracellular cAMP is already increased 85-fold in $G_s\alpha$ -QL cells (7), consistent with forskolin's negligible effect. Intracellular cAMP is expected to be high in CT+ cells also, owing to persistent activity of CT. These findings suggest that the correlation of $G_s\alpha$ activity with CD44v9 expression is cAMP-dependent. An association between cAMP and CD44 expression has also recently been described in lung cancer (29). D. α -tubulin loading control. E. 250,000 cells were exposed to the forskolin dose causing maximal CD44v9 expression (100 μ M for LnCaP; 10 μ M for PC-3, $G_s\alpha$ -QL, and CT+) or no forskolin. Numbers of unstimulated cells that invaded were 10, 3712, 5229, and 5451 respectively, and forskolin increased mean invasion compared with controls to 370%, 158%, 129%, and 109%. F. $G_s\alpha$ -QL prostate cancer cells were exposed to G-protein inhibitory drug GDP- β S. Dose-dependent inhibition of CD44v9 expression was achieved by use of GDP- β S at 0-200 μ M, reducing the level of expression to that of simultaneously incubated PC-3 cells. We conclude that $G_s\alpha$ activity alone is responsible for the higher CD44v9 expression. G. α -tubulin loading control. Functionally, Matrigel invasion of 250,000 $G_s\alpha$ -QL cells exposed to 200 μ M GDP- β S is reduced to $68.0 \pm 22.6\%$ of that of controls.

metastasis. Prostatic neuroendocrine cells lack androgen receptors and thus it has been suggested that tumors with greater neuroendocrine cell populations display faster and more autonomous growth (17).

The receptor for CT is coupled to the $G_s\alpha$ protein (2, 4), suggesting a role for its signal transduction cascade (on the inner cytoplasmic membrane) in modulating tumor development. A local increase in secretion of CT in

aggressive prostate cancer may cause persistent stimulation of $G_s\alpha$ -coupled receptors. To study this phenomenon, $G_s\alpha$ -QL transfectants were established (by G.V.S.) from PC-3M cells. Cells were transfected to express the *gsp* mutant of $G_s\alpha$ protein that lacks GTPase activity and thus is constitutively active. Prostate cancer cells transfected with this $G_s\alpha$ show increased cAMP accumulation, increased DNA synthesis (7), and greater anchorage-independent growth, basement matrix invasiveness, and metastasis formation (8). Increased $G_s\alpha$ protein activity in prostate cancer growth and invasion has been well-documented (7, 8, 11). The PC-3M-derived cell line CT+ was produced by stably transfecting the cloned cDNA for CT (7).

In our experiments, CT stimulated cancer cell invasion more than two-fold in the LnCaP and CT+ cells and marginally in PC-3. The stimulation in LnCaP cells is in agreement with the finding that, although this cell line does not make endogenous CT, it is positive for CT receptor (7). The weaker stimulation of invasion in PC-3 cells agrees with data that this cell line makes CT mRNA but is negative for CT receptor (7). Finally, PC-3M and its derived cell lines such as $G_s\alpha$ -QL and CT+ cells are positive for CT receptor (7). Baseline CD44v9 expression was elevated in these two cell lines compared to PC-3 cells. Only the CT+ cells had markedly increased invasion in response to exogenous CT; the refractoriness of $G_s\alpha$ -QL cells may be consistent with their having the highest baseline invasion as well as the finding that baseline cAMP is elevated 85-fold after transfection with $G_s\alpha$ -QL cDNA (7).

Our most notable finding was the dose-dependent relationship of CT to CD44v7-10 expression, with maximal 7-fold mRNA response at 100 nM and increased protein levels at 50 nM. CT+ and $G_s\alpha$ -QL cells have the strongest endogenous expression of CD44v9 protein. Further, the addition of exogenous CT increased CD44v9 protein in four cell lines tested, though not necessarily in proportion to the increase in Matrigel invasion which was greatest for LnCaP and CT+. This is, to our knowledge, the first description of transcriptional activation of a cell adhesion protein resulting from the hormonal signal transduction complex. CD44 has a well-documented interaction with the cytoskeleton. G-protein activity, by altering CD44 expression, may influence the cytoskeleton to change cell shape and increase cell motility. The CT system could be a model for other factors that stimulate prostate cancer growth and invasion, including muscarinic cholinergics (19), epidermal growth factor (20), and insulin-like growth factors I and II (21). Whether these factors also affect cell adhesion molecule expression remains to be tested.

We previously investigated the significance of CD44v7-10 in prostate cancer. CD44 standard as well as most other cell adhesion molecules are down-regulated or unchanged in prostate cancer, the sole exceptions being increased

expression of MUC18 (22, 23), N-cadherin and cadherin-11 (24). Increased and disorganized expression of CD44 variants characterizes various cancer tissues but not benign tissue. This includes breast, lung, and pancreatic carcinomas, colon cancer (25) and bladder cancer (26, 27), for which it is a serum, urinary, and tissue marker. We have observed, using immunohistochemical staining, Western blotting, *in situ* hybridization and reverse transcriptase-PCR, that the CD44v7-v10 portion of the variant sequence is overexpressed in prostate cancer, while expression of the CD44v2-v6 isoforms is down-regulated (9-11). Furthermore, this CD44v7-10 overexpression has functional significance; by targeting RNA interference against CD44v9, thus abrogating CD44v7-10 expression, we greatly reduced invasiveness into a Matrigel artificial basement membrane. RNA interference against MUC18, another overexpressed molecule, showed it to be of minor importance in invasion (11).

A possible linkage between $G_s\alpha$ activity and expression of any cell adhesion molecule, including CD44, had remained largely unexplored. CD44v7-10 overexpression in prostate cancer (11) plays a role in invasion analogous to CD44 standard in lung cancer (28). One recent piece of evidence for a link between an autocrine/paracrine factor, its $G_s\alpha$ -coupled receptor, intracellular cAMP, and CD44 standard expression was described in non-small cell lung cancer. The COX-2 product prostaglandin E2 (PGE2) in these cells initiates signaling through the EP4 receptor, alters MMP-2 expression, and increases invasion. CD44 expression was assessed using flow cytometry with a fluorescent antibody. EP4 signaling raised intracellular cAMP, and treatment with PGE2 or agents capable of increasing cAMP was noted to increase CD44 standard protein markedly; mRNA was not studied (29). Our findings with real-time RT-PCR and *in situ* hybridization provide the first evidence that CD44 is a target gene of CT.

Autocrine factors such as osteopontin can also influence CD44 expression through signal transduction pathways other than $G_s\alpha$: RET/PTC kinase in transformed thyrocytes (30). Thus, in order to establish that the effects of CT on CD44v9 expression were mediated by $G_s\alpha$, we performed experiments in which cell lines were subjected to a G-protein stimulatory or inhibitory agent. The normally low expression of CD44v9 in PC-3 cells was elevated to the level of concomitantly grown $G_s\alpha$ -QL by the stimulatory drug Gpp(NH)p, and the high expression in $G_s\alpha$ -QL cells was lowered to that comparable to PC-3 cells with the inhibitory drug GDP- β S. Thus, the effects of CT were largely reproducible by modulating $G_s\alpha$.

We also investigated the intracellular effectors of CT, since its receptor is capable of independently stimulating both the $G_s\alpha$ -mediated 3'5'-cAMP and the inositol phosphate/ Ca^{2+} signaling pathways (2, 31). Forskolin promoted a dose-dependent increase in CD44v9 expression in LnCaP and PC-3

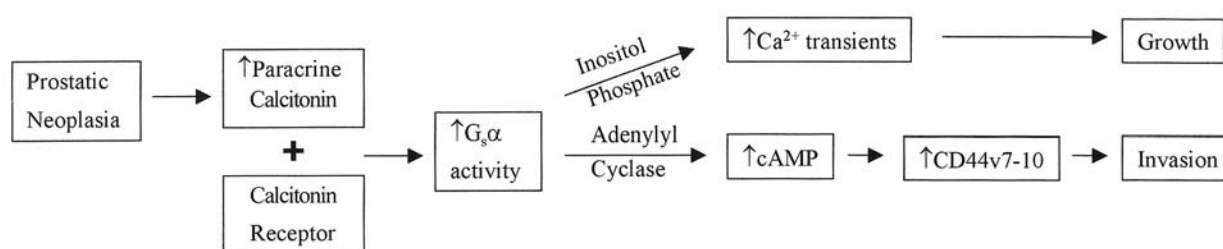


Figure 6. Our data, together with prior findings (6, 8), allow us to postulate a mechanism of interactions antecedent to and following CD44 variant activation in prostate cancer.

cell lines that normally have low expression of this molecule, and increased invasion the most in these two cell lines. Others have studied the role of cAMP in prostate cancer using dibutyryl cAMP and found an increase in DNA synthesis, *in vitro* cell migration, and invasiveness of LnCaP cells (32). In $G_s\alpha$ -QL and CT+ cells, CD44v9 expression was strong and relatively refractory to forskolin. This result may reflect the aforementioned 85-fold elevation in baseline cAMP after transfection for $G_s\alpha$ -QL cDNA (7). The CT+ cell line is also likely to have high cAMP, making it refractory to forskolin. This suggests that $G_s\alpha$ -mediated cAMP elevation is the predominant intracellular effector mediating CT-stimulated CD44v9 expression. Likewise, Chien and Shah found that forskolin could not mimic the growth-promoting actions of $G_s\alpha$ -QL transfectants, but the calcium channel antagonist nifedipine did potently inhibit DNA synthesis in these cells (6). Taken together with our results, it appears that $G_s\alpha$ increases CD44 variant expression by a cAMP-mediated mechanism but promotes growth by regulating voltage-gated Ca^{2+} channels (Figure 6). The influence on CD44 transcription suggests involvement of a cAMP-binding response element on the CD44 promoter. These response elements regulate the transcription of a number of proteins, including some that are important to cancer growth and are susceptible to inhibition by introducing oligonucleotide decoys to the response element (33).

In conclusion, CT peptide stimulation, $G_s\alpha$ activity, and adenylyl cyclase activity correlate positively with prostate cancer's overexpression of CD44v7-10, *in vivo* and *in vitro*. This provides novel evidence that this signal transduction system can interact with a cell adhesion molecule to alter its expression. Since CD44 variant expression determines growth and invasiveness, its regulation by this hormonal transduction system has functional significance.

Acknowledgments

This work was supported by the American Cancer Society Research Scholar Grant RSG-02-157-01-CCE and a Veterans Administration Merit Review Grant. We thank Ron Irby for his

assistance with computer graphics, and Karen Beyer and Pat Glenton for use of their protein measurement reagents and protocol.

References

- 1 Iczkowski KA, Pantazis CG, Long J, Noble MJ and Shah GV: Expression of calcitonin-like peptide is increased in prostatic adenocarcinoma. *J Urol Pathol* 8: 135-147, 1998.
- 2 Shah GV, Rayford W, Noble MJ, Austenfeld M, Weigel J, Vamos S and Mebust WK: Calcitonin stimulates growth of human prostate cancer cells through receptor-mediated increase in cyclic adenosine 3', 5'-monophosphates and cytoplasmic Ca^{2+} transients. *Endocrinology* 134: 596-602, 1994.
- 3 Ritchie CK, Thomas KG, Andrews LR, Tindall DJ and Fitzpatrick LA: Effects of the calcitrophic peptides calcitonin and parathyroid hormone on prostate cancer growth and chemotaxis. *Prostate* 30: 183-187, 1997.
- 4 Lin HY, Harris TL, Flannery MS, Aruffo A, Kaji EH, Gorn A, Kolakowski LF Jr, Lodish HF and Goldring SR: Expression cloning of an adenylyl cyclase-coupled calcitonin receptor. *Science* 254: 1022-1024, 1991.
- 5 Hanna FW, Smith DM, Johnston CF, Akinsanya KO, Jackson ML, Morgan DG, Bhogal R, Buchanan KD and Bloom SR: Expression of a novel receptor for the calcitonin peptide family and a salmon calcitonin-like peptide in the alpha-thyrotrophin thyrotroph cell line. *Endocrinology* 136: 2377-2382, 1995.
- 6 Chien J and Shah GV: Role of stimulatory guanine nucleotide binding protein ($G_s\alpha$) in proliferation of PC-3M prostate cancer cells. *Int J Cancer* 91: 46-54, 2001.
- 7 Chien J, Ren Y, Wang YQ, Bordelon W, Thompson E, Davis R, Rayford W and Shah G: Calcitonin is a prostate epithelium-derived growth stimulatory peptide. *Mol Cell Endocrinol* 181: 69-79, 2001.
- 8 Chien J, Wong E, Nikes E, Noble MJ, Pantazis CG and Shah GV: Constitutive activation of stimulatory guanine nucleotide binding protein ($G_s\alpha$)-mediated signaling increases invasiveness and tumorigenicity of PC-3M prostate cancer cells. *Oncogene* 18: 3376-3382, 1999.
- 9 Iczkowski, KA, Pantazis CG and Collins J: The loss of expression of CD44 standard and variant isoforms is related to prostatic carcinoma development and tumor progression. *J Urol Pathol* 6: 119-129, 1997.

- 10 Iczkowski KA, Bai S and Pantazis CG: Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. *Anticancer Res* 23: 3129-3140, 2003.
- 11 Omara-Opyene AL, Qiu J, Shah G and Iczkowski KA: Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18. *Laboratory Investigation* 84: 894-907, 2004.
- 12 Iczkowski KA, Omara-Opyene AL and Klösel R: Metafectene is superior to Lipofectamine in the transfection of G_α prostate cancer cells. *Mol Biotech* 28: 97-103, 2004.
- 13 Stephenson RA, Dinney CP, Gohji K, Ordonez NG, Killion JJ and Fidler IJ: Metastatic model for human prostate cancer using orthotopic implantation in nude mice. *J Natl Cancer Inst* 84: 951-957, 1992.
- 14 Livak KL and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta C_T$ method. *Methods* 25: 402-408, 2001.
- 15 Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680-685, 1970.
- 16 Colantonio CM, Kwan W-K, Czerwinski W, Mitchell J and Schimmer BP: Altered G protein activity in a desensitization-resistant mutant of the Y1 adrenocortical tumor cell line. *Endocrinology* 139: 626-633, 1998.
- 17 di Sant'Agnese PA: Calcitonin-like immunoreactive and bombesin-like immunoreactive endocrine-paracrine cells of the human prostate. *Arch Pathol Lab Med* 110: 412-415, 1986.
- 18 Shah GV, Noble MJ, Austenfeld M, Weigel J, Deftos LJ and Mebust WK: Presence of calcitonin-like immunoreactivity (iCT) in human prostate gland: evidence for iCT secretion by cultured prostate cells. *Prostate* 21: 87-97, 1992.
- 19 Rayford W, Noble MJ, Austenfeld MA, Weigel J, Mebust WK and Shah GV: Muscarinic cholinergic receptors promote growth of human prostate cancer cells. *Prostate* 30: 160-166, 1997.
- 20 Kim HG, Kassis J, Souto JC, Turner T and Wells A: EGF receptor signaling in prostate morphogenesis and tumorigenesis. *Histol Histopathol* 14: 1175-1182, 1999.
- 21 LeRoith D and Roberts CT: The insulin-like growth factor system and cancer. *Cancer Lett* 195: 127-137, 2003.
- 22 Wu GJ, Wu MW, Wang SW, Liu Z, Qu P, Peng Q, Yang H, Varma VA, Sun QC, Petros JA, Lim SD and Amin MB: Isolation and characterization of the major form of human MUC18 cDNA gene and correlation of MUC18 over-expression in prostate cancer cell lines and tissues with malignant progression. *Gene* 279: 17-31, 2001.
- 23 Wu GJ, Varma VA, Wu MW, Wang SW, Qu P, Yang H, Petros JA, Lim SD and Amin MB: Expression of a human cell adhesion molecule, MUC18, in prostate cancer cell lines and tissues. *Prostate* 48: 305-315, 2001.
- 24 Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ and Schalken JA: Cadherin switching in human prostate cancer progression. *Cancer Res* 60: 3650-3654, 2000.
- 25 Yoshida K, Goodison S, Sugino T, Bolodeoku J, Churchman M, Warren BF and Tarin D: Semiquantitative detection of abnormal CD44 transcripts in colon carcinomas by reverse transcription-polymerase chain reaction enzyme-linked immunosorbent assay (RT-PCR ELISA). *Mol Diagn* 1: 167-173, 1996.
- 26 Matsumura Y, Sugiyama M, Matsumura S, Hayle AJ, Robinson P, Smith JC and Tarin D: Unusual retention of introns in CD44 gene transcripts in bladder cancer provides new diagnostic and clinical oncological opportunities. *J Pathol* 177: 11-20, 1995.
- 27 Okamoto I, Morisaki T, Sasaki J, Miyake H, Matsumoto M, Suga M, Ando M and Saya H: Molecular detection of cancer cells by competitive reverse transcription-polymerase chain reaction analysis of specific CD44 variant RNAs. *J Natl Cancer Inst* 90: 307-315, 1998.
- 28 Zhang Y, Thant AA, Machida K, Ichigotani Y, Naito Y, Hiraiwa Y, Senga T, Sohara Y, Matsuda S and Hamaguchi M: Activation of pro-gelatinase B by Endometase/Matrilysin-2 promotes invasion of human prostate cancer cells. *J Biol Chem* 278: 15056-15064, 2003.
- 29 Dohadwala M, Batra RK, Luo J, Lin Y, Krysan K, Pold M, Sharma S and Dubinett SM: Autocrine/paracrine prostaglandin E2 production by non-small cell lung cancer cells regulates matrix metalloprotease-2 and CD44 in cyclooxygenase-2-dependent invasion. *J Biol Chem* 277: 50828-50833, 2002.
- 30 Castellone MD, Celetti A, Guarino V, Cirafo AM, Basolo F, Giannini R, Medico E, Kruhoffer M, Orntoft TF, Curcio F, Fusco A, Melillo RM and Santoro M: Autocrine stimulation by osteopontin plays a pivotal role in the expression of the mitogenic and invasive phenotype of RET/PTC-transformed thyroid cells. *Oncogene* 23: 2188-2196, 2004.
- 31 Chabre O, Conklin BR, Lin HY, Lodish HF, Wilson E, Ives HE, Catanzariti L, Hemmings BA and Bourne HR: A recombinant calcitonin receptor independently stimulates 3'5' cyclic adenosine monophosphate and Ca²⁺/inositol phosphate signaling pathways. *Mol Endocrinol* 6: 551-556, 1992.
- 32 Hoosein NM, Logothetis CJ and Chung LWK: Differential effects of peptide hormones bombesin, vasoactive intestinal polypeptide and somatostatin analog RC-60 on the invasive capacity of human prostatic carcinoma cells. *J Urol* 149: 1209-1213, 1993.
- 33 Cho-Chung YS, Park YG, Nesterova M, Lee YN and Cho YS: CRE-decoy oligonucleotide inhibition of gene expression and tumor growth. *Mol Cell Biochem* 212: 29-34, 2000.

Received February 11, 2005

Accepted March 1, 2005