Calcitonin promotes in vivo metastasis of prostate cancer cells by altering cell signaling, adhesion, and inflammatory pathways

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Abstract

Expression of calcitonin (CT) and its receptor (CTR) is elevated in advanced prostate cancer (PC). Although the significance of CT–CTR axis in PC cell growth, invasion, and epithelial to mesenchymal transition has been established, its role in tumor metastasis has not been examined. To examine the role of CT–CTR axis in tumor metastasis, we employed stable CT–CTR activated and silenced system of three PC cell lines, LNCaP cells that lack endogenous CT, PC-3 cells that lack endogenous CTR, and PC-3M cells that co-express CT and CTR. Enforced expression of CT in LNCaP cells and CTR in PC-3 cells increased their ability to form orthotopic tumors and distant metastases in multiple organs. By contrast, silencing of CT expression in PC-3M cells not only reduced their tumorigenicity, but also completely abrogated their metastatic potential. To investigate the effect of in vivo silencing of CT expression on tumor growth, we employed recombinant adeno-associated virus (rAAV) to deliver anti-CT ribozymes in preexisting tumors of nude mice and large probasin promoter (LPB)-Tag transgenic mice. rAAV-CT– treatment not only abrogated the growth of pre-implanted tumors in nude mice, but also significantly reduced the growth of spontaneous tumors in LPB-Tag mice. Analysis of CT upregulated and silenced PC-3M transcriptomes revealed 105 genes affected by the modulation of CT expression. These CT signature genes generated survival, adhesion, pro-inflammatory, and pro-metastatic pathways. Added together, these data indicate a pivotal role for CT–CTR axis in PC metastasis and may serve as a potential therapeutic target for advanced PC.

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Introduction

The population of prostate cells expressing neuro-peptides such as calcitonin (CT) increases during prostate cancer (PC) progression (Shah et al. 1992, Abrahamsson & di Sant’Agnese 1993, Jongsma et al. 2002). Earlier results from this laboratory have shown that CT and its receptor (CTR) are exclusively localized in the basal compartment of normal prostate epithelium. However, their expression is deregulated during malignancy, resulting in increased abundance of CT/CTR transcripts in whole epithelia (Chien et al. 2001). Moreover, the abundance of CT/CTR mRNA correlates positively with Gleason grade of primary PCs. In addition, exogenous CT or enforced activation of CT–CTR autocrine axis causes increased tumorigenicity, invasiveness, and resistance to apoptosis in several PC cell lines (Ritchie et al. 1997, Nagakawa et al. 1998, Sabbisetti et al. 2005b, Thomas & Shah 2005, Thomas et al. 2006). However, the pathological significance of CT expression in progression of PC from localized tumor to its metastatic form has not been established.
Recent studies from this laboratory have shown that CT may induce multiple molecular events to increase tumorigenicity and invasiveness of PC cell lines including the loss of cell–cell adhesion, increase in the surface activity of αvβ3 or αvβ5 integrins, and increase in the secretion of matrix metalloproteinases 2 and 9 and urokinase-type plasminogen activator (Sabbisetti et al. 2005a,b, Thomas et al. 2007). Although cyclic AMP-dependent protein kinase plays a key role in the actions of CT, CT also activates the PI3-kinase-Akt-survivin and the β-catenin pathways (Chien & Shah 2001, Sabbisetti et al. 2005b, Thomas et al. 2006).

The objective of the present study was to examine the significance of CT in tumorigenicity/metastatic potential of PC cell lines and to identify potential pathway(s) associated with CT-stimulated tumor growth and metastasis by a combination of in vitro, in vivo, and transcriptomic studies. The results suggest that modulation of CT expression in PC cell lines significantly alters their tumorigenicity and ability to form distant metastases. These CT actions may be mediated by gene clusters that undergo significant changes in their transcriptional profile in response to modulation of CT expression.

Materials and Methods

Animals

Nu/nu mice
Male balb/c nu/nu mice (6–8 weeks old) were purchased from Harlan (Madison, WI, USA), and housed two per cage in microisolator units under 70% humidity and temperature-controlled conditions. The animals were fed ad lib on a standard sterilizable laboratory diet (Teklad Lab chow, Harlan Teklad), and quarantined for 1 week prior to their use in the study.

LPB-Tag transgenic mouse line
LPB-Tag transgenic mice lines 12T-7fast were provided by Dr Robert J Matusik (Vanderbilt University, Nashville, TN, USA). The colony of these mice was established in our facility, and newborn mice were identified by genotyping as previously described (Wang et al. 2006). Positive adult mice of this line were used in the present study.

Surgical orthotopic implantation (SOI)
All animal procedures were conducted in accordance with the principles and procedures outlined by the NIH and Institutional Animal Care and Use Committee at University of Louisiana at Monroe. The SOI was performed as previously described (Stephenson et al. 1992). Animals were regularly monitored for tumor growth and metastasis by fluorography using Kodak 4000 MM imaging station. The animals were killed 60 days after orthotopic tumor implantation, and organs were examined for tumor metastasis.

Cell culture
PC-3M PC cell line was provided by Dr Isiah Fidler (MD Anderson Cancer Center, Houston, TX, USA), and LNCaP and PC-3 cells were obtained from ATCC (Manassas, VA, USA). The cells were maintained in the complete medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin G, and 100 mg/ml streptomycin) under standard culture conditions.

CT/CTR expression in PC cell lines; generation of PC sublines with modulated CT expression
LNCaP (CT−, CTR+) cells were stably transfected with either the vector (pcDNA3.1, V) or CT (recombinant pcDNA3.1 containing full-length CT cDNA, expression driven by cytomegalovirus (CMV) promoter) to obtain LNCaP-V and LNCaP-CT cell lines respectively. Similarly, constitutive CTR expression in PC-3 (CT+/CTR−) cells was enforced by stable transfection of either the vector (pcDNA3.1, V) or CTR (recombinant pcDNA3.1 containing full-length CTR cDNA, expression driven by CMV promoter).

CT expression in PC-3M (CT+, CTR+) cells was elevated with stable transfection of recombinant pcDNA3.1 containing full-length CT cDNA (PC-3M-CT+). Selection of stable transfectants was carried out with Geneticin (800 µg/ml for 3 weeks). CT-deficient PC-3M subline (PC-3M-CT−) was generated by stable co-transfection of hammerhead ribozymes against CT mRNA and pcDNA3.1-Zeo in PC-3M cells as described recently (Thomas et al. 2006). LNCaP-V cells lacked CT mRNA and did not secrete CT in the conditioned media, but LNCaP-CT cells displayed high CT mRNA abundance and secreted CT in the conditioned media. PC-3-V cells were unresponsive to exogenously added CT in Matrigel invasion assay. However, PC-3-CTR cells responded to 50 nM CT with a threefold increase in cell invasion. PC-3M-CT+ cells displayed almost threefold increase in CT mRNA abundance when compared with PC-3M-V cells. PC-3M-CT− cells displayed greater than 90% decrease in the abundance of endogenous CT mRNA and CT secretion when compared with their vector controls (PC-3M-V). The sublines were extensively characterized in recently published studies (Thomas et al. 2006).
Stable expression of red fluorescence protein (RFP) in PC cell lines

To detect implanted tumor cells in mice, we stably transfected all PC sublines with DsRed-MCherry-Hyg-N1, a mammalian expression vector that encodes DsRed-MCherry, a derivative of red fluorescent protein (Clontech). Hygromycin resistant colonies of double-tranfectants were selected, and observed over a period of 4 weeks. All cell lines expressed strong red fluorescence at a steady level over the entire observation period of 8 weeks.

In vivo suppression of CT expression: in vivo delivery of anti-sense CT ribozymes with recombinant adeno-associated virus (rAAV)

Construction of rAAV Plasmids (rAAV2-CT− and rAAV2-C)

The rAAV2 plasmid was constructed by the replacement of sequences between the CMV promoter and BGHpolyA signal in pAAV2 by the expression cassette consisting of U6 polIII promoter, followed by the anti-sense CT ribozyme oligo-duplex (sense: 5'-GAAGATCTTC/AGCTTCTAG/TTTCGTCCTC-ACGCACTCATCAG/ATCTGGCT/CCGCTCGAGCGG-3'), followed by a polIII polymerase termination signal (four thymidine residues) to generate ribozymes with very little extraneous sequences (Thomas et al. 2006). The expression cassette was cloned into pAAV2 plasmid at Xba1 and MluI sites (Neyns et al. 2001, Thomas et al. 2006). The corresponding rAAV2-C (control) plasmid was generated by replacing anti-sense CT ribozyme duplex with inactive ribozyme oligo-duplex, which had the following sequence: sense- 5'-GAAGATCTTC/CTGGGCACG/AAAGCAGGAGUGCCUGAGUAGUC/CACACAAG/CCGCTCGAGCGG-3').

Preparation of recombinant AAV stock

rAAV2 viruses were produced by triple transfection of 293 cells followed by two rounds of CsCl2 purification (Mahadevan et al. 2007). For each viral preparation, physical titers (GC/ml) were determined by dot blot analysis and Taq-Man quantitative PCR using (Applied Biosystems, Foster City, CA, USA) with two different probes (Anderson et al. 2000, Drittanti et al. 2000).

rAAV infection in PC-3 cells

To test the efficacy of rAAV-CT− in silencing of endogenous CT expression, we first used PC-3 cells as model because the cells endogenously secrete CT in the conditioned media. In T-75 flask, 2 × 10⁶ PC-3 cells were plated. After overnight serum starvation, the cells were transfected with either 0–20 μl (1 × 10¹² particles/ml) of rAAV-CT− or rAAV-C in Opti-MEM media and incubated for 48 h. The Opti-MEM was then replaced with the complete medium (RPMI1640 containing 10% fetal bovine serum), and transfected cells were plated in six-well plates at the density of 200 000 cells/well overnight. Complete medium was then replaced with serum-free basal incubation medium for 24 h. Conditioned media were collected and assayed for CT with specific CT RIA (Thomas et al. 2006).

Administration of r-AAV in mice

Nude mice

Recombinant viral particles (rAAV2-CT− or rAAV2-C) were injected intratumorally once every week as described in the Results section at three different doses: 5 (containing ~ 10 genomic particles of rAAV-CT− and rAAV-C), 10, and 20 μl. The viral dose was diluted to the final volume of 100 μl with normal saline and injected with a 30 g needle.

LPB-Tag mice

Approximately, 10¹¹ genomic particles of AAV-CT and AAV-C cells were injected intraperitoneally using a 30 g needle three times a week. The treatment began at the age of 30 days, and continued until day 90.

Histology

At the termination of experiment, primary tumor and other organs (as described in the Results section) were harvested, weighed, and wet sections of the tumors were examined for the presence of RFP. Fluorescent images of RFP expressing cells were acquired with a charge-coupled device Retiga 2000 RT digital camera connected to a microscope (Nikon Optiphot 2) and a computer. The images were then processed with the IPLab Image Analysis software (BD Biosciences, San Jose, CA, USA).

Affymetrix microarray analysis

PC-3M-V, PC-3M-CT−, and PC-3M-CT+ cells were grown to confluence in 100 mm dishes, and total RNA was isolated using total RNA isolation kit (Qiagen). The RNA quality was assessed by capillary electrophoresis using an Agilent 2100 bioanalyzer (Agilent, Wilmington, DE, USA), and quantified by absorbance at A260. One hundred nanograms of total RNA from each cell line were biotinylated with Affymetrix’s eukaryotic small sample target labeling assay, version II. This protocol is designed to reproducibly amplify 10–100 ng total RNA by performing two cycles of

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double-stranded cDNA synthesis and in vitro transcription reactions using T7 RNA polymerase. Biotinylated target cRNA was then hybridized to an Affymetrix Focus array according to the manufacturer’s instructions and gene expression data were obtained using the Affymetrix Microarray Analysis Suite, version 5.0. The expression data were normalized to the target value of 150 by global scaling. This procedure uses a constant scaling factor for every gene on an array, where the scaling factor is obtained from a trimmed average signal of the array after excluding the 2% of the probe sets with the highest and the lowest values. After normalization, the expression profiles were imported into a Microsoft Excel database.

Microarray data analysis

Affymetrix data following initial filtering were retrieved and analyzed in GeneSpring (Asirvatham et al. 2006). The statistically significant list was subjected to a three-way analysis to identify differentially expressed genes (DEG) between CT⁺ versus V, CT⁺ versus CT⁻, and V versus CT⁻ groups. The DEGs were filtered using the Cross gene error model using the following criteria: mean signal intensity ≥ 300 (one of three conditions), fold change ≥ 3 (two of the three conditions), flag = P (when two of the three conditions are present), default statistical validation and normalizations to global mean/chip.

Unique and common gene expression patterns between treatments were evaluated using Venn diagrams. We focused on unique DEG between CT and CT⁻ group as potentially true CT responsive genes (CT gene set). The CT gene set was filtered against CT⁺ versus V and CT⁻ versus V as shown in Fig. 5 and subjected to cluster analysis. Patterns of gene expression were identified using unsupervised cluster analysis within the set of differentially expressed transcripts in the filtered list. Clustering algorithms allow for the separation of distinct patterns of expression based on the similarity of expression profiles between different genes. In this analysis, a hierarchical clustering algorithm utilizing a standard correlation with the default parameters was utilized in order to isolate distinct, non-repetitive patterns of expression within treatments.

Pathway analysis

To understand functional relationships and mechanisms of differential gene expression in response to CT modulation and to derive probable CT-modulated signaling pathways, we used Ingenuity pathway analysis software (IPA version 6, Redwood city, CA, USA). The gene lists were imported into IPA and core analysis performed as per default software settings. The IPA core analysis identifies molecular networks and biological processes that are most significantly perturbed in a dataset. The network score is expressed as the negative log of the p-value (score of 2 or higher indicates a 99% confidence of not being generated by a random chance alone).

Results

Modulation of CT expression alters their in vivo growth and ability to form distant metastases

To examine the action of CT on PC metastasis, we tested three PC cell lines with different androgen-responsiveness and oncogenic characteristics. PC-3M cells, originally derived from metastasis of PC-3 xenografts, are androgen-refractory and highly metastatic (Stephenson et al. 1992); PC-3M cells also co-express CT and CTR (Chien et al. 2001). PC-3 cells are androgen-refractory but moderately metastatic (Kaighn et al. 1979); PC-3 cells express CT, but lack endogenous CTR expression (Chien et al. 2001). LNCaP cells, originally derived from lymph node metastasis of a PC patient, are androgen responsive and indolent (Horoszewicz et al. 1983); LNCaP cells express CTR but lack endogenous CT expression (Chien et al. 2001). PC-3M-V (CT⁺/CTR⁻) cells formed orthotopic tumors and distant metastases in several organs as depicted in Figs 1A and 2A and Table 1. Overexpression of CT in PC-3M (PC-3M-CT⁺) cells led to even larger orthotopic tumors and larger metastases in most distant organs except mesentery and liver. Moreover, PC-3M-CT⁺ cells acquired the ability to penetrate blood–brain barrier and establish colonies in the brain. By contrast, stable knock-down of CT expression (PC-3M-CT⁻) led to remarkable loss in tumorigenicity and metastatic activity of PC-3M cells as suggested by 89% decline in orthotopic tumor mass (when compared with PC-3M-V cells), and the absence of metastases in distant organs except for a few colonies in lymph nodes. To ensure that the metastatic action of CT is not PC-3M cell specific, we tested this process in LNCaP (CT⁻/CTR⁺) and PC-3 (CT⁺/CTR⁻) cells. As expected, LNCaP-V cells displayed poor orthotopic growth, and were localized within the prostate with no metastatic activity in any of the organs examined. Enforced CT expression enabled LNCaP (LNCaP-CT) cells to grow much faster within the prostate, and also formed metastases in lymph node, lung, femur bone, and testes (Figs 1B and 2B, and Table 1). PC-3 cells
formed orthotopic tumors and metastatic colonies as presented in Figs 1C and 2C, and Table 1. Constitutive expression of CTR in PC-3 (PC-3-CTR) cells increased the size of orthotopic tumors by twofold, and metastases in most organs were visibly larger (Fig. 2C). Moreover, PC-3-CTR cells formed metastatic colonies in mesentery, liver, and kidneys, where PC-3-V cells could not establish the colonies.

**In vivo knock-down of CT expression with rAAV-CT**

Knock-down of endogenous CT expression in PC-3 cells

We first tested the efficacy of rAAV preparations to knock-down CT expression in the cultures of PC-3 cells. As depicted in Fig. 3A, rAAV-C treatment did not affect CT secretion. However, rAAV-CT effectively knocked down endogenous CT expression as suggested by a remarkable decline in CT secretion.

In vivo knock-down of endogenous CT expression

To examine whether CT-stimulated tumor growth can be sustained by its expression during early phase of tumor implantation/growth or it requires continued CT expression, we administered rAAV-CT (5–20 μl) intratumorally to nude mice 8 days after PC-3M-CT+ cell implantation. Subcutaneous xenografts of implanted PC-3M cells could be palpated at this time. We chose PC-3M-CT+ cell line because it is more tumorigenic than PC-3M cells, and may represent most advanced cases of PC (Thomas et al. 2006). The animals received three additional weekly injections of same doses on Day 15, 22, and 29. The controls received equivalent doses of rAAV-C at the same times. The animals were killed on day 35 and the tumors were harvested and weighed. As shown in Fig. 3, administration of rAAV-CT significantly attenuated the in vivo tumor growth in a dose-dependent manner. More than 80% decline in tumor mass (when compared with vehicle control) was achieved at the highest tested concentration of 20 μl (Fig. 3B–D). Immunofluorescence of paraffin-embedded tumor sections shows that the tumors treated with rAAV-CT lacked CT-immunopositive cells, whereas cells of those receiving rAAV-C displayed intense CT expression (Fig. 3E), suggesting that rAAV-CT effectively knocked-down CT expression in tumor cells.

**rAAV-CT attenuates tumor growth and metastasis in LPB-Tag transgenic mice**

Having seen potent inhibition of in vivo xenograft growth of PC-3M-CT+ cells by rAAV-CT−, we
wanted test whether this treatment will also prove effective in heterogeneous tumors. We examined the effect of rAAV-CT\(^-\) in LPB-Tag transgenic mice, which develop spontaneous prostate tumors around day 30 post-natal (Kasper et al. 1998). The mice were administered either 20 µl rAAV-CT\(^-\) or rAAV-C intraperitoneally, three times a week beginning at day 30. The treatment was continued for the period of 60 days. The mice were killed on day 90, and their reproductive organs were harvested and weighed. rAAV-CT\(^-\) treatment reduced the tumor growth by \(\sim\)60% when compared with rAAV-C-treated mice (Fig. 4A and B). Once again, the effectiveness of rAAV-CT\(^-\) in suppressing endogenous CT expression was tested by CT immunofluorescence of tumor sections. The results demonstrate a remarkable reduction in CT-immunopositive cells in the prostates of rAAV-CT\(^-\)-treated mice when compared with the prostates of those treated with rAAV-C (from 469 cells/400 \(\times\) field or 88.5% CT-positive cells in rAAV-C-treated mice to 54 cells/400 \(\times\) field or 10% CT-positive cells in rAAV-CT\(^-\)-treated mice, a decrease of 78.5%; Fig. 4C and D).

**Figure 2** Fluorescent images of tumor micrometastases. RFP-expressing cells (1 \(\times\) \(10^6\)) were orthotopically implanted into the prostate of nude mice. Animals were killed 5 weeks after implantation, and fresh organs were removed from mice and were observed directly under fluorescent microscope. The images were captured at 100 \(\times\) magnification. (A) Metastases of PC-3M-V, PC-3M-CT\(^+\), and PC-3M-CT\(^-\) xenografts. (B) Metastases of LNCaP-V and LNCaP-CT xenografts. (C) Metastases of PC-3-V and PC-3-CTR xenografts.

### Altered gene expression in response to modulation of CT expression

Since modulation of endogenous CT expression in PC-3M cells produced remarkable changes in their tumorigenicity and metastasizing potential, we used them as an experimental model to identify critical clusters of CT-regulated genes that may mediate tumor growth and metastasis in advanced PC. We examined the transcriptomes of PC-3M-V, PC-3M-CT\(^+\), and PC-3M-CT\(^-\) cells with Affymetrix Focus cDNA microarrays. We hypothesized that filtering the CT-regulated genes against PC-3M-V and PC-3M-CT\(^-\) sample set would be an ideal approach. Three-way Venn analysis of the gene expression data identified 105 unique CT-regulated genes (Fig. 5A). The genes demonstrated a remarkable reciprocal pattern of expression between PC-3M-CT\(^+\) and PC-3M-CT\(^-\) cells as determined by cluster analysis (Fig. 5B). In the absence of CT (CT\(^-\)), the expression of 57 genes increased and 48 genes decreased. The detailed list of these genes with fold change in expression is shown in Supplementary Table 1, which can be viewed online at http://erc.endocrinology-journals.org/.

### Table 1 Tumor cell populations in ectopic organs

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<thead>
<tr>
<th>Organs</th>
<th>PC-3M-V</th>
<th>PC-3M-CT(^+)</th>
<th>PC-3M-CT(^-)</th>
<th>LNCaP-V</th>
<th>LNCaP-CT</th>
<th>PC-3-V</th>
<th>PC-3-CTR</th>
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<td>Lymph node</td>
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+ occasional cells; + + few colonies; + + + several colonies; + + + + colonies throughout organ.
Based on the magnitude of change, the transcription factor Krüppel-like factor 9 (KLF9/ BTEB1, sixfold decrease in CT−) and Toll like receptor 7 (TLR7, sixfold increase in CT−) could represent novel targets of CT in PC cells, although it is unknown at this stage if these represent direct target genes.

To understand the biological significance of CT-regulated gene expression in PC-3M cells and to identify their molecular functions, we performed pathway analysis using Ingenuity pathway analysis software (Supplementary Table 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/). A total of seven networks were generated. The first four networks (networks 1–4) with scores of 39, 25, 25, and 20 indicated that genes within these pathways regulated cell–cell signaling, cell cycle, DNA replication and...
tumor biology respectively. High scores of these networks suggest that they were generated by including the data of larger number of CT-regulated genes, thus raising the probability that CT regulates prostate tumor growth and metastasis through one or more of these networks.

Discussion

Earlier results from this laboratory have shown that the expression of CT/CTR is deregulated in secretory cells of malignant prostates (Chien et al. 2001). Although we and others have also demonstrated stimulatory effect of CT on in vitro invasiveness of PC cell lines, we did not examine whether this translates into the formation of distant metastases (Ritchie et al. 1997, Sabbisetti et al. 2005b, Thomas et al. 2006). To examine the role of CT in the process of tumor metastasis, we first stably activated or silenced CT–CTR axis in three PC cell lines by either enforcing the expression of CT in LNCaP cells and CTR in PC-3 cells or by knocking down CT expression in PC-3M cells. As expected, PC-3M cells expressing the carrier plasmid formed large orthotopic tumors and distant metastases in multiple organs of nude mice. Although CT increases invasiveness of PC cell lines, the formation of distant metastases require additional abilities like the capacity to survive during the process of intravasation into blood and lymphatic vasculature, extravasation into parenchyma of distant tissues, and the ability to grow in ectopic environment (Waltregny & Castronovo 1996, Sabbisetti et al. 2005b, Albini 2008). Overexpression of CT in PC-3M cells increased these abilities as demonstrated by not only larger metastases than those of PC-3M-V cells, but also the formation of new metastases in the brain. These results suggest that the overexpression of CT enabled PC-3M cells to penetrate blood–brain barrier and establish colonies in the brain. Although there is evidence of brain metastasis of PC in humans, it is rare and is considered to be a terminal event of advanced systemic PC (Tremont-Lukats et al. 2003, Salvati et al. 2005). This, when combined with the present results, suggests that overexpression of CT may have increased tumorigenicity and metastatic potential of PC-3M cells to the level of tumor cells of systemic PC in terminal patients. The ability of CT to stimulate the mechanisms required for tumor metastasis was demonstrated more convincingly in LNCaP cells,

Figure 4 Treatment of LPB-Tag mice with rAAV. Adult LPB-Tag mice were injected with ~ 10\(^{11}\) genomic particles of either rAAV-CT- or rAAV-C ip three times a week between age of 30–90 days. The mice were killed at 90 days of age and the tumors were removed, cleaned and weighed. (A) Representative pictures of gross tumors of rAAV-C and rAAV-CT\(^{-}\)-treated mice at necropsy. (B) The bar graphs present final weights of tumors at necropsy (mean g tumor weight \(\pm\) S.E.M. for \(n=6\)). *Significant change from rAAV-C; \(P<0.05\) (one-way ANOVA and Newman–Keuls test). (C) CT immunoreactivity in tumors of mice receiving rAAV. Paraffin-embedded tumor sections of rAAV-treated LPB-Tag mice were analyzed for CT immunofluorescence as described in the Materials and Methods section. Representative photomicrographs of CT immunofluorescence of rAAV-C and rAAV-CT\(^{-}\)-treated tumor sections captured in 400× field were merged with DAPI immunofluorescence from the same field. (D) The bar graphs represent the average number of CT-immunopositive cells versus total number of cells in 400× fields (average number of cells \(\pm\) S.E.M. from \(n=6\) representative 400× fields per section \(\times 6\) sections per tumor \(\times 6\) tumors per group). *Significant change from rAAV-C; \(P<0.05\) (one-way ANOVA and Newman–Keuls test).
which are indolent and have low tumorigenic and metastatic potential (Stephenson et al. 1992). Stable expression of CT transformed them into aggressive cells as demonstrated by their ability to form larger orthotopic xenografts and distant metastases in multiple organs including lymph nodes and femur bone. In contrast, silencing of CT expression in highly metastatic PC-3M cells not only reduced their tumorigenicity, but also almost abrogated their metastatic potential.

Recent evidence suggests that cancer stem cells, which are speculated to be resistant to cancer therapy, are a major cause of tumor relapse and metastasis (Dontu et al. 2005, Allan et al. 2006, Vaidya, 2007, Zhao et al. 2008). It has also been suggested that important pathways regulating cell self-renewal and cell fate such as Wnt,
Notch and Hedgehog, and tumor suppressor genes such as phosphatase and tensin homolog on chromosome 10 and tumor protein p53, are believed to be deregulated in cancer stem cells, leading to uncontrolled self-renewal and generation of tumors that are resistant to conventional therapies (He & Jablons 2006, Ailles & Weissman 2007, Clark et al. 2007, Dreesen & Brivanlou 2007, Grinstein & Wernet 2007, Katoh & Katoh 2007, Campbell et al. 2008, Eyler & Rich 2008, Lee & Vasioukhin 2008). Considering that the modulation of CT–CTR axis in three different PC cell lines remarkably altered their metastatic potential raises a critical question: does active CT–CTR autocrine axis increases the stemness of PC cells? Our previous and present results support this possibility. For example, prostate cells expressing CT/CTR in benign prostate are exclusively localized in basal epithelium where prostate stem cells are also localized (Chien et al. 2001, Lam & Reiter 2006). Moreover, CT expression in normal secretory prostate cells is inhibited by androgens, but this control is lost in malignant cells (Shah et al. 1992, Chien et al. 2001). Secondly, we have shown that CT activates the β-catenin pathway, an important pathway for cell self-renewal (He & Jablons 2006, Katoh & Katoh 2007, Campbell et al. 2008). Thirdly, in PC cell lines, CT increases the resistance to cytotoxic drugs-induced apoptosis by activating the PI3kinase-Akt-survivin pathway (Thomas & Shah 2005, Rossi & Weissman 2006). CT also induces the expression of CD44, the most frequently used marker for stem cells (Iczkowski et al. 2005, Hurt et al. 2008, Pries et al. 2008, Zeilstra et al. 2008). Finally, the present results demonstrate that an activated CT–CTR axis enables PC cells to escape the orthotopic environment of the prostate, and increases their survival on their journey to distant organs. Indeed, additional studies will be necessary to link CT–CTR axis with stemness, but taken together, these results are certainly suggestive of this link.

If CT increases tumorigenicity of PC cells, we then asked the next question: will silencing of CT expression attenuate the growth of already implanted tumors? We addressed this question by generating rAAV to deliver anti-CT ribozymes in the tumor to knock-down CT expression in vivo. Considering that rAAV-CT− therapy remarkably attenuated in vivo growth of highly metastatic and chemoresistant PC-3M-CT− xenografts, the present results pose a second important question: can the metastatic activity of heterogeneous prostate tumors be reduced by the modulation of CT expression? Considering that metastasis is the primary cause of cancer mortality, a more thorough understanding of the factors that regulate the process of metastasis is critical for understanding tumor progression and to develop novel therapeutic approaches for the treatment of advanced PC. Since rAAV-CT− therapy attenuated PC-3M-CT+ xenografts growth, it can be potentially useful for the treatment of patients with high CT prostate tumors. However, human tumors are not homogenous like PC-3M-CT+ xenografts. Therefore, we tested rAAV-CT− therapy in LPB-Tag mice, a transgenic model developed to study prostate carcinogenesis (Kasper 2005). Prostate tumors of LPB-Tag mice display significant similarities with human tumors including tumor heterogeneity, neuroendocrine features, and relatively slower tumor growth (Kasper 2005). Our results show that rAAV-CT− therapy was effective in LPB-Tag mice as assessed by a remarkably reduced growth of heterogeneous tumors and their metastasis in reproductive organs, decreased morbidity and increased survival of the mice. These results increase the potential usefulness of rAAV-CT− for therapeutic purposes, especially in the subset of patients with aggressive tumors demonstrating high level of CT expression.

Since manipulation of CT expression produced dramatic changes in tumorigenicity/metastatic activity of PC-3M cells, we used them as a tool to identify critical gene clusters associated with CT-stimulated tumorigenicity/metastasis. The careful analysis of PC-3M-CT+, PC-3M-V, and PC-3M-CT− transcriptomes revealed a list of 105 genes that may have been directly affected by CT overexpression or knock-down. These genes were either induced or suppressed in PC-3M-CT+ cells, and the opposite profile of these genes was observed in PC-3M-CT− cells. Further break-down of these CT signature genes on the basis of their function, revealed that 13 genes were associated with cell–cell adhesion and six genes were associated with inflammation, suggesting that inflammation and loss of cell–cell adhesion may be early responses of PC cells and may constitute early events associated with CT-mediated metastasis. These results are consistent with proinflammatory phenotype of human PC stem cells, further supporting a possible role of CT–CTR axis in maintaining stemness of PC cells (Untergasser et al. 2005, Birnie et al. 2008).

To further understand the function of CT-regulated genes and their potential contribution to tumor growth/metastasis, we attempted to fit them in the networks through bioinformatics approaches. Since genes have multiple functions and cross-talk across pathways, it is conceivable that the pathways identified by bioinformatics may not always entirely agree with those identified by experimental studies. However, our earlier results validate at least networks 1 and 4 generated by the microarray analysis of the present data (Supplementary Table 2, which can be viewed online at http://erc.endocrinology-journals.org-supplemental/).
For example, the network 1 as well as our previous studies demonstrated that CT activates PI3K-Akt-survivin pathway inducing apoptosis resistance. Interestingly, this pathway also involves NFκB and associated proinflammatory signaling. We have also shown that CT regulates the expression of cadherins, and activates β-catenin and Wnt signaling pathways in PC cell lines, which is confirmed by the network 4. Thus, the pathway analysis of the microarray data provides further insight into CT-stimulated tumor growth/metastasis, and raise a possibility that the nodal point(s) of these networks may potentially provide new targets to interfere with the process of tumor growth/metastasis in cases of advanced PC. Indeed, additional studies will be necessary to determine which of the nodal points are critical for CT-mediated tumor growth and metastasis. However, present results have identified a novel experimental approach of combining biological studies and gene therapy with microarray analysis to identify novel markers and critical signaling pathways associated with tumor growth and metastasis.

Declaration of interest
We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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