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TITLE: Alternate Splicing of CD44 Messenger RNA in Prostate
Cancer Growth

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14. ABSTRACT <u>Aim 1:</u> Loss of CD44 standard and increased splice variant form CD44v7-10 facilitate prostate cancer (PC) invasion. First Sub-Aim: A manuscript was published (2008) on the role of Mitogen-activated protein kinase (MAPK) pathways and paracrine calcitonin, both of which dysregulate CD44. Second Sub-Aim: Metabolic labeling studies of CD44 total and CD44v7-10 protein were pursued over about a 6-month period, but the findings were not publishable. <u>Aim 2:</u> Instead of adeno-associated virus for altering expression of CD44 prior to in vitro and in vivo studies, retroviruses were used. The focus was on PC-3M PC cells. Confirmation of re-expression of CD44s as a 1) Fusion protein (with luciferase) or 2) Separate protein, or 3) RNAi knockdown of CD44v7-10, was achieved using qRT-PCR, western blot analysis, and IVIS visualization of luminescence after adding luciferin substrate, in a flask or mouse tumor. Cells re-expressing CD44s had decreased growth, decreased Matrigel migration and invasion, decreased anchorage-independent colony formation, and restoration of adhesion to hyaluronan (a benign feature). RNAi against CD44v7-10 caused decreased Matrigel invasion and markedly increased Docetaxel chemosensitivity, as the only in vitro changes. All 3 treatments had mild non-significant anti-growth effects on mouse subcutaneous xenografts. A manuscript is under review (BMC Cancer). <u>Other:</u> 3 manuscripts published on the effect of Silibinin, microRNAs 373 and 520c, and hydantoin compounds, on CD44 expression.					
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INTRODUCTION:

By the end of 2008, we had **three** paper publications (1, 2, 3) on various aspects of CD44 function in prostate cancer. These were reported on in the April 2009 progress report. The six-month cost-free extension enabled completion of work leading to **two more** manuscripts (Appendix). The first, “Stable alterations of CD44 isoform expression in prostate cancer cells decrease invasion and growth and alter ligand binding and chemosensitivity” fulfills Aim 2 and is under review by *BMC Cancer*. The second was not the result of a specific aim or sub-aim in the original proposal but relates to the effects of hydantoin derivatives on CD44 expression and CD44-related functions such as adhesion. It was accepted by *American Journal of Translational Research*.

CD44 is a transmembrane cell adhesion glycoprotein whose alternative splicing produces proteins that enable divergent functions in benign and cancerous prostate.

In Aim 1, we used real-time RT-PCR and western blot analysis to show significant alterations of CD44 transcription, splicing, or both, caused by 1) exogenous calcitonin and 2) inhibition of certain components of the mitogen-activated protein (MAP) kinase pathway (1). In Aim 1, we also attempted pulse-chase experiments to study the turnover of CD44 variant versus standard protein isoforms, but low abundance of these isoforms and technical complications were prohibitive.

For Aim 2, we decided to establish lentivector (rather than plasmid or AAV—as proposed earlier) prostate cancer cell transfectants to over-express CD44 standard isoform (anti-tumorigenic). We established other transfectants bearing RNA interference against the pro-tumorigenic CD44 variant. These transfectants have been used in a series of *in vitro* assays as well as in mouse xenografts, and packaged into a submitted manuscript.

BODY:

Note on Timing and Personnel: My progress in the first year was limited because I had recently moved from the Gainesville, Florida, Veterans Affairs Medical Center and University of Florida to the University of Colorado, with a start date at work of March 23, 2007. Although funding became effective April 1, 2007, it took 3 months to get a Research Assistant.

Delays were due to the transfer of the award from my old institution (University of Florida) to my new one (University of Colorado Health Science Center); to the physical necessities of the move; and to the need to recruit by advertising; and to administrative start date constraints. **Mr. Eric Robbins**, M.S. (Microbiology) served from July, 2007, to July, 2008 as *Research Assistant*.

Using my start-up funds, **Dr. Emily Travanty**, Ph.D. (Microbiology) was hired as a *Fellow* September 1, 2007. Dr. Travanty had completed a 3-year fellowship in virology and remained with our laboratory for four months, at which time she left to take a Research Scientist position at another institution. In April, 2008, **Dr. Kui Yang**, Ph.D., was recruited as a *Fellow* and in March 2009 tapered to part time work, had a leave of absence in July-August, and resigned in mid-October to accept a full-time position in biostatistics. **Dr. Alina Handorean** was a *Fellow* from July 2008 to March 2009. A 6-month cost-free extension was obtained from March 31 to September 30, 2009. **Ms.**

Yaqiong Tang, B.S. served as *Research Assistant* from June-August 2009. Ms. Tang's special expertise with western blot analysis was crucial during this time.

Aim 1: Mitogenic Pathway Effects on Splicing: **Aim 1** was chiefly accomplished by Mr. Robbins, from July 1—December 1, 2008, particularly the first sub-Aim involving MAP kinase components which, based on prior literature, showed evidence of involvement in CD44 splicing (4), which we published (1).

Our CD44 variant primer/probe set (I brought from Florida) was suboptimal because the primers were too short and had a suboptimal G+C percent. The weeks from August to mid-September 2007 were taken up re-designing primer/probe sets for TaqMan for CD44 total (standard plus CD44v) and for CD44v7-10; with several experiments optimizing annealing temperatures and concentrations of probe and primer for the CD44s, CD44v, and the 18S ribosomal RNA control; and gaining proficiency making non-degraded RNA preparations. This involved some regular RT-PCR. RNA was isolated from treated or mock-treated cells in each experiment and examined by electrophoresis and optical densitometry.

The paper (1) broadened this Aim to include not only testing of MEK and JNK inhibitors, but also p38 inhibitor, listed in the Alternatives section of the Aim. Further, involvement of the enzyme upstream to these three pathways, protein kinase A (PKA), was tested using its specific inhibitor H89.

Another expansion of Aim 1 was that we studied the effect of exogenous or endogenous calcitonin (CT) and its receptor (CTR). The rationale for this is my decade-long collaboration with Dr. Girish Shah (5-9) (Univ. of Louisiana) and his compelling demonstration of functional roles of CT and CTR in prostate cancer growth and invasion (10-20). As depicted in Fig. 7 of my proposal, we had proposed an action of CT-CTR axis on PKA. The response of CD44 total (mostly CD44s) and CD44v7-10 to exogenous CT was elucidated (1).

Metabolic labeling of proteins: This sub-Aim was pursued by Dr. Alina Handorean from September 2008 to February 2009. It was a metabolic study attempting to explain our observation that overexpression of CD44s caused an inhibition of CD44v (9). It involved radio-labeling the endogenous CD44v in cells in which re-expression of CD44s was (or was not) enforced.

The entire fall of 2008 and winter of 2009 were spent on this project, but there were two insurmountable problems. First, we did not have a reliable antibody for CD44v9. We had some of the v9 supernatant from ATCC's HB-258 left over from my previous lab in Florida from 2005-06, and it worked for the purpose of our MAP Kinase/calcitonin paper (1) but then ceased to work after several uses. Two purchases of the same hybridoma cell line from ATCC, in late 2008, yielded a supernatant that failed by dot blot and western blot analyses to detect CD44v9 in cells that should have been strongly positive for it, such as PC-3 and PC-3M.

The second problem was that the amount of CD44v7-10 protein was probably not abundant enough to immunoprecipitate.

A future alternative approach to examining this problem might use the luciferase vector that we developed for CD44 promoter (altered transcription) and, if we can make it work,

the one for CD44v splicing. They might reveal a direct effect of CD44s overexpression on CD44v splicing.

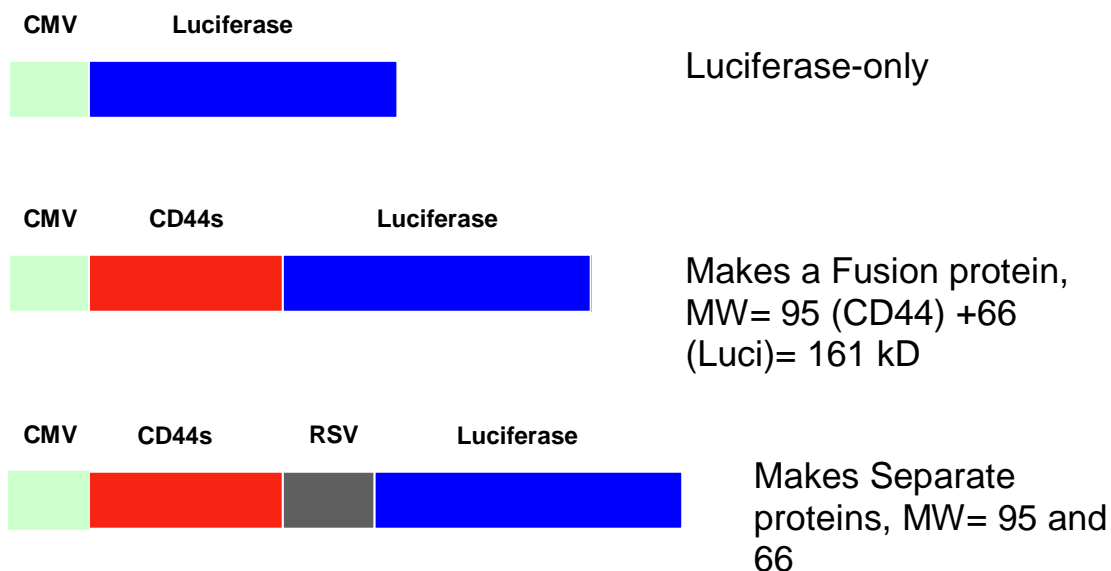
Dr. Handorean's write-up of the project is appended (**Appendix 1**).

Aim 2: Approaches to this Aim using adeno-associated virus 2 (AAV2) approach were begun in 2007 by Dr. Travanty and Mr. Robbins. Subsequently, lentivirus (or other retrovirus) constructs were fully developed by Dr. Kui Yang in the summer of 2008. Using these constructs, we have performed a number of *in vitro* assays, and grown transfected cells (or controls) as tumors in 36 mice.

Our efforts to use either a plasmid delivery system or AAV for transient or stable alteration of CD44, were described in the April 2008 progress report. Briefly, after spending several months in this way, we decided by March 2008 when we realized the viral construct was too cytotoxic.

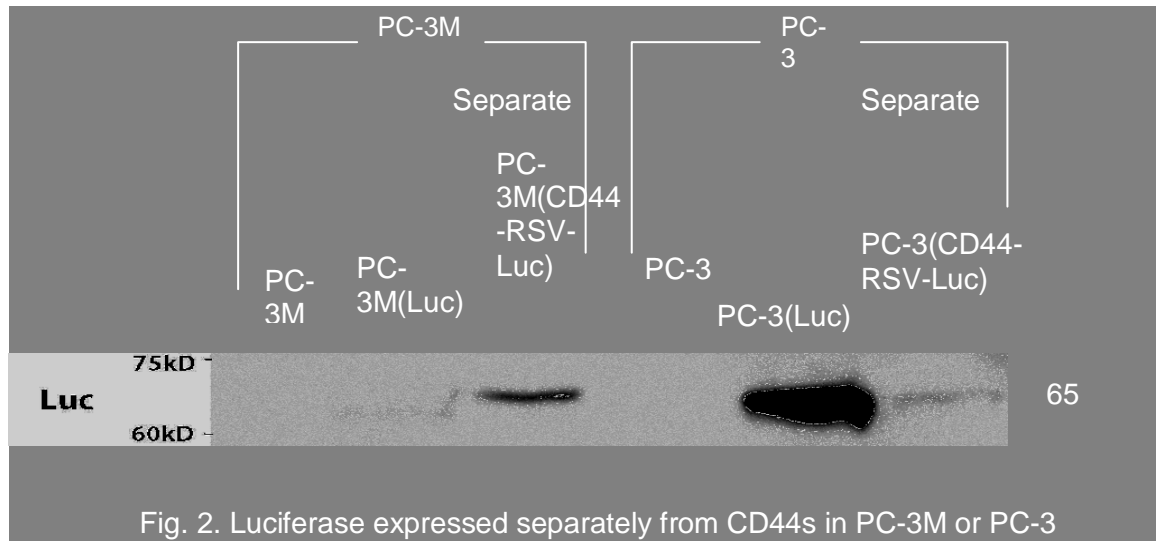
After performing some experiments with our old, non-viral approach using PC-3 cells and transient pTracer plasmid, we switched to lentivirus vectors. Three constructs were made in Lentivector pLEX-MCS (Open Biosystems, Huntsville, AL), namely: Luciferase-only, Lenti-CD44s-luciferase, and Lenti-CD44s-RSV-luciferase (**Fig. 1**) as below. Lentivector contains the cytomegalovirus promoter, associated sequences, and puromycin resistance gene.

Figure. 1 CD44s luciferase constructs in Lentivector



Four shRNA constructs were made for knocking down CD44 variant 9 (of CD44v7-10). Three sequences were generated by Extractor 4 computer program, and the fourth was from our prior publication (7). Retrovirus was produced from pSuper-RETRO (OligoEngine, Seattle) derived from pSuper. The RNAi construct, like the overexpression construct, contained a puromycin gene for selection. However, it lacked luciferase for detection. Thus, the only means of confirming knocked down CD44v7-10 were qRT-PCR (**Table 1**) or western blot (**Figs. 2-4**). Once these constructs were made, we confirmed the altered expression.

One way of demonstrating altered expression of luciferase, and by extension, of the accompanying protein, was to do western blot analysis directly for luciferase (**Fig. 2**).



A second approach was to perform qRT-PCR with probe and primer sets specific for CD44 total directed against a CD44 standard exon (standard plus variant), or a set specific for CD44v9 (detects CD44v7-10).

Table 1. qRT-PCR confirmation of altered CD44 expression

Cells:	Normalized* CD44 total	Normalized*CD44 v7-10
PC-3M Fusion	1.80	0.79
PC-3M Sep	4.35	1.0
1519 v7-10 RNAi	0.33	0.55
1522 v7-10 RNAi	0.58	0.21

*Normalized to untreated cells

As **Table 1** shows, CD44 total, most of which is CD44s, appeared more increased in PC-3M Separate cells than Fusion cells, but western blots showed both approaches were effective. RNAi against CD44v7-10 appeared more effectively achieved by the 1522 construct than the 1519 one, and this was borne out in western blot analysis (**Figs. 3-4**):

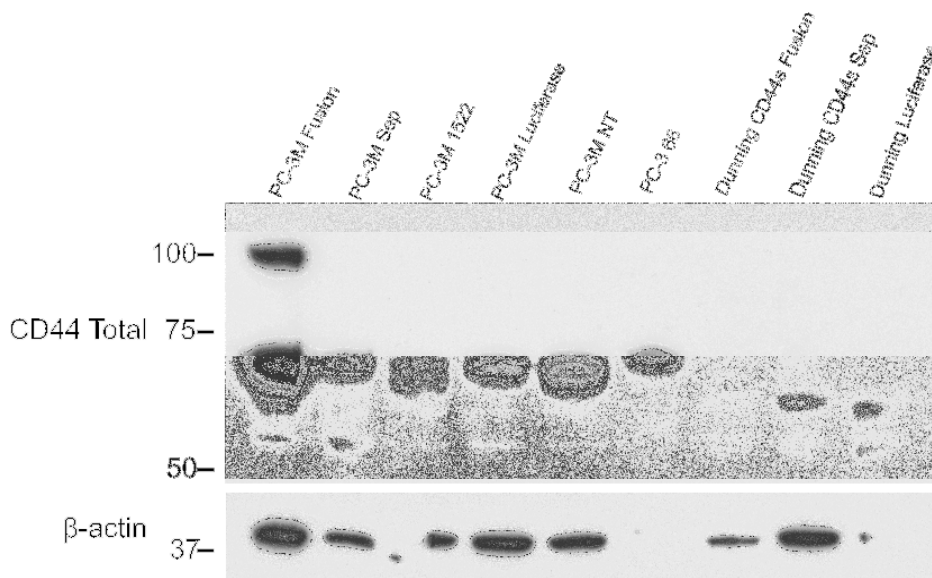


Fig. 3. Antibody to CD44 Total detects a predominant signal at 70 kD,
 ←
 corresponding to CD44s. In the Fusion construct, CD44s-luciferase is noted at 105 kD, but there also is some CD44s as a separate protein.

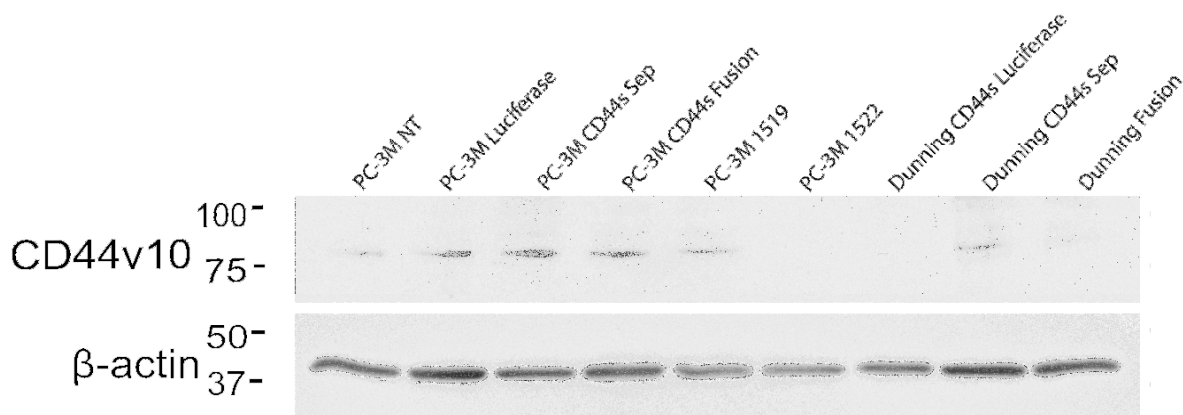


Fig. 4. To detect CD44v7-10, antibody to CD44v10 was used in lieu of the ineffective hybridoma supernatant for CD44v9. The detection of a predominant cleavage product at 80 kD rather than the >100 kD uncleaved form, is consistent with our experience (1, 6-9). The re-expression of CD44s as a separate (Sep) or Fusion protein (lanes 3-4) was shown to have no effect on CD44v7-10. Two of our RNAi constructs, 1519 and 1522, were tested for knockdown of CD44v7-10. The knockdown was most successful in 1522, so 1522 was used for all future in vitro and in vivo assays.

RIGHT: The Dunning (rat) cells, containing the luciferase construct only, do not express CD44v7-10 detectable by the anti-human antibody we used.

The attached manuscript, submitted to *BMC Cancer*, details our findings in a series of in vitro experiments (Matrigel invasion and migration, growth, soft agar colony formation, adhesion assays, and chemosensitization assays using the common agent Docetaxel). The manuscript also covers our findings with growth of xenografts in mice, explained in more detail below. The western blot analysis *expertise of Ms. Yaqiong Tang* who spent 3 months in our laboratory, was critical to documenting alterations in CD44 and its

downstream effector, merlin, for submission of our “Stable alterations of CD44 isoform expression...” manuscript.

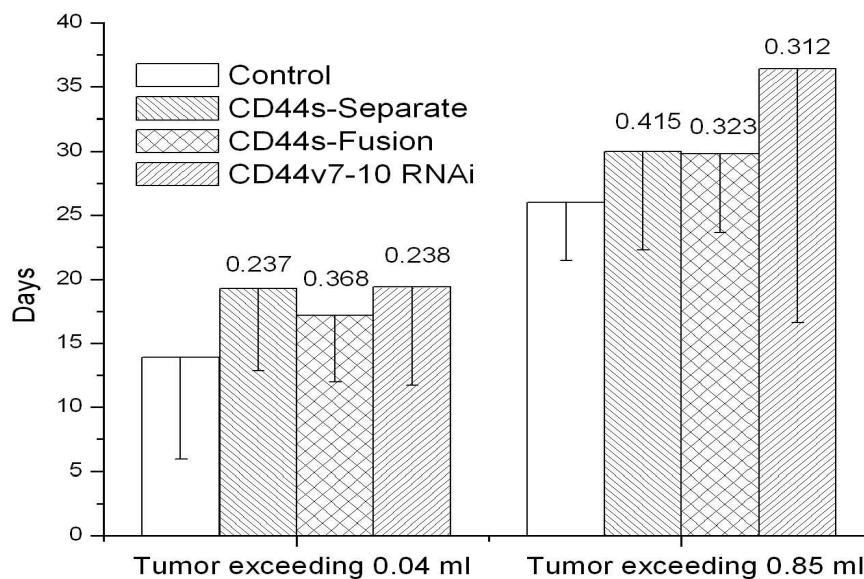
6. *In vivo* growth assays in mice. Dr. Kui Yang was largely responsible for this work. Either pretreated PC-3 cells or Dunning rat prostate cells, were grown subcutaneously in nude mice. The cells were imaged with an IVIS (In Vivo Imaging System, Xenogen Corp., Hopkinton, MA). No metastatic tumor was found from the PC-3M cells (as expected) nor from the Dunning cells (in which metastases *were* expected). Even more importantly, our overexpression and RNAi constructs in the Dunning cells were not effective by western blot, probably because CD44s and CD44v7-10 exons differ from a few base pairs from human CD44. Thus we discontinued all work with Dunning cells. We have used 36 mice to date (of 108 for which we were authorized), as detailed in in Table 2. The results for the PC-3M cell transfectants are shown in **Figure 11**, and those for the Dunning cell transfectants—not worthy of inclusion in the manuscript—in **Figure 12**.

Table 2. Use of mice for Aim 2 of the project

Mouse	Date	Cells used	Cumulative total used, each type
1	8/8/08	PC-3M	1
2	8/8/08	PC-3M scrambled mock RNAi	1
2	9/19/08	PC-3M fusion: no growth	1
2	10/13/08	PC-3M: no growth	Don't count
3	10/2/08	PC-3M fusion	2
4	10/2/08	PC-3M	2
5	11/11/08	PC-3M	3
6	11/11/08	PC-3M separate	1
7	11/21/08	PC-3M fusion	3
7	12/16/08	PC-3M CD44v-RNAi: no growth	1
8	12/24/08	PC-3M CD44v-RNAi	2
9	12/24/08	PC-3M luciferase	1
10	12/24/08	PC-3M fusion: no growth	4
11	2/4/09	PC-3M fusion: no growth	5
11	3/9/09	PC-3M separate	2
12	2/25/09	Dunning-luciferase	1
13	2/25/09	Dunning-luciferase	2
14	2/25/09	Dunning-fusion	1
15	2/25/09	Dunning-fusion	2
16	3/11/09	Dunning-separate	1
17	3/24/09	Dunning-separate	2
18	3/24/09	Dunning-separate	3
19	3/24/09	Dunning-luciferase	3
20	3/24/09	Dunning-luciferase	4
21	4/9/09	PC-3M	4

22	4/9/09	PC-3M luciferase	2
23	4/9/09	PC-3M fusion	6
24	4/15/09	PC-3M separate	3
25	4/9/09	PC-3M separate	4
26	4/27/09	PC-3M CD44v-RNAi	3
27	4/27/09	PC-3M CD44v-RNAi	4
28	4/27/09	PC-3M CD44v-RNAi	5
29	4/27/09	PC-3M separate	5
30	4/27/09	PC-3M	5
31	5/19/09	PC-3M fusion	7
32	5/20/09	PC-3M CD44v-RNAi	6
33	5/20/09	PC-3M CD44v-RNAi	7
34	8/3/09	PC-3M CD44v-RNAi	8
35	8/3/09	PC-3M CD44v-RNAi	9
36	8/10/09	PC-3M CD44v-RNAi	10

Figure 11.
PC-3M CELL GROWTH IN MICE



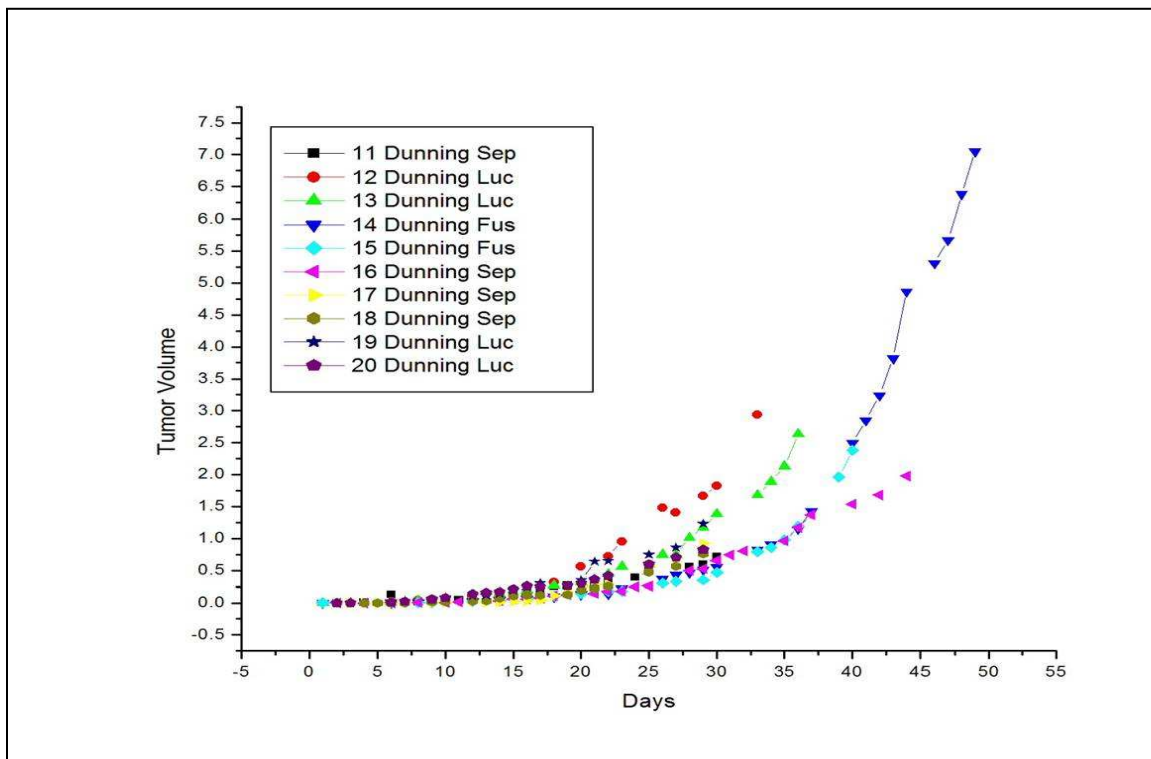
Cell Type:	PC-3M control	CD44s-Separate	CD44s-Fusion	CD44v7-10 RNAi
Tumor take	9/12 (75%)	7/8 (88%)	4/7 (57%)	4/7 (57%)

With the PC-3M cells, it became apparent that a Kaplan-Meier analysis would be most likely to disclose possible significant differences in growth rates. Tumor volumes of 0.04 and 0.85 ml were chosen because these represented the minimum palpable tumor, and the minimum fully grown tumor in any mouse at sacrifice. Several conclusions were apparent:

- 1) In PC-3M controls (untreated cells or those with luciferase only or mock scrambled RNAi), the tumor 'take' was 75%, while with PC-3M-CD44s-Fusion or CD44v7-10 RNAi cells, the tumor take was 57%.
- 2) When we had reached $n \geq 5$ mice in each group, there were only nonsignificant trends toward slower growth rate with all three treatments. The wide variability within each treatment regimen *discouraged us from using more mice* (of 108 possible) as it became apparent that significant differences between treatment groups would not be achievable.
- 3) There are still some important disparities in growth rates within a given cell transfectant type, but experiments are now in progress toward the goal mice for each group.

We also performed experiments using Dunning rat cells (**Figure 12**). We had been sufficiently convinced on the basis of just a weak-to-moderate luciferase signal emitted by transfected cells that we had achieved expression of the desired sequences. However, after repeating several western blot analyses we came to the conclusion that we failed to alter CD44s (re-expression) or CD44v7-10 (knockdown) noticeably in the transfected cells. That essentially invalidated the growth results with Dunning cells; however, the results are still presented below.

Figure 12. DUNNING RAT CELL TUMOR GROWTH IN MICE



As of April, the focus shifted to PC-3M cells only. Between late May and early August, we decided to focus mostly on *in vitro* experiments. Preliminary data, however, suggested that CD44v7-10 knockdown might be the most effective of the three main treatments we tried, so in August we initiated 3 more experiments with CD44v7-10 RNAi

In conclusion, **Table 3** below summarizes our observations regarding the transfected PC-3M cells.

Table 3. Summary of *in vitro* and *in vivo* observations in PC-3M cells stably transfected with three different treatments, in comparison to PC-3M-luciferase-only cells.

Characteristic Assessed:	CD44s-Sep	CD44s-Fus	RNAi to CD44v7-10
Growth	↓	↓	No effect
Matrigel migration & invasion	↓	↓	↓
Soft agar colony formation	↓		No effect
Hyaluronan adhesion	↑	No effect	No effect
Chemosensitivity to Docetaxel	↑ (weak)	↑ (weak)	↑ (marked)
Xenograft tumor take and growth	Probably no effect	Probably no effect	Nonsignificant trend of ↓

Our project is complete, thanks to the 6-month cost-free extension, allowing sufficient time for the ancillary experiments for manuscript submission.

KEY RESEARCH ACCOMPLISHMENTS:

1. Completion of Aim 1's sub-Aim 1; publication of manuscript (1).
2. Completion of Aim 2, submission of manuscript (Appendix).
3. The scope of Aim 2 was expanded: Because of the importance the ezrin-moesin-merlin (ERM) complex in mediating the cytoplasmic signaling of CD44 (23-25), we were interested in how the C-terminal cytoplasmic tail of CD44 interacted with Merlin, a cytoplasmic protein that increases growth in the phosphorylated state and prevents growth in the unphosphorylated state. We postulated that CD44s-Separate protein functions differently from the Fusion protein. The Fusion protein has luciferase bound to its cytoplasmic C-terminus, and so would be unable to interact with Merlin. We considered growing cells on coverslips and directly visualizing the cytoplasmic versus cell membrane localization of the luciferase. This would provide some indication of whether CD44s-Fusion inserts itself in the cell membrane. However, technical constraints were limiting so this was not done.

Plasmid pUHD10-3 that contained *EcoR*I fragments for either wild-type merlin cDNA or for ineffective, mutant merlin, was a gift of Dr. D. H. Gutmann of Washington University in St. Louis. We grew larger amounts of both, sufficient for transfection. We successfully overexpressed Merlin, and then tested its effect in PC-3M cells with altered CD44 expression. CD44s overexpression as a Separate but not a Fusion protein, increased a hypophosphorylated form of P-merlin and total merlin expression. Merlin overexpression, likewise, increased CD44s, suggesting the two molecules stabilize each other through binding. The inclusion of merlin studies expanded the scope of Aim 2.

4. Based on the discovery that miR-373 and miR-520c had important interactions with CD44 in breast cancer (21), we investigated their effects in prostate cancer (2). We used the *luciferase promoter construct* to test further the effects of these miRs and found them to act somewhat differently in prostate cancer.

5. Because of the interest at Univ. of Colorado in the nutritional chemoprevention of prostate cancer by the anti-growth compound Silibinin, we collaborated with Dr. Rajesh Agarwal, of the Pharmacology Department, and found an inhibitory effect on CD44 total and CD44 variant; and this work was published (3).

This has led to a collaboration with Dr. Thomas Flaig (Urologic Oncology) who has access to prostatectomy tissues from patients treated with Silibinin. We used the *luciferase promoter construct* to test further the effects of Silibinin. Silibinin did decrease total CD44 and CD44v7-10, suggesting a transcriptional site of action (3).

6. Preparation of luciferase constructs for CD44 promoter activation (using a construct from Dr. Jim Lambert of our Department) and for CD44 variant splicing. These have great potential to supplement our work to come. These constructs could be used to verify the extent of CD44 alteration in our cell lines, although we have relied mainly on visualization for the CD44s re-expression transfectants.

In February 2008, the CD44 variant splicing pET construct and empty pET vector were obtained from Dr. Harald Koenig in Germany. They had a CD44v5 splicing construct for use with lymphocytes (22), but we removed it and replaced it with CD44 v7-10 in the multiple cloning site. These constructs were prepared but have not proven effective in our hands and work on this project was discontinued in early 2008. The 8 weeks of work done in preparation of the luciferase constructs were detailed in last year's report, Appendix 2.

7. Because of the interest of some of our colleagues in the Univ. of Colorado Pathology Department's Prostate Cancer Research Laboratory (26) in the tumor suppressive effects of vitamin D (27, 28), we tested the effect of vitamin D and an analog on CD44 expression in G_sα-QL and ALVA-3 (a PC-3 derivative) cells. This work was done in 9/07-11/07. Effects of vitamin D or its butyl ester (BE) analog (1 α ,3 β ,24(OH)₃-22-ene-24-cyclopropyl-25-n-butyl-ester-vitamin D₃) at 10⁻⁷ or 10⁻⁸ M doses, or of the vitamin D receptor antagonist ZK159222 were tested for 24 hr. TaqMan assays for CD44s or CD44v levels gave some hints of a change but results were not consistent. With the use of the above-mentioned *luciferase promoter construct*, we tested whether vitamin D

caused CD44 transcription changes in prostate cancer cells. There were no changes. We had thought of including the vitamin D data with the silibinin data (3), but because the result was negative, we limited the paper to Silibinin only.

8. Using my colleague Dr. Girish Shah's discovery that hydantoin derivatives PMH and S-PMH had the capacity to affect invasion, growth, and aggressiveness features of prostate cancer cells, we postulated that these phenotypic changes would be accompanied by, and perhaps mediated by, CD44. Performance of adhesion assays and CD44s and CD44v7-10 expression studies showed that this was true, and this resulted in a new, accepted manuscript (Appendix).

REPORTABLE OUTCOMES:

Manuscripts: Three manuscripts have been published (1-3). The PDFs are in the Appendix. Two others not yet in print are in the Appendix.

Presentations: The content of the paper entitled "Stable alterations of CD44 isoform expression in prostate cancer cells decrease invasion and growth and alter ligand binding and chemosensitivity" has been accepted for poster presentation at the Society for Basic Urologic Research meeting in New Orleans, November 5-8, 2009. Dr. Iczkowski was also the invited speaker at the Hormone-Related Malignancies conference of the Oncology Department, Univ. of Colorado Denver on April 29, 2009. A presentation of most of these data was also made then by Dr. Iczkowski.

Cell lines: We have established stable cell lines with CD44 variant RNAi, and as controls, CD44 variant RNAi with 4 conservative mutations. We have cells overexpressing CD44s as a separate protein or as a fusion protein with luciferase. All are frozen in liquid nitrogen for possible future use or sharing with other investigators.

Grant proposals submitted: A DOD Prostate Cancer Research Program pre-proposal was submitted in March 2009, proposing to pursue the microRNA project, on the strength of our published findings (2). However, based on the pre-proposal, we were not invited to submit a full proposal.

Trainees: Two college students spent summers as interns in my laboratory, learning basic research techniques: Mr. Dan Howard (Northwestern University) in 2008, and Mr. Gabriel Habermehl (University of Colorado Boulder) in 2009.

CONCLUSION: During the grant funded period, we accomplished most of Aim 1. We finished all of Aim 2 and expanded its original sub-aims and extended their scope through several *in vitro* invasion and proliferation assays and Docetaxel sensitivity assays.

We also widened our studies to address three topics of contemporary interest as they relate to CD44: the influences of microRNA, Silibinin, and hydantoin compounds on CD44 expression in prostate cancer.

The *in vitro* experiments suggest that re-expression of CD44s holds promise for gene therapy, alter morphology and cell adhesion, and can reduce prostate cancer growth and invasion. Why the *in vivo* experiments failed to show a similar magnitude of effect is not

certain, but could have been the result of using subcutaneous xenografts rather than the more technically difficult orthotopic technique, as discussed in our submitted manuscript.

REFERENCES

1. Robbins EW, Travanty EA, Yang K, **Iczkowski KA**. MAP kinase pathways and calcitonin influence CD44 alternate isoform expression in prostate cancer cells. *BioMed Central Cancer* 8:260, 2008.
2. Yang K, Handorean AM, and **Iczkowski KA**. MicroRNA 373 and 520c are downregulated in prostate cancer, suppress CD44 translation, and modulate prostate cancer cell invasion. *International J Clinical and Experimental Pathology* 2:361-9, 2009.
3. Handorean AM, Yang K, Flaig T, Robbins EW, and **Iczkowski KA**. Silibinin suppresses CD44 expression in prostate cancer cells. *American Journal of Translational Research* 1:80-7, 2009.
4. Lee HY, Oh SH, Suh YA, Baek JH, Papadimitrakopoulou V, Huang S, Hong WK. Response of non-small cell lung cancer cells to the inhibitors of phosphatidylinositol 3-kinase/Akt- and MAPK kinase 4/c-Jun NH2-terminal kinase pathways: an effective therapeutic strategy for lung cancer. *Clin Cancer Res* 2005;11:6065-74.
5. Iczkowski KA, Pantazis CG, Long J, Noble MJ, Shah, GV. Expression of salmon calcitonin-like peptide is increased in prostatic adenocarcinoma. *Journal of Urologic Pathology* 1998;8:135-147.
6. Iczkowski KA, Bai S, Pantazis CG. Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. *Anticancer Research* 2003;23:3129-40.
7. Omara-Opyene AL, Qiu J, Shah G, Iczkowski KA. Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18. *Laboratory Investigation* 2004;84:894-907. Editorial Comment pages 811-813.
8. Iczkowski KA, Omara-Opyene AL, Kulkarni TR, Pansara M, Shah GV. Paracrine calcitonin in prostate cancer is linked to CD44 variant expression and invasion. *Anticancer Res* 2005;25:2075-83.
9. Iczkowski KA, Omara-Opyene AL, Shah GV. The predominant CD44 splice variant in prostate cancer binds fibronectin, and calcitonin stimulates its expression. *Anticancer Res* 2006;26:2863-72.
10. Chien J, Shah GV. Role of stimulatory guanine nucleotide binding protein ($G_s\alpha$) in proliferation of PC-3M prostate cancer cells. *Int J Cancer* 2001;91:46-54.
11. Chien J, Wong E, Nikes E, Noble MJ, Pantazis CG, Shah GV. Constitutive activation of stimulatory guanine nucleotide binding protein ($G_s\alpha$ -QL)-mediated signaling increases invasiveness and tumorigenicity of PC-3M prostate cancer cells. *Oncogene* 1999;18:3376-82.
12. Chien J, Ren Y, Qing Wang Y, Bordelon W, Thompson E, Davis R, Rayford W, Shah G. Calcitonin is a prostate epithelium-derived growth stimulatory peptide. *Mol Cell Endocrinol* 2001;181:69-79.
13. Sabbisetti VS, Chirugupati S, Thomas S, Vaidya KS, Reardon D, Chiriva-Internati M, Iczkowski KA, Shah GV. Calcitonin increases invasiveness of prostate cancer cells: role for cyclic AMP-dependent protein kinase A in calcitonin action. *Int J Cancer*. 2005;117:551-60.
14. Shah GV, Noble MJ, Austenfeld M, Weigel J, Deftos LJ, Mebust WK. Presence of calcitonin-like immunoreactivity in human prostate gland: evidence for iCT secretion by cultured prostate cells. *Prostate* 2007;21:87-97.
15. Berenguer C, Boudouresque F, Dussert C, Daniel L, Muracciole X, Grino M, Rossi D, Mabrouk K, Figarella-Branger D, Martin PM, Ouafik L. Adrenomedullin, an autocrine/paracrine factor induced by androgen withdrawal, stimulates 'neuroendocrine phenotype' in LNCaP prostate tumor cells. *Oncogene* 2008; 27:506-18.

16. Shah GV, Rayford W, Noble MJ, Austenfeld M, Weigel J, J, Vamos S, Mebust WK. Calcitonin stimulates growth of human prostate cancer cells through receptor-mediated increase in cyclic adenosine 3', 5'-monophosphates and cytoplasmic Ca²⁺ transients. *Endocrinology* 1994;134:596-602.
17. Thomas S, Chigurupati S, Anbalagan M, Shah G. Calcitonin increases tumorigenicity of prostate cancer cells: evidence for the role of protein kinase A and urokinase-type plasminogen receptor. *Mol Endocrinol.* 2006;20:1894-911.
18. Sabbisetti V, Chigurupati S, Thomas S, Shah G. Calcitonin stimulates the secretion of urokinase-type plasminogen activator from prostate cancer cells: its possible implications on tumor cell invasion. *Int J Cancer* 2006;118:2694-702.
19. Chigurupati S, Kulkarni T, Thomas S, Shah G. Calcitonin stimulates multiple stages of angiogenesis by directly acting on endothelial cells. *Cancer Res* 2005;65:8519-29.
20. Thomas S, Chiriva-Internati M, Shah GV. Calcitonin receptor-stimulated migration of prostate cancer cells is mediated by urokinase receptor-integrin signaling. *Clin Exp Metastasis* 2007;24:363-77.
21. Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Puré E, Agami R. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. *Nat Cell Biol* 2008;10:202-10.
22. Weg-Remers S, Ponta H, Herrlich P, Konig H. Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway. *EMBO J* 2001;20:4194-203.
23. Martin TA, Harrison G, Mansel RE, Jiang WG. The role of the CD44/ezrin complex in cancer metastasis. *Crit Rev Oncol Hematol* 2003;46:15-186.
24. Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* 2001;15:968-80.
25. Horiguchi A, Zheng R, Shen R, Nanus DM. Inactivation of the NF2 tumor suppressor protein merlin in DU145 prostate cancer cells. *Prostate* 2008;68:975-94.
26. Lambert JR, Young CD, Persons KS, Ray R. Mechanistic and pharmacodynamic studies of a 25-hydroxyvitamin D3 derivative in prostate cancer cells. *Biochem Biophys Res Commun.* 2007;361:189-95.
27. Banach-Petrosky W, Ouyang X, Gao H, Nader K, Ji Y, Suh N, DiPaola RS, Abate-Shen C. Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3.1;Pten mutant mice. *Clin Cancer Res.* 2006;12:5895-901.
28. Yang ES, Maiorino CA, Roos BA, Knight SR, Burnstein KL. Vitamin D-mediated growth inhibition of an androgen-ablated LNCaP cell line model of human prostate cancer. *Mol Cell Endocrinol* 2002;186:69-79.

APPENDICES:

Appendix 1. Metabolic Labeling Study. By Dr. Alina Handorean

Appendix 2. Our five papers (three published, one accepted, one under review)

Research report

Metabolic labeling of proteins

The metabolic labeling of proteins of PC3M cells nontreated and PC3M cells overexpressing CD44s was used as a tool to discriminate the expression level of proteins in the two cell types. CD44s overexpressing cells should have a lower level of expression of CD44v9 proteins, fact that can be illustrated by using phosphor imaging that will provide information on the decrease in the band corresponding to this protein in PC3M cells overexpressing CD44s.

From the information available from the PDB (Protein Data Bank) we found out that the hyaluronan binding domain of human CD44(1poz) that consists of 150 amino acids has 3 methionine residues and 6 cysteine residues that can be potentially labeled by using the [35S] Met [35S]Cys mixture from Perkin Elmer. The whole protein contains 742 amino acids, and consists of 14 residues of methionine and 9 residues of cysteine. (Protein ID P16070).

The cells overexpressing CD44s and the PC3M control cells were grown in 6-well plates in RPMI medium + 10% dialyzed fetal bovine serum (FBS) until they were 80-90% confluent. Prior to the radiolabeling step, the medium was replaced with methionine-deficient RPMI 1640 medium +10 % dialyzed FBS and the cells were allowed to grow for 30 minutes at 37°C in a 5% CO₂ incubator. 25µCi of the radioactive mixture were added to 1 mL of RPMI medium and allow the cells to incorporate radioactive methionine and cysteine. After 1 hour, the radioactive medium was replaced by RPMI depleted medium that contained 100µg/mL cold cysteine and 100µg/mL cold methionine. The proteins were extracted using RIPA buffer and the amount of radioactivity incorporated was checked by running an SDS-PAGE gel followed by exposure to the cassette and phosphor imaging. No band could be detected, so we concluded that the amount of time the cells were in contact with the radioactive mixture was insufficient for the incorporation of radioactive material during protein synthesis.

We performed a time course reaction permitting the cells to be in contact with the radioactive mixture for a longer time and we determined by phosphor imaging that the one hour incubation used previously was insufficient and the optimum time for labeling is 16 or 24 hours. (Figure 1)

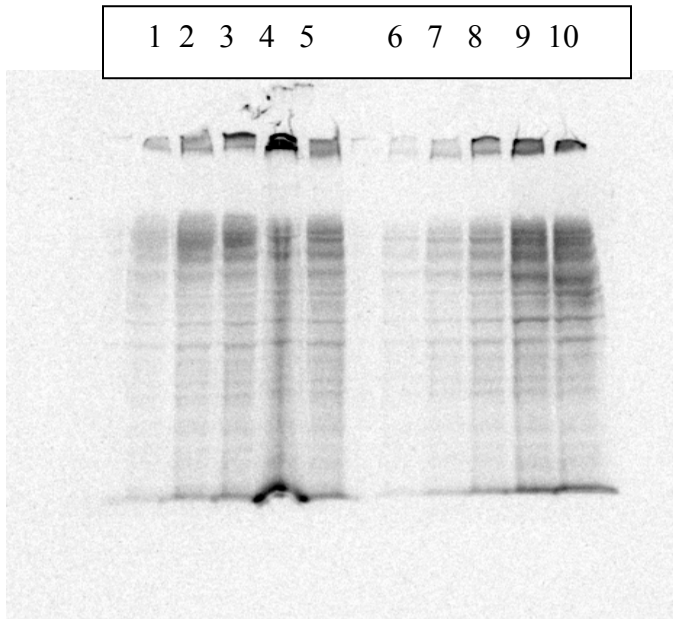


Figure 1 legend:

Lane 1-5: PC3M untreated cell lysate

Lane 6-10: PC3M overexpressing CD44s (luciferase construct)

Lanes 1 and 6: 1 hr exposure

Lanes 2 and 7: 2 hrs exposure

Lanes 3 and 8: 6 hrs exposure

Lanes 4 and 9: 16 hrs exposure

Lanes 5 and 10: 24 hrs exposure

We also optimized the amount of radioactivity added to the cell culture. Initially, we added 25 μCi of radioactively labeled mixture to 1 mL of culture medium. We treated cells with 10, 25, 35 and 50 $\mu\text{Ci/mL}$ but no significant difference in terms of the radioactive labeling of proteins was observed for these samples compared to the 25 $\mu\text{Ci/mL}$ we initially used.

For the next step, we decided to use the 24 hours cell lysate extracts for the immunoprecipitation (IP) assay. 50-100 μg of cell lysate were incubated overnight at 4°C with 100 μL of the CD44v9 culture supernatant previously grown according to the manufacturer's procedure. The protein-antibody complex was further incubated with 70 μL of protein G agarose beads, overnight at 4°C with end-over-end mixing. The solution is centrifuged at maximum speed for 1 minute and the supernatant is discarded. The precipitate is washed two times in washing solution and eluted with 4X NuPAGE buffer. Following the centrifugation, the beads will precipitate and the protein-antibody complex remains in the supernatant and it is ready to be loaded on SDS-PAGE. Following the exposure to the cassette and the phosphor imaging of the screen, no band could be detected for the expected 70 kDa molecular weight. (Figure 2)

1 2 3 4 5 6 7 8

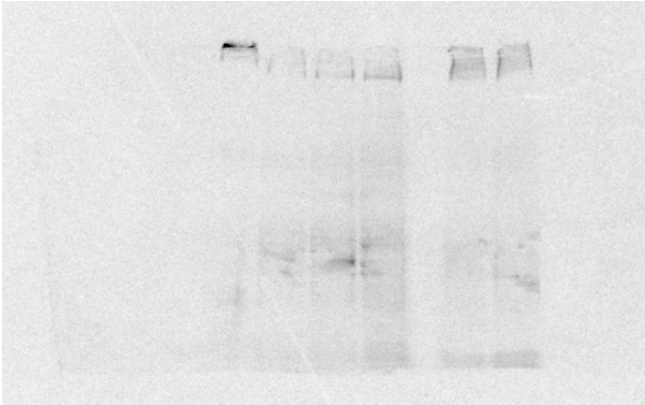


Figure 2 legend:

Lane 1-4: PC3M non treated cell lysate

Lane 1: 10 μ Ci radioactive mix

Lane 1: 25 μ Ci radioactive mix

Lane 1: 35 μ Ci radioactive mix

Lane 1: 50 μ Ci radioactive mix

Lane 5: PC3M non treated cell lysate 8 hrs exposure to radioactive mixture

Lane 6: PC3M overexpressing CD44s cell lysate 8 hrs exposure to radioactive mixture

Lane 7: PC3M non treated IP sample 8 hrs exposure to radioactive mixture

Lane 8: PC3M overexpressing CD44s IP sample 8 hrs exposure to radioactive mixture

We definitely proved that the proteins of both type of cells were radioactive labeled and we know from dot-blot experiments and other western blots run in the lab that the antibody against CD44v9 is working properly and is able to bind the protein of interest. Our conclusion is that the amount of radioactivity for this particular protein we expect to show as a 70 kDa band is not enough to discriminate between the treated and untreated cells. To confirm this conclusion, we performed measurements of the amount of radioactivity incorporated in cell lysate by liquid scintillation counter. For all samples, treated and untreated, the calculated amount of radioactivity incorporated ranged from 2-10%, that corresponds to the amount reported in the literature.(1). However, this low percentage prevented us to distinguish the treated from untreated samples relative to the expression levels of CD44v9.

References:

1.Chen, L.-C., Casadevall, A. – “Labeling of proteins with [35S] Methionine and/or [35S] Cysteine in the Absence of the Cells”, Anal. Biochem, 269, 179-188 (1999) –it’s in my folder of references paper for CD44v9 project, on the shelf.

Research article

Open Access

MAP kinase pathways and calcitonin influence CD44 alternate isoform expression in prostate cancer cells

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Abstract

Background: Dysregulated expression and splicing of cell adhesion marker CD44 is found in many types of cancer. In prostate cancer (PC) specifically, the standard isoform (CD44s) has been found to be downregulated compared with benign tissue whereas predominant variant isoform CD44v7-10 is upregulated. Mitogen-activated protein kinase pathways and paracrine calcitonin are two common factors linked to dysregulated expression and splicing of CD44 in cancer. Calcitonin has been found to increase proliferation and invasion in PC acting through the protein kinase A pathway.

Methods: In androgen-independent PC with known high CD44v7-10 expression, CD44 total and CD44v7-10 RNA or protein were assessed in response to exogenous and endogenous calcitonin and to inhibitors of protein kinase A, MEK, JNK, or p38 kinase. Benign cells and calcitonin receptor-negative PC cells were also tested.

Results: MEK or p38 but not JNK reduced CD44 total RNA by 40%–65% in cancer and benign cells. Inhibition of protein kinase A reduced CD44 total and v7-10 protein expression. In calcitonin receptor-positive cells only, calcitonin increased CD44 variant RNA and protein by 3 h and persisting to 48 h, apparently dependent on an uninhibited p38 pathway. Cells with constitutive CT expression showed an increase in CD44v7-10 mRNA but a decrease in CD44 total RNA.

Conclusion: The MEK pathway increases CD44 RNA, while calcitonin, acting through the protein kinase A and p38 pathway, facilitates variant splicing. These findings could be used in the formulation of therapeutic methods for PC targeting CD44 alternate splicing.

Background

CD44, a transmembrane glycoprotein, is the product of a gene that can undergo extensive alternate splicing. The standard (CD44s) isoform is ubiquitous but tissue-specific isoforms may include an assortment of 10 variant (v) exons (CD44v). CD44 facilitates multiple cellular functions. CD44 enables cell-cell and cell-matrix adhesion –

primarily to its main ligand hyaluronan, and links the cell membrane to the actin cytoskeleton, modulating motility. CD44 is universally dysregulated in human cancer, and this imbalance of isoforms allows tumor growth and invasion [1-8]. CD44v are expressed in prostatic secretory cells while CD44s is found in the whole epithelium. About 30% of cases of prostate cancer (PC) undergo a

transition from quiescent to aggressive. Altered CD44 and other adhesion molecules permit this transition in which tumor cells detach, interact with proteins that digest stromal matrix, migrate through matrix, and intravasate into lymphovascular channels.

By isolating RNA from clinical PC specimens, we discovered that the major variant isoform expressed in PC is CD44v7-10. This PC signature was consistently present in both primary and metastatic PC [1-3]. Interference against this CD44v caused a 69% reduction in invasion index compared to untreated control cells[3]. Moreover, PC loses the splicing ability to produce the CD44s expressed in benign prostate[3,9,10]. CD44 must oligomerize to bind matrix ligands or to cause metastasis[11] and variant isoforms, with longer extracellular tails, have altered ability to complex[12]. We found that the CD44v7-10 isoform makes PC cells preferentially bind to fibronectin rather than hyaluronan; re-expression of CD44s causes the predominant ligand to revert from fibronectin back to hyaluronan[4]. In mouse xenografts of PC-3 prostate tumor, forced expression of CD44s reduced growth *in vitro* and tumorigenicity[5], and our use of RNAi against CD44v7-10 in xenografts yielded similar effects (unpublished results).

In PC, calcitonin (CT) acts as a paracrine growth factor that up-regulates CD44 variant[4,6]. In histologic specimens PC, but not benign secretory epithelium, contains CT[13] and its receptor (CTR)[14], and CT exerts paracrine effects that promote proliferation[15], invasion[16], and metastasis[17]. CTR, essential for prostate cancer tumorigenicity[18], is coupled to the transduction protein $G_s\alpha$. We have shown that CT promotes alternate splicing leading to CD44v7-10 mRNA and protein[4,6] by acting through $G_s\alpha$ signaling[3]. $G_s\alpha$ stimulates the cyclic AMP signaling cascade[17,19] and protein kinase A (PKA)[16].

PKA, in turn, acts on the 3 main MAPK pathways: a growth factor-responsive pathway that uses MAP2K (also called MEK) as key downstream effector; and two stress-activated pathways, c-jun N-terminal kinase (JNK), and p38 kinase, that respond to stress including cytokines, osmotic shock, and irradiation. CD44 variants activate MAPK pathways[20], sometimes by functioning as co-receptors for growth factors[21]. MAPK pathways, in turn, can cause CD44 alternative splicing to include variant exons[22]. Oncogenes such as ras[7,23] and mitogens using the MEK-ERK MAP kinase (MAPK) pathway[7], but not the p38 pathway[24], induce CD44 promoter activity and increase expression of certain CD44v. To test whether these influences modulate RNA levels and alternative splicing of CD44 in PC, we studied the CT signaling system, PKA, and MAPK pathways. CD44 mRNA and protein levels were measured.

Methods

Cell lines

PC-3 cells (American Type Culture Collection, Manassas, VA) were incubated in F12-K medium, 10% fetal calf serum, and antibiotics at 37°C in a 5% CO₂ incubator. $G_s\alpha$ -QL cells, CT+, CT-, and CTR-cells were gifts of Dr. Girish Shah, Univ. of Louisiana-Monroe[17]. The $G_s\alpha$ -QL cells were derived from metastasizing PC-3M cells stably transfected by a plasmid that directs expression of mutant, constitutively active $G_s\alpha$ [17,19]. These three cell lines were grown in RPMI 1640 with L-glutamine, 5% fetal calf serum, 15% horse serum and antibiotics. Benign BPH-1 cells (from Dr. Simon Hayward, Vanderbilt Univ., Nashville, TN) were grown in RPMI with 10% fetal calf serum and antibiotics. For each experiment, cells in a flask were trypsinized and saline washed to remove trypsin. 200,000 cells were plated per well on a 6-well plate. Cells were adherent and 80% confluent for all experiments. Each treatment was applied to three wells, with three other wells as mock treated controls.

Effect of exogenous calcitonin

PC-3M cells express both CT and CT-receptor (CTR)[16]. To test the effect of solely exogenous CT on CD44, we used the derivative, CT-minus (CT-). CT-cells have endogenous CT stably knocked down to undetectable levels using anti-CT hammerhead ribozymes[25]. Salmon CT (BAChem, Torrance, CA) was used at a physiologic 50 nM dose[14,16], which effectively alters CD44[6], or at 250 nM. The K_d of CTR is 4–21 nM[15]. To detect acute versus long-acting effects on RNA and protein levels, cells were treated with 50 nM CT and harvested after 3 h or 48 h. To determine whether CT effects on CD44 proteins resulted from *de novo* protein synthesis versus protein stabilization, cycloheximide (10 nM) was given to CT-cells after 3 hr of CT exposure, and cells harvested 1 hr, 3 hr, 6 hr, and 9 hr subsequently, similar to a prior CD44 study[26]. Also tested were highly invasive, CT positive $G_s\alpha$ -QL cells[17]. Finally, to rule out non-CTR-mediated CT effects, two negative controls were tested: PC-3 cells (shown to be negative for CT receptor[14]), and cells called CTR-, derived from PC-3M cells after anti-CT receptor ribozyme knock-down of CTR[18]. CTR-cells have very low levels of CD44v protein[4].

Effect of endogenous calcitonin

To test the effect of endogenous CT on CD44 RNA, we used PC-3M cells expressing CT-pcDNA 3.1 plasmid (CT+), which constitutively express CT[25]. 125,000 PC-3M or CT+ cells per well were plated and allowed to grow for 72 hours.

Inhibition of protein kinase A and MAPK components

$G_s\alpha$ -QL cells were chosen for these studies because they have the highest baseline CD44v[3]. Protein kinase A

inhibitor H-89 (in 50% ethanol) (Calbiochem, La Jolla, CA) was added to cells in fresh medium (1 mL/well) at 1 μ M, previously shown effective in nerve cells[23] or at a 10 μ M dose. Cells were incubated with H-89 for 24 hours[27] then harvested.

In similar assays, either 10 μ l of 1 mM JNK inhibitor (SP600125, Calbiochem) or 12.5 μ l of 2 mM MEK inhibitor (PD98059, Calbiochem) in water were added, yielding concentrations previously shown effective: 10 μ M for JNK[28] and 25 μ M for MEK (personal communication, Dr. Bolin Liu). p38 kinase inhibitor (SB203580, Calbiochem) was used at 10 μ M[29,30] in DMSO, and control cells received DMSO only. Cells were incubated for the optimum time of 48 h[28] to show effects.

Interaction of CT with MAPK pathways

Based on results above, we tested the effect of pretreatment with MEK or p38 kinase inhibitors on CT-mediated alteration of CD44 expression. 25 μ M MEK inhibitor or 10 μ M p38 kinase inhibitor was added to CT-cells 4 hours prior to administering 50 nM CT. Cells were harvested after 48 h as above.

Real time TaqMan RNA analysis

Total RNA was prepared from cell pellets using Trizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNase-free water, and its concentration measured. Complementary DNA (cDNA) was synthesized from 4 μ g total RNA in 20 μ l reaction mixture as we did previously[9]. At least triplicate samples were run using a primer/probe set for all CD44v that brackets the entire variant region[6], one for CD44 total that binds a standard exon, and 18S ribosomal RNA. Quantitative PCR reactions were optimized to 4 μ g cDNA (0.16 μ g with 18S) plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 μ l. 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 ABI Prism 7700 cycler (Perkin-Elmer, Waltham, MA). Primer/probe sets for CD44v were: forward, AACGCTTCAGCCTACTGCAAA; reverse, TCITCCAAGCCTTCATGTGATG; probe, GATTTGGACAGGACAGGACCTCTTTCAATG. For CD44 total we used forward, CAACTCCATCTGTGCAGCAAA; reverse, GTAACCTCCTGAAGTGCTGCTC; probe, CATATTGCTTCAATGCTTCAGCTCCACCTG. Primer and probe sets for 18S were proprietary to the manufacturer.

Western blot analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) with protease inhibitor Complete-mini tablet (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate

was estimated by Bradford method. Samples were resolved on SDS-PAGE using 25 μ g sample/lane with the NuPAGE system (Invitrogen, Carlsbad, CA). 5 μ l of Rainbow protein marker (RPN 756, Amersham, Piscataway, NJ) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Three primary antibodies were used. To assess CD44v9 (the largest component of the overexpressed CD44v7-10) the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 standard was assessed using anti-HCAM (DF1485, Santa Cruz Biologicals, Santa Cruz, CA, 1:2000), which binds all CD44 isoforms. Anti- β -actin antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 \times 15 min in TBS with 20 mM Tris pH 7.5 and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) was added at 1:9000 dilution in 5% skim milk for 1 hr. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Statistical analysis

TaqMan data were analyzed by the $2(-\Delta\Delta C_T)$ method [31] to determine fold change in gene expression (mock treated cells = 1.00). The ΔC_T was taken as the difference between the CD44v or CD44 total and the 18S ribosomal RNA C_T s. The $\Delta\Delta C_T$ was obtained using the mean ΔC_T of mock treated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test. Statistical significance was set at $p < 0.05$.

Results

Calcitonin increases CD44v

In the PC-3M-derived CT-cells, a 50 or 250 μ M CT dose after 48 h had little effect on the total amount of CD44 RNA, but the CD44v was tripled (Fig. 1a). Although different binding affinities of primer/probe sets preclude determining CD44v as a percent of CD44 total, the *relative* percent of CD44v RNA can be calculated by the $2(-\Delta\Delta C_T)$ method, as increasing fivefold after 50 μ M CT. The same response, but less marked, was seen in $G_s\alpha$ -QL cells, at 50 and 250 μ M doses. In CTR-cells and PC-3 cells – both lacking CTR – exogenous CT had little effect. Similarly, BPH-1 cells responded to CT with very slight stimulatory effect on CD44v, and no effect on CD44 total. At the protein level, however, the CT-cells treated with CT showed increases in both total and variant CD44 after just 3 h (Fig. 1b) and at 48 h (Fig. 1c). The stimulation of CD44v protein was attenuated by cycloheximide up to 9 h after CT (data not shown). This suggests that *de novo* protein synthesis is required and that upregulation of CD44v is not simply a result of protein stabilization.

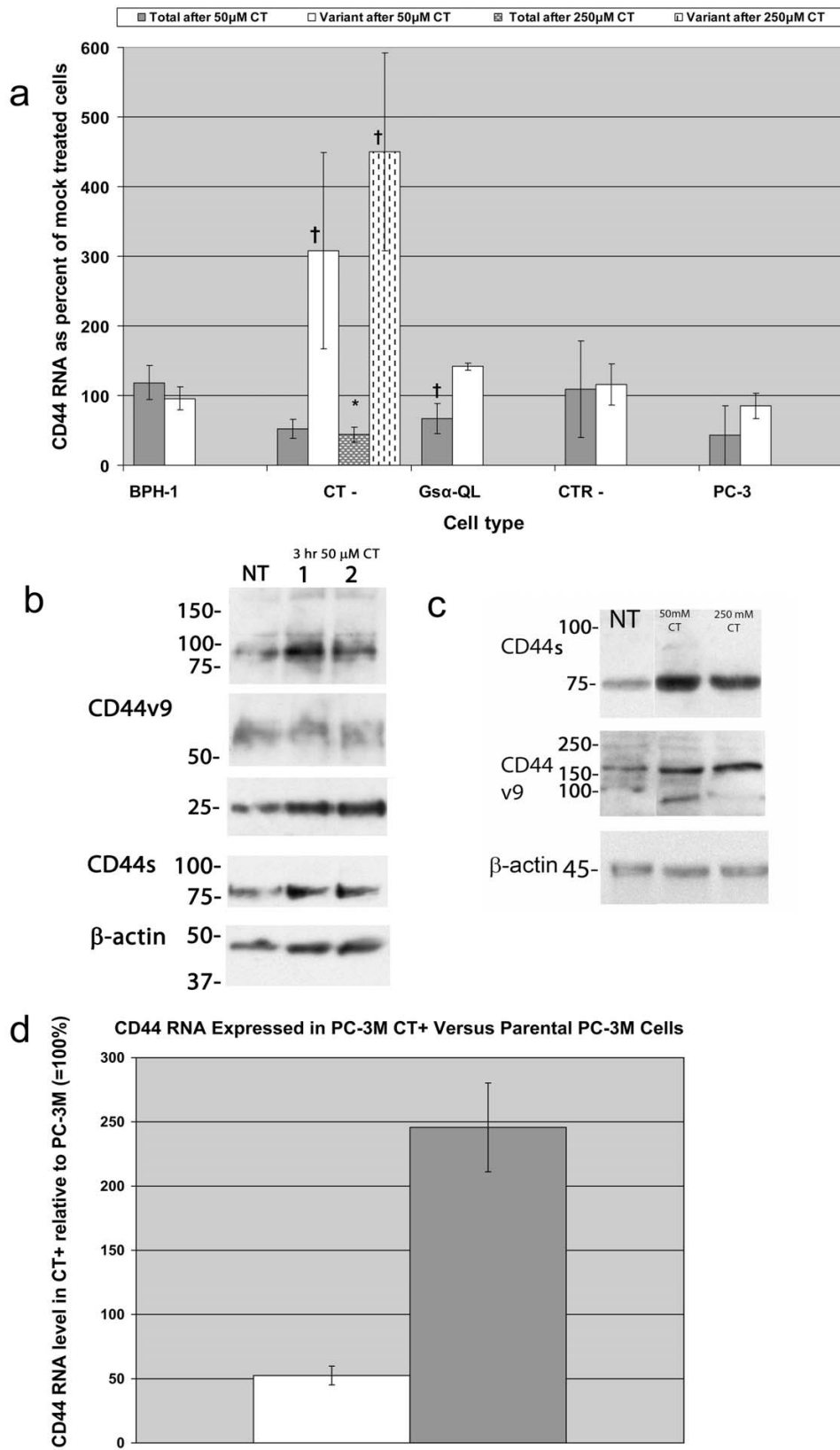


Figure 1

Figure 1

1a. RNA response to exogenous CT at 48 h is calcitonin (CT) receptor-dependent. CD44 total and variant RNA are quantified by triplicate TaqMan RT-PCR experiments. In benign BPH-1 cells, no response is observed to exogenous calcitonin (CT). CT-cells, a PC-3M derivative that lack endogenous CT but have CT receptor, showed a decrease in CD44 total RNA but an increase in variant RNA. The $G_s\alpha$ -QL cell derivative showed the same trend. In CT-receptor-negative CTR- and PC-3 cells, there is no significant response to CT. Error bars are standard deviation. * $p = 0.01$; † $p = 0.05$ with respect to mock treated controls. **1b.** Western blot analysis. Exogenous calcitonin given to the CT-cells for 3 h stimulates expression of CD44s at 75 kD (top), and CD44v at 180 kD and its cleavage products below 97 kD. Comparison is shown to cells with no treatment (NT). β -actin analysis control confirms equal protein loading (bottom). **1c.** This stimulatory effect persists at 48 h and at doses of 50 mM or 250 mM exogenous calcitonin. Also, a time-dependent reduction in protein was observed after cycloheximide treatment, suggesting that CT effect on CD44 variant requires *de novo* protein synthesis and does not result from protein stabilization. **1d.** CT+ cells, endogenously expressing CT, are derived from PC-3M cells and have increased RNA for CD44v ($p = 0.02$) but a decrease in CD44 total ($p = 0.008$).

In CT+ cells, CD44v mRNA doubled compared to PC-3M while CD44 total expression was cut in half (Fig. 1d). This suggests that endogenous CT exerts an increase on CD44 variant similar to exogenous CT.

Protein kinase A and MAP kinase pathways and their interaction with calcitonin

$G_s\alpha$ -QL cells have high basal levels of CD44v7-10; for this reason, these cells were used to examine the effects of protein kinase A (PKA) and MAPK pathway inhibitors. PKA inhibitor lowered CD44 total and CD44v mRNA (Fig. 2a) and dose-dependently decreased protein for both (Fig. 2b). Downstream to PKA, inhibition of MEK significantly decreased CD44 total ($p = 0.001$) and non-significantly decreased CD44v. In contrast, inhibition of JNK had no significant effects. p38 inhibitor led to a larger, significant decrease in CD44 variant and a smaller significant decrease in CD44 total (Fig. 2a). MEK and JNK inhibitors were also tested in PC-3 cells and had no effect (data not shown). MEK inhibitor was also tested in BPH-1 cells, in which it reduced CD44 total and variant RNA.

To examine the dependence of CT effects on MAPK pathways, the CT-cells were pretreated with p38 inhibitor 4 h prior to administration of CT. Results were similar to p38 inhibitor alone: more than 50% decrease in CD44 total but none in CD44v (Fig. 3). This lack of CD44v suppression contrasts with p38 inhibitors marked CD44v suppression in $G_s\alpha$ -QL cells (Fig. 2a), which have far higher CD44v[4]. This suggests that CT mediated splicing is through p38 kinase. In further support of this, the expected CT induced tripling of CD44v mRNA in CT-cells (Fig. 1a) was prevented by p38 inhibitor pretreatment. Pretreatment with MEK inhibitor before CT also blunted the expected rise in CD44 variant mRNA seen in Fig. 1a, and JNK inhibitor pretreatment had no effect (data not shown).

Discussion and conclusion

Here, we demonstrate that calcitonin (CT) causes CT receptor-dependent increases in CD44 alternate splicing in prostate cancer (PC), apparently mediated through p38 kinase. Furthermore, transcription but not splicing appears to require the MEK/ERK (MAPK) pathway. Proposed interactions are shown (Fig. 4).

Paracrine CT is among several growth factors that interact with CD44[22]. In our prior *in vitro* studies up to 100 nM exogenous CT[6], or CT originating endogenously (in a PC-3 derivative called CT+ [4]), increased CD44v7-10 expression at the mRNA and protein levels. This was also observed in LnCaP, PC-3, and PC-3M derived cells; however, we had not examined total CD44 previously. Here, we used CT-minus (CT-) cells, an androgen-independent PC-3M derivative, to exclude all endogenous CT influence, so any effects would be attributable solely to exogenous CT. In CT-cells, the aberrant splice product is CD44v7-10[6]. This action occurred also in $G_s\alpha$ -QL cells, which are CTR+. Finally, CT+ cells showed an increase in CD44v compared with PC-3M cells. Supporting the view that this stimulation was CT-receptor mediated and not nonspecific, administering CT to CT-receptor negative PC-3 or CTR-cells did not have this effect. In further support of this interpretation, with benign BPH-1 cells, which are also negative for CT and CTR (personal communication, GV Shah), exogenous CT exerted no effect.

It is a novel observation that CT increases CD44v mRNA and protein as early as 3 h in cells that are CTR+, and has little effect on CD44 total. A response to CT should occur in the first several hours (personal communication, GV Shah), and indeed these increases were evident in CD44 protein levels at 3 h and 48 h.

Since the MEK/ERK pathway and the two stress-activated MAPK pathways are implicated in androgen-independent prostate cancer growth[32], we tested all three for modulation of CD44. We had found that CD44v7-10 protein

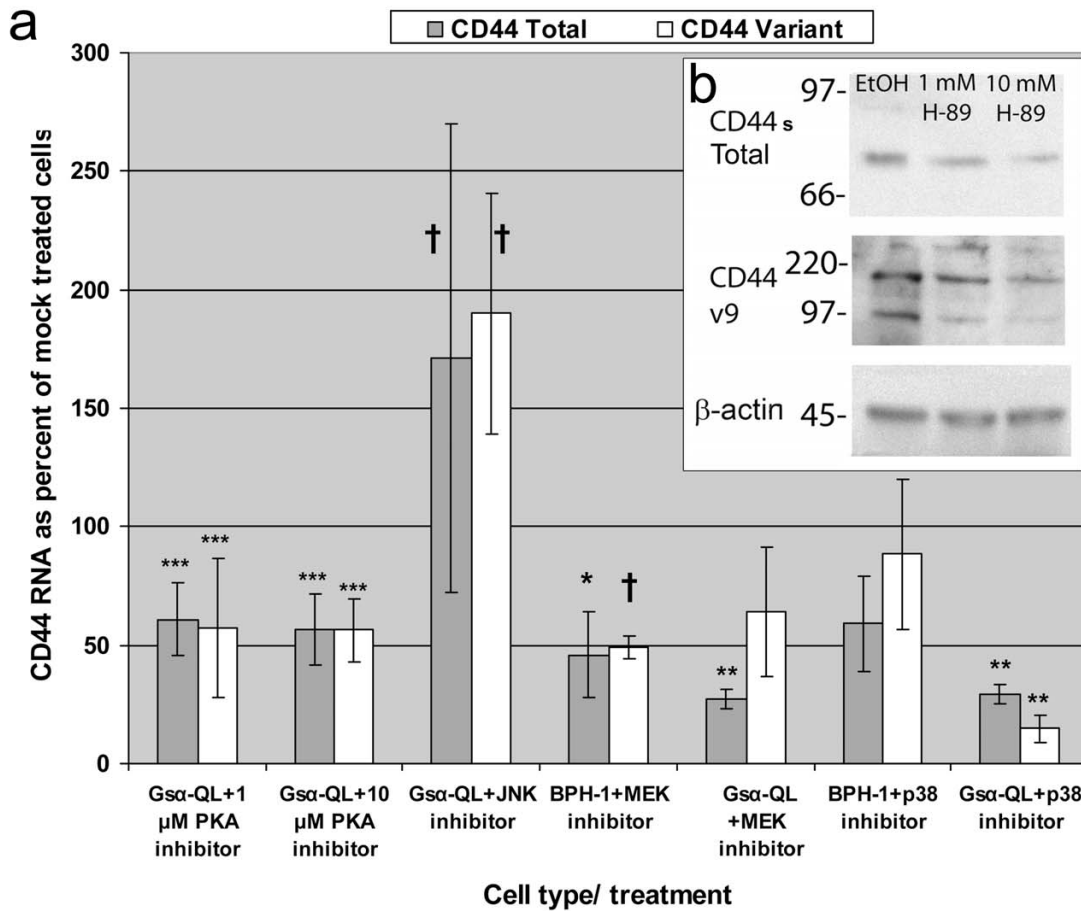


Figure 2

2a. RNA response to molecular inhibitors. CD44 total and variant RNA in triplicate TaqMan RT-PCR experiments. In subconfluent G α -QL prostate cancer cells, significant decreases of about 50% or more were observed after protein kinase A (PKA) inhibitor H-89, or inhibitors of downstream signaling pathways MEK or p38 kinase, but not JNK inhibitor. Error bars are standard deviation. *p = 0.01; **p = 0.001; †p = 0.0002; ***p = 0.0001 with respect to mock treated controls. **2b.** Western blot analysis. Protein kinase A inhibitor exerts a dose-dependent decrease on CD44s and CD44v compared with EtOH control in G α -QL cells.

was overexpressed in xenografts of G α -QL compared to PC-3M[3]; and *in vitro*, pharmacologic stimulation of G α or adenylyl cyclase raised CD44v7-10[6]. Using a PKA inhibitor, we found reduced total and CD44v7-10 mRNA, suggesting involvement of MEK pathway. Over half of PC cases have activated MEK-ERK signaling, shown by immunohistochemistry for p44/ERK1 and p42/ERK2[33].

To assess MAPK inhibitor effects, we chose G α -QL cells, derived from metastasizing PC-3M cells that stably express *gsp* mutant, constitutively active G α [17,19] because they have high baseline CD44v7-10[3,6]. We found MEK inhibitor caused similar percent decreases in CD44 total and CD44 variant, implicating MEK in transcription if not CD44v splicing. Similar effects have been noted with ras oncoprotein, which acts on the MEK-ERK

pathway[24]. Ras activation can induce CD44 promoter activity in fibroblasts, as shown using transient cotransfection of c-ras expression constructs and CD44 promoter reporter gene constructs[7]. Leakiness of splice control is proposed to lead to increased CD44v[7]. In activated T-lymphocytes during the immune response, mutant *ras* stimulation of MEK-ERK pathway increases CD44 total mRNA and triggers inclusion of CD44v exons in the mature RNA[24]; from our experiments, MEK seems to be active at least in CD44 transcription in G α -QL cells.

Some studies have suggested a positive feedback loop coupling MEK/ERK pathway and CD44v splicing. Activation of *ras* oncogene in rat fibroblasts[7] and HeLa cervical cancer[23] and of its effector, the MAP kinase pathway in T-cells[24] both upregulate CD44v splicing. CD44 vari-

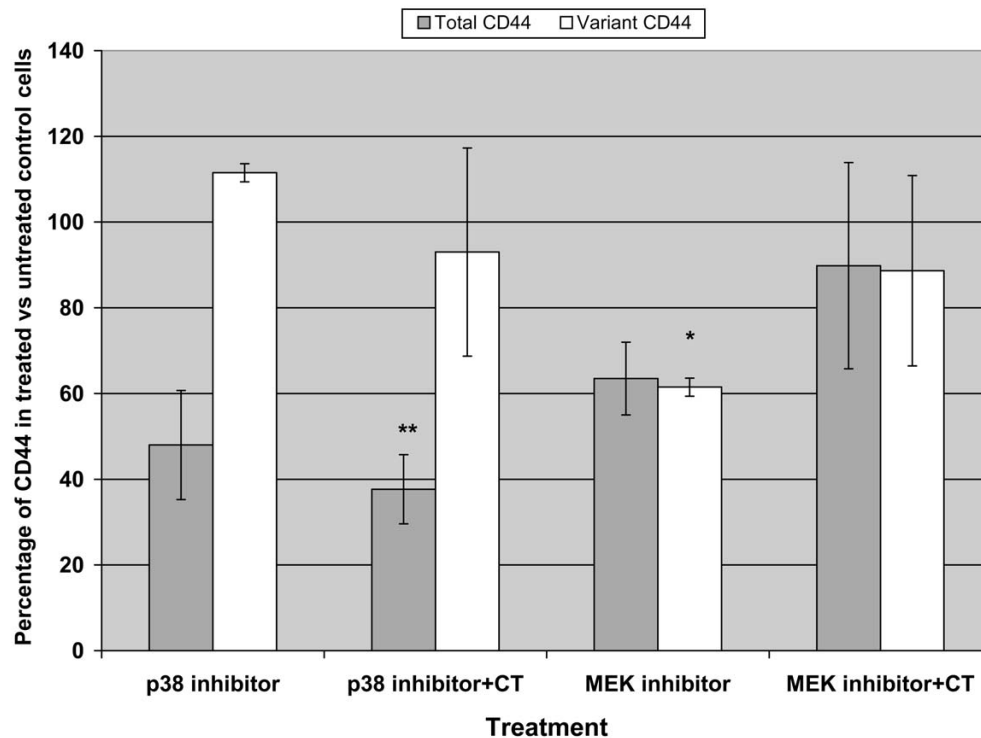


Figure 3

In CT-cells, the blockage of p38 kinase counteracts exogenous calcitonin (CT) stimulatory effect on CD44 variant RNA expression. Triplicate TaqMan RT-PCR experiments. While p38 blockade does not affect the CT-induced decrease in CD44 total, it does abrogate the expected tripling (Fig. 1) in CD44 variant. Conversely, MEK inhibitor moderately reduced total and variant CD44, and this effect was not counteracted by CT, suggesting CT does not act downstream of MEK. * $p = 0.02$; ** $p = 0.006$ with respect to mock treated controls.

ants, in turn, serve as coreceptors for growth factor receptors that activate ras[23] or form complexes with receptor tyrosine kinases such as c-met[8,21] to mediate cell signaling. Moreover, CD44v6 promotes T-cell proliferation by persistently activating MAP kinases[20], and CD44v8-10 causes apoptosis resistance in small cell lung cancer by activating Rho-stimulated focal adhesion kinase (FAK)[34].

We examined p38 kinase in benign and PC cells. In BPH-1, CD44 total RNA decreased with p38 kinase inhibition but variant form was unchanged. Since benign prostate lacks the aberrant splicing leading to CD44v7-10[3], but CD44v3-10 expression is present[35], the latter may be the form detected in benign cells. In $G_s\alpha$ -QL cells, a more marked effect on total and variant CD44 was seen. However, p38 may have CT-independent actions. Similar to our current and previous[6] findings with CD44 and $G_s\alpha$, p38 and MEK (but not JNK) were responsive to G-protein-coupled P2Y purinoceptor agonist ATP in PC-3 cells (CTR-negative), and these 2 pathways were required for invasion[30]. p38 has recently been recognized as a cell prolifer-

ation and survival factor in PC[36] partly by regulating IL-6 secretion[32]. Taken together with our findings about the MEK/ERK role in CD44 transcription, this could reflect convergence of the ERK1/2 and p38 systems in activating the MNK1 kinase, which enhances transcription of certain targets[37], suggesting a common final pathway that stimulates CD44 expression in PC.

We tested possible JNK pathway effects on CD44, not previously examined in the literature. JNK appears mainly important in PC apoptosis[29,38] and promoting chemotherapy susceptibility. JNK inhibitor slightly decreased CD44 total protein and did not change CD44v mRNA or protein in $G_s\alpha$ -QL cells. Our data suggest that JNK is a minor influence on CD44 expression.

Inhibition of p38 and MEK pathways affected CD44 in $G_s\alpha$ -QL cells. To investigate whether either might mediate CT's effects on CD44, we administered CT to CT-cells after blocking either one of these pathways. CT-cells have low baseline CD44v[6], and p38 inhibitor did not suppress the CD44v, but it blocked the expected stimulation of

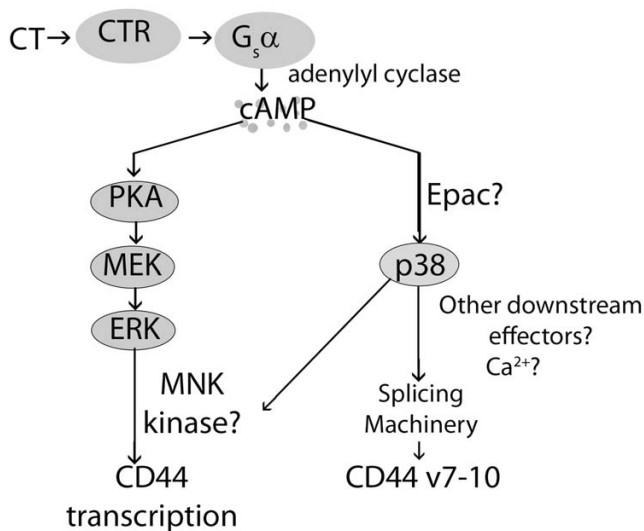


Figure 4
Proposed effects of calcitonin and MAP kinase pathways on CD44 expression in androgen-independent prostate cancer. Calcitonin (CT) binds to its receptor (CTR), which is coupled to the $G_s\alpha$ transduction protein. $G_s\alpha$ activity, mediated through cAMP, activates protein kinase A (PKA) [19,25]. PKA activates the MAPK kinase (MEK)-extracellular regulated kinase (ERK) pathway, that facilitates CD44 transcription. CT also induces splicing of CD44 to include v7-10, dependent on p38 but not on PKA. p38 may be induced by Exchange protein activated by cAMP (Epac). p38 could affect splicing machinery directly, through other downstream effectors, or by causing release of intracellular Ca^{2+} .

CD44v by CT, suggesting that p38 mediates CT-stimulated alternative splicing. The marked CD44v suppression seen in $G_s\alpha$ -QL cells, which have endogenous CT and high baseline CD44v, adds support for this interpretation. It is tempting to speculate that CT signaling, raising cAMP, may act through the effector, "exchange factor directly activated by cAMP" (Epac, Fig. 4). Epac has been shown to activate p38 kinase and mobilize intracellular calcium in neurons[39]. This PKA-independent mechanism would explain why PKA affected primarily CD44 transcription, yet p38 showed evidence of an additional effect on splicing.

To our knowledge, this is the first report in PC of how interactions between CT, and MAP kinase pathways, dysregulate the expression and splicing of the CD44 molecule. CD44 variant isoforms, probably through alterations in multimerization[12] and ligand binding[4], allow prostate cancer invasion[3,6]. This knowledge may find application in targeting the aberrant splicing of CD44 in PC by gene therapy, molecular inhibitor therapy, or for sensitization to radiotherapy.

Abbreviations

CD44: cell determinant 44; CT: calcitonin; CTR: calcitonin receptor; ERK: extracellular signal-regulated kinase; JNK: Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; PC: prostate cancer; PKA: protein kinase A.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EWR and EAT conducted experiments; EAT performed statistical analysis; KY did some western blot analyses; KAI conceived of the study and wrote much of the manuscript.

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References

1. Iczkowski KA, Pantazis CG, Collins J: **The loss of expression of CD44 standard and variant isoforms is related to prostatic carcinoma development and tumor progression.** *J Urol Pathol* 1997, **6**:119-129.
2. Iczkowski KA, Bai S, Pantazis CG: **Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level.** *Anticancer Res* 2003, **23**:3129-3140.
3. Omara-Opyene AL, Qiu J, Shah GV, Iczkowski KA: **Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18.** *Lab Invest* 2004, **84**:894-907. Editorial Comment: 811-813.
4. Iczkowski KA, Omara-Opyene AL, Shah GV: **The predominant CD44 splice variant in prostate cancer binds fibronectin, and calcitonin stimulates its expression.** *Anticancer Res* 2006, **26**:2863-2872.
5. Miyake H, Hara I, Okamoto I, Gohji K, Yamanaka K, Arakawa S, Saya H, Kamidono S: **Interaction between CD44 and hyaluronic acid regulates human prostate cancer development.** *J Urol* 1998, **160**:1562-1566.
6. Iczkowski KA, Omara-Opyene AL, Kulkarni TR, Pansara M, Shah GV: **Paracrine calcitonin in prostate cancer is linked to CD44 variant expression and invasion.** *Anticancer Res* 2005, **25**:2075-2083.
7. Hofmann M, Rudy W, Gunthert U, Zimmer SG, Zawadzki V, Zoller M, Lichtner RB, Herrlich P, Ponta H: **A link between ras and metastatic behavior of tumor cells: ras induces CD44 promoter activity and leads to low-level expression of metastasis-specific variants of CD44 in CREF cells.** *Cancer Res* 1993, **53**:1516-1521.
8. Jothy S: **CD44 and its partners in metastasis.** *Clin Exp Metastasis* 2003, **20**:195-201.
9. Vis AN, van Rhijn BW, Noordzij MA, Schroder FH, Kwast TH van der: **Value of tissue markers p27(kip1), MIB-1, and CD44s for the pre-operative prediction of tumour features in screen-detected prostate cancer.** *J Pathol* 2002, **197**:148-154.
10. Vis AN, Noordzij MA, Fitoz K, Wildhagen MF, Schroder FH, Kwast TH van der: **Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer.** *J Urol* 2000, **164**:2156-2161.
11. Peng ST, Su CH, Kuo CC, Shaw CF, Wang HS: **CD44 crosslinking-mediated matrix metalloproteinase-9 relocation in breast tumor cells leads to enhanced metastasis.** *Int J Oncol* 2007, **31**:1119-1126.
12. Lesley J, Kincade PW, Hyman R: **Antibody-induced activation of the hyaluronan receptor function of CD44 requires multivalent binding by antibody.** *Eur J Immunol* 1993, **23**:1902-1909.
13. Shah GV, Noble MJ, Austenfeld M, Weigel J, Deftos LJ, Meubst WK: **Presence of calcitonin-like immunoreactivity in human prostate gland: evidence for iCT secretion by cultured prostate cells.** *Prostate* 2007, **21**:87-97.

14. Chien J, Ren Y, Qing Wang Y, Bordelon W, Thompson E, Davis R, Rayford W, Shah G: **Calcitonin is a prostate epithelium-derived growth stimulatory peptide.** *Mol Cell Endocrinol* 2001, **181**:69-79.
15. Shah GV, Rayford W, Noble MJ, Austenfeld M, Weigel J, Vamos S, Mebust WK: **Calcitonin stimulates growth of human prostate cancer cells through receptor-mediated increase in cyclic adenosine 3',5'-monophosphates and cytoplasmic Ca²⁺ transients.** *Endocrinology* 1994, **134**:596-602.
16. Sabbisetti VS, Chirugupati S, Thomas S, Vaidya KS, Reardon D, Chiriva-Internati M, Iczkowski KA, Shah GV: **Calcitonin increases invasiveness of prostate cancer cells: role for cyclic AMP-dependent protein kinase A in calcitonin action.** *Int J Cancer* 2005, **117**:551-560.
17. Chien J, Wong E, Nikes E, Noble MJ, Pantazis CG, Shah GV: **Constitutive activation of stimulatory guanine nucleotide binding protein (G_α-QL)-mediated signaling increases invasiveness and tumorigenicity of PC-3M prostate cancer cells.** *Oncogene* 1999, **18**:3376-3382.
18. Thomas S, Muralidharan A, Shah GV: **Knock-down of calcitonin receptor expression induces apoptosis and growth arrest of prostate cancer cells.** *Int J Oncol* 2007, **31**:1425-1437.
19. Chien J, Shah GV: **Role of stimulatory guanine nucleotide binding protein (G_α) in proliferation of PC-3M prostate cancer cells.** *Int J Cancer* 2001, **91**:46-54.
20. Marhaba R, Bourouba M, Zoller M: **CD44v6 promotes proliferation by persisting activation of MAP kinases.** *Cell Signal* 2005, **17**:961-973.
21. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H: **CD44 is required for two consecutive steps in HGF/c-Met signaling.** *Genes Dev* 2002, **16**:3074-3086.
22. Ponta H, Sherman L, Herrlich PA: **CD44: from adhesion molecules to signaling regulators.** *Nat Rev Mol Cell Biol* 2003, **4**:33-45.
23. Cheng C, Yaffe MB, Sharp PA: **A positive feedback loop couples Ras activation and CD44 alternative splicing.** *Genes Dev* 2006, **20**:1715-1720.
24. Weg-Remers S, Ponta H, Herrlich P, Konig H: **Regulation of alternative pre-mRNA splicing by the ERK MAP-kinase pathway.** *EMBO J* 2001, **20**:4194-4203.
25. Thomas S, Chigurupati S, Anbalagan M, Shah G: **Calcitonin increases tumorigenicity of prostate cancer cells: evidence for the role of protein kinase A and urokinase-type plasminogen receptor.** *Mol Endocrinol* 2006, **20**:1894-1911.
26. Forster-Horváth C, Bocsi J, Rásó E, Orbán TI, Olah E, Timár J, Ladányi A: **Constitutive intracellular expression and activation-induced cell surface up-regulation of CD44v3 in human T lymphocytes.** *Eur J Immunol* 2001, **31**:600-608.
27. Leemhuis J, Boutillier S, Schmidt G, Meyer DK: **The protein kinase A inhibitor H89 acts on cell morphology by inhibiting Rho kinase.** *J Pharmacol Exp Ther* 2002, **300**:1000-1007.
28. Lee HY, Oh SH, Suh YA, Baek JH, Papadimitrakopoulou V, Huang S, Hong WK: **Response of non-small cell lung cancer cells to the inhibitors of phosphatidylinositol 3-kinase/Akt- and MAPK kinase 4/c-Jun NH2-terminal kinase pathways: an effective therapeutic strategy for lung cancer.** *Clin Cancer Res* 2005, **11**:6065-6074.
29. Davoodpour P, Landstrom M: **2-Methoxyestradiol-induced apoptosis in prostate cancer cells requires Smad7.** *J Biol Chem* 2005, **280**:14773-14779.
30. Chen L, He HY, Li HM, Zheng J, Heng WJ, You JF, Fang WG: **ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion.** *Cancer Lett* 2004, **215**:239-247.
31. Livak KL, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC_T) method.** *Methods* 2001, **25**:402-408.
32. Shida Y, Igawa T, Hakariya T, Sakai H, Kanetake H: **p38MAPK activation is involved in androgen-independent proliferation of human prostate cancer cells by regulating IL-6 secretion.** *Biochem Biophys Res Commun* 2007, **353**:744-749.
33. Burger M, Denzinger S, Hammerschmid C, Tannappel A, Maderstorfer A, Wieland WF, Hartmann A, Stoehr R: **Mitogen-activated protein kinase signaling is activated in prostate tumors but not mediated by B-RAF mutations.** *Eur Urol* 2006, **50**:1102-1109.
34. Fujita Y, Kitagawa M, Nakamura S, Azuma K, Ishii G, Higashi M, Kishi H, Hiwasa T, Koda K, Nakajima N, Harigaya K: **CD44 signaling through focal adhesion kinase and its anti-apoptotic effect.** *FEBS Lett* 2002, **528**:101-118.
35. Harrison GM, Davies G, Martin TA, Mason MD, Jiang WG: **The influence of CD44v3-v10 on adhesion, invasion and MMP-14 expression in prostate cancer cells.** *Oncol Rep* 2006, **15**:199-206.
36. Ricote M, Garcia-Tunon I, Bethencourt F, Fraile B, Onsurbe P, Paniagua R, Royuela M: **The p38 transduction pathway in prostatic neoplasia.** *J Pathol* 2006, **208**:401-407.
37. Raught B, Gingras AC: **eIF4E activity is regulated at multiple levels.** *Int J Biochem Cell Biol* 1999, **31**:43-57.
38. Cho SD, Ahn NS, Jung JW, Yang SR, Park JS, Lee YS, Jo EH, Hwang JW, Lii J, Kang KS: **Critical role of the c-JunNH2-terminal kinase and p38 mitogen-activated protein kinase pathways on sodium butyrate-induced apoptosis in DU145 human prostate cancer cells.** *Eur J Cancer Prev* 2006, **15**:57-63.
39. Ster J, De Bock F, Guerineau NC, Janossy A, Barrere-Lemaire S, Bos JL, Bockaert J, Fagni L: **Exchange protein activated by cAMP (Epac) mediates cAMP activation of p38 MAPK and modulation of Ca²⁺-dependent channels in cerebellar neurons.** *Proc Natl Acad Sci USA* 2007, **104**:2519-2524.

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Original Article

MicroRNAs 373 and 520c Are Downregulated in Prostate Cancer, Suppress CD44 Translation and Enhance Invasion of Prostate Cancer Cells *in vitro*

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Abstract: Prostate cancer (PCa), like most human cancers, features dysregulated CD44 expression. It loses expression of CD44 standard (CD44s), present in benign epithelium, and overexpresses a less abundant splice isoform, CD44v7-10. MicroRNAs 373 and 520c putatively regulate CD44. The levels of these two microRNAs were measured in matched benign and malignant patient tissues and in prostate cell lines. The effects of their transfection on CD44 mRNA and protein were documented. Whether these miRNAs act on CD44 promoter, or its 3' untranslated region (UTR), was studied with luciferase reporter constructs and their influences on migration and invasion were determined in PC-3M cells. *miR-373* and *miR-520c* expression were decreased in PCa cell lines and tissues, in proportion to their decreases in total CD44 mRNA. Exogenous *miR-373* caused a dose-dependent increase in total CD44 RNA, but a decrease in CD44v7-10 RNA, with an optimal dose at 6 nM. At the protein level, however, both microRNAs suppressed CD44. Both migration and invasion were stimulated by *miR-373* and *miR-520c*. The microRNAs had no effect on the CD44 promoter, but did exhibit 3'UTR binding. In conclusion, *miR-373* and *miR-520c* exert their effect in PCa by preventing the translation of CD44 RNA, rather than by degrading the RNA. Despite this observation, they exert pro-invasive functional effects, as previously described in breast cancer cells. Their effects are mediated by binding CD44 3'UTR.

Keywords: MicroRNA, miR-373, miR-520c, prostatic neoplasms, CD44, invasion

Introduction

About 30% of cases of prostate cancer (PCa) undergo a transition from quiescent to aggressive. This transition requires changes in adhesion glycoproteins such as CD44 that allow tumor cells to detach, interact with proteins that digest stromal matrix, and migrate through matrix and intravasate into lymphovascular channels. CD44 is a transmembrane, cell adhesion glycoprotein that mediates cell-cell and cell-stromal interactions, binds hyaluronan and several other matrix substrates, and controls cell shape through the cytoskeleton. CD44 is expressed as a ubiquitous standard (CD44s) isoform, but in epithelial cells, gene products of variant (CD44v) exons get included that lengthen the extracellular portion of CD44. In either isoform, oligomerization at the extracellular domain is required for CD44 function. Global dysregulation of alternate

splicing is common in cancer, and in PCa CD44 expression is lost while splicing is altered in favor of a variant isoform which causes invasion [1]. The probable mechanism is that inclusion of abnormal variant sequences alters CD44's ability to oligomerize and its ligand binding [2], potentiating tumor growth, invasion, and metastasis.

CD44, along with 30% of human genes [3], is regulated by at least 851 human microRNAs (miRNAs). Dysregulation of this recently discovered class of noncoding RNAs is also common in PCa [4]. miRNAs that may interact with CD44 have been studied only in breast cancer, not PCa. Recently, Huang *et al* described *miR-373* and *miR-520c*, members of the same miRNA family sharing similar seed sequences [5], as functional oncomiRs in breast cancer [6]. They bound specifically to the CD44 3' untranslated region (3'UTR) and suppressed CD44; both CD44 knockdown and

miR-373 stimulated tumor invasion and migration. This action can be explained because CD44, particularly CD44s, is a tumor suppressor in breast cancer as it is in PCa. However, CD44 functional implications differ by tumor type: a CD44v confers invasive ability in PCa [1], but CD44s has pro-invasive properties in some tumors such as colon cancer [7]. Thus, we tested whether similar mechanisms and effects were operative in PCa. Because *miR-373* targets the E-cadherin promoter [8] and might also target the CD44 promoter according to our sequence analysis, we explored whether its mode of action was by the promoter or 3'UTR.

Materials and Methods

Cell Lines and Tissues

Benign PrEC and BPH-1, and LNCaP and PC-3 prostate cancer lines and MCF-7 breast cancer cells were from American Type Culture Collection (Manassas, VA). The culture medium for all these cell lines was RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal calf serum. PC-3M cells, a metastasis-derived variant of PC-3, were obtained from Dr. Girish Shah, U. of Louisiana—Monroe. They were grown in RPMI 1640 (Invitrogen) with 12% horse serum, 5% fetal calf serum and antibiotics. Cells were grown in 5% CO₂ incubator at 37°C. For each experiment, cells in a flask were trypsinized, washed with sterile PBS to remove trypsin, resuspended in basal medium, and counted after dilution with Trypan blue dye using the grid method [2].

Laser Capture Microdissection

We isolated pure benign glandular cells and benign stromal cells. RNA from cryostat sections was prepared using the PicoPure RNA Isolation Kit for frozen tissues (Arcturus, Mountain View, CA) with columns designed to capture short RNA.

Quantitative RT-PCR (qRT-PCR)

Total RNA was prepared from cell pellets using Trizol (Invitrogen) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNase-free water, and its concentration measured. Complementary DNA (cDNA) was synthesized from 4 µg total RNA in 20 µl reaction mixture as described previously [1].

qRT-PCR reactions used 4 µg cDNA plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 µl.

For miRNA, the qRT-PCR was performed in two different ways for selected samples. In the first (primer + probe) approach, cDNA was prepared with MultiScribe (Applied Biosystems), with a special recombinant Moloney murine leukemia virus (rMoMuLV) reverse transcriptase in an optimal buffer. Unlike mRNAs, miRNAs are not polyadenylated, so hairpin-loop forming primer + probe sets for *miR-373* and *miR-520c* (Applied Biosystems) were used. The cycling conditions for both mRNA and miRNA were: hold 50°C 2 min, 95°C 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min, as previously described [9], using the ABI Prism 7500 cycler (Perkin-Elmer, Waltham, MA).

A second, confirmatory qRT-PCR approach used the NCode kit (Invitrogen). After RT, RNA was tailed with poly (A) polymerase, not normally present on miRNA, then a forward primer specific to each miRNA was used together with universal poly-T reverse primer. Detection was done by SYBR green without a probe. Cycling settings were: 50°C for 2 min, and then 40 cycles of 95°C for 25 sec and 60°C for 1 min.

For CD44, samples were run in triplicates with a primer/probe set for all CD44v that brackets the entire variant region [1], one for CD44 total whose probe binds a standard exon, and 18S ribosomal RNA. Primer/probe sets for CD44v were: forward, 5'-AACGCTTCAGCCTACTGCAAA-3'; reverse, 5'-TCTTCCAAGCCTTCATGTGATG-3'; probe, 5'-GATTTGGACAGGACAGGACCTCTTCAATG-3'. For CD44 total, we used forward, 5'-CAACTCCATCTGTGCAGCAAA-3'; reverse, 5'-GTAACCTCCTGAAGTGCTGCTC-3'; probe, 5'-CATATTGCTTCAATGCTTCAGCTCCACCTG-3'. Primer and probe sets for 18S were proprietary to the manufacturer.

MicroRNA Transfection

A flask of PC-3M cells at 80% confluence was lipofected with *miR-373* or *miR-520c-3p* (IDT, Coralville, IA) or the irrelevant miR Negative Control #2 (Ambion, Austin, TX) for 6 hours using Trans-IT (Mirus, Madison, WI). Cells were allowed at least 24 hours to recover from the

transfection before experiments.

Western Blot Analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 µg sample/lane according to the Laemmli method using the NuPAGE system (Invitrogen, Carlsbad, CA). 5 µl of Kaleidoscope Precision Plus Protein Standards (Bio-Rad) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Three primary antibodies were used. To assess CD44v9 (the largest component of the overexpressed CD44v7-10), the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 total (standard + variant) was assessed using anti-HCAM (DF1485, Santa Cruz Biologicals, Santa Cruz, CA). Anti-β-actin-HRP antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in TBS with 0.1% Tween 20% and 1:1000 dilution of goat anti-mouse IgG antibody labeled with HRP (Bio-Rad, Hercules, CA) was added in 5% skim milk + TBST for 1 hr. Reactivity was detected using the SuperSignal West Pico Substrate chemiluminescent system (Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

CD44 Promoter and 3'UTR Luciferase Constructs

Using the PXP2 plasmid, 1150 bases of CD44 sequence including the start site, and beginning 964 bases upstream to the start site, was cloned in between *Xho* I and *Hind* III sites. Because one of the most effective positive controls to test the promoter responsiveness is *HOXC6* expression [10], we obtained a plasmid to overexpress *HOXC6* in prostate cells (Gift of Dr. Jim Lambert). The overexpression was documented by Western blot analysis using an anti-HoxC6 rabbit polyclonal antibody (Aviva Systems Biology, San Diego, CA) at 1:500 dilution, with goat anti-rabbit secondary antibody at 1:10,000 (Bio-Rad, Hercules, CA), as above. The CD44 3'UTR luciferase reporter plasmid was a gift of Dr. Qihong Huang from Wistar Institute, Philadelphia [6]. Luciferase activity was

measured 48 hours after transfection using the firefly luciferase assay (Gold Biotechnologies, St. Louis, MO). The cells were harvested in 20 mM K₂HPO₄ pH 7.8 with 5 mM MgCl₂ and 0.5% Triton X-100 buffer for 15 min on ice, and the mixture was centrifuged for 10 min at 4°C. 50 µl of lysate plus 350 µl of luciferase assay buffer as per the manufacturer. The relative luciferase units (RLU) were normalized to protein concentration as determined by Bradford assay.

Invasion and Migration Assays

Invasion was assessed with triplicate 24-well Matrigel two-tier invasion chambers, and migration was assessed with triplicate control inserts, both with 8.0 µm pores (Collaborative Biomedical Products, Bedford, MA) [1, 9]. Cells were seeded at 30,000 per well, untreated or after microRNA transfection. Cells in the upper insert were in serum-free basal medium (RPMI 1640 with 4 mM L-glutamine, 100 µg/mL each of penicillin G and streptomycin). The lower chamber contained chemoattractant medium consisting of 10% fetal bovine serum, 20% conditioned medium from subconfluent culture, and 70% complete medium. The incubation was carried out 24 h in 5% CO₂ incubator at 37°C. The medium from the upper inserts, together with any residual cells were removed off the upper Matrigel surface. The membrane was fixed in methanol and stained with May-Grunwald stain (Sigma, St. Louis, MO) according to manufacturer's protocol. Experiments were repeated twice.

Results

Laser Capture Microdissection

The prior study of *miR-373* and *miR-520c* in breast tissue samples did not distinguish its origin from breast glandular cells or stromal cells [6]. To make this determination in prostate, benign glandular cells and benign stromal cells were microdissected. Taking the log₂ of $-(C_T \text{ value normalized to } \beta\text{-actin})$, *miR-373* glandular expression was 0.003377; stromal expression was 0.005048. *miR-520c* was below detection limits in glands and stroma.

Quantitative RT-PCR (qRT-PCR)

To correlate CD44 standard and v7-10

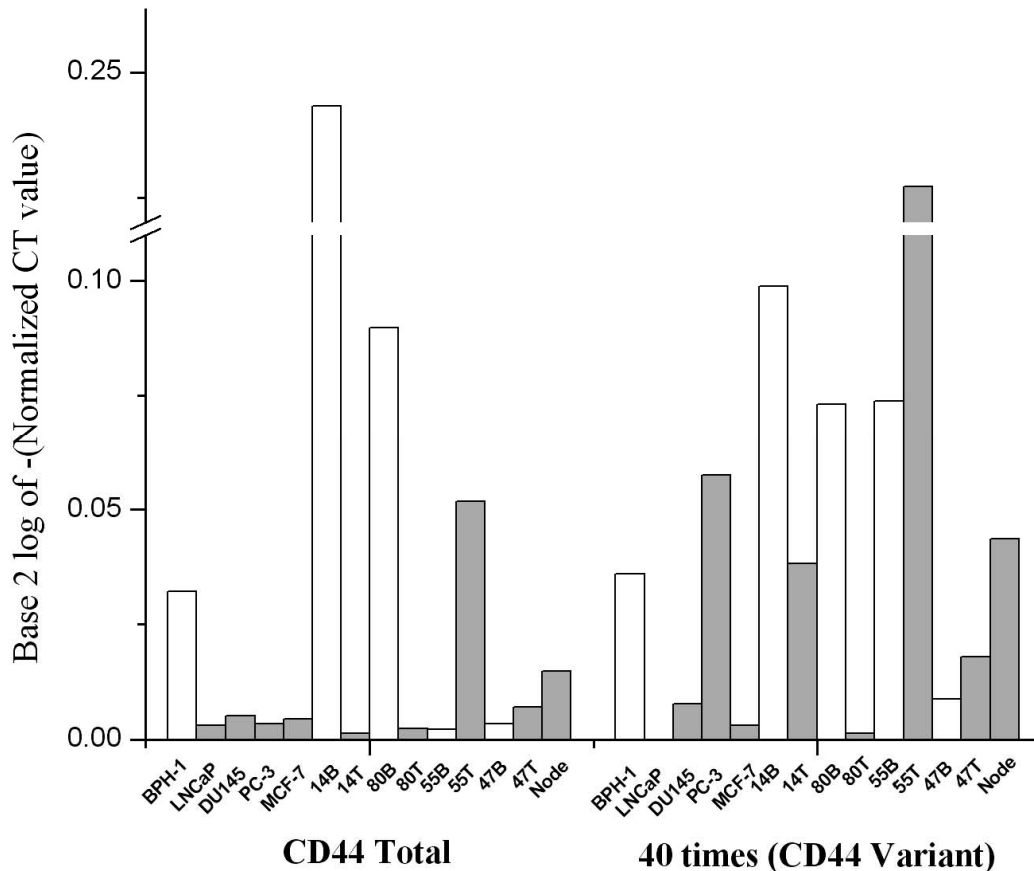


Figure 1 qRT-PCR of CD44 total (left) and variant (right) in 5 cell lines and 5 clinical prostatectomy specimens normalized to 18S RNA. Left: CD44 total, comprising the more abundant CD44s isoform, is higher in benign BPH-1 cell line than in androgen-sensitive LNCaP cancer or androgen-independent DU-145 or PC-3, consistent with previous findings of ours [13] and others [15, 16]. In MCF-7 breast cancer cells, it is low as expected [6]. In matched, frozen section-confirmed, benign (B) and tumor (T) tissues from 4 patients, CD44 is downregulated in the tumor component of 2. In microdissected metastatic prostate cancer from a lymph node (Node), CD44 is low. Right: The less abundant CD44 v7-10 is expressed in androgen-independent prostate cell lines but very low in MCF-7. In 5 tissues, CD44v7-10 is highly expressed in most tumor (T) components and in lymph node with metastasis (Node), as expected from our prior work [1, 12].

expression with miRNA expression in cells and tissues, qRT-PCR was performed. Total CD44 mRNA was lower in all cancer cell lines than benign ones, and in most cancer tissue samples compared with benign tissue from the same patient (**Figure 1**).

By probe + primer method, *miR-373* was highest in the BPH-1 cell line (**Figure 2a**), intermediate in androgen-sensitive, slow-growing LNCaP cells, and very low in androgen-independent PCa as well as in MCF-7, confirming the finding of Huang *et al* who used MCF-7 as a negative control [6]. In matched tissues, *miR-373* was again generally higher in benign tissue than in the tissue sample with cancer (**Figure 2b**).

The differences were greater than could be accounted for merely by *miR-373* being 1.5 times as prevalent in stroma as in glands, and the presence of proportionally less stroma in tumor. These trends were confirmed in separate SYBRgreen qRT-PCR experiments (**Figure 2c**). A similar trend applied to *miR-520c* in cell lines and tissues. *miR-520c* RNA was decreased in primary tumors and metastasis using both a SYBRgreen method (**Figure 2d**) and by using primers with a specific probe (not shown).

Effects on CD44 Promoter and 3'UTR

PC-3M cells were transfected with our CD44 luciferase construct that tests for binding to

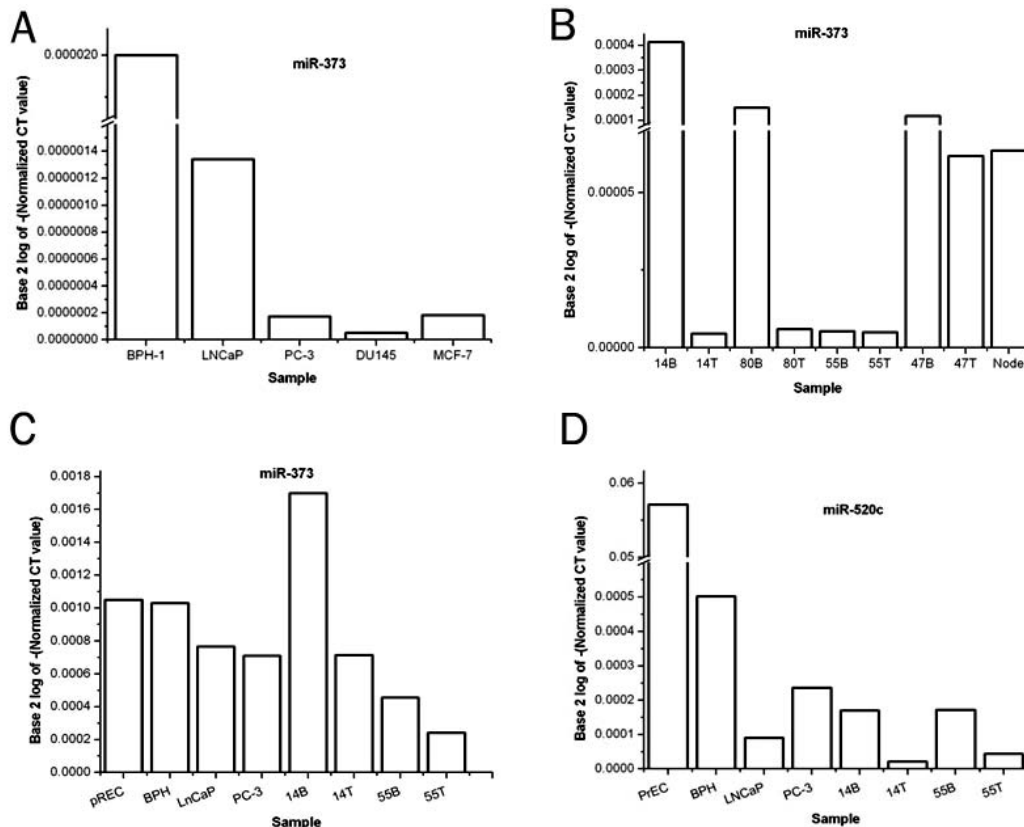


Figure 2 qRT-PCR for *miR-373* by primer and probe method normalized to 18S rRNA (**A** and **B**). **A**. Among cell lines, benign BPH-1 cells have the most *miR-373*, while slow-growing, androgen-dependent LNCaP cancer cells have decreased but detectable *miR-373*. Androgen-independent cell lines PC-3 and DU145 have nearly absent *miR-373*. Level is low in breast cancer cell line MCF-7, consistent with others' findings [5]. **B**. Matched (same patient) tissue specimens. *miR-373* is higher in the benign (B) component than in tumors (T) for 3 of 4 cases. **C**. qRT-PCR by SYBRgreen method for confirmation of *miR-373* expression. Values are higher because they are normalized to β -actin which has lower copy number. *miR-373* is downregulated in cancer cell lines compared to benign PrEC and BPH cells. In 2 patients' tissues, there is downregulation of *miR-373* in the tumor (T) component. **D**. qRT-PCR for *miR-520c* by SYBRgreen method normalized to β -actin. Average expression levels of *miR-520c* were at 1/5 or less the level of *miR-373*. 2 malignant cell lines have less *miR-520c* than 2 benign ones. In 2 patients' tissues, there is downregulation of *miR-520c* in the tumor (T) component.

the promoter region. HOXC6 is documented to regulate the CD44 promoter in PCa cells [10]. We achieved its overexpression by transfection (**Figure 3a**). Cells that were co-transfected with CD44 promoter and HOXC6 demonstrated a strong inhibition of promoter activity by HOXC6 (**Figure 3b**). Overexpression of *miR-373* or *miR-520c* caused ~85% decreases in luminescence readings generated by the 3'UTR construct (**Figure 3c**), signifying that both microRNAs target the CD44 3'UTR.

Effects on CD44 Expression

Transfection of 6 nM synthetic *miR-373* was optimal for detecting its expression (**Figure 4a**).

Doses up to 6 nM caused dose-dependent overexpression of CD44 total RNA normalized to untreated cells (using a primer set and probe that detects CD44 total = standard + variants). Thus 6 nM was considered an optimal dose. At the RNA level, divergent effects were noted on total CD44 (stimulation) and on CD44 variant (inhibition, **Figure 4a**). Because miRNA can either degrade mRNA or reversibly inhibit its translation [11], we used western blot analyses to determine whether the directionality of the protein effect was the same. *miR-373* and *miR-520c* both suppressed total CD44 protein compared with untreated positive control or with transfection of an irrelevant, scrambled miRNA (**Figure 4b**).

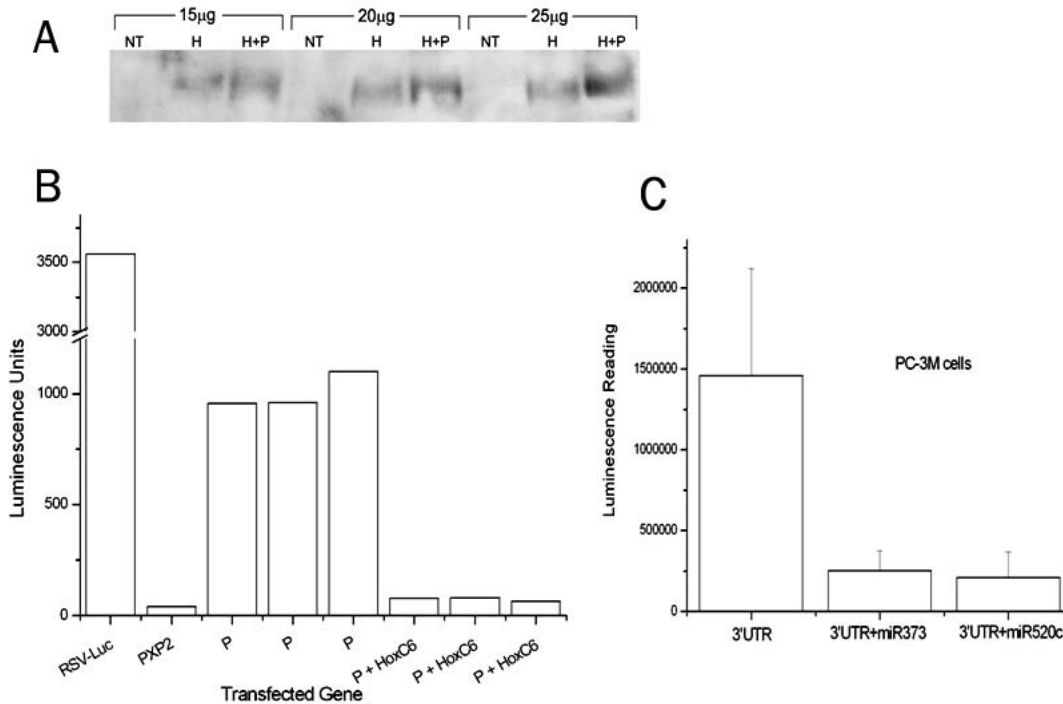


Figure 3 **A.** Successful overexpression of HOXC6 (primary Ab) by Western blot analysis using 15-25 µg protein. PC-3M cells were transfected with either HOXC6 (H) alone or H + CD44 promoter (P), compared to no treatment (NT). **B.** Cells from **A** were subjected to luciferase activity assay for CD44 promoter (P) activity. RSV is the positive control and PXP2 is the negative control. Promoter activity is inhibited by HOXC6. Experiments were performed in triplicates. **C.** Optimized doses of *miR-373* or *miR-520c* were transfected into PC-3M cells subsequent to transfection of a luciferase construct containing the 3'UTR of CD44. Both suppressed gene expression, indicating that CD44 is a direct target of *miR-373* and *miR-520c*.

Effects on Invasion and Migration

Overexpression of *miR-373* increased migrating PC-3M cells more than 5 folds and invading cells more than 3 folds. Moreover, the invaders as a percent of migrators increased from 85% to >100% after *miR-373* transfection. Overexpression of *miR-520c* nearly doubled invasion and migration (**Figure 4c**).

Discussion

Both PCa cells and tumor from prostatectomy show downregulation of *miR-373* and *miR-520c* compared with their benign counterparts. Exogenous doses cause an increase in total CD44 RNA. At the protein level, however, both *miR-373* and *miR-520c* suppress total CD44. This means that *miR-373* and *miR-520c*, like most miRNAs, inhibit, not stimulate, their target; and since CD44 is a tumor suppressor, *miR-373* and *miR-520c* behave as oncomiRs.

The protein finding in our study is concordant with that of Huang et al [6], which demonstrated that miRNAs 373 and 520c exerted marked CD44 suppression in MCF-7 breast cancer cells by western blot analysis. Thus, *miR-373* and *miR-520c* function by fundamentally different mechanisms in PC-3M and MCF-7 cells but cause the same phenotypic effect on CD44 protein. The findings of Huang et al suggest RNA degradation, whereas our findings suggest the opposite mechanism: RNA accumulation but paradoxically translational repression. It has been noted that mRNA whose repression is mediated by miRNA gets stored in P bodies and conditions of stress can reverse the repression [11]. In hepatoma cells, cationic amino acid transporter 1 (CAT-1) mRNA and reporters bearing the CAT-1 3'UTR or its fragments were able to be relieved from the miRNA *miR-122*-induced inhibition by subjection to different stress conditions [11]. Whether placing prostate cancer cells under

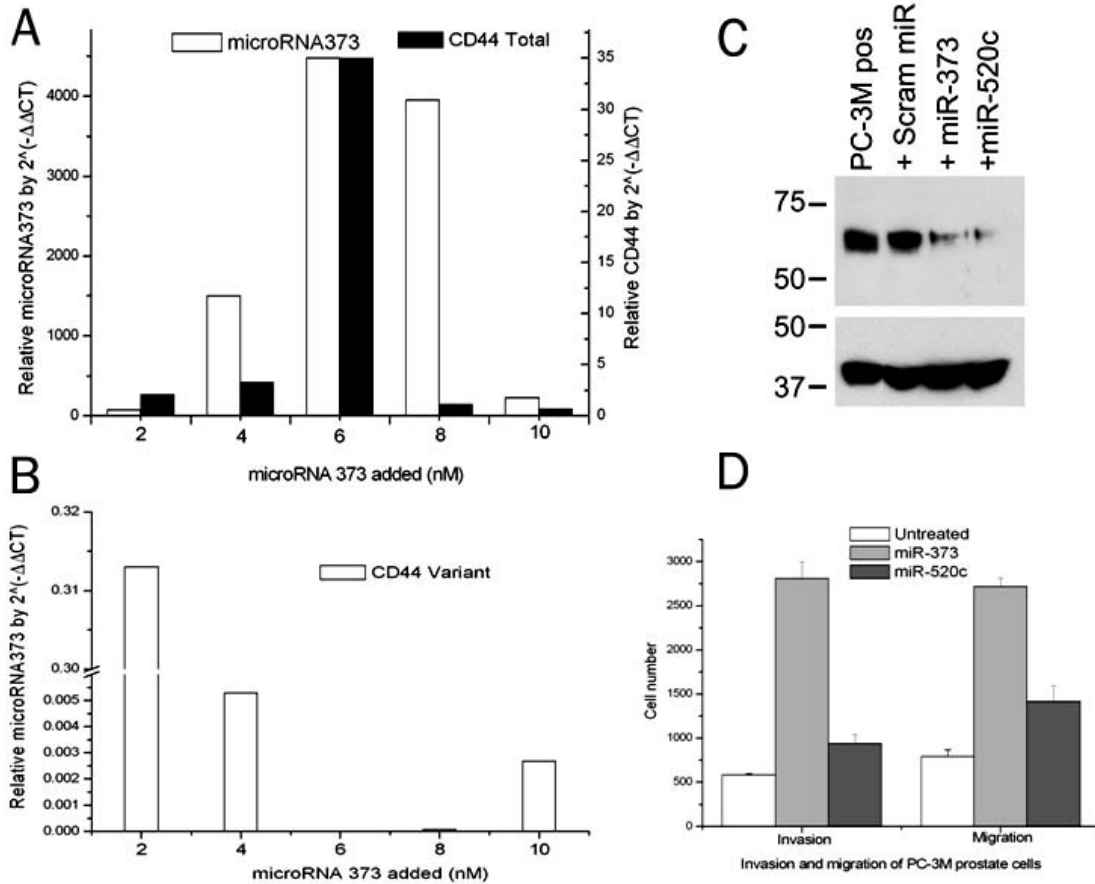


Figure 4 **A.** Left y-axis: Transfection of 6 nM dose of synthetic miR-373 maximized its expression by qRT-PCR. Right y-axis: Doses up to 6 nM caused dose-dependent 35-fold overexpression of CD44 total RNA, normalized to untreated cells (using a primer and probe set that detects CD44 total = standard + variants). **B.** The same dose suppressed CD44v7-10 variant expression. **C.** Top: Western blot analysis using primary antibody for total CD44. Lane 1: PC-3M cells as positive (pos) control, with strong CD44. Lane 2, PC-3M cells transfected with scrambled (+Scram), irrelevant microRNA. Lanes 3 and 4: PC-3M cells transfected with *miR-373* or *miR-520c* respectively, showing CD44 suppression. Bottom: β-actin loading control. **D.** 6 nM dose of exogenous *miR-373* also has functional effects: more than 5-fold increase in migration and more than 3-fold increase in invasion of 30,000 PC-3M cells compared to untreated cells. Migration and invasion almost doubled with *miR-520c*.

stress might affect *miR-373* and *miR-520c* effects on CD44 would be of interest.

Most of CD44 is normally CD44s, a tumor suppressor lost in PCa [2, 13-16]. A curious finding was that *miR-373* stimulated total CD44 RNA but also suppressed the pro-invasive CD44v7-10 variant. This suggests an effect occurring prior to splicing, or a direct effect on splicing. This effect also bespeaks a role of these miRNAs as oncomiRs, like their role in breast cancer, as studied with non-invasive MCF-7 cells and in tissues. In those studies, *miR-373* and *miR-520c* suppressed RNA and protein of the anti-invasive CD44 (total versus variant was not examined),

increased migration and invasion, and were upregulated in metastases; expression levels in benign breast were not examined [6]. We have not examined *miR-373* and *miR-520c* in metastases, except for just one case.

Functionally, *miR-373* and *miR-520c* also behave as oncomiRs in PCa despite their downregulation. They stimulated cells to migrate and invade. miRNA downregulation is in fact the trend in PCa, applying to 76 of 85 detectable miRNAs in one study [4]. There are only a few reportedly upregulated miRNAs in PCa. The pro-invasive effect of these miRNAs suggests that, although downregulated in PCa, they target other invasion-promoting genes [6].

We determined by microdissection of whole, benign prostate that at least *miR-373* is present in both glandular and stromal cells of prostate tissue. This distinction was not addressed in breast tissue [6], but suggests that the stromal contribution of miRNA in glandular organs is not negligible. Of course in tumor, the glandular component is much more prevalent, so a lesser stromal contribution might be expected.

Most miRNAs bind a response element in the 3' untranslated region (3'UTR) of targeted mRNA. This either reversibly represses its translation or directs sequence specific degradation [11]. Because some influences on *miR-373* and *miR-520c* and CD44 were fundamentally different from those described before, we examined the possibility that their actions were mediated by the CD44 promoter, using a promoter luciferase construct. We demonstrated that this interaction is through the CD44 3'UTR, again suggesting a unique role in PCa pathophysiology. Certain miRNAs can target complementary DNA promoter sequences to induce gene expression [8]. A recent paper, using a prostate cancer model, identified a *miR-373* binding site in the promoter of E-cadherin [8]. Our sequence analysis also suggested a theoretical *miR-373* binding site in the CD44 promoter; but, we ruled out a promoter site of action of *miR-373* and *miR-520c* in PCa cells. Their effect seems to be exerted only by binding to the 3'UTR.

Our prior work demonstrated a unique role for CD44v7-10 in PCa. By isolating RNA from clinical PCa specimens, we discovered that expression of CD44v7-10 constitutes a unique PCa signature, consistently expressed in both primary PCa and PCa metastatic to other tissues [1, 12, 13]. Interference against CD44v caused a 69% reduction in invasion index compared to untreated control cells [13]. Moreover, PCa loses the splicing ability to produce the standard isoform expressed in benign prostate [1, 14-16]. The discovery of downregulation of *miR-373* and *miR-520c* in PCa is consistent with the dysregulated expression of miRNAs in PCa [3, 17] and the upregulation of the enzyme Dicer [18].

In conclusion, in benign prostate, higher *miR-373* and *miR-520c* correlate with the predominance of CD44s isoform. In prostate cancer, the downregulation of total CD44 and upregulation of its less abundant isoform

CD44v correlated with loss of *miR-373* and *miR-520c*. With *miR-373*, an exogenous dose increased CD44 RNA. However, at the protein level *miR-373* and *miR-520c* suppressed total CD44, indicating translational repression. These actions are most likely mediated through the CD44 3'UTR. The loss of CD44 total protein, most of which is CD44s, is probably functionally important in mediating pro-invasive effects of *miR-373* and *miR-520c*. Further work is needed to determine whether these miRNAs prevent or target CD44 splicing factors or splicing.

Acknowledgements

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References

- [1] Omara-Opyene AL, Qiu J, Shah GV and Iczkowski KA. Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18. *Lab Invest* 2004;84:894-907.
- [2] Iczkowski KA, Omara-Opyene AL and Shah GV. The predominant CD44 splice variant in prostate cancer binds fibronectin, and calcitonin stimulates its expression. *Anticancer Res* 2006;26:2863-2872.
- [3] Hwang HW and Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. *Br J Cancer* 2007;96 Suppl:R40-44.
- [4] Ozen M, Creighton CJ, Ozdemir M and Ittmann M. Widespread deregulation of microRNA expression in human prostate cancer. *Oncogene* 2008;27:1788-1793.
- [5] Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH and Agami R.. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* 2006;124:1169-1181.
- [6] Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Puré E and Agami R. The microRNAs *miR-373* and *miR-520c* promote tumour invasion and metastasis. *Nature Cell Biol*

- 2008;10:202-210.
- [7] Harada N, Mizoi T, Kinouchi M, Hoshi K, Ishii S, Shiiba K, Sasaki I and Matsuno S. Introduction of antisense CD44s cDNA down-regulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells. *Int J Cancer* 2001;91:67-75.
- [8] Place RF, Li LC, Pookot D, Noonan EJ and Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci USA* 2008;105:1608-1613.
- [9] Iczkowski KA, Omara-Opyene AL, Kulkarni TR, Pansara M and Shah GV. Paracrine calcitonin in prostate cancer is linked to CD44 variant expression and invasion. *Anticancer Res* 2005;25:2075-2083.
- [10] McCabe CD, Spyropoulos DD, Martin D and Moreno CS. Genome-wide analysis of the homeobox C6 transcriptional network in prostate cancer. *Cancer Res* 2008;68:1988-1996.
- [11] Bhattacharyya SN, Habermacher R, Martine U, Closs EI and Filipowicz W. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harb Symp Quant Biol* 2006;71:513-521.
- [12] Iczkowski KA, Bai S and Pantazis CG. Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. *Anticancer Res* 2003;23:3129-3140.
- [13] 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* 1997;6:119-129.
- [14] Vis AN, van Rhijn BW, Noordzij MA, Schroder FH and van der Kwast TH. Value of tissue markers p27(kip1), MIB-1, and CD44s for the pre-operative prediction of tumour features in screen-detected prostate cancer. *J Pathol* 2002;197:148-154.
- [15] Vis AN, Noordzij MA, Fitoz K, Wildhagen MF, Schroder FH and van der Kwast TH. Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer. *J Urol* 2000;164:2156-2161.
- [16] Kallakury BV, Sheehan CE and Ross JS. Co-downregulation of cell adhesion proteins alpha and beta-catenins, p120CTN, E-cadherin, and CD44 in prostatic adenocarcinomas. *Hum Pathol* 2001;32:849-855.
- [17] Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, Bartucci M, Muto G, Peschle C and De Maria Ruggero. The mir-15a-mir-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nat Med* 2008;14:1271-1277.
- [18] Chiosea S, Jelezcova E, Chandran U, Acquafondata M, McHale T, Sobol RW and Dhir R. Up-regulation of dicer, a component of the microRNA machinery, in prostate adenocarcinoma. *Am J Pathol* 2006;169:1812-1820.

Original Article

Silibinin suppresses cd44 expression in prostate cancer cells

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Abstract: Prostate cancer (PCa), like most human cancers, features dysregulated CD44 expression. Expression of CD44 standard (CD44s), present in benign epithelium, is lost in PCa while pro-invasive splice variant isoform CD44v7-10 is overexpressed. The role of CD44 in silibinin's anti-growth effects was uncertain. To assess silibinin's effects on CD44 promoter activity, PC-3M PCa cells were transfected with luciferase-CD44 promoter construct 24 h prior to 25-200 μ M silibinin treatment for 48 h. Also, cells' expression of CD44 RNA (by qRT-PCR) and protein (Western blot analysis) was studied. Silibinin was further tested preoperatively on a pilot cohort of 6 men with PCa compared with 7 matched placebo-treated men, with immunostaining for CD44v7-10 in their prostates. In PC-3M cells, silibinin dose-dependently inhibited CD44 promoter activity up to 87%, caused a 90% inhibition of total CD44 and 70% decrease in CD44v7-10 RNA, and at the protein level, decreased total CD44 at 100-200 μ M dose and decreased CD44v7-10 after 3 days. Silibinin decreased adhesion to hyaluronan and fibronectin. Silibinin at 100-200 μ M inhibited Egr-1, a regulator of CD44 promoter activity. Men treated with silibinin did not differ in tissue CD44v7-10 expression. In conclusion, CD44 inhibition is one mechanism by which silibinin reduces PCa tumorigenicity.

Key Words: Silibinin, prostatic neoplasms; CD44; plasmid; alternate splicing; adhesion

Introduction

About 30% of prostate cancer (PCa) cases undergo transition from quiescent to aggressive, and this transition relies on altered expression of adhesion glycoproteins such as CD44 that allow tumor cells to detach, interact with proteins that digest stromal matrix, migrate through matrix and intravasate into lymphovascular channels. CD44 enables cell-cell and cell-matrix adhesion, primarily to hyaluronan but also to other ligands, and links the cell membrane to the actin cytoskeleton, modulating motility. CD44 is a transmembrane molecule encoded by an alternately spliced gene. It is expressed as ubiquitous, standard (CD44s) isoform but inclusion of one or more of 10 variant (v) exons produces tissue-specific (CD44v) isoforms. Moreover, a unique variant isoform of CD44 is overexpressed in PCa and

facilitates PCa cell growth and invasion [1-6].

Silibinin is a nutritional supplement that has gained attention as intervention to prevent or treat cancer. Silibinin is a polyphenolic flavonolignan isolated from milk thistle and artichoke that arrests PCa proliferation *in vitro* [7] with cell cycle arrest in G1 [8], potentiates a chemotherapeutic drugs [9], and *in vivo* increases apoptosis and inhibits angiogenesis [10]. It inhibits cancer invasion by inactivating the PI3K-Akt and MAPK signaling pathways [11] as well as decreasing production of urokinase-plasminogen activator (uPA) and matrix metalloprotease-2 [12]. The early growth response-1 (Egr-1) transcription factor binds to and activates CD44 promoter [13]. Here we investigate these effects of silibinin on CD44 total and variant, adhesion, and Egr-1 expression.

Materials and Methods

Cell lines and treatment

PC-3M cells, a metastasis-derived variant of PC-3, were obtained from Dr. Girish Shah, U. of Louisiana—Monroe. LNCaP and DU145 cells were from ATCC (Manassas, VA). Cells were grown in RPMI 1640 (Invitrogen) with 10% fetal calf serum and antibiotics. Cells were grown in 5% CO₂ incubator at 37°C. For each experiment, cells in a flask were trypsinized, washed with sterile PBS to remove trypsin, resuspended in basal medium, and counted after dilution with Trypan blue dye using the grid method.

Cells were treated with a dose of 25 up to 200 µM silibinin; based on the fact that 100 µM most effectively inhibited invasion, MMP-2, and uPA [11,12]. Vehicle for both agents was DMSO, which was applied to control cells.

CD44 promoter luciferase assays

Using the PXP2 plasmid, 1150 bases of CD44 sequence including the start site, and beginning 964 bases upstream to the start site, was cloned in between Xho I and Hind III sites. The efficacy of the promoter construct had been validated based on its inhibition by HOXC6 [14]. Luciferase activity was measured 48 h after transfection using the firefly luciferase assay (Gold Biotechnologies, St. Louis, MO). The cells were harvested in 20 mM K₂HPO₄ pH 7.8 with 5 mM MgCl₂ and 0.5% Triton X-100 buffer for 15 min on ice, and the mixture was centrifuged for 10 min at 4°C. 50 µl of lysate were added to 350 µl of luciferase assay buffer as per the manufacturer. The relative luciferase units (RLU) were normalized to protein concentration as determined by Bradford assay.

Real time TaqMan RNA analysis

Total RNA was prepared from cell pellets using Trizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNase-free water, and its concentration measured. Complementary DNA

(cDNA) was synthesized from 4 µg total RNA in 20 µl reaction mixture as we did previously [15]. At least triplicate samples were run using a primer set that brackets the entire variant region with a probe for CD44v9 [15], primers for CD44 total with a probe that binds a standard exon, and 18S ribosomal RNA. Quantitative PCR reactions were optimized to 4 µg cDNA (0.16 µg with 18S) plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 µl. 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 ABI 7500 cycler (Perkin-Elmer, Waltham, MA). Primer/probe sets for CD44v were: forward, AACGCTTCAGCCTACTGCAAA; reverse, TCTTCCAAGCCTTCATGTGATG; probe, GATTTGGACAGGACAGGACCTCTTTCAATG. For CD44 total we used forward, CAACTCCATCTGTGCAGCAAA; reverse, GTAACCTCCTGAAGTGCTGCTC; probe, CATATTGCTTCAATGCTTCAGCTCCACCTG. Primer and probe sets for 18S were proprietary to the manufacturer.

Western blot analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Roche, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 µg sample/lane according to

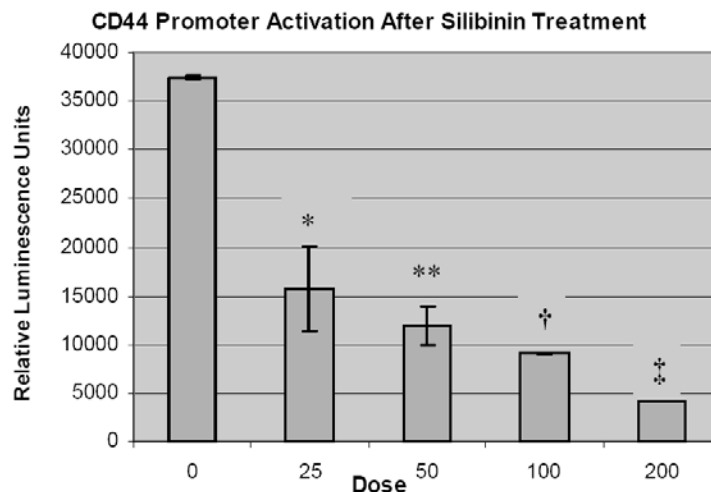


Figure 1. CD44 promoter assays. Transfected PC-3M cells overexpress PXP2 plasmid containing cloned in CD44 promoter + luciferase. Cells were incubated with silibinin for 48 hr after promoter transfection. There is a dose-dependent inhibition of promoter activity. Below: β-actin is shown as a normalizer.

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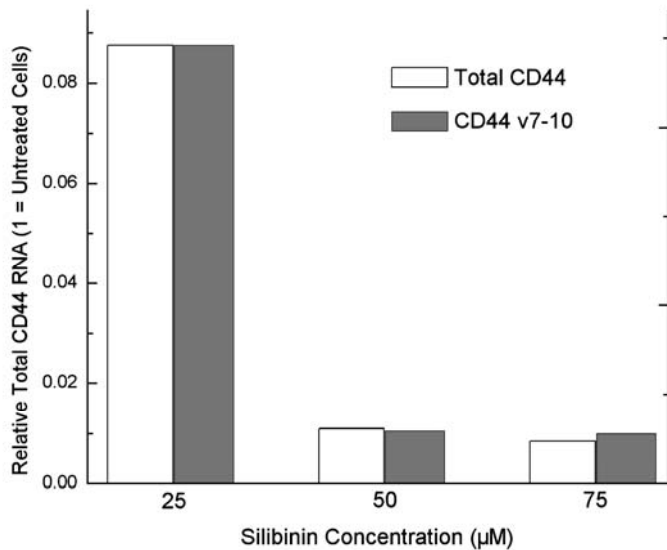


Figure 2. qRT-PCR demonstrates mean CD44 RNA levels after 25-100 µM silibinin treatment, normalized to untreated cells. Total CD44 was 91-99% inhibited. CD44v7-10 was 2.4x control value after 25 µM dose but inhibited at higher doses.

Laemmli method using the NuPAGE system (Invitrogen, Carlsbad, CA). 10 µl of Kaleidoscope Precision Plus Protein Standards (Bio-Rad) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to nitrocellulose. Primary antibodies were used. Polyclonal anti-Egr-1 C-terminus (Santa Cruz) was used at 1:200. To assess the overexpressed CD44v7-10 the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 total (standard + variant) was assessed using monoclonal antibody 156-3C11 (Cell Signaling Technology, Danvers, MA) at 1:1000. Anti-β-actin-HRP antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in TBS with 0.1% Tween 20% and 1:2000 dilution of goat anti-mouse IgG antibody labeled with HRP (Bio-Rad, Hercules, CA) was added in TBS + 5% skim milk for 1 hr. Reactivity was detected using the SuperSignal West Pico Substrate chemiluminescent system (Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Cell adhesion assays

Cellular adhesion assays as described [15],

were carried out using trypsinized confluent untreated, PC-3M cells after 48 hour 100 µM silibinin treatment or vehicle only. Each test condition was set in 8 wells. 96-well black-edged, clear flat bottom Costar plates (Cole-Parmer, Vernon Hills, IL) were coated with 20 µg/ml fibronectin (Becton Dickinson, Bedford, MA) or 2 mg/ml hyaluronan (Sigma, St. Louis, MO) [15] at 37°C overnight. To measure baseline nonspecific binding, other wells were coated with 1 mg/ml BSA. 1x10⁶ cells suspended in 1 mL PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo) for 15 minutes at 37°C [16]. After two washes of the cells with PBS, cells were added to plates at a density of 3x10⁴ per well and incubated at 37°C for 90 min. Fluorescence intensities at 530 nm were measured using a Bio-Tek FL-600 fluorescent plate reader. Nonadherent cells were removed with successive PBS washes, reading fluorescence intensities with PBS in

the wells after each. Adhesion was calculated [15] as % cells bound=(100) fluorescence intensity after second wash / fluorescence intensity of total cells plated. The assay was repeated twice.

Immunostaining for CD44v9 in prostate cancer patients

Under written informed consent, six men planning to undergo radical prostatectomy received 13 g silibinin-phytosome daily in 3 divided doses for a 2-4 week duration, and 7 men were untreated controls. Plasma levels of silibinin peaked at 25-150 µM with a half-life ranging from 2-5 hours [17]. The primary antibody was neat supernatant from the hybridoma cell line HB-258 (ATCC) secreting mouse anti-human antibody to CD44v9. The secondary antibody was goat anti-mouse IgG antibody labeled with HRP (Bio-Rad, Hercules, CA).

Statistical analysis

CD44 promoter activity data were analyzed by ANOVA and post t-test, comparing activity at various doses. TaqMan data were analyzed by the 2(-ΔΔCT) method [18] to determine fold change in gene expression (untreated

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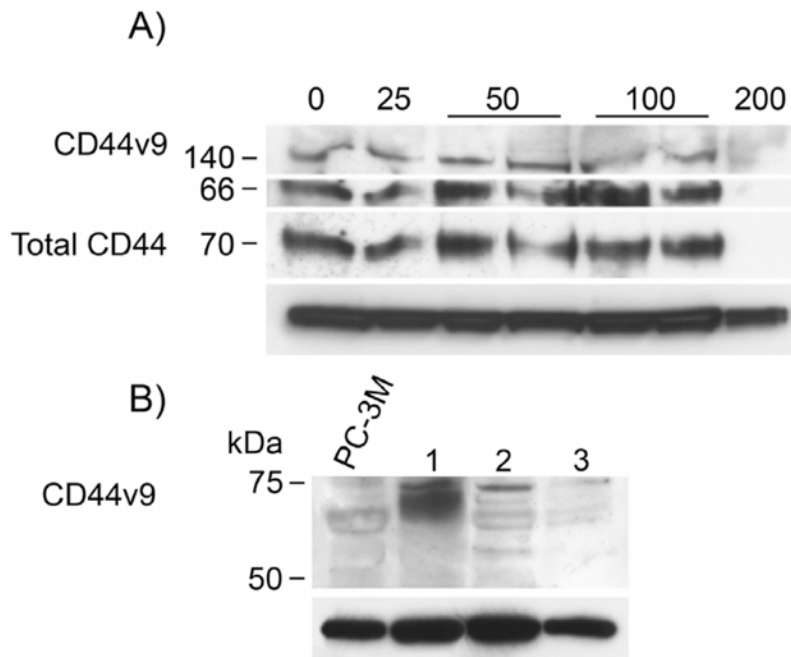


Figure 3. A) Western blot analysis of 25-200 μ M silibinin-treated PC-3M cells at 24 h, using monoclonal anti-CD44v9. Predominant band is 66kDa, characteristic for CD44 variant cleavage product [3] although there is some full-length protein at 140 kDa. Monoclonal anti-total CD44, predominantly CD44s, is also detected. At 24 hr, total CD44 and CD44v9 are decreased only at 200 μ M dose. Below: β -actin is shown as a normalizer. B) Extending the silibinin exposure of cells to 3 days caused a most marked inhibitory effect on CD44v9.

cells=1.00). The Δ CT was taken as the difference between the CD44v or CD44 total and the 18S ribosomal RNA CTs. The $\Delta\Delta$ CT was obtained using the mean Δ CT of untreated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test. Statistical significance was set at $p < 0.05$. Adhesion assay data are expressed as mean \pm SD. The significance of differences among group means was tested by 2-tailed paired t-test.

Results

Silibinin suppresses CD44 promoter activity, RNA and protein

Transfection of our CD44 promoter construct into PC-3M prostate cancer cells for 24 h was followed by silibinin treatment for 48 h to assess the effects of these agents. Silibinin significantly dose-dependently inhibited promoter activity, up to 86.7% ($p < 0.001$) at 200 μ M (**Figure 1**). Doses from 25 μ M to 200 μ M differed significantly from no treatment; and a dose of 200 μ M caused significantly less promoter activity than all lower doses. Mean RNA levels after all doses of silibinin treatment, normalized to untreated cells, were more than 90% lower for total CD44. CD44v7-10 was noted to be increased at 25 μ M dose but decreased at higher doses (**Figure 2**). At

the protein level, silibinin's effect at the protein level was minimal after 1 day, with western blot analysis (**Figure 3**) demonstrating inhibition of total CD44 and CD44 variant mainly at the highest, 200 μ M dose. We then performed a time course experiment, however, and noted that the earlier CD44v7-10 stimulation turned to a strong inhibition at 3 days, consistent with silibinin's inhibition of CD44v7-10 RNA.

Adhesion assays

Compared to untreated cells, PC-3M cells after 100 μ M silibinin had a 66% reduction in rate of adhesion to hyaluronan and 47% reduction in adhesion to fibronectin (**Figure 4**).

Clinical treatment with silibinin

Prostate tissues from 6 men with prostate cancer treated with silibinin and 7 treated with placebo were immunostained for the functionally important CD44 v7-10 using anti-CD44v9, directed against the largest portion of the included protein product CD44v7-10. Immunoreactivity was scored on a scale of 0-3+. The mean reactivity in treated and untreated men respectively were 1.0 and 1.3 for cancer, 2.9 and 2.6 for benign basal cells, 0.7 and 0.8 for benign secretory cells, and 0.8 and 0.7 for atrophic glands ($p = \text{NS}$). No

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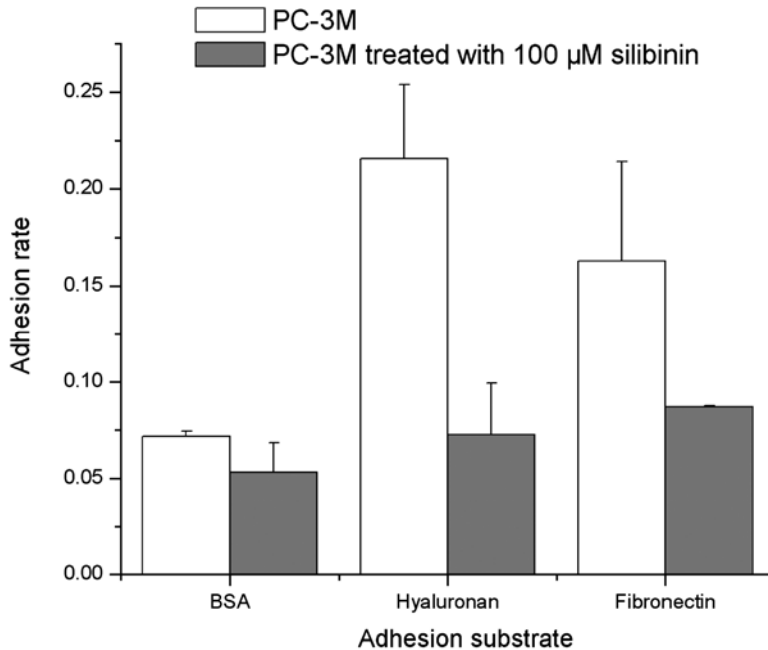


Figure 4. Adhesion assay with PC-3M cells after 100 μM silibinin treatment. Adhesion to both hyaluronan and fibronectin is diminished to baseline level, near that of BSA-coated wells, after silibinin treatment.

significant differences were noted.

Candidate mediators of silibinin effect

Silibinin dose of 100 or 200 μM inhibited Egr-1 expression (**Figure 5**). Bands were observed at 55 kD consistent with the theoretical monomeric form of Egr-1, with dimeric form bands near 110 kD [19]. 100 μM concentration equals the plasma level attained in patients [17]. As a negative control, slow growing, androgen-sensitive LNCaP cells were negative, while more aggressive DU145 cells expressed Egr-1. Immunoblots against EGFR and phospho-EGFR disclosed no silibinin effect (data not shown).

Discussion

We discovered that the nutrient silibinin reduces CD44 promoter activity, resulting in decreased total CD44 RNA. Inhibition of CD44v7-10 was less marked; however, at the protein level, this inhibition was strongly evident after 3 days of treatment. Our interest in CD44v7-10 variant isoform expression in PCa extends back several years. By isolating RNA from clinical PCa specimens, we found

CD44v7-10 to be a unique PCa signature, consistently overexpressed in androgen-independent cells such as PC-3, primary PCa, and PCa metastatic to other tissues [1-3]. Interference against CD44v7-10 reduced invasion index by 69% compared to untreated control cells [3]. Moreover, PCa loses the splicing ability to produce the standard isoform expressed in benign prostate that constitutes most of total CD44 [3-6] along with moderate levels of CD44v3-10 variant [6]. In PC-3 prostate tumor, CD44s transfection reduced invasion *in vitro* [20] and its tumorigenicity in mouse xenografts [21]. Silibinin's ability to reduce pro-invasive CD44v7-10 undoubtedly contributes to its effects.

CD44's adhesive affinities depend on its ability to cluster and multimerize, which is altered by a change in CD44s

to CD44 variant ratio. Thus we tested PCa cell adhesion to hyaluronan and fibronectin, two ligands that are most functionally important for CD44 in PCa cells. Binding to these two ligands was most strongly altered in PCa cells compared to benign BPH-1 cells [20], which bound hyaluronan strongly and fibronectin

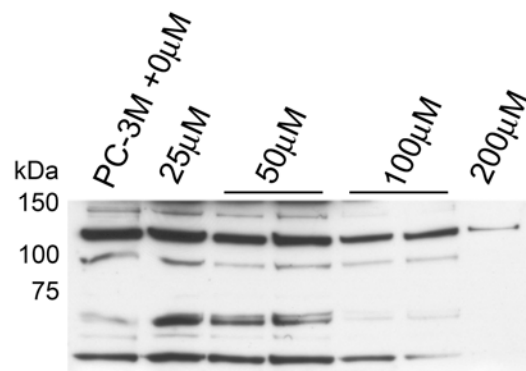


Figure 5. Western blot analysis of 25-200 μM silibinin-treated PC-3M cells using polyclonal anti-Egr-1. LNCaP and DU145 whole cell lysates are used as negative controls. Monomeric 55-kD and dimeric 110-kD cytoplasmic Egr-1 bands are seen, as well as a 75-kD band that had been described as specific to nuclear Egr-1 [19].

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minimally. The androgen-sensitive LNCaP cells bound both ligands equally, while PC-3 and their derivatives bound most strongly to fibronectin, probably representing an integrin-independent mechanism of binding to fibronectin. Moreover, re-expression of CD44s significantly increased adhesion to hyaluronan and decreased adhesion to fibronectin. Here, we observed a loss of total CD44 (which is largely CD44s) after silibinin treatment, consistent with our decreased hyaluronan binding, and lower CD44v7-10 which would mediate less fibronectin binding. Because of the decreased hyaluronan binding, silibinin does not strictly cause reversion to the benign phenotype but is consistent with the decreased migration [7], invasion [11,12] and possibly decreased *in vivo* growth [8] of cancer cells after silibinin treatment. The only prior study of silibinin's effects on PCa cell adhesion were done with type I collagen, showing inhibited adhesion of PC-3 cells to that ligand [7].

By immunostaining, prostatectomy tissue from silibinin-treated men did not differ in CD44v9 expression from that of untreated men. This lack of effect has two good explanations. First, the treatment was only for 2-4 weeks, minimizing the opportunity for the agent to alter CD44 expression in the tumor. Second, samples from this cohort of men are being tested to determine the tissue absorption into the prostate. Despite the high serum levels achieved, we have not confirmed reliable tissue penetration.

Silibinin's suppression of CD44 promoter activity which diminishes total CD44 RNA and protein is probably indirect. Factors that regulate CD44 expression by binding its promoter include Egr-1 [13,22], epidermal growth factor (EGF) [23], activating protein-1 (AP-1) [24], and H-ras [25]. Early growth response (EGR) genes encode zinc finger DNA-binding nuclear transcription factors, control cell proliferation, and show divergent expression in various human tumors. In PCa, Egr-1 binds to the androgen receptor [26] with important physiologic implications. One of us [23] demonstrated increased Egr-1, but not Egr-2 or EGR- α expression, in malignant prostate tissue as compared with weak expression in benign tissue. By *in situ* hybridization, the expression of Egr-1 was highest in tumors of Gleason score 8-10 than in those of lower Gleason score. Here we

showed for the first time that the therapeutically relevant 100 μ M dose of silibinin suppresses the high level of Egr-1 in prostate cancer (PCa) cells, suggesting that Egr-1 modulates, at least in part, the effects of silibinin on CD44.

Our findings are not meant to imply that Egr-1 is the sole mediator of silibinin's effects on CD44. Silibinin was reported to act on the MAP kinase pathway [11]. We have shown that MEK and p38 components of the MAP kinase pathway are functionally important for PCa cell expression of CD44 as well as possibly in its splicing [27]. Silibinin also inhibits the PI3K-Akt signaling pathway [11]. Further, it is likely that Egr-1, by binding to GC-rich motifs of other genes, exerts pro-growth effects relevant to PCa that are not dependent on CD44. These include activation of ICAM-1/CD54 [13], which is relevant to PCa growth [28], repression of Fas/CD95 gene [13], required for apoptosis, and repression of transforming growth factor- β Type II receptor, shutting off a growth-inhibitory pathway [29].

In conclusion, silibinin exerts functional effects on CD44 through the CD44 promoter, leading to reduced CD44 total and CD44v7-10 expression. These changes are accompanied by altered adhesion to known extracellular matrix substrates of CD44.

Acknowledgement

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References

- [1] Iczkowski KA, Pantazis CG, Collins, J. The loss of expression of CD44 standard and variant isoforms is related to prostatic carcinoma development and tumor progression. *J Urol Pathol* 1997;6:119-129.
- [2] Iczkowski KA, Bai S, Pantazis CG. Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. *Anticancer Res* 2003;23:3129-40.
- [3] Omara-Opyene AL, Qiu J., Shah GV, Iczkowski

Silibinin suppresses cd44 expression

- KA. Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18. *Lab Invest* 2004;84:894-907.
- [4] Vis AN, van Rhijn BW, Noordzij MA, Schroder FH., van der Kwast TH. Value of tissue markers p27(kip1), MIB-1, and CD44s for the pre-operative prediction of tumour features in screen-detected prostate cancer. *J Pathol* 2002;197:148-54.
- [5] Vis AN, Noordzij MA, Fitoz K, Wildhagen MF, Schroder FH, van der Kwast TH. Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer. *J Urol* 2000;164:2156-61.
- [6] Harrison GM, Davies G, Martin TA, Mason, MD, Jiang, WG. The influence of CD44v3-v10 on adhesion, invasion and MMP-14 expression in prostate cancer cells. *Oncol Rep* 2006;15:199-206.
- [7] Mokhtari MJ, Motamed N, Shokrgozar MA. Evaluation of silibinin on the viability, migration and adhesion of the human prostate adenocarcinoma (PC-3) cell line. *Cell Biol Int* 2008;32:888-92.
- [8] Xi X, Agarwal R. Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. *Proc Natl Acad Sci USA* 1999;22:96:7490-5.
- [9] Flaig TW, Su LJ, Harrison G, Agarwal R, Glodé LM. Silibinin synergizes with mitoxantrone to inhibit cell growth and induce apoptosis in human prostate cancer cells. *Int J Cancer* 2007;120:2028-33.
- [10] Raina K, Rajamanickam S, Singh RP, Deep G, Chittezhath M, Agarwal R. Stage-specific inhibitory effects and associated mechanisms of silibinin on tumor progression and metastasis in transgenic adenocarcinoma of the mouse prostate model. *Cancer Res* 2008;68:6822-30.
- [11] Chen PN, Hsieh YS, Chiou HL, Chu SC. Silibinin inhibits cell invasion through inactivation of both PI3K-Akt and MAPK signaling pathways. *Chem Biol Interact* 2005;20;156:141-50.
- [12] Chu SC, Chiou HL, Chen PN, Yang SF, Hsieh YS. Silibinin inhibits the invasion of human lung cancer cells via decreased productions of urokinase-plasminogen activator and matrix metalloproteinase-2. *Mol Carcinog* 2004;40:143-9.
- [13] Abdel-Latif MM, Windle HJ, Fitzgerald KA, Ang YS, Eidhin DN, Li-Weber M, Sabra K, Kelleher D. *Helicobacter pylori* activates the early growth response 1 protein in gastric epithelial cells. *Infect Immun* 2004;72:3549-60.
- [14] Yang K, Handorean AM, Iczkowski KA. MicroRNA 373 and 520c are downregulated in prostate cancer, suppress CD44 translation, and modulate prostate cancer cell invasion. *Int J Clin Exp Pathol* 2008, in Press. *Prostate* 1997;31:77-83.
- [15] Iczkowski KA, Omara-Opyene AL, Kulkarni TR, Pansara M, Shah GV. Paracrine calcitonin in prostate cancer is linked to CD44 variant expression and invasion. *Anticancer Res* 2005;25:2075-83.
- [16] Harada N, Mizoi T, Kinouchi M, Hoshi S, Shiiba K, Sasaki I, Matsuno S: Introduction of antisense CD44s cDNA downregulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells. *Int J Cancer* 2001;91:67-75.
- [17] Flaig TW, Gustafson DL, Su LJ, Zirrolli JA, Crighton F, Harrison GS, Pierson AS, Agarwal R, Glodé LM. A phase I and pharmacokinetic study of silybin-phytosome in prostate cancer patients. *Invest New Drugs* 2007;25:139-46.
- [18] Livak KL, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(- $\Delta\Delta CT$) method. *Methods* 2001;25:402-8.
- [19] Vollmann H, Wolfel S, Ohneseit P, Stransky E, Vonthein R, Wick W, Meyermann R, Simon P. Differential expression of Egr1 and activation of microglia following irradiation in the rat brain. *Strahlenther Onkol* 2001;183:248-55.
- [20] Iczkowski KA, Omara-Opyene AL, Shah GV. The predominant CD44 splice variant in prostate cancer binds fibronectin, and calcitonin stimulates its expression. *Anticancer Res* 2006;26:2863-72.
- [21] Miyake H, Hara I, Okamoto I, Gohji K, Yamanaka K, Arakawa S, Saya H, Kamidono, S. Interaction between CD44 and hyaluronic acid regulates human prostate cancer development. *J Urol* 1998;160:1562-6.
- [22] Fitzgerald KA, O'Neill LA. Characterization of CD44 induction by IL-1: a critical role for Egr-1. *J Immunol* 1999;162:4920-7.
- [23] Eid MA, Kumar V, Iczkowski KA, Bostwick DG, Tindall DJ. Expression of early growth response genes in human prostate cancer. *Cancer Res* 1998;58:2461-8.
- [24] Foster LC, Wiesel P, Huggins GS, Panares R, Chin MT, Pellacani A, Perrella MA. Role of activating protein-1 and high mobility group-I(Y) protein in the induction of CD44 gene expression by interleukin-1beta in vascular smooth muscle cells. *FASEB J* 2000;14:368-78.
- [25] Orian-Rousseau V, Morrison H, Matzke A, Kastilan T, Pace G, Herrlich P, Ponta H. Hepatocyte growth factor-induced Ras activation requires ERM proteins linked to both CD44v6 and F-actin. *Mol Biol Cell* 2007;18:76-83.
- [26] Yang SZ, Abdulkadir SA. Early growth response gene 1 modulates androgen receptor signaling in prostate carcinoma cells. *J Biol Chem* 2003;278:39906-39911.
- [27] Robbins EW, Travanty EA, Yang K, Iczkowski

Silibinin suppresses cd44 expression

- KA. MAP kinase pathways and calcitonin influence CD44 alternate isoform expression in prostate cancer cells. *BioMed Central Cancer* 2008;8:260.
- [28] Brooks KJ, Coleman EJ, Vitetta ES. The antitumor activity of an anti-CD54 antibody in SCID mice xenografted with human breast, prostate, non-small cell lung, and pancreatic tumor cell lines. *Int J Cancer* 2008;123:2438-45.
- [29] Du B, Fu C, Kent KC, Bush H Jr, Schulick AH, Kreiger K, Collins T, McCaffrey TA. Elevated Egr-1 in human atherosclerotic cells transcriptionally represses the transforming growth factor-beta type II receptor. *J Biol Chem* 2000;275:39039-47.

**STABLE ALTERATIONS OF CD44 ISOFORM EXPRESSION IN PROSTATE CANCER CELLS
DECREASE INVASION AND GROWTH AND ALTER LIGAND BINDING AND
CHEMOSENSITIVITY**

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Abstract

Background: Dysregulated CD44 expression characterizes most human cancers, including prostate cancer (PCa). PCa loses expression of CD44 standard (CD44s) that is present in benign epithelium, and overexpresses the novel splice variant isoform, CD44v7-10.

Methods: Using retroviral gene delivery to PC-3M PCa cells, we expressed luciferase-only, enforced CD44s re-expression as a fusion protein with luciferase at its C-terminus or as a protein separate from luciferase, or knocked down CD44v7-10 by RNAi. Invasion, migration, proliferation, soft agar colony formation, adhesion, xenograft growth, and Docetaxel sensitivity assays were carried out. Expression responses of merlin, a CD44 binding partner, and growth-permissive phospho-merlin, were assessed by western blot.

Results: Compared to luciferase-only PC-3M cells, all three treatments reduced invasion and migration. Growth and soft agar colony formation were reduced only by re-expression of CD44s as a separate or fusion protein but not CD44v7-10 RNAi. Hyaluronan and osteopontin binding were greatly strengthened by CD44s expression as a separate protein, but not a fusion protein. CD44v7-10 RNAi in PC-3M cells caused marked sensitization to Docetaxel; the two CD44s re-expression approaches caused minimal sensitization. In limited numbers of mouse subcutaneous xenografts, all three alterations produced only nonsignificant trends toward slower growth compared with luciferase-only controls. The expression of CD44s as a separate protein, but not a fusion protein, caused emergence of a strongly-expressed, hypophosphorylated species of phospho-merlin.

Conclusion: Stable re-expression of CD44s reduces PCa growth and invasion in vitro, and possibly in vivo, suggesting CD44 alterations have potential as gene therapy. When the C-terminus of CD44s is fused to another protein, most phenotypic effects are lessened, particularly hyaluronan adhesion. Finally, CD44v7-10, although it was not functionally significant for growth, may be a target for chemosensitization.

Key Words: alternate splicing; CD44; chemosensitization; invasion; lentivirus; merlin; prostate cancer

Background

About 30% of cases of prostate cancer (PCa) undergo transition from quiescent to aggressive. In this transition, altered expression of adhesion glycoproteins such as CD44 occurs allowing tumor cells to detach, interact with proteins that digest stromal matrix, migrate through matrix, and intravasate into lymphovascular channels. CD44 is a transmembrane molecule encoded by an alternately spliced gene. The standard (**CD44s**) isoform is ubiquitous, but inclusion of one or more of 10 variant (**v**) exons lengthens the extracellular stem, producing tissue-specific (**CD44v**) isoforms. CD44 is involved in multiple cellular functions. Its N-terminus enables cell-cell adhesion and binds hyaluronan and other matrix ligands, while the C-terminus links the cell's membrane to actin and ankyrin in the cytoskeleton, modulating shape and motility.

In prior work, we isolated RNA from clinical PCa tissues and discovered that expression of CD44v7-10 variant isoform constitutes a unique PCa signature, consistently expressed in primary and metastatic PCa. Androgen-independent PCa cell lines also strongly expressed it[1-3]. Interference against CD44v7-10 caused a 69% reduction in invasion index compared to untreated control cells[3] and altered ligand-binding affinities[4]. Moreover, PCa loses the splicing ability to produce the standard isoform expressed in benign prostate[3, 5, 6], and certain variants other than CD44 v7-10[1,7]. We enacted transient CD44v7-10 RNAi in PC-3 cell variants[3] using the plasmid U6pBS which lacks a drug-resistance selection gene, and later, using pTracer with drug resistance and GFP signal genes[4,8], and observed several phenotypic changes. Others have enacted transient plasmid transfection of CD44s into PC-3 cells, causing reduced growth *in vitro* and tumorigenicity in mouse xenografts[9].

Here, we achieve stably altered CD44 expression by cloning into retroviruses a CD44v7-10 RNAi sequence or one of two CD44s re-expression sequences—one translating to a fusion protein comprising CD44s with luciferase at its cytoplasmic, C-terminus, and the other expressing these as two separate proteins. We assess the functional effects of these stable alterations, and their potential for human gene therapy by testing *in vitro* invasion, attached growth, anchorage-independent growth, and chemosensitization, and by creating subcutaneous xenografts. Dephosphorylated merlin binds CD44 and

inhibits growth, whereas merlin is inactivated by phosphorylation to a growth-permissive state. Because the two re-expression transfectants differ in the freedom of their cytoplasmic tail, we assessed whether merlin, a binding partner of CD44's –COOH tail, may be sterically hindered from interacting with cells expressing CD44s-Fusion.

Methods

Cell lines

Benign BPH-1 and 293T cells were from American Type Culture Collection (Manassas, VA). PC-3M cells, a metastasis-derived variant of PC-3, were from Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX. The culture medium for PC-3M cells was RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) and antibiotics. 293T cells were in Dulbecco's modified Eagle medium with FCS. Cells were grown in 5% CO₂ incubation at 37°C. For cell set-up, cells in a flask were trypsinized, medium with serum was added to neutralize trypsin, and cells were stained in Trypan blue and counted by grid method[4].

Viral (Stable) CD44 Transfectants; Merlin Transfectants

Three constructs were made in Lentivector pLEX-MCS (Open Biosystems, Huntsville, AL), which contains the cytomegalovirus promoter, associated sequences, and puromycin resistance gene. These were Lenti-luciferase, Lenti-CD44s-luciferase, and Lenti-CD44s-RSV-luciferase (**Figure 1**). Lenti-luciferase was made by inserting the luciferase fragment from a plasmid (gift of Dr. J. Shen, Department of Radiation Oncology, Univ. of Colorado Denver) behind CMV promoter. A "Fusion" construct, Lenti-CD44s-luciferase, was then made by PCR-amplifying the CD44s fragment from our pTracer construct[7] and subcloning it into Lenti-luciferase. The fusion protein expressed comprises CD44s with luciferase at its C-terminal end. The "Separate" construct, Lenti-CD44s-RSV-luciferase, was made by inserting an RSV promoter between CD44s and luciferase sequences to drive luciferase expression. This construct expresses separate CD44s and luciferase proteins. PC-3M cells were infected

with the 3 respective constructs to yield PC-3M-luciferase cells, PC-3M-CD44s-Fusion cells, or PC-3M-CD44s-Separate cells. Cells were grown in 1 $\mu\text{g}/\text{mL}$ puromycin medium. Persistent expression was confirmed by adding luciferin substrate and imaging the flask on an IVIS 200 system (Xenogen, Alameda, CA), and by western blot analysis prior to use.

Four shRNA constructs were made for knocking down CD44 variant 9 (longest exon of CD44v7-10). Three sequences were generated by Extractor 4 computer program, and the fourth was from our prior publication[3]. Sequences were cloned into pSuper-RETRO (OligoEngine, Seattle) derived from pSuper[10].

2×10^6 293T cells were plated per 10 cm dish, and grown to 80% confluence in complete growth medium the next day. For luciferase-only and CD44s, 293T cells were transfected with Lentivector, packaging plasmid (psPAX2), and envelope plasmid (pMD2.G) using 3:1 PEI:DNA. For RNAi against CD44v7-10, the pSuper-RETRO with the construct was transfected similarly, but with 3 packaging vectors pJK3, pTAT2, and pVSVG, to produce retrovirus. After 48 h, supernatant was harvested and filtered with a 0.45 μm syringe filter. Incubation with viral supernatant for 2 days was used to infect the PC-3M cells. Cells were passaged into a 75 cm^2 flask and supplied with puromycin to final concentration of 1 $\mu\text{g}/\text{ml}$. A stable cell line was maintained by changing this selection medium every 3-7 days.

Plasmid pUHD10-3 that contained *EcoR1* fragments for wild-type merlin cDNA was a gift of Dr. D. H. Gutmann of Washington University in St. Louis[11]. PCa cells were transfected using PEI in the same manner as the 293T cells. To alter p21-activated kinase-2 (PAK-2), on which merlin phosphorylation depends in DU145 cells[12], sense and antisense nucleotides were transfected as described[13] with PEI.

Real Time Quantitative RT-PCR

To confirm altered CD44 expression, we used a primer + probe set that detects CD44 total, or a set that detects CD44v7-10 (Applied Biosystems, Foster City, CA) as described[14]. Detection of 18S ribosomal RNA was done simultaneously as a normalizer. TaqMan data were analyzed by the $2^{(-\Delta\Delta C_T)}$

method[14] to determine fold change in gene expression (untreated cells=1.00). The ΔC_T was taken as the difference between the CD44v or CD44 total C_T and the 18S ribosomal RNA C_T . The $\Delta\Delta C_T$ was obtained using the mean ΔC_T of untreated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test.

Migration and invasion assays

We used a population of PC-3M cells stably infected with lentivirus (for CD44s) or pSUPER-derived retrovirus (for CD44v RNAi). Invasion was assessed with triplicate 24-well Matrigel two-tier invasion chambers, and migration was assessed with control inserts, both with 8.0 μm pore diameter (Collaborative Biomedical Products, Bedford, MA)[4]. Untreated cells or those expressing a construct were seeded at 30,000 per well. Cells in the upper insert were in serum-free basal medium (RPMI 1640 with 0.1% BSA, 4 mM L-glutamine, 100 $\mu\text{g}/\text{mL}$ each of penicillin G and streptomycin). The lower chamber contained chemoattractant medium consisting of 10% fetal bovine serum, 20% conditioned medium from subconfluent culture, and 70% complete medium. The incubation was carried out 24 h in 5% CO_2 incubator at 37°C. The medium from the upper inserts, together with non-invading cells were removed off the upper Matrigel surface. The lower surface was fixed in methanol and stained with May-Grunwald stain (Sigma) according to manufacturer's protocol.

Soft agar colony formation and growth assays

A bottom layer was formed using 2 ml of complete medium with 1% agarose that was poured into each well of 6-well plates and solidified at 4°C. PC-3M cells with or without viral infection, 5000/well, were mixed in complete medium with 0.5% agarose and seeded as a top layer. The agarose was solidified at 4°C and then incubated at 37°C. On day 14, the colonies were stained with 1 ml of PBS containing 0.5 mg/ml of p-iodonitrotetrazolium violet (Sigma, St. Louis, MO). Only live cells convert this into a colored product. The total number of colonies from 20, 100x microscopic fields was counted.

For growth assays, we seeded 30,000 cells per 6-well plate well and harvested and counted cells 3 days later by grid method.

Cellular adhesion assays

Assays were carried out as described[15] using trypsinized confluent untreated or virally treated cells. Each test condition was set in 8 wells and each experiment repeated twice. 96-well black-edged clear flat bottom Costar plates (Cole-Parmer, Vernon Hills, IL) were coated with optimal concentrations of ligands[15] using 8 wells to test each one, at 37°C overnight. As controls, 8 wells were coated with 1 mg/ml BSA to measure baseline nonspecific binding. 1×10^6 cells suspended in 1 ml PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo) for 15 min at 37°C. After two washes of the cells with PBS, cells with serum-free basal medium were added to plates at a density of 3×10^4 per well and incubated at 37°C for 90 min. Fluorescence intensities at 530 nm were measured using a Bio Tek FL-600 plate reader. Nonadherent cells were removed with 2 PBS washes. Fluorescence intensities with PBS in the wells were measured. Adhesion was calculated[15] as % cells bound = (100) fluorescence intensity post-wash / fluorescence intensity of total cells plated.

Western blot analysis

Cultured cells were directly lysed in dishes using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 µg sample/lane according to the Laemmli method[16] using the NuPAGE system (Invitrogen, Carlsbad, CA). 10 µl of Kaleidoscope protein marker (Bio-Rad, Hercules, CA) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Mouse monoclonal antibodies all used at 1 µg/mL were: to assess overexpressed CD44v7-10, CD44v10 antibody (Bender MedSystems, Burlingame, CA); CD44 total (standard + variant 156-3C11), CD44s (LabVision, Fremont, CA), and rabbit merlin and phospho-merlin (Cell Signaling) were used at 1:1000. Anti-β-actin antibody (Sigma, St. Louis) was used at a

dilution of 1:5,000. Membranes were washed 3 x 10 min in TBS with 0.1% Tween-20 (TBST) and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) or 1:1,250 goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) was added in 5% skim milk for 1 h. After washing membrane with 1x TBST, reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Docetaxel Sensitivity Assays

Cells were seeded on a 96-well plate containing 5000 cells/well for 6 h. Docetaxel (Taxotere, Sanofi Aventis, Bridgewater, NJ) was administered to wells at doses from 0-50 nM. After 72 hours, MTS assay (CellTiter 96AQ, Promega, Madison, WI) for proliferation was performed and read on the Bio Tek. The plate was read at hourly intervals and the representative interval was chosen for each cell type so as to normalize intensity at time zero. The assay was repeated twice with similar results.

Xenograft Growth

1.5×10^6 PC-3M-luciferase cells, CD44s-Separate cells, CD44s-Fusion cells, or CD44v7-10 RNAi cells, mixed 1:1 with Matrigel, were injected subcutaneously into the flank of nude mice. Presence or absence of tumor 'take' was recorded, and tumor length, width, and height were measured daily. When tumor reached 2.0 cm in greatest dimension, mice were imaged on the IVIS 200 (Xenogen) to confirm persistent signal in all tumors, prior to mouse sacrifice.

Statistical Analyses

For in vitro assays and mice growth, data were expressed as mean \pm SD. The significance of differences among group means was tested by two-tailed 2-sample Student *t*-test. Statistical significance was set at $p < 0.05$.

Results

Establishment of altered CD44 expression

Western blot analyses for total human CD44 (**Figure 2**) in CD44s-Separate cells and CD44s-Fusion cells confirmed CD44s overexpression. Western blot analysis for CD44v7-10 was inadequate, since the antibodies we tested were not reliable. By Real Time RT-PCR, CD44 total was increased 1.80-fold in CD44-Fusion cells and 4.35-fold in CD44-Separate cells compared to untreated cells. CD44s re-expression as a fusion or separate protein did not affect CD44 variant. CD44v7-10 re-expression was reduced to 0.21 (79% silencing) in the 1522 viral RNAi construct. In this transfectant, knockdown was the best among 3 RNAi constructs, so we used it for all further work.

Migration and invasion

PC-3M CD44s-Fusion cells, CD44s-Separate cells, and CD44v7-10 RNAi cells all displayed >50% decreases in both migration and invasion compared with the luciferase-only PC-3M control (**Figure 3**). These effects were strongest in CD44s-Separate cells compared with the other two, with $\geq 75\%$ decreases.

Attached cell growth and anchorage-independent growth

Compared to PC-3M-luciferase only cells, attached cell growth at 3 days was decreased in CD44s-Separate cells and CD44s-Fusion cells (both $p < 0.001$) but not in CD44v7-10 RNAi cells ($p = 0.22$, **Figure 4**). In soft agar assays, the same trends were evident, with 25% decreases in colonies formed from CD44s-Separate ($p < 0.023$) and CD44s-Fusion ($p < 0.002$) but not CD44v7-10 RNAi cells ($p = 0.156$) (**Figure 5**).

Cell adhesion assay

Increases of about 4-fold in percent cells binding to hyaluronan, and 3-fold in percent cells binding to osteopontin were noted with the CD44s-Separate cells ($p < 0.001$) compared to luciferase-only

controls. The CD44s-Fusion cells showed a divergent response, with a minimal but significant increase in hyaluronan binding but no increase in osteopontin binding. Both CD44s infected cell lines and CD44v7-10 RNAi cells had slightly increased adhesion to fibronectin (**Figure 6**).

Docetaxel sensitivity assay

Compared to PC-3M luciferase-only control cells, all the treated cells had increased sensitivity to Docetaxel doses of 0-50 nM. PC-3M CD44s-Separate cells and PC-3M CD44s-Fusion cells showed minimal sensitivity, while PC-3M CD44v7-10 RNAi cells were extremely sensitive. Benign BPH-1 cells, as a positive control, were also sensitive (**Figure 7**).

Xenograft growth

Tumor take for PC-3M control cells was 9/12 (75%), 7/8 (88%) for CD44s-Separate cells, but only 4/7 (57%) for CD44s-Fusion cells, and 4/7 (57%) for PC-3M CD44v7-10 RNAi cells. Tumor latency was defined as days until appearance of a 0.04 ml palpable mass. Appearance of a fully-formed mass was defined according to the smallest of the masses that developed among animals with tumor take, giving a set point of 0.85 ml. Mean number of days until these 2 points were reached was calculated for each cell type (**Figure 8**). There was no significant difference among the 4 groups by log-rank test ($p=0.89$), but pairwise comparisons showed non-significant trends toward efficacy for all three therapies, particularly CD44v7-10 RNAi.

Relationship of CD44 with Merlin

Enforced expression of merlin increased CD44 total in CD44s-Separate cells (**Figure 2**). We also examined the degree of overexpression of merlin and phospho-merlin achievable in CD44s-Separate cells, compared to luciferase-only or CD44s-Fusion cells (**Figure 9**). Total merlin content was increased the most in CD44s-Separate cells. Immunoblotting for phospho-merlin, confirmed by the manufacturer to be phospho-specific to P-Ser 518, demonstrated a great increase in a band representing hypophosphorylated,

75 kD merlin in CD44s-Separate cells. The luciferase-only cells and CD44s-Fusion cells, in contrast, had less 75-kD, hypophosphorylated form as well as a band for the 85-kD form, reflecting phosphorylation at multiple sites.

Dependence of Phospho-merlin on PAK2

To determine whether expression of the phospho-merlin were dependent on PAK2, as is the case in DU145 cells[12], PC-3M cells were transfected with merlin and with PAK2 antisense oligonucleotide or a sense sequence. Antisense against PAK2 in luciferase-only PC-3M cells was effective, and profoundly decreased phospho-merlin (**Figure 10**).

Discussion

We have shown that in aggressive PC-3M PCa cells, re-expression of CD44s greatly reduced invasion, migration, and tumor cell proliferation, suggesting a number of functional effects in PCa and possibly those other cancers such as bladder, for which CD44s functions as a tumor suppressor.

Growth in vitro, and to a lesser extent, soft agar colony formation, were inhibited more effectively by CD44s-Separate than any other treatment. This effect of CD44s was also described in colon cancer cells[15] and is consistent with previous studies in which the overexpression of the CD44s cytoplasmic tail was able to inhibit pro-growth Met-Ras-ERK signaling[17]. It also explains why CD44s-Fusion was not associated with increased merlin, since the C-terminus luciferase would render inaccessible the amino acids 292-300 that bind merlin (and ERM proteins). Furthermore, this blockage of binding would explain the lack of increase in growth-inhibiting hypophosphorylated merlin after merlin transfection of CD44s-Fusion (**Figure 8**).

Knockdown of CD44v7-10 was effective in reducing PCa invasion and migration, as we showed previously[3] but CD44s overexpression was even more effective. Invasion was assessed through Matrigel membranes, which have no hyaluronan but do have fibronectin, consistent with the observation that CD44s suppression of invasion and metastasis does not rely on hyaluronan binding[18].

Altered CD44 expression also affected ligand binding, with increased hyaluronan binding in the CD44s-Separate cells compared to CD44s-Fusion. As an explanation, the mature form of human CD44s lacks the protein product of exon 19 but contains most of exon 20 at its cytoplasmic, C-terminus. A functional C-terminus is required not only for CD44 binding to ankyrin[19] but also for hyaluronan binding by the “link” domain at the N-terminus[20] which involves oligomerization[17], and for formation of the pericellular matrix[20].

Merlin is activated by phosphorylation under growth-promoting conditions and dephosphorylated under growth-inhibiting conditions including CD44-hyaluronan binding[11, 21]. Dephosphorylation inactivates it by a conformational change to a circularized form. In CD44s-Separate cells, a strong band representing hypophosphorylated merlin was detected by us with a phospho-specific antibody. Moreover, this form was PAK2-dependent and correlated with increased hyaluronan binding. Fully phosphorylated, active merlin inhibits CD44-hyaluronan binding[21] so our detection of this form suggest that in PC-3M cells, CD44s stabilizes merlin in a hypophosphorylated state that is growth-inhibitory but permits CD44s-hyaluronan binding. The CD44s-Fusion cells have their C-terminus blocked by the attached luciferase, precluding stimulation of either merlin expression or hyaluronan binding. The increased binding to osteopontin by CD44s-Separate, but not CD44s-Fusion, suggests that a functional C-terminus is also required for osteopontin binding. Interestingly, the increased osteopontin adhesion in CD44s-Separate cells is at variance with a previous conclusion that CD44 variants, but not CD44s, facilitated osteopontin binding to allow migration[22]; however, these data were based on rat pancreatic carcinoma, rat fibrosarcoma, and mouse melanoma cells, and pertained to CD44v4-7, not CD44v7-10.

Horiguchi et al.[12] found that silencing of CD44 dephosphorylates merlin in benign cells. Moreover, both merlin and phospho-merlin were strongly expressed in DU145 PCa cells, but were weak to absent in LNCaP, PC3, 22RV1, and LAPC-4. Studying only the DU145 cells in further detail, they found that silencing total CD44 had no effect on these high levels of merlin and phospho-merlin, although another CD44 binding partner, the ERM (ezrin-radixin-moesin) proteins, had markedly reduced phosphorylation. This implied constitutive merlin phosphorylation. We, conversely, enforced merlin expression in PC-3M-

luciferase cells, resulting in increased CD44s. Enforced CD44s-Separate, in turn, increased the hypophosphorylated merlin compared to the minimal, mostly hyperphosphorylated amount in PC-3M-luciferase cells (**Figure 2**). This may reflect mutual stabilization of CD44s and merlin upon binding. In CD44s-Separate cells, the overexpressed merlin manifested predominantly as a 70-kD hypophosphorylated form, as opposed to the expected 75 kD. That the cytoplasmic end of CD44 is required for this interaction is proven by relative the lack of effect of CD44s-Fusion expression on total merlin or phospho-merlin. Taken together with our observation of decreased growth of CD44s-Separate cells, this suggests that not only dephosphorylated merlin, but also this hypophosphorylated form, has anti-growth properties. The serine/threonine kinase PAK-2 normally phosphorylates and activates merlin. Antisense inhibition of PAK-2, however, did not decrease this hypophosphorylated merlin (**Figure 9**). This suggests that in PC-3M cells, merlin is preferentially phosphorylated to this hypophosphorylated state. In cells with CD44v7-10 silencing, increased hypophosphorylated merlin was not evident (**Figure 8**). We previously determined that CD44v7-10 was functionally significant for PCa invasion.³ CD44 variants are known to oligomerize, causing pro-growth signaling but in the current studies, the CD44v7-10 did not appear to have been significant for in vitro, anchorage-dependent or independent growth or xenograft growth. CD44v7-10 RNAi did not affect merlin or phospho-merlin, consistent with this lack of an observed effect on growth. However, CD44v7-10 expression was most effective for chemosensitization of PC-3M cells to the widely used drug, docetaxel.

We considered the attractive prospect of altering both CD44s and CD44v7-10. It was not possible, under our system, to establish doubly-infected cells for CD44s and CD44v7-10-RNAi, because both rely on puromycin for selection, and flow cytometry for luciferase is not feasible.

CD44s-Separate cells and CD44v7-10 RNAi cells, despite demonstrable anti-growth or pro-chemosensitivity effects in vitro, showed only a non-significant trend toward slower mice subcutaneous xenograft growth.. Altered expression of other cell adhesion molecules have been described to cause divergent in vitro and in vivo effects. For example, in thyroid cancer, expression of CEACAM1, a molecule also involved in both adhesion and cell signaling of growth pathways, facilitated invasion but

slowed growth of tumor xenografts[23]. However, two limitations of our study are sample size (n=7 or 8 per group with altered CD44), and not having tested orthotopic xenografts. Orthotopic grafts, in a different stromal and matrix metalloprotease microenvironment, are often slower-growing, non-metastatic, and respond to certain therapies that subcutaneous ones do not[24]. We tried to overcome this limitation by use of Dunning rat PCa cells, in which subcutaneous grafts can metastasize to lungs[18] but re-expression of human CD44s as a fusion or separate protein could not be documented on western blot analysis, and human CD44v was absent (data not shown), possibly because a few CD44 bases differ between human and rat. Thus, our use of the subcutaneous approach may have precluded assessment of relevant therapeutic effects.

Conclusions

The stable re-expression of CD44s reduces PCa growth and invasion in vitro, and shows a non-significant growth reduction in the subcutaneous xenograft model. When the C-terminus of CD44s is fused to another protein, some phenotypic changes are lessened. Finally, CD44v7-10, although it was not functionally significant for growth, may be a target for chemosensitization. These findings suggest the potential for CD44 alterations in gene therapy, perhaps in conjunction with enforced expression alterations of other molecules.

List of Abbreviations Used: CD44: Cell determinant 44; CD44s: CD44 standard; CD44v: CD44 variant; PEI: polyethyleneimine; RNAi: RNA interference.

Competing Interests: None

Authors' Contributions: KY, YT, and GKH performed experiments; KY performed statistics; KAI and KY wrote the paper.

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REFERENCES

- 1 Iczkowski KA, Pantazis CG Collins, J: **The loss of expression of CD44 standard and variant isoforms is related to prostatic carcinoma development and tumor progression.** *J Urol Pathol* 1997;**6**:119-129.
2. Iczkowski KA, Bai S Pantazis, CG: **Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level.** *Anticancer Res* 2003;**23**: 3129-3140.
3. Omara-Opyene AL, Qiu J, Shah GV, Iczkowski KA: **Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18.** *Lab Invest* 2004;**84**:894-907.
4. Iczkowski KA, Omara-Opyene AL Shah GV: **The predominant CD44 splice variant in prostate cancer binds fibronectin, and calcitonin stimulates its expression.** *Anticancer Res* 2006;**26**:2863-2872.
5. Vis AN, van Rhijn BW, Noordzij MA, Schröder FH, van der Kwast TH: **Value of tissue markers p27(kip1), MIB-1, and CD44s for the pre-operative prediction of tumour features in screen-detected prostate cancer.** *J Pathol* 2002;**197**:148-154.
6. Vis AN, Noordzij MA, Fitoz K, Wildhagen MF, Schröder FH, van der Kwast TH: **Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer.** *J Urol* 2000;**164**:2156-2161.
7. Harrison GM, Davies G, Martin TA, Mason MD, Jiang WG: **The influence of CD44v3-v10 on adhesion, invasion and MMP-14 expression in prostate cancer cells.** *Oncol Rep* 2006;**15**:199-206.
8. Iczkowski KA, Omara-Opyene AL, Kulkarni TR, Pansara M, Shah GV: **Paracrine calcitonin in prostate cancer is linked to CD44 variant expression and invasion.** *Anticancer Res* 2005;**25**:2075-2083.
9. Miyake H, Hara I, Okamoto I, Gohji K, Yamanaka K, Arakawa S, Saya H, Kamidono S: **Interaction between CD44 and hyaluronic acid regulates human prostate cancer development.** *J Urol* 1998;**160**:1562-1566.
10. Brummelkamp TR, Bernards R, Agami R: **Stable suppression of tumorigenicity by virus-mediated RNA interference.** *Cancer Cell* 2002;**2**:243-247.

11. Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P: **The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44.** *Genes Dev* 2001;**15**:968-980.
12. Horiguchi A, Zheng R, Shen R, Nanus DM: **Inactivation of the NF2 tumor suppressor protein merlin in DU145 prostate cancer cells.** *Prostate* 2008;**68**:975-994.
13. Chan WH, Wu HJ, Shiao N-H: **Apoptotic signaling in methylglyoxal-treated human osteoblasts involves oxidative stress, c-Jun N-terminal kinase, caspase-3, and p21-activated kinase 2.** *J Cell Biochem* 2007;**100**:1056-1069.
14. Robbins EW, Travanty EA, Yang K, Iczkowski KA: **MAP kinase pathways and calcitonin influence CD44 alternate isoform expression in prostate cancer cells.** *BioMed Central Cancer* 2008;**8**:260.
15. Harada N, Mizoi T, Kinouchi M, Hoshi K, Ishii S, Shiiba K, Sasaki I, Matsuno S.: **Introduction of antisense CD44s cDNA downregulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells.** *Int J Cancer* 2001;**91**:67-75.
16. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970;**227**:680-685.
17. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H: **CD44 is required for two consecutive steps in HGF/c-Met signaling.** *Genes Dev* 2002;**16**:3074-86.
18. Gao AC, Lou W, Sleeman JP, Isaacs JT: **Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate.** *Cancer Res* 1998;**58**:2350-2352.
19. Lokeshwar VB, Bourguignon LY: **The lymphoma transmembrane glycoprotein GP85 (CD44) is a novel guanine nucleotide-binding protein which regulates GP85 (CD44)-ankyrin interaction.** *J Biol Chem* 1992;**267**:22073-22078.
20. Jiang H, Peterson RS, Wang W, Bartnik E, Knudson CB, Knudson W: **A requirement for the CD44 cytoplasmic domain for hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells.** *J Biol Chem* 2002;**277**:10531-10538.

21. Bai Y, Liu Y, Wang H, Xu Y, Stamenkovic I, Yu Q: **Inhibition of the hyaluronan-CD44 interaction by merlin contributes to the tumor-suppressive activity of merlin.** *Oncogene* 2007;26:836-850.
22. Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF, Uede T: **CD44 variants but not CD44s cooperate with β 1-containing integrins to permit cells to bind to osteopontin independently of agrinine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis.** *Cancer Res* 1999;59:219-226.
23. Liu W, Wei W, Winer D, Bamberger AM, Bamberger C, Wagener C, Ezzat S, Asa SL: **CEACAM1 impedes thyroid cancer growth but promotes invasiveness: a putative mechanism for early metastases.** *Oncogene* 2007;26:2747-2758.
- 24 Jantscheff P, Esser N, Graeser R, Ziroli V, Kluth J, Unger C, Massing U: **Liposomal gemcitabine (GemLip)–efficient drug against hormone-refractory DU145 and PC-3 prostate cancer xenografts.** *Prostate* 2009;69:1151-1163.

Figure Legends

Figure 1. Constructs used for CD44s re-expression in Lentivector. CD44s was expressed as a fusion protein with luciferase, or as a separate protein. CD44s in human prostate includes exons 1-5 and 16-18, with a small portion of 20, according to our sequencing[3].

Figure 2. Detection of CD44 total, by monoclonal antibody in PC-3M cells. Appearance of a predominant cleavage product at 75 kD (lane 1) rather than the >100 kD uncleaved form, is consistent with our experience [1-4, 14]. The re-expression of CD44s as a Fusion protein with luciferase (lanes 2-3) produced a 137 kD form. The 75-kD form may be partly cleaved off after trypsinizing, giving the same 75 kD CD44s form noted when CD44s was expressed as a Separate protein (lane 4). In CD44s-Separate cells, the forced expression of CD44's intracellular binding partner merlin potentiated the increased CD44s (lane 5).

Figure 3. Invasion (Matrigel insert) and migration (control insert) assays of PC-3M cells. Compared to the luciferase-only control cells, the CD44s-Separate cells had 86% less migration and 74% less invasion ($p<0.001$). The CD44s-Fusion cells showed 78% decreased migration and 55% decreased invasion ($p<0.001$). CD44v7-10 RNAi cells also had significantly decreased migration and invasion.

Figure 4. Growth assays of 30,000 cells after 3 days. The PC-3M-CD44s-Separate cells and CD44s-Fusion cells, grown in Puromycin-containing medium, both showed decreased growth ($p<0.001$) compared to luciferase-only cells. CD44v7-10 RNAi cells did not show decreased growth ($p=0.218$). Untreated cells grew similarly to luciferase-only cells if grown in medium without puromycin.

Figure 5. Colony formation of PC-3M in soft agar after 14 days. The CD44-Separate ($p < 0.023$) and CD44 Fusion cells ($p < 0.002$), but not CD44v7-10 RNAi cells ($p = 0.156$), grew significantly fewer colonies than luciferase-only cells.

Figure 6. Cell adhesion assay of PC-3M cells. p values indicated above bars. CD44s-Separate cells showed by far the greatest increases in hyaluronan binding (4x) and osteopontin binding (3x) compared to luciferase-only cells. CD44s-Fusion cells showed a less marked but significant increase in hyaluronan binding, but not osteopontin binding. All infected cells showed slight but significant increases in fibronectin binding.

Figure 7. Docetaxel assays. PC-3M cells with CD44v7-10 RNAi were maximally sensitive to Docetaxel doses up to 50 nM. Re-expression of CD44s as a Separate or Fusion protein moderately increased the sensitivity of PC-3M cells. As a control, benign BPH-1 prostate cells were also quite sensitive.

Figure 8. Kaplan-Meier analysis of growth of subcutaneous xenografts in nude mice. Daily tumor volume was measured, and two cut points were chosen: 0.04 ml for formation of a palpable mass, and, based on the smallest tumor among mice with tumor take, 0.85 ml for a fully-formed tumor. Noted trends were non-significant. n= number of mice with tumor take.

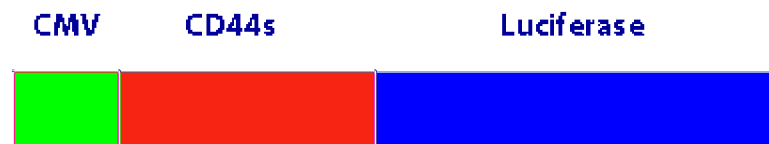
Figure 9. Enforced expression of merlin in virally-infected CD44s-Separate cells markedly increases CD44 total (Figure 2). It also greatly increases total merlin and the predominant, lower molecular weight, hypophosphorylated form of p-merlin (lane 3). These increases are only minimal in CD44s-Fusion cells (lane 4). This suggests that CD44s-merlin binding reduces degradation of both molecules.

Figure 10. PAK2 is an enzyme that phosphorylates (and activates) merlin. In PC-3M cells, antisense inhibition of PAK2 was carried out, causing a decrease in p-merlin. Thus, the preferentially expressed hypophosphorylated form of merlin in PC-3M cells depends on PAK2, as it does in DU145 cells[12].

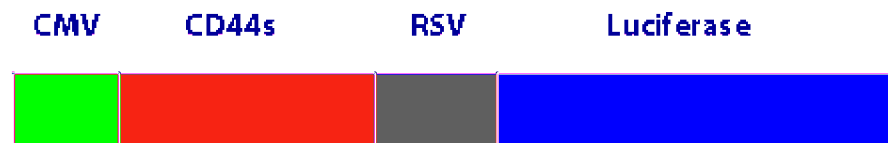
For CD44s Re-expression: Luciferase Constructs in Lentivector



Luciferase only



Makes a **Fusion** protein, MW= 137, including CD44s (75 kD) and luciferase (62 kD)



Makes Separate proteins for CD44s (75 kD) and luciferase (62 kD)

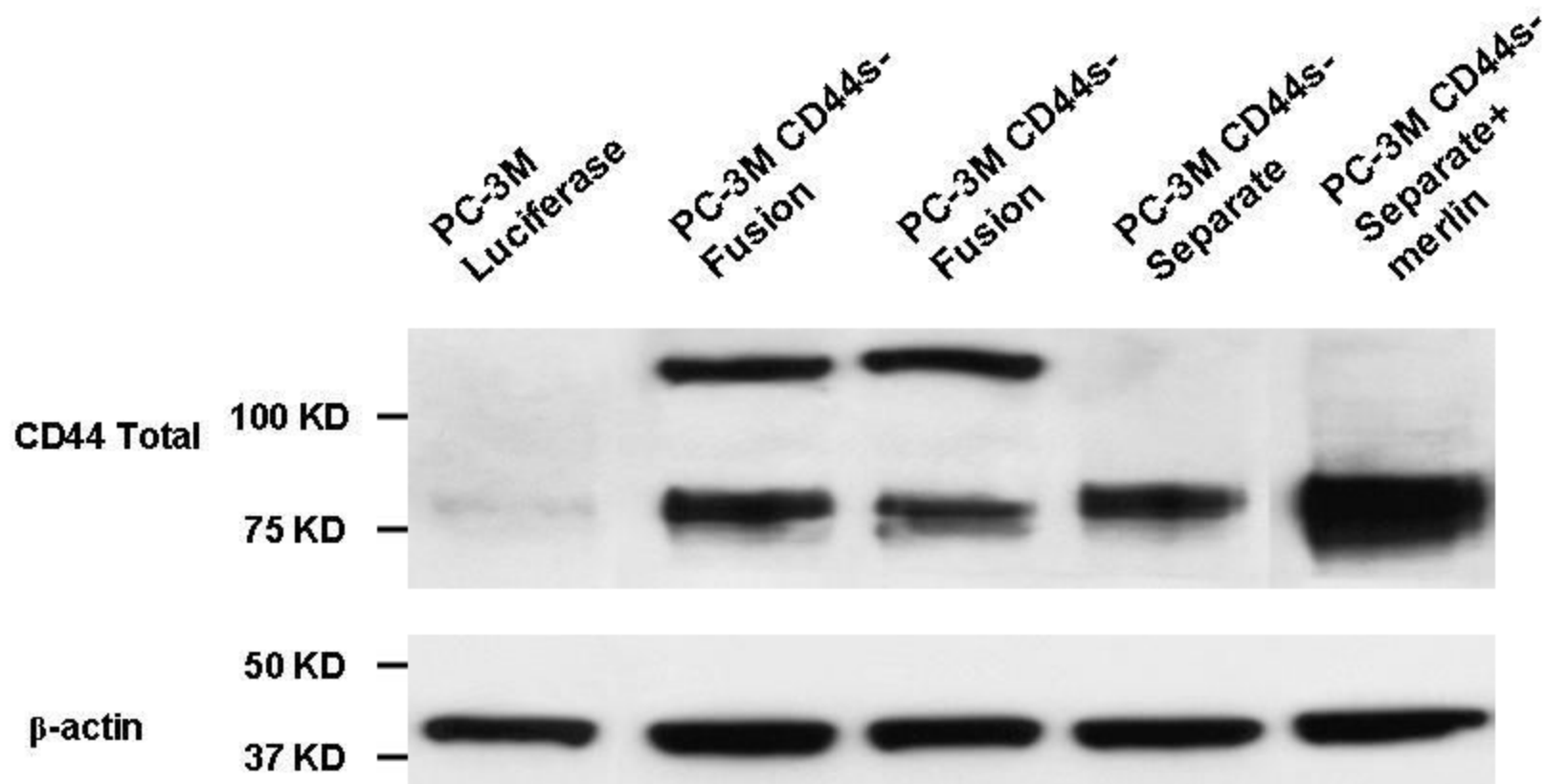


Figure 2

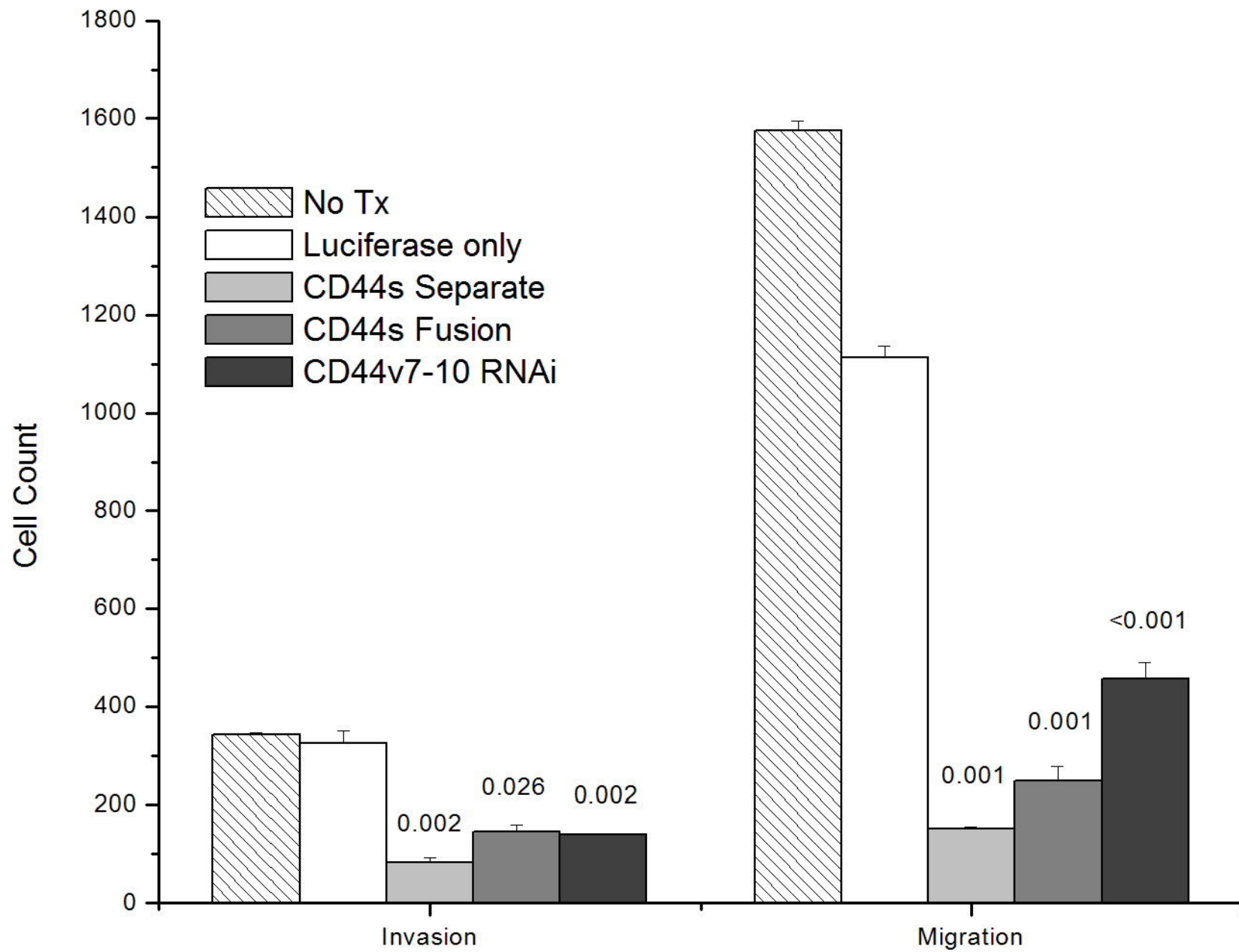


Figure 3

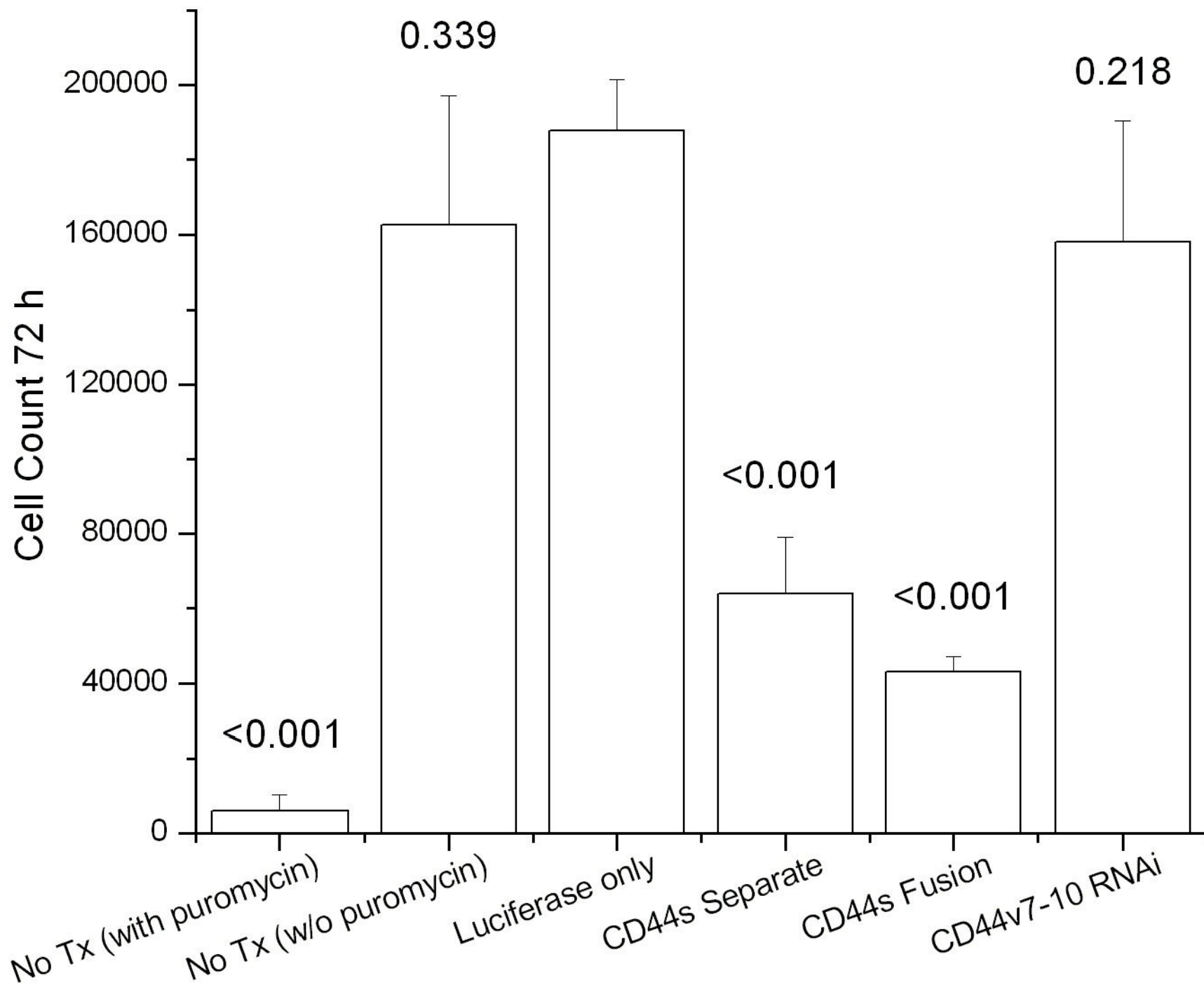


Figure 4

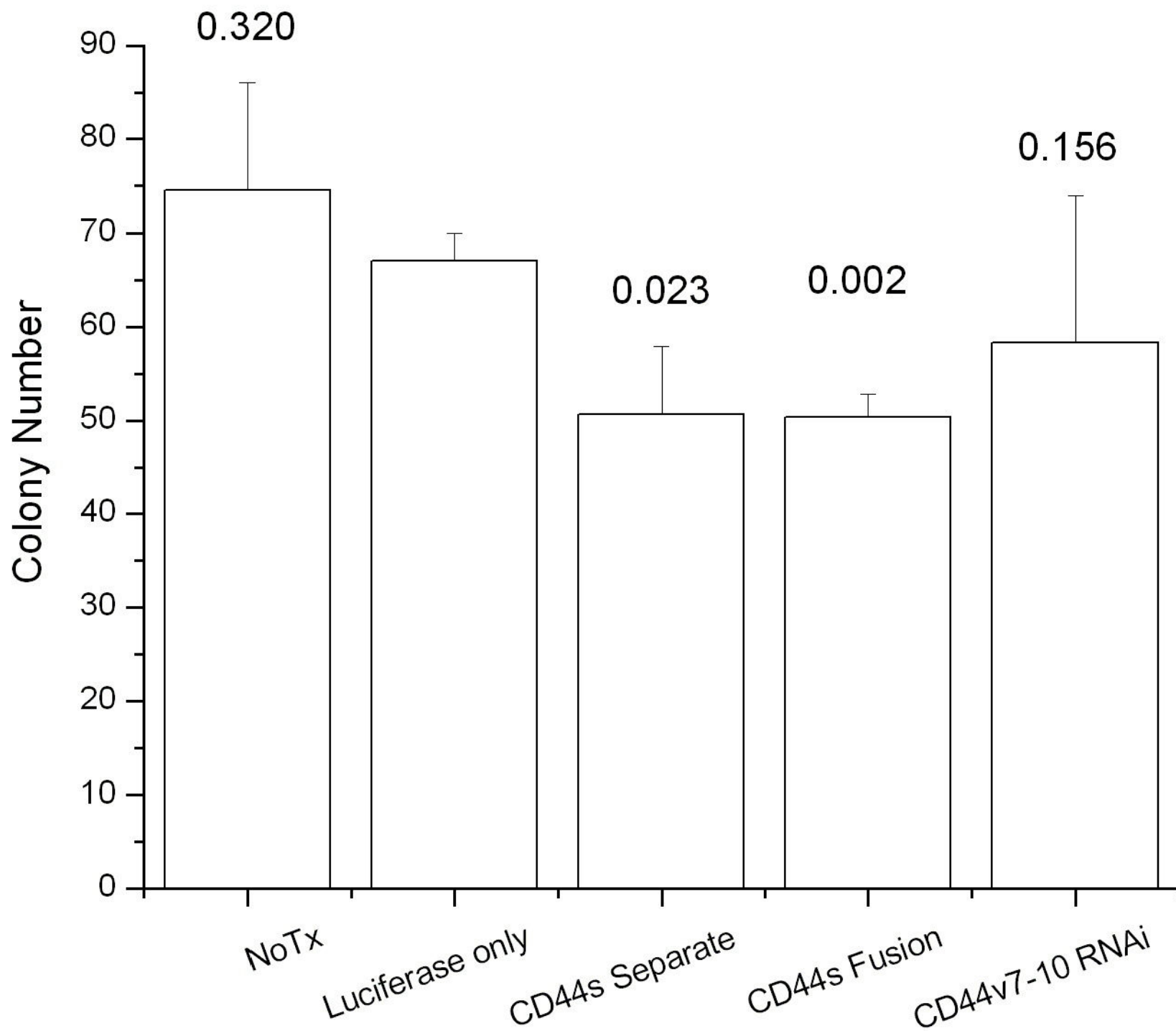


Figure 5

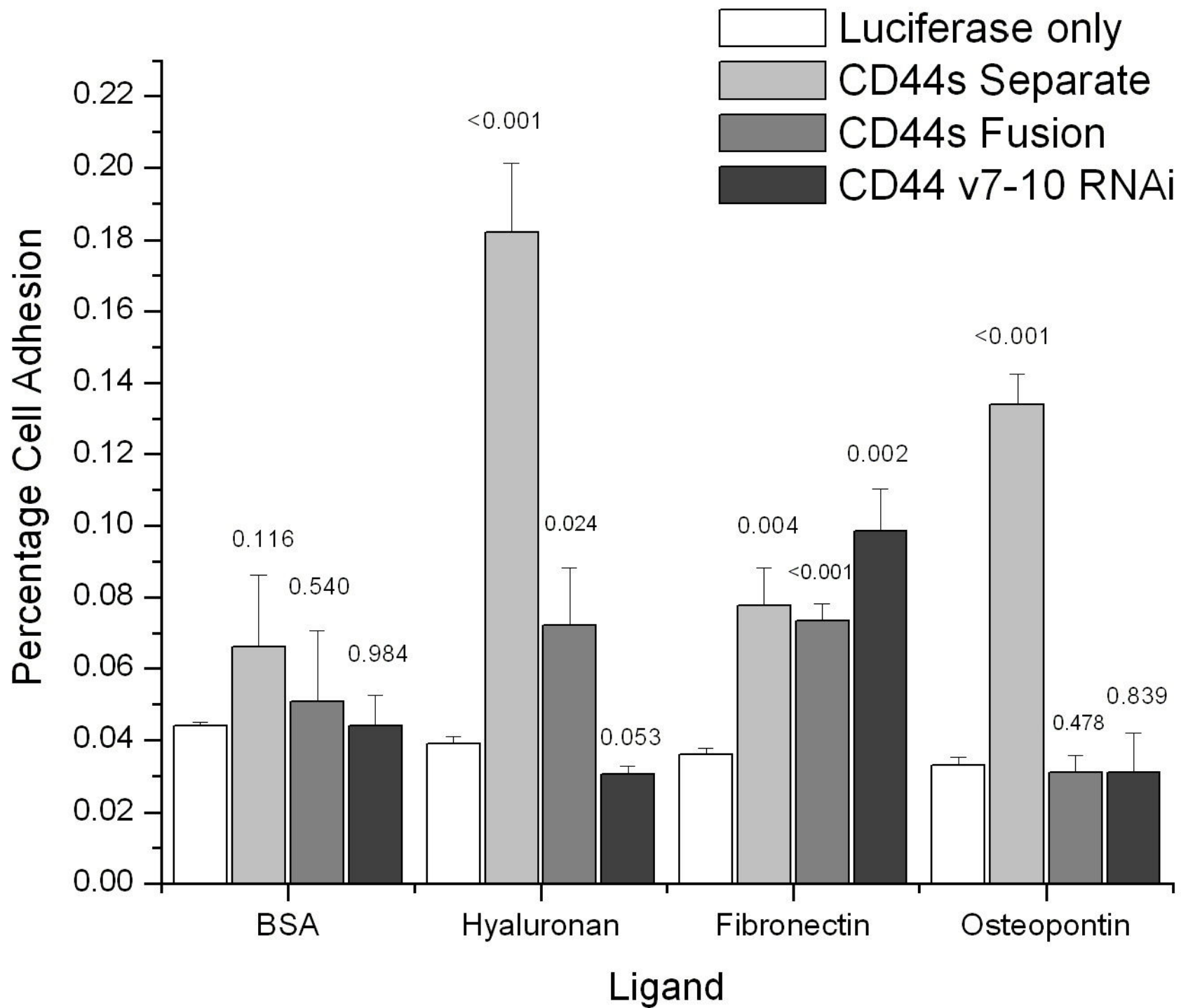


Figure 6

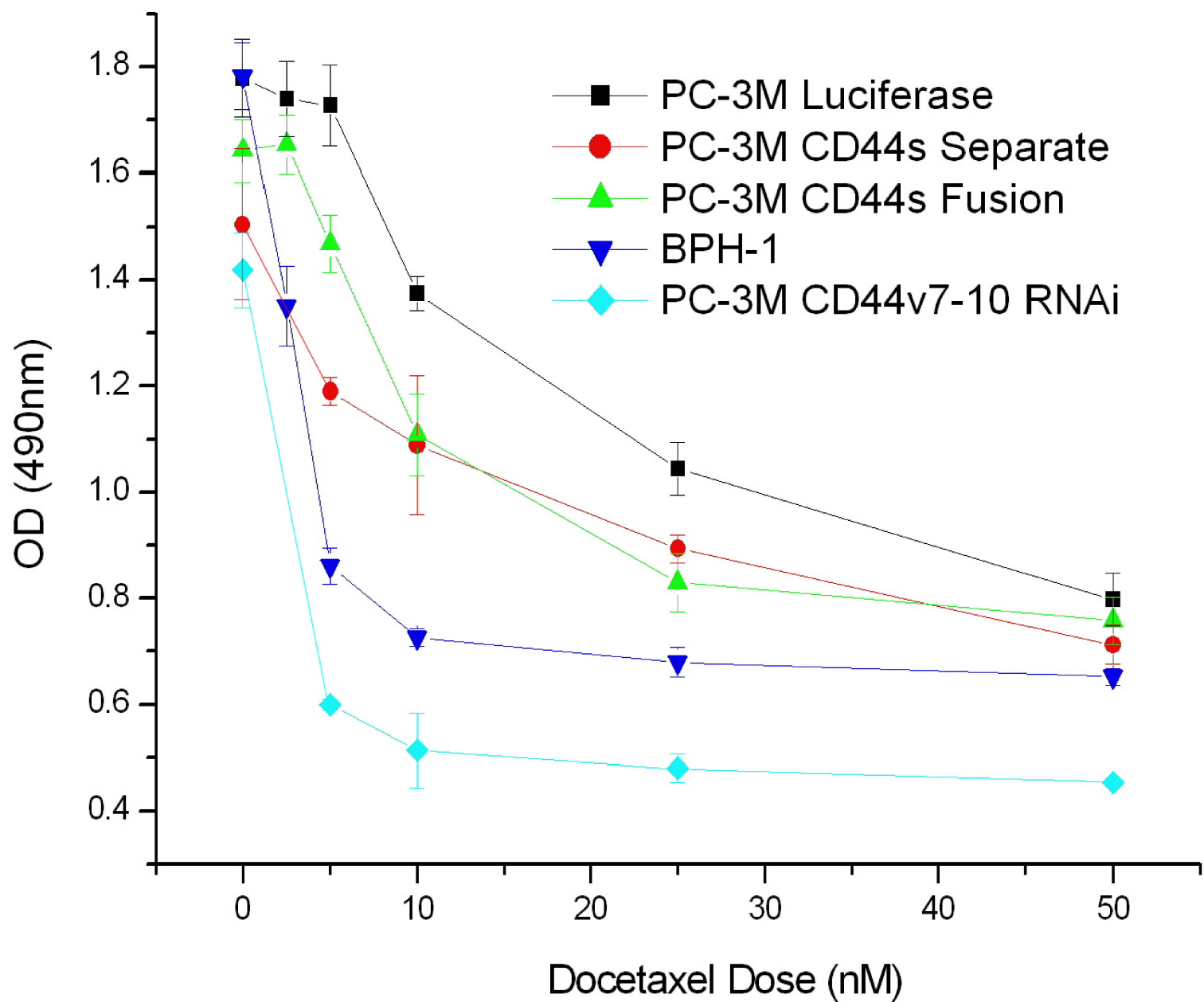


Figure 7

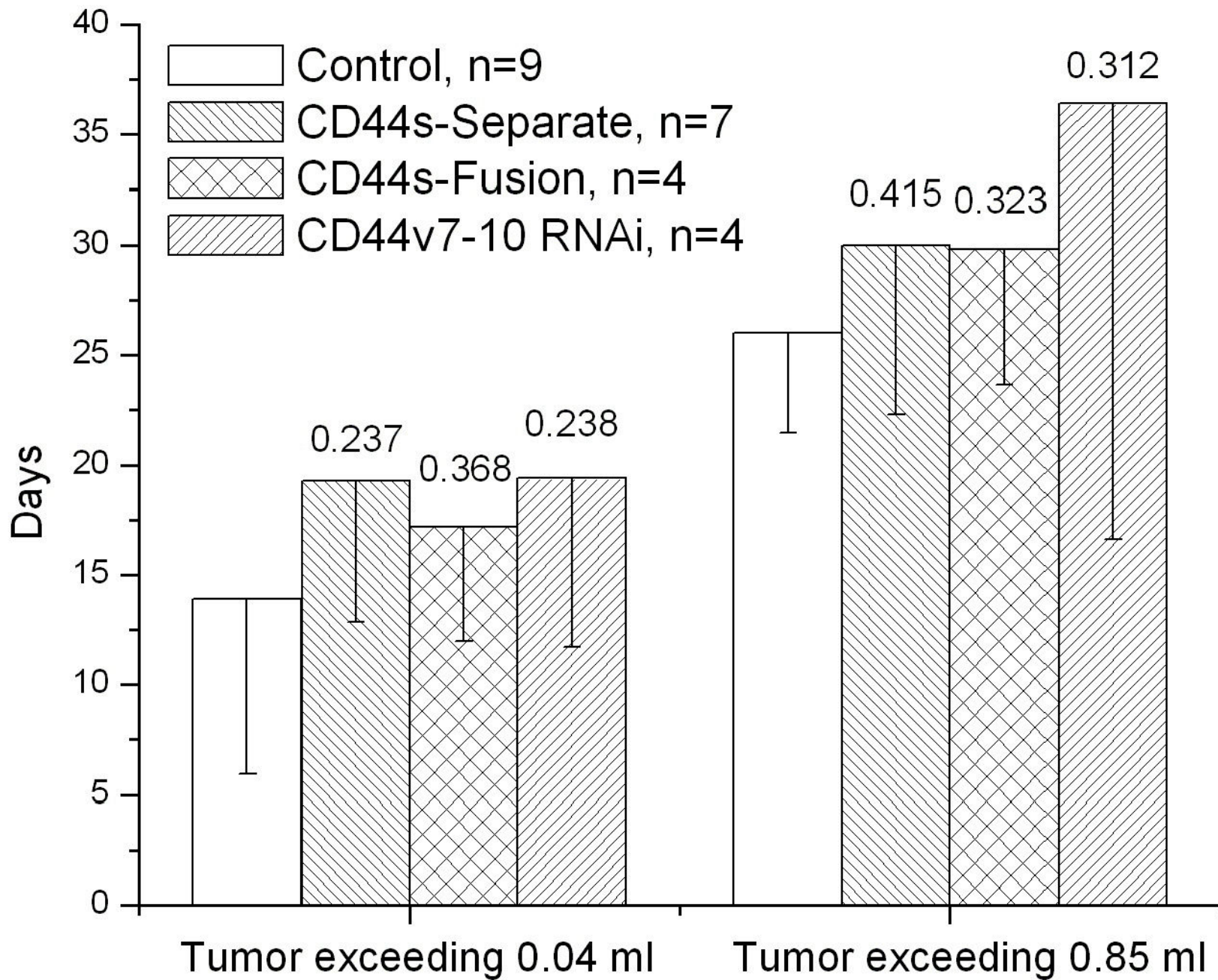


Figure 8

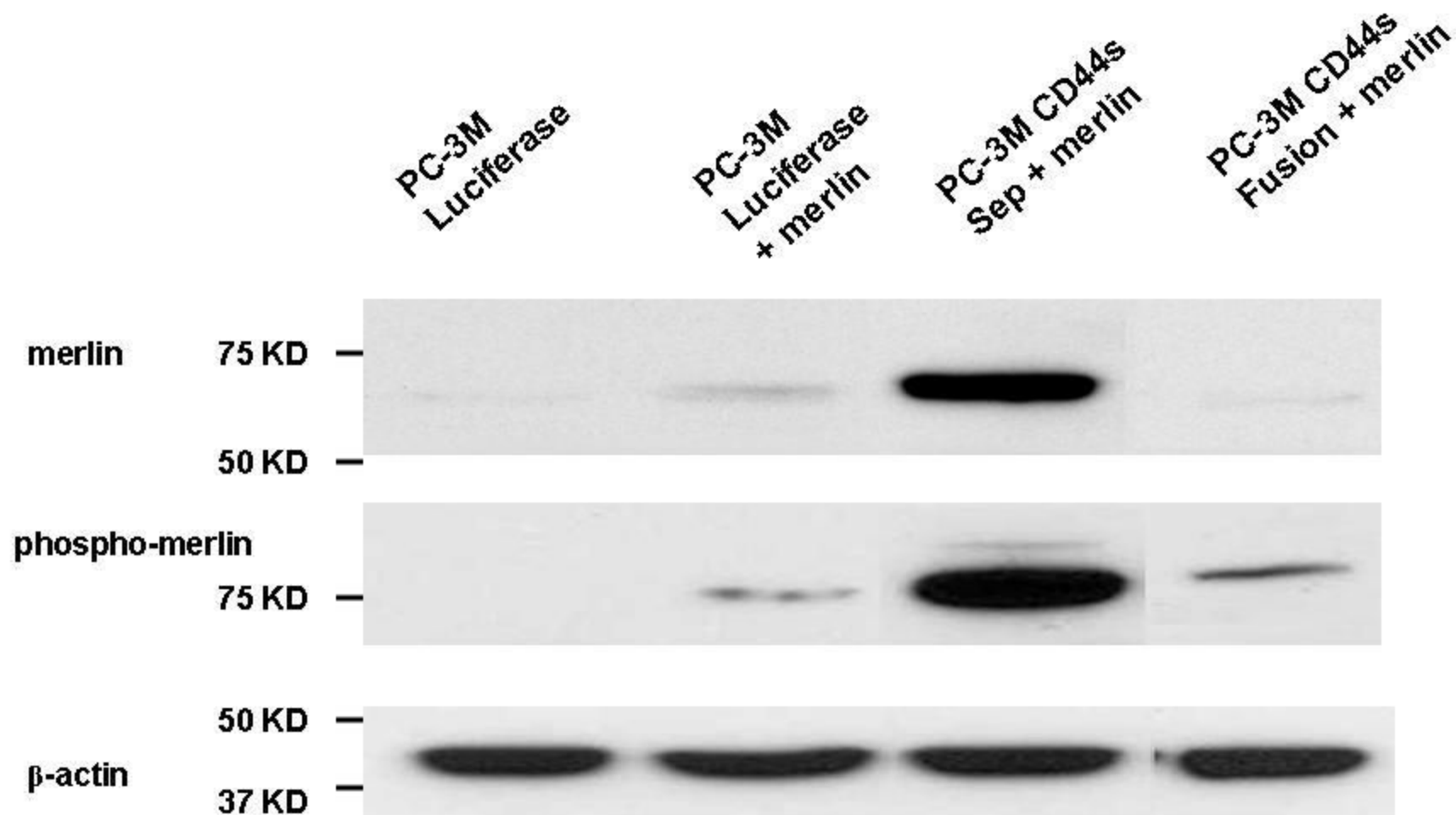


Figure 9

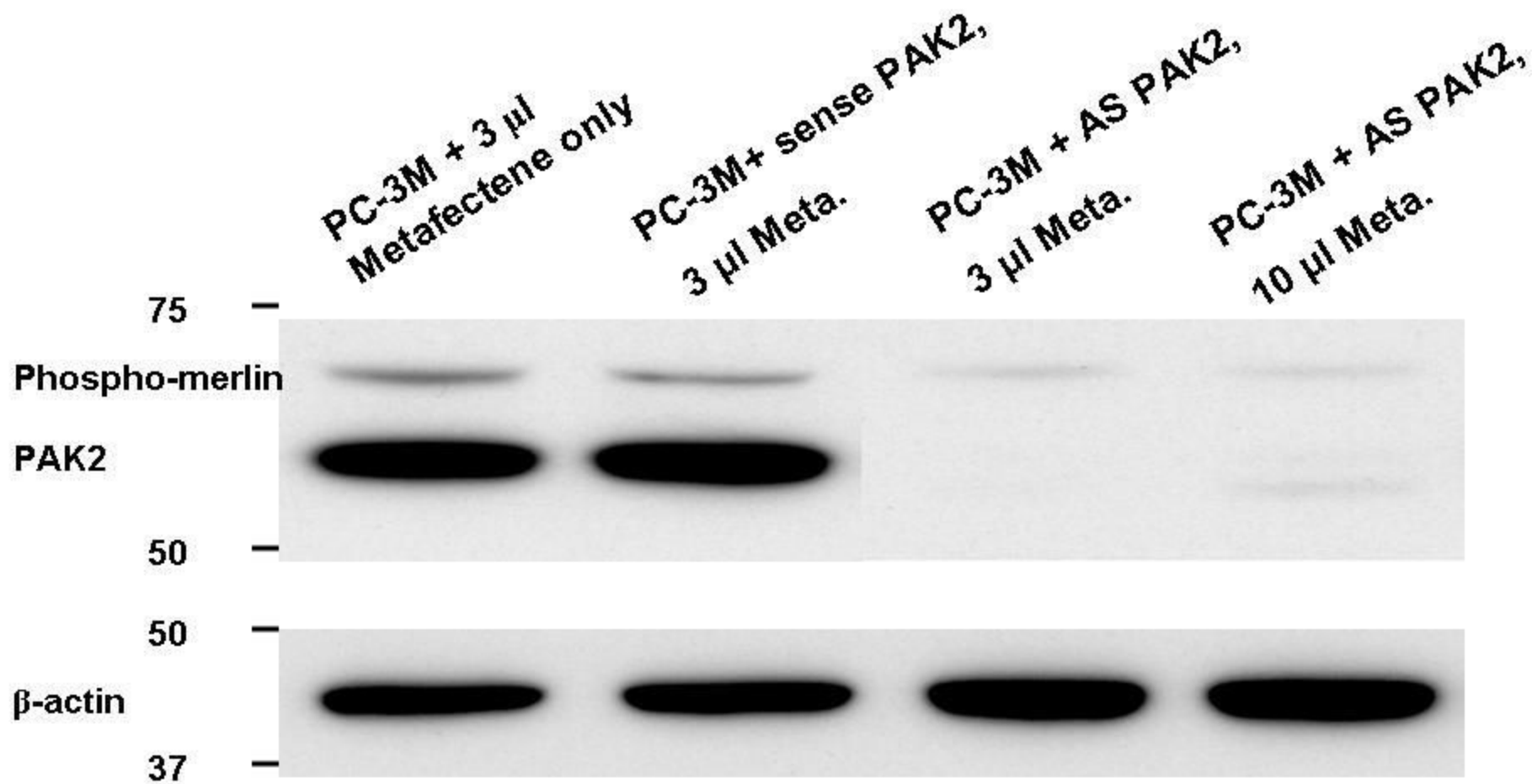


Figure 10

**PHENYL-METHYLENE HYDANTOINS ALTER CD44-SPECIFIC LIGAND BINDING OF
BENIGN AND MALIGNANT PROSTATE CELLS AND SUPPRESS CD44 ISOFORM
EXPRESSION**

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Abstract

Dysregulated CD44 expression is a feature of most human cancers, including prostate cancer (PCa). PCa loses expression of CD44 standard (CD44s) which is present in benign epithelium, and overexpresses a novel splice variant isoform, CD44v7-10, specifically facilitating fibronectin binding and invasion. Naturally-occurring or synthetic phenyl-methylene hydantoin (PMH) and S-ethyl PMH (S-PMH) can reportedly augment cell-cell adhesion, and reduce invasion and growth of PCa. Benign BPH-1 and malignant PC-3M prostate cells were treated with PMH or S-PMH for 36 h and cells were harvested. Cell adhesion assays were carried out. Cancer cells' expression of total CD44 and CD44v7-10 were tested by western blot analysis and real-time RT-PCR. Compared to BPH-1 or PC-3M cells treated with vehicle only, PMH-or S-PMH-treated benign and malignant cells had decreased adhesion to hyaluronan ($p=0.001$ to 0.007) and fibronectin ($p<0.001$ to 0.047). Both compounds decreased PCa expression of CD44 total mRNA (representing mostly CD44s, to 0.076 ± 0.033 and 0.254 ± 0.123 of control) and CD44v7-10 (to 0.386 ± 0.279 and 0.115 ± 0.037 of control). S-PMH but not PMH decreased CD44 total protein, while both decreased CD44v7-10 protein. Both hydantoins lowered β -catenin, as reported previously. Both only slightly decreased $\beta 1$ -integrin, the definitive receptor for fibronectin. In conclusion, the ability of PMH and S-PMH to decrease hyaluronan adhesion appears to be mediated through decreased CD44s, while the decrease in fibronectin adhesion correlates with, and may be mediated by, decreased CD44v7-10.

Key words: Alternate splicing; CD44; hydantoin; phenyl-methylene hydantoin; prostate cancer; hyaluronan; fibronectin

Introduction

About 30% of cases of prostate cancer (PCa) undergo transition from quiescent to aggressive. In this transition, altered splicing of the hyaluronan receptor CD44 occurs that allows tumor cells to detach, interact with proteins that digest stromal matrix, migrate through matrix, and intravasate into lymphovascular channels. CD44 is a transmembrane molecule encoded by an alternately spliced gene. The standard (**CD44s**) isoform is ubiquitous, but inclusion of one or more of 10 variant (**v**) exons lengthens the extracellular stem, producing tissue-specific (**CD44v**) isoforms. CD44 is involved in multiple cellular functions. Its N-terminus enables cell-cell adhesion and binds hyaluronan and other matrix ligands, while the C-terminus links the cell's membrane to actin and ankyrin in the cytoskeleton, modulating shape and motility.

In prior work, we isolated RNA from clinical PCa tissues and discovered that expression of CD44v7-10 variant isoform constitutes a unique PCa signature, consistently expressed in primary and metastatic PCa [1], and most strongly expressed in androgen-independent PCa cell lines [2-3]. RNA interference against CD44v7-10 caused a 69% reduction in invasion index compared to untreated control cells [3] and altered ligand-binding affinities [4]. Moreover, benign prostate strongly expresses CD44s and CD44v6, while PCa loses ability to splice pre-mRNA into CD44s [3, 5, 6], or certain variants other than CD44v7-10 [1,7]. CD44v7-10 was important for the increased fibronectin binding of cancer cells [4].

Recently, Mudit et al [8] characterized guanidine alkaloid compounds derived from Red Sea sponge *Hemimycale arabica*, that have antitumor, antiviral, antifungal, and anticonvulsant properties. Potent anti-growth and anti-invasive properties were noted against PC-3M prostate cells. Shah et al. [9] reported that phenyl-methylene hydantoin (PMH) and its S-ethyl derivative, S-ethyl PMH (S-PMH)—equally potent whether extracted from sponges or synthesized—could augment cell-cell adhesion, and reduce Matrigel invasion, including invasion stimulated by calcitonin which functions as a pro-invasive paracrine hormone. PMH compounds could also reduce spheroid disaggregation in an *in vitro* assay that measures metastatic potential of tumor cells, and significantly inhibit growth and metastasis of PCa in orthotopic

tumor xenografts [9]. Since CD44 is a key player in PCa invasion, we tested PMH compounds' effects on adhesion to known ligands of CD44 and on total and variant CD44 expression.

Materials and Methods

Cell lines

Benign BPH-1 prostate cells were from American Type Culture Collection (Manassas, VA). PC-3M cells, a metastasis-derived variant of PC-3, were from Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX. The culture medium for PC-3M cells was RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) and antibiotics. Cells overexpressing CD44s as a protein "Separate" from luciferase, were made by infecting PC-3M with virus packaged in 293 cells after inserting CD44s-RSV promoter-luciferase sequence behind the cytomegalovirus promoter of Lentivector pLEX-MCS (Open Biosystems, Huntsville, AL), and selecting through its puromycin resistance gene.

For cell set-up, cells in a flask were trypsinized, medium with serum was added to neutralize trypsin, and cells were stained in Trypan blue and counted by grid method [4]. Cells were treated with 50 $\mu\text{mol/L}$ of synthetic PMH or S-PMH (generous gifts of Dr. Girish V. Shah, Univ. of Louisiana-Monroe) in DMSO, since this dose was most effective for enhancement of tight junction and adherens junction function, and for attenuation of calcitonin-stimulated invasion [9].

Cellular adhesion assays

At least 2 repeat assays were carried out [10] using trypsinized confluent untreated or virally treated cells. Each test condition was set in 5 wells and each experiment repeated with similar results. 96-well black-edged clear flat bottom Costar plates (Cole-Parmer, Vernon Hills, IL) were coated with optimal concentrations of ligands [10] using 8 wells to test each one, at 37°C overnight. As controls, 8 wells were coated with 1 mg/ml BSA to measure baseline nonspecific binding. 1×10^6 cells suspended in 1 ml PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo) for 15 min at 37°C. After two washes of the cells with PBS, cells with serum-free basal medium were added to plates at a density of 3×10^4 per well

and incubated at 37°C for 90 min. Fluorescence intensities at 530 nm were measured using a BioTek FL-600 plate reader. Nonadherent cells were removed with 2 PBS washes. Fluorescence intensities with PBS in the wells were measured. Adhesion was calculated [10] as % cells bound=(100) fluorescence intensity post-wash / fluorescence intensity of total cells plated.

Real Time Quantitative RT-PCR

To confirm altered CD44 expression, we used a primer + probe set that detects CD44s, or a set that detects CD44v7-10 (Applied Biosystems, Foster City, CA) as we described [3,11]. Detection of 18S ribosomal RNA was done simultaneously as a normalizer. TaqMan data were analyzed by the $2^{(-\Delta\Delta C_T)}$ method [12] to determine fold change in gene expression (untreated cells=1.00). The ΔC_T was taken as the difference between the CD44v7-10 or CD44 total and the 18S ribosomal RNA C_{Ts} . The $\Delta\Delta C_T$ was obtained using the mean ΔC_T of untreated cells as calibrator. Mean \pm standard deviation of normalized amount of RNA was calculated based on triplicates.

Western blot analysis

Cultured cells were directly lysed in dishes using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 μ g sample/lane according to the Laemmli method using the NuPAGE system (Invitrogen, Carlsbad, CA). 10 μ l of Kaleidoscope protein marker (Bio-Rad, Hercules, CA) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Mouse monoclonal antibodies were all used at 1 μ g/mL including CD44v7/8 antibody (Bender MedSystems, Burlingame, CA) to assess CD44v7-10; CD44 total (standard + variant, Bender, 156-3C11), CD44s (LabVision, Fremont, CA), rabbit phospho-merlin (Cell Signaling), β 1 integrin (R&D, Minneapolis, MN), or β -catenin. Anti- β -actin antibody (Sigma, St. Louis) was used at a dilution of 1:5,000. Membranes were washed 3 x 10 min in TBS with 0.1% Tween-20 (TBST) and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) or 1:1,250

goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) was added in 5% skim milk for 1 h. After washing membrane with 1x TBST, reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Statistical Analyses

For all *in vitro* assays, data were expressed as mean \pm SD. The significance of differences among group means was tested by two-tailed paired Student *t*-test. Statistical significance was set at $p < 0.05$.

Results

Cell adhesion assay

PMH or S-PMH treatments reduced the adhesion of BPH-1 and PC-3M cells to both hyaluronan ($p = 0.001$ to 0.007) and fibronectin ($p < 0.001$ to 0.047) by one-third to one-half (**Figure 1**) compared to DMSO-only controls.

PMH and S-PMH effect on total CD44, phospho-merlin, and β -catenin

Western blot analyses in PC-3M cells showed that S-PMH, but not PMH, decreased total CD44 (**Figure 2**). Also, the phosphorylated, pro-growth form of merlin, a downstream mediator of the effects of CD44 signaling on proliferation, was decreased. Using an antibody to β -catenin, we duplicated the observation of Shah et al. [9] that both hydantoins decreased β -catenin. Real Time RT-PCR was used to demonstrate relative amounts of CD44 total mRNA (representing mostly CD44s). mRNA was decreased by PMH to 0.076 ± 0.033 , and by S-PMH to 0.254 ± 0.123 of control.

PMH and S-PMH effect on CD44v7-10 and β 1-integrin

The definitive ligand for fibronectin is β 1-integrin, particularly as part of integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$. To determine whether the altered fibronectin adhesion in the presence of the hydantoins was the result of

abatement of high CD44v7-10 expression versus β 1-integrin (CD29), western blot analyses were performed for both (**Figure 3**). PMH and S-PMH virtually abolished CD44v7/8-reactive protein, representing CD44v7-10, with only minimal effect on β 1-integrin. The re-expression of CD44s somewhat suppressed CD44v7-10, consistent with what we showed previously [4]. By Real Time RT-PCR, CD44v7-10 mRNA after PMH was 0.386 ± 0.279 of control and after S-PMH was 0.115 ± 0.037 of control. Together, these results implicate suppression of splice isoform CD44v7-10 in the reduced fibronectin binding.

Discussion

We have shown that in aggressive PC-3M PCa cells, phenyl-methylene hydantoin (PMH) and S-ethyl PMH (S-PMH) significantly alter CD44 expression and cell adhesion to ligands that are known binding partners of CD44 isoforms. Overall, both seemed equally potent, although in prior work, S-PMH was 3 times more potent than PMH in inhibiting PC-3M cell spheroid dispersal, and more effective at extending mouse survival and preventing metastases [9].

CD44s, according to Miyake et al. [13], is the sole ligand in PCa that binds hyaluronan. The introduction of CD44s in PC-3 cells markedly enhanced the binding and migration of these cells to hyaluronan, but not to other extracellular matrix molecules. Thus, a reduced binding to hyaluronan correlates with the treatment effect of less total CD44 RNA and protein. CD44s is considered a metastasis suppressor in PCa. However, we noted that lymph node metastases of PCa seemed to re-express CD44s [1], and CD44 positivity is associated with basal cell/ stem phenotypes and high proliferation in prostate [14] and breast [15] cancers. Thus a reduction of total CD44, consisting mostly of CD44s, could partly explain the antimetastatic potential [9] of PMH and S-PMH.

Fibronectin facilitates cell attachment via α 4 β 1, α V β 3, and α 5 β 1 integrins, with the latter being decreased in some cancers, but is also a ligand for CD44v. We showed previously that the predominant CD44v7-10 in PCa cells specifically binds to fibronectin [4]. The ability of the tested hydantoins to reduce fibronectin binding appears primarily attributable to altered CD44v7-10 expression (detected by

anti-CD44v7/8), although β 1-integrin expression minimally changed in their presence. Interestingly, PMH caused increased cell-cell adhesion of PC-3M cells, measured by the transepithelial resistance method [9]—standing in contrast to the decreased cell-extracellular matrix adhesion to these two important ligands reported herein.

Like Shah et al. [9], we performed western blot analysis for E-cadherin, and obtained no signal. E-cadherin was detectable by them only in the insoluble fraction (cell pellet), unless cells were stimulated by calcitonin, thus our use of the soluble proteins for our studies gave the expected result. As in Shah et al., β -catenin was decreased by treatment with PMH, and although they did not study S-PMH, we found S-PMH to exert the same effect.

Given that metastasis suppression by CD44s is not entirely dependent on hyaluronan [16], the intracellular binding partners of CD44 may be implicated. We examined merlin, an intracellular protein that mediates the action of CD44 in a phosphorylation-dependent manner. Merlin is activated by phosphorylation under growth-promoting conditions and dephosphorylated under growth-inhibiting conditions including CD44-hyaluronan binding which inactivates it and circularizes it [17,18]. Using antibody to phospho-merlin, we detected PMH- and S-PMH-induced decreases in both a hyperphosphorylated and a lower molecular weight, hypophosphorylated form of merlin. Since P-merlin is considered growth-promoting, this result suggests that merlin dephosphorylation plays a role in the growth-inhibitory effects of these hydantoin, perhaps orchestrated by less CD44s and less hyaluronan binding. In benign BPH-1 cells, the same trend was evident, consistent with Horiguchi et al. [19] who found that silencing of CD44 dephosphorylates merlin in benign cells.

The hydantoin compounds PMH and S-PMH are known to have antitumor, antiviral, antifungal, and anticonvulsant effects, reviewed by Mudit et al. [8], can be cost-effectively synthesized, and show minimal side effects in mice [9]. More recent investigations have found hydantoin derivatives also effective in inhibiting angiogenesis *in vitro* from cultured endothelial cells and inhibiting secretion of VEGF by osteosarcoma cells [20]. Here we show that both PMH and S-PMH reduce CD44 total and CD44 v7-10 expression in PC-3M cells, of which the latter isoform facilitates invasion [3]. These two

changes, respectively, correlate with reduced adhesion to hyaluronan and fibronectin, and are likely to relate to the compounds' ability to inhibit PC-3M cell invasiveness [9].

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REFERENCES

- [1] Iczkowski KA, Pantazis CG Collins, J. The loss of expression of CD44 standard and variant isoforms is related to prostatic carcinoma development and tumor progression. *J Urol Pathol* 1997;6:119-129.
- [2] Iczkowski KA, Bai S Pantazis, CG. Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. *Anticancer Res* 2003;23: 3129-3140.
- [3] Omara-Opyene AL, Qiu J, Shah GV, Iczkowski KA. Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18. *Lab Invest* 2004;84:894-907.
- [4] Iczkowski KA, Omara-Opyene AL Shah GV. The predominant CD44 splice variant in prostate cancer binds fibronectin, and calcitonin stimulates its expression. *Anticancer Res* 2006;26:2863-2872.
- [5] Vis AN, van Rhijn BW, Noordzij MA, Schröder FH, van der Kwast TH. Value of tissue markers p27(kip1), MIB-1, and CD44s for the pre-operative prediction of tumour features in screen-detected prostate cancer. *J Pathol* 2002;197:148-154.
- [6] Vis AN, Noordzij MA, Fitoz K, Wildhagen MF, Schröder FH, van der Kwast TH. Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer. *J Urol* 2000;164:2156-2161.
- [7] Harrison GM, Davies G, Martin TA, Mason MD, Jiang WG. The influence of CD44v3-v10 on adhesion, invasion and MMP-14 expression in prostate cancer cells. *Oncol Rep* 2006;15:199-206.
- [8] Mudit M, Khanfar M, Muralidharan A, Thomas S, Shah GV, van Soest RW, El Sayed KA. Discovery, design, and synthesis of anti-metastatic lead phenylmethylene hydantoins inspired by marine natural products. *Bioorg Med Chem* 2009;17:1731-1738.
- [9] Shah GV, Muralidharan A, Thomas S, Gokulgandhi M, Mudit M, Khanfar M, El Sayed K. Identification of a small molecule class to enhance cell-cell adhesion and attenuate prostate tumor growth and metastasis. *Mol Cancer Ther* 2009;8:509-520.
- [10] Harada N, Mizoi T, Kinouchi M, Hoshi K, Ishii S, Shiiba K, Sasaki I, Matsuno S. Introduction of antisense CD44s cDNA downregulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells. *Int J Cancer* 2001;91:67-75.

- [11] Iczkowski KA, Omara-Opyene AL, Kulkarni TR, Pansara M, Shah GV. Paracrine calcitonin in prostate cancer is linked to CD44 variant expression and invasion. *Anticancer Res* 2005;25:2075-2083.
- [12] Livak KL, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. *Methods* 2001;25:402-408.
- [13] Miyake H, Hara I, Okamoto I, Gohji K, Yamanaka K, Arakawa S, Saya H, Kamidono S. Interaction between CD44 and hyaluronic acid regulates human prostate cancer development. *J Urol* 1998;160:1562-1566.
- [14] Palapattu GS, Wu C, Silvers CR, Martin HB, Williams K, Salamone L, Bushnell T, Huang LS, Yang Q, Huang J. Selective expression of CD44, a putative prostate cancer stem cell marker, in neuroendocrine tumor cells of human prostate cancer. *Prostate*. 2009;69:787-798.
- [15] Buess M, Rajski M, Vogel-Durrer BML, Herrmann R, Rochlitz C. Tumor-endothelial interaction links the CD44+/CD24- phenotype with poor prognosis in early-stage breast cancer. *Neoplasia* 2009;11:987-1002.
- [16] Gao AC, Lou W, Sleeman JP, Isaacs JT. Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate. *Cancer Res* 1998;58:2350-2352.
- [17] Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. *Genes Dev* 2001;15:968-980.
- [18] Bai Y, Liu Y, Wang H, Xu Y, Stamenkovic I, Yu Q. Inhibition of the hyaluronan-CD44 interaction by merlin contributes to the tumor-suppressive activity of merlin. *Oncogene* 2007;26:836-850.
- [19] Horiguchi A, Zheng R, Shen R, Nanus DM. Inactivation of the NF2 tumor suppressor protein merlin in DU145 prostate cancer cells. *Prostate* 2008;68:975-994.
- [20] Basappa, Ananda Kumar CS, Nanjunda Swamy S, Sugahara K, Rangappa KS. Anti-tumor and anti-angiogenic activity of novel hydantoin derivatives: Inhibition of VEGF secretion in liver metastatic osteosarcoma cells. *Bioorg Med Chem* 2009;17:4928-4934.

Figure Legends

Figure 1. Cell adhesion assays. p values indicated above bars. In both benign (BPH-1) and cancer (PC-3M) cells, 50 μ M PMH or S-PMH significantly reduced binding to hyaluronan and fibronectin compared to DMSO-only treated control cells. Baseline binding was assessed by coating plates with bovine serum albumin (BSA).

Figure 2. Detection of total CD44, CD44's intracellular effector phospho-merlin, and β -catenin, a subunit of the cadherin protein complex, as influenced by PMH or S-PMH in PC-3M prostate cancer cells. In lane 5, the CD44s-Sep cells were designed by us to overexpress CD44s and luciferase as separate proteins (Yang et al., unpublished observations), providing a positive control for high total CD44.

Figure 3. CD44v7/8 and β 1-integrin alterations due to PMH or S-PMH in PC-3M prostate cancer cells. Detection at 85 kD of a cleaved form of CD44v7/8, a proxy for CD44v7-10, although decreased by overexpression of CD44s (lane 2), is almost abolished by PMH and S-PMH (lanes 3-4). β 1-integrin is only slightly decreased by the compounds.

Percentage Cell Adhesion

