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PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Bone metastases are a debilitating and devastating complication for patients with advanced prostate cancer. Unfortunately, treatment options for patients with bone metastases are limited. Both hyaluronan synthase (HAS) and hyaluronic acid (HA) are upregulated in metastatic prostate cancer cells. 7-Hydroxy-4-Methyl Coumarin (HMC) is an inhibitor of hyaluronan synthase. It is commonly available in herbal supplements and, up to now, has been utilized mainly for digestion complaints. We propose that it may be efficacious in the prevention and treatment of prostate cancer. Our hypothesis is that hyaluronic acid (HA) is utilized by prostate cancer cells to facilitate metastasis. Thus, reducing the production of HA should reduce the metastatic potential of prostate cancer cells making HA an ideal target for preventing and treating metastatic disease. The goal of this current research proposal is to determine whether reduction of HAS, via treatment with HMC, will prevent prostate cancer metastasis to bone and other organs or serve as a viable treatment for established prostate cancer bone metastasis. To date, we have in vivo evidence that HA protein levels in vitro correlate with metastatic potential and that HA levels can be modulated in vitro using HMC. Furthermore, we have shown the in vitro growth of prostate cancer cells is slowed by inhibition of HA with HMC. In addition, we now have demonstrated that HMC can slow in vivo prostate cancer growth. We are currently beginning to assess the efficacy of HMC on in vivo metastasis, particularly to bone.					
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## **INTRODUCTION**

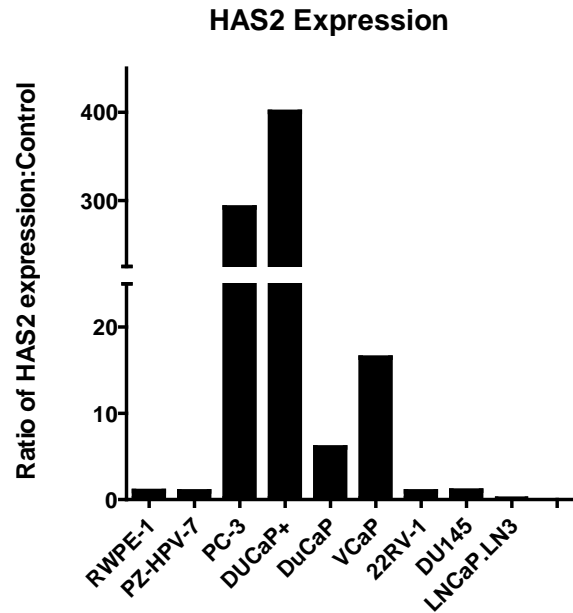
Bone metastases are a debilitating and devastating complication for patients with advanced prostate cancer. Unfortunately, treatment options for patients with bone metastases are limited. Both hyaluronan synthase (HAS) and hyaluronic acid (HA) are upregulated in metastatic prostate cancer cells. 7-Hydroxy-4-Methyl Coumarin (HMC) is an inhibitor of hyaluronan synthase. It is commonly available in herbal supplements and, up to now, has been utilized mainly for digestion complaints. We propose that it may be efficacious in the prevention and treatment of prostate cancer. Our hypothesis is that hyaluronic acid (HA) is utilized by prostate cancer cells to facilitate metastasis. Thus, reducing the production of HA should reduce the metastatic potential of prostate cancer cells making HA an ideal target for preventing and treating metastatic disease. The goal of this current research proposal is to determine whether reduction of HAS, via treatment with HMC, will prevent prostate cancer metastasis to bone and other organs or serve as a viable treatment for established prostate cancer bone metastasis. To date, we have *in vivo* evidence that HA protein levels *in vitro* correlate with metastatic potential and that HA levels can be modulated *in vitro* using HMC. Furthermore, we have shown the *in vitro* growth of prostate cancer cells is slowed by inhibition of HA with HMC. We also now have evidence that HMC has some effects on *in vivo* tumor growth and have some preliminary evidence that HMC will be useful for the prevention and treatment of prostate cancer bone metastases. However, the drug in its current formulation, while not as hard on the animals as the acidic form of HMC, does not appear to be well tolerated by the animals.

## **BODY**

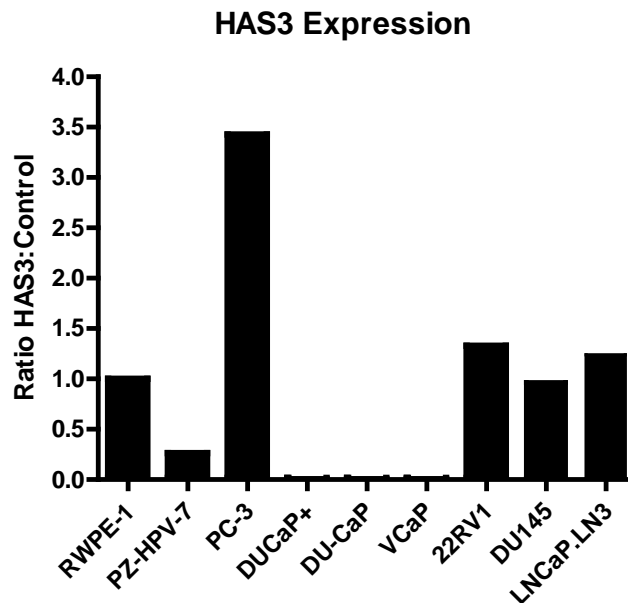
**TASK 1: Determine whether hyaluronan synthase (HAS) expression and hyaluronic acid (HA) production in prostate cancer cells correlates with increased growth both *in vitro* and *in vivo* and whether modulation of HAS expression by 7-Hydroxy-4-Methyl Coumarin (HMC) will inhibit tumor growth in the primary (subcutaneous) site. (Months 1-12)**

## **RESEARCH ACCOMPLISHMENTS:**

- a. Levels of HAS2 and HAS3 expression in established prostate cancer cell lines, PC-3, LN.CAP-LN3, VCaP, DuCaP, DU-145 and 22RV1 has been determined by quantitative expression analysis and compared to expression levels in non-tumor prostate epithelial cell lines, PZ-HPV-7 and RWPE-1. HAS2 and HAS3 levels were determined in each of the cells lines listed above by quantitative expression analysis. Results are shown in Figures 1 and 2 below. HAS1 expression was undetectable in the cancer cell lines. We have now repeated these experiments with LN.CaPLN3 cells included.



**Figure 1. Quantitative Analysis of HAS2 Expression levels by Real-Time PCR.** HAS2 expression is reported relative to expression in the RWPE-1 prostate epithelial cell line. Note that all of the prostate cancer cell lines except for LNCaP-LN3 express HAS2 at higher levels than prostate epithelial cell lines, RWPE-1 and PZ-HPV-7. DuCaP+ indicates DuCaP cells harvested with a feeder layer. DuCaP indicates only the isolated DuCaP cells.

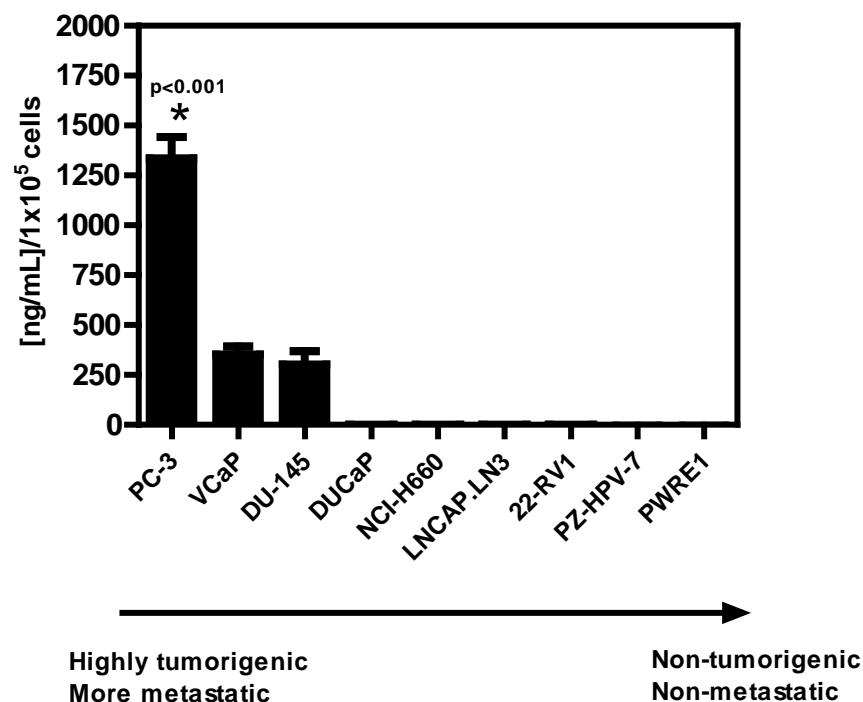


**Figure 2. Quantitative Analysis of HAS3 Expression levels by Real-Time PCR.** HAS3 expression is reported relative to expression in the RWPE-1 prostate epithelial cell line. Note that not all of the prostate cancer cell lines express HAS3. DuCaP+ indicates DuCaP cells harvested with a feeder layer. DuCaP indicates only the isolated DuCaP cells.

PC-3, the most aggressive of the prostate cancer cell lines, in vivo, expresses both HAS2 and HAS3 at much higher levels than the prostate epithelial cell lines, RWPE-1 and PZ-HPV-7 and the other prostate cancer cell lines. These differences are not nearly as

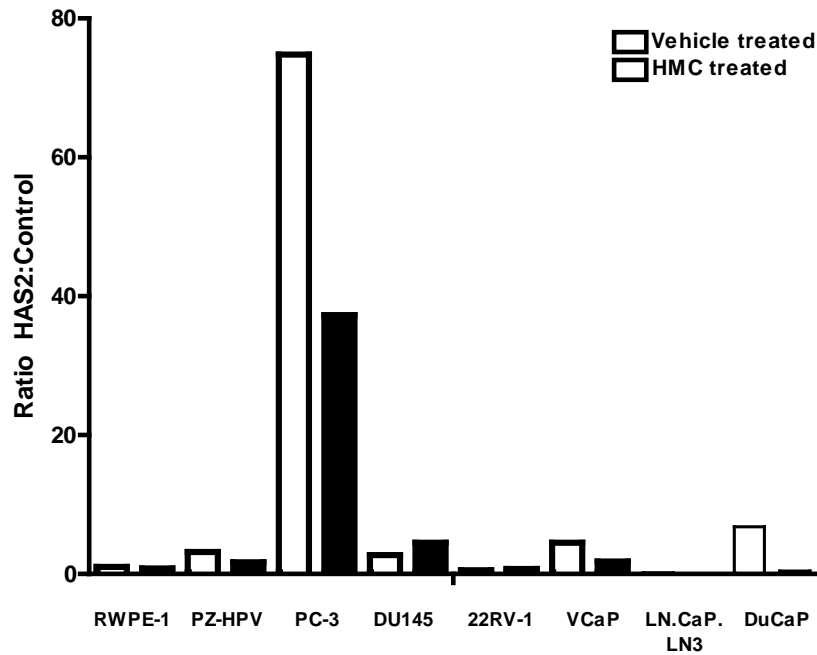
remarkable as those observed with HAS2 expression. This indicates that HAS2 likely plays a much more important role in HA production in these cell lines. Interestingly, these results do not correlate with our initial studies which indicated increased HAS3 expression in DU-145 and VCaP cell lines as well. This data have now been reproduced for verification and inclusion of LN.CaP-LN3 data, which had not previously been analyzed for expression.

- b. HA synthesis was quantitated in the same cell lines examined in sub-task 1a using a competitive binding assay specific for HA. Again, non-tumor prostate epithelial cell lines, PZ-HPV-7 and RWPE-1 were utilized as controls (Figure 3). These results correlate with *in vivo* tumorigenicity and metastatic potential which has been previously determined in our laboratory.

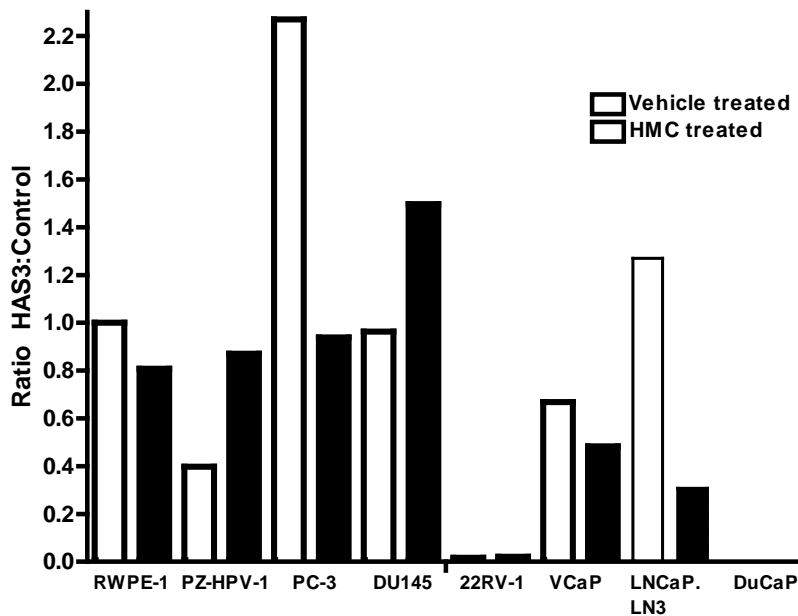


**Figure 3. HA synthesis by prostate cancer cell lines.** Note that the metastatic prostate cancer cell lines, PC-3, VCaP and DU-145 make HA. PC-3 cells make significantly more HA than other prostatic cancer cell lines and the prostate epithelial cell lines, PZ-HPV-7 and PWRE1. The other prostate cancer cell lines make very low levels or undetectable levels of HA. These results correlate with *in vivo* tumorigenicity and metastatic potential.

- c. The prostate cancer cell lines were treated *in vitro* with HMC, a known inhibitor of HAS. Conditioned media was collected after 48 hours of incubation and quantitative expression analysis of HAS2 and HAS3 (Figures 4 and 5), HA synthesis (Figure 6) and *in vitro* growth rate (Figures 7-13) were examined in both HMC- and vehicle-treated cells. Cytotoxicity assays were performed using commercial colorimetric cell proliferation assays (Promega), based on the cleavage of tetrazolium salts by mitochondrial dehydrogenases (MTS) in viable cells, but were uninformative due to interference of HA with the assay.

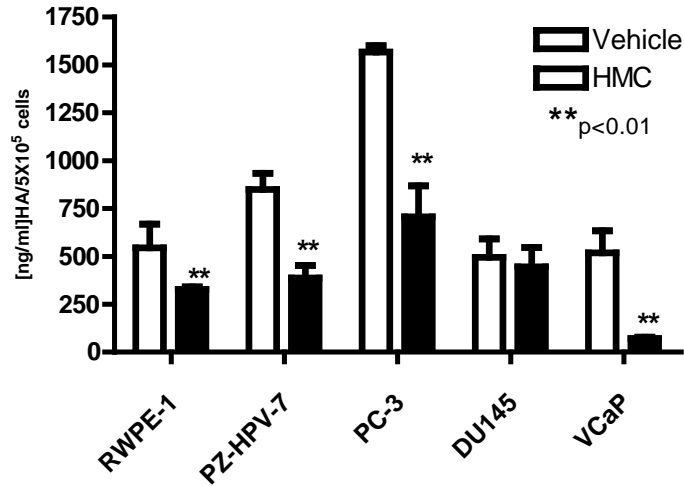


**Figure 4. Quantitative Analysis of HAS2 Expression levels by Real-Time PCR.** HAS2 expression is reported relative to expression in the vehicle treated RWPE-1 prostate epithelial cell line. HMC treatment reduced HAS2 expression levels in all of the cell lines except DU145. We have repeated this experiment to obtain data with DuCaP and LN.CaP-LN3 cells lines which were not available when this experiment was first performed and to verify the other data.



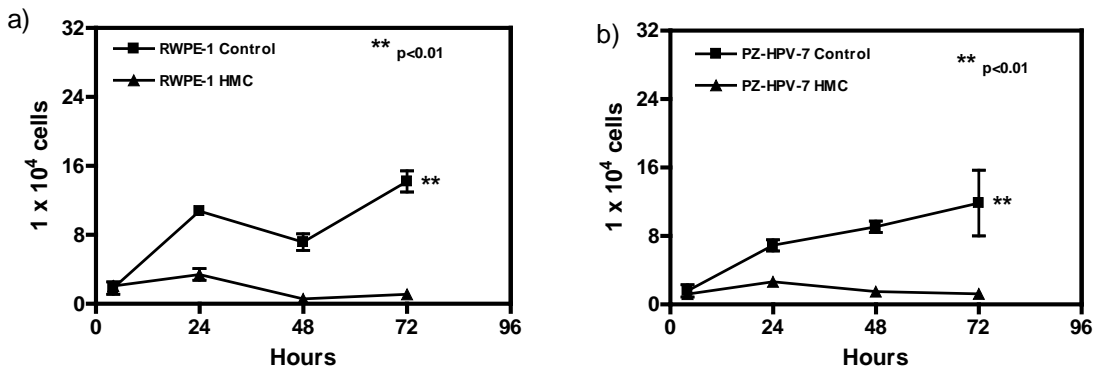
**Figure 5. Quantitative Analysis of HAS3 Expression levels by Real-Time PCR.** HAS3 expression is reported relative to expression in the vehicle-treated RWPE-1 prostate epithelial cell line. HMC treatment reduced HAS3 expression levels in all of the cell lines except DU145 and the prostate epithelial cell line PZ-HPV-7. We are currently investigating the reasoning for this. We

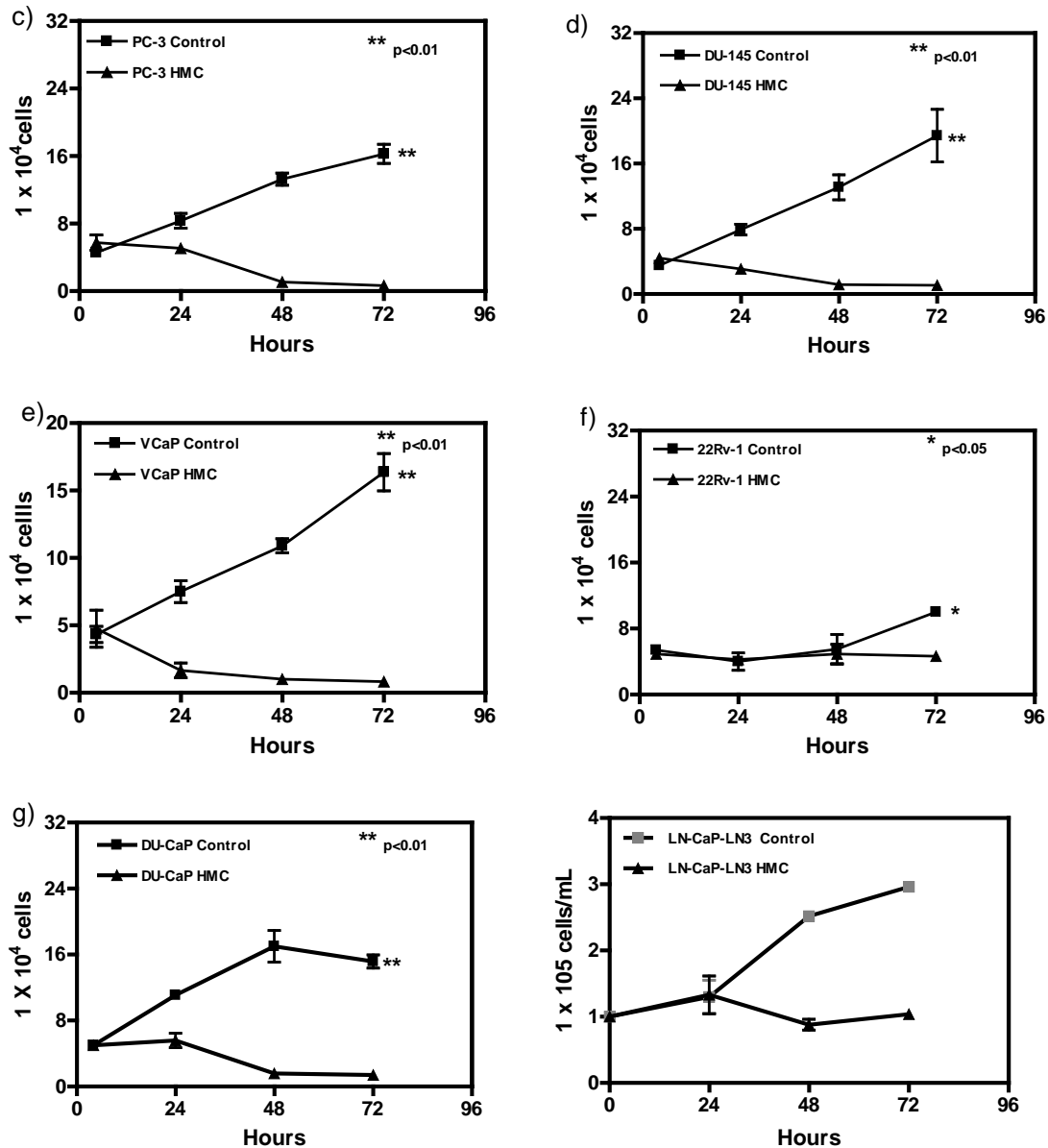
have now repeated this experiment to obtain data with DuCaP and LN.CaP-LN3 cells lines which were not available when this experiment was first performed. Note that DuCaP cells do not express HAS3.



**Figure 6. HA synthesis by prostate cancer cell lines.** Note that the metastatic prostate cancer cell lines, PC-3, VCaP and DU145 make HA and thus were included in this experiment. HA production was significantly reduced in both PC-3 and VCaP but not in DU145 cells, consistent with the results of HAS2 and HAS3 expression analysis (Figures 4 and 5). The other prostate cancer cell lines make very low levels or undetectable levels of HA by this assay and thus were not included here.

These results are very interesting particularly since it appears that HA levels and HAS expression in DU145 may not be affected by inhibition of HAS by HMC. This warrants further investigation and will be explored outside the confines of this grant.



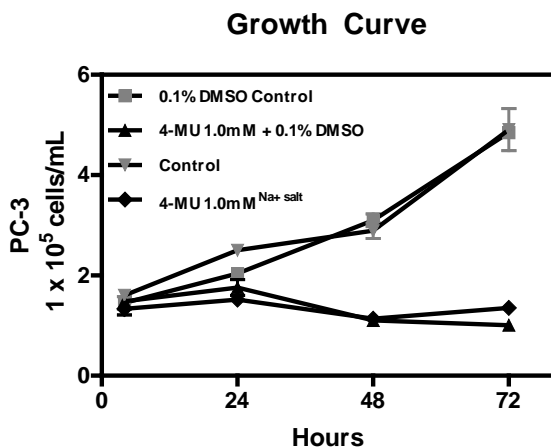


**Figure 7 (a-h). In vitro growth of vehicle-treated and HMC-treated prostate cancer cell lines.** Growth curves were generated by plating  $1 \times 10^4$  cells in media supplemented with either HMC or vehicle and counting at 4h, 24h, 48h, and 72h. The growth of all cell lines including nontransformed prostate epithelial cell lines, RWPE-1 and PZ-HPV-7, was significantly reduced by treatment with HMC.

d. To determine the safest dose of HMC in athymic nude mice, animals were divided into three groups and each group treated daily with a different concentration (100 mg/kg, 250 mg/kg and 500 mg/kg respectively) of HMC. This was to allow us to identify the highest dose that is well-tolerated by the animals.

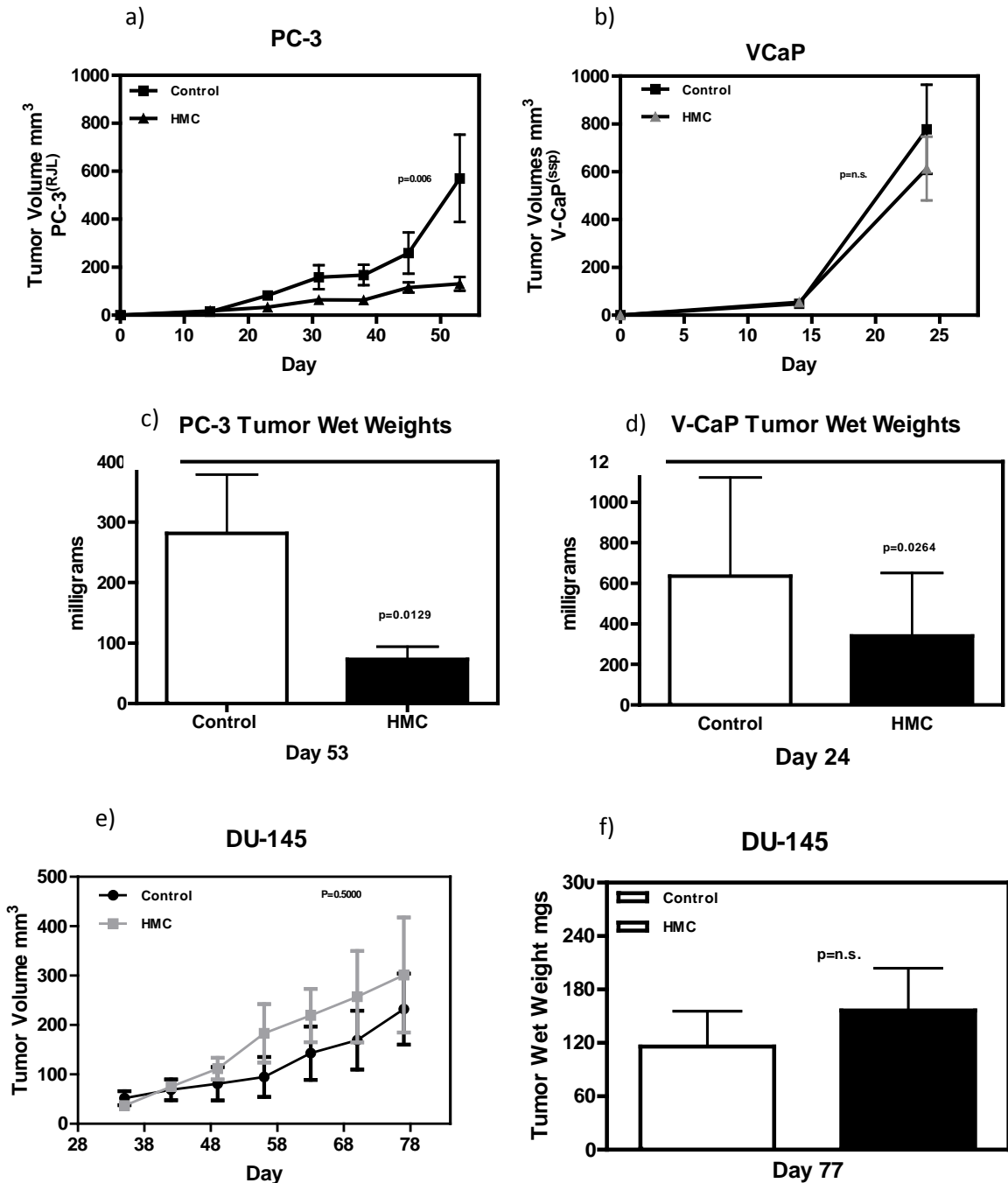
These experiments were performed using an acidic form of HMC. The mice tolerated this form reasonably well even at the highest dose proposed, even though the solution was thick

and chalky. However, little if any change was seen in HA levels. After some discussion with Dr. Leach, the co-investigator on this grant, regarding the bioavailability of HMC, we scaled up on the doses of HMC and tested it at 3 higher concentrations (1.5g/kg, 2.5g/kg and 3.0g/kg). The bioavailability of HMC is very low and thus, an increase in dosage should increase the actual bioavailable HMC. Because the acidic form of HMC was difficult to get into solution, particularly so at higher concentrations, it was decided very recently to try to use the salt form of HMC. We have tested this form in mice at the dose of 1g/kg/mouse/day, which has been used previously in other studies. This dose was well tolerated by the animals as were the lower doses. Thus, we proceeded with the remainder of the experiments using this form once we verified that there is a change in HA levels in our test mice. The data on HAS2 and HAS3 expression and HA production has already been confirmed using the salt solution of HMC. The remainder of the in vitro data has also been repeated to verify that this form will be as effective as the acidic form on the growth of prostate tumor cells. To illustrate how similar the two compounds are in vitro behavior, we performed a side by side comparison in vitro (see Figure 8 for example).



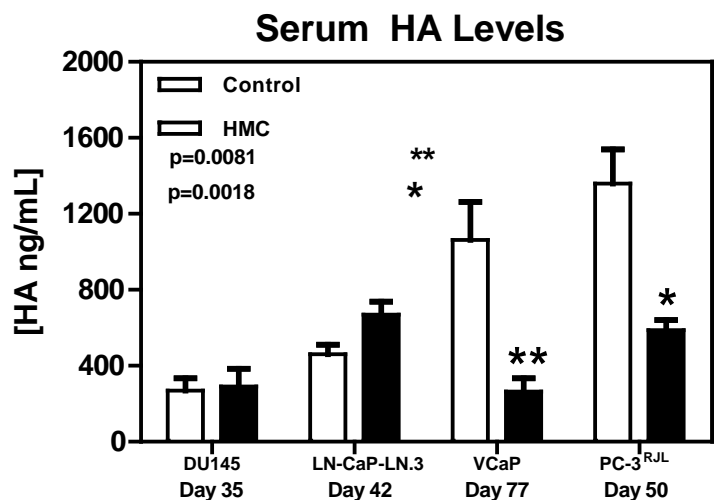
**Figure 8. Growth curve of PC-3 cells comparing HMC-salt solution with the acidic form of HMC.** Note that there is no difference between the two compounds.

- e. To determine the effects of HMC on the growth of prostate cancer cells in vivo, nude mice were inoculated with each of the prostate cancer tumor cell lines (n=16 mice per cell line; 8 per treatment group) and treated with the 1mg/kg/day dose of the HMC salt solution or with vehicle. (8 mice per group X 6 cell lines X 2 groups = 96 mice total X 2 experiments\*\*). This has been modified to five cell lines. Prostate cancer cell lines, PC-3, DU-145, VCaP, LNCaP.LN3 have been examined thus far. DuCaP cells are no longer being used as they will not consistently form tumors in nude mice. 22RV1 cells are currently being analyzed in this assay.



**Figure 9: HMC effects on tumor volumes and tumor wet weight at sacrifice. a) In vivo growth curve of PC-3 tumors treated with control or HMC. Note HMC slows the growth of the tumors. b) In vivo growth curve of VCaP tumors treated with control or HMC. Again, note that HMC slows the growth of the tumors. c) PC-3 tumor weight at sacrifice following in vivo treatment with control or HMC. Note that HMC significantly reduced the tumor weight ( $p=0.021$ ). d) VCaP tumor wet weight at sacrifice following in vivo treatment with control or HMC. Note that HMC also significantly reduced the tumor weight ( $p=0.0204$ ). e) In vivo growth curve of DU-145 tumors treated with**

**control of HMC.** Note that HMC increased tumor growth and tumor wet weight (**f**) at sacrifice in DU-145 tumors. This is consistent with the *in vitro* data. There was no difference in the growth curve or final tumor wet weight with LN.CaP.LN3 cells (data not shown).



**Figure 10.** Serum HA levels at sacrifice in mice bearing tumors as indicated and treated with either HMC or control. Note that both VCaP and PC-3 tumor bearing mice had a significant decrease in HA levels consistent with the significant decrease in tumor size.

**Outcome:** Expression of HAS and HA production has been established and shown to correlate with *in vivo* tumorigenicity and metastatic capability. Dosage of HMC for *in vivo* studies has been established and effect of the compound on tumor growth determined. HMC had significant effects on the growth of both VCaP and PC-3 cells *in vivo*. These two cell lines have been selected for further study on the effect of this compound on bone metastases.

**TASK 2: Determine whether inhibition of HAS expression and HA production with HMC will prevent metastases of prostate cancer cells to bone and other organs in mouse models of bone metastases.** Mice will be pre-treated with HMC to mimic the clinical scenario whereby a patient is diagnosed with advanced prostate cancer and may be at risk for bone metastases, but has no evidence yet of skeletal lesions at diagnosis. HMC could then be used to augment standard of care to prevent metastases. These experiments are currently in progress. We are a bit behind because of some issues we had with tissue culture contamination. However, these problems have been solved and we are now working on these experiments. **(Months 13-24)**

- a. Athymic nude mice were treated with the optimal dose of HMC or vehicle daily for two weeks prior to tumor cell inoculation (n=12 per group X 2 cell lines X 2 treatments X 2 experiments \*\*for a total of plus 96 mice)

- b. Originally, we planned to treat PC-3 and VCaP prostate cancer cells with HMC or vehicle *in vitro* prior to inoculation into mice. However, because HMC has such significant effects on growth of these cells *in vitro*, we are not pre-treating the cells.
- c. Mice were inoculated with either PC-3 cells or VCaP cells via intra-cardiac inoculation. Mice are being treated with the 1mg/kg/day dose of HMC or vehicle and treatment will continue on a daily basis for the duration of the experiment.
  - a. Mice were examined by radiography at baseline and are being examined weekly post heart injection for the development of bone metastases in the case of PC-3, where skeletal lesions will develop more rapidly and most likely will be osteolytic in nature. VCaP cells cause a more osteoblastic phenotype and take longer for the development of bone metastases so these mice are being x-rayed once per month until there is evidence of bone metastases in control animals and then more frequently. This will allow us to track the development of skeletal metastases over the course of the experiment.
  - b. Serum samples have been harvested at baseline and continue to be harvested once per month until sacrifice so that we can measure HA levels over the course of the experiment, as well as markers of bone turnover as indicators of bone metastases. These will be evaluated at the end of the experiments.
  - c. Half of the mice in each group (n=6 per group X 2 cell lines X 2 treatments) will be examined by <sup>18</sup>F-FDG MicroPET at sacrifice for the identification of metastases to other organs. However, due to circumstances beyond our control, the PET scans cannot be performed. The University's microPET scanner is broken and we are exploring alternative imaging options.
  - d. At sacrifice, tissues from each mouse will be harvested for histological preparation. Quantitative bone histomorphometry will be performed on sections of long bones to determine the effects of HA on bone metastases. Sections will also be stained for HA as previously described, to examine the effect of HMC on tumor cell and host HA production in the bone metastatic site.

**Outcomes:** We will have both radiographic and histological evidence in two *in vivo* models that targeting the production of HA using HAS is a viable treatment option to prevent prostate cancer metastases to bone and other organs. Thus far, our data indicates that HMC treatment results in a delay in the development of the prostate cancer bone metastases from PC-3 cells. The data with VCaP also supports this but is not as dramatic. Initial histological data is currently being processed and should be completed soon. We expect this task to be completed (including data analysis) in the next six months.

**TASK 3: Determine whether inhibition of HAS via treatment with HMC will be beneficial in animals with established prostate cancer bone metastases utilizing the same model used in Specific Aim 2.** In this case, mice will be monitored radiographically for the development of bone metastases, and treatment with HMC will not begin until 75% of the mice have evidence of bone metastases. This would mimic the clinical scenario in which patients present with bone metastases at the time of diagnosis. (20 mice per group X 2 cell lines X 2 treatments X 2 experiments\*\*160 mice total. [Note: more mice are used per group because of the nature of this experiment. Some

animals may need to be euthanized before treatment begins and our analysis indicates that n=20 is the minimum number needed to observe a statistical difference.] (Months 25-36)

- a. PC-3 and VCaP prostate cancer cells will be treated with HMC or vehicle *in vitro* for 48 hours prior to inoculation into mice.
- b. Athymic nude mice will then be inoculated with either PC-3 cells or VCaP cells via intracardiac inoculation.
- c. Mice will be examined by radiography at baseline and then weekly beginning at 2 weeks post heart injection for the development of bone metastases in the case of PC-3, where skeletal lesions will most likely be osteolytic in nature and develop more rapidly. VCaP cells will have a more osteoblastic phenotype and take longer to develop bone metastases so these mice will be x-rayed once per month until there is evidence of bone metastases in control animals. This will allow us to track the development of skeletal metastases over the course of the experiment and to determine when approximately 75% of the mice have evidence of bone metastases.
- d. Daily treatments with the optimal dose of HMC or vehicle will begin when 75% of the mice have evidence of bone metastases by radiography.
- e. Serum samples will be collected at baseline and once per month until sacrifice so that we can measure HA levels over the course of the experiment as well as markers of bone turnover as indicators of bone metastases.
- f. Half of the mice in each group (n=6 per group X 2 cell lines X 2 treatments) will be examined by <sup>18</sup>F<sup>18</sup>FDG MicroPET at sacrifice for the identification of metastases to other organs. As noted in Task 2, the MicroPET scanner at UTHSCSA is broken and we are currently pursuing other imaging options for the remainder of the experiments.
- g. At sacrifice, tissues from each mouse will be harvested for histological preparation. Quantitative bone histomorphometry will be performed on sections of long bones to determine the effects of HA on bone metastases. Sections will also be stained for HA to examine the effect of HMC on tumor cell and host HA production in the bone metastatic site.

**Outcomes:** These experiments are underway. Our first round of experiments with PC-3 cells indicates that *in vivo* systemic treatment with HMC to treat established bone metastases may help reduce the progression of the bone metastases and thus HMC may be a viable treatment option for patients with already established bone metastases. However, our work also indicates that HMC delivered via oral gavage is not well-tolerated by the animals. We have just euthanized animals from this first experiment and are analyzing the data as well as processing the histological specimens. In addition, the lead technician on this project, Barry Grubbs, retired in March 2011, following some health issues. We have recently identified and hired his replacement, Ms. Teresa Frosto-Burke. We are in the process of training her. Ms. Burke has more than 20 years of research experience and is quickly getting up to speed on the required techniques. While this has hampered our progress this year, we are confident we will complete the aims in a timely manner.

\*\*Each *in vivo* experiment will be performed twice for reproducibility and consistency of results and 3 unmanipulated controls are always included in each experiment to ensure reliability of our techniques.

All of the experiments proposed here are designed to provide pre-clinical evaluation of HMC as a potential agent for the prevention and treatment of prostate cancer bone metastases. These are important experiments which could lead to clinical trials of HMC in patients with prostate cancer.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Levels of HAS1, HAS2 and HAS3 expression in prostate cancer cell lines has been determined. HAS1 expression is virtually undetectable in all of the cell lines. HAS2 expression is upregulated in PC-3, DuCaP, VCaP cell lines. HAS3 expression levels are substantially lower than HAS2 in all of the cell lines. HAS3 is expressed most abundantly in PC-3, 22RV1 and DU145 cells. This indicates that HAS2 is likely to be responsible for the bulk of HA production in prostate cancer cells.
- Levels of HA production by prostate cancer cells has been established and has been shown to correlate with tumorigenicity and metastatic behavior. Cell lines that have a more aggressive phenotype in mouse models, such as PC-3, VCaP and DU-145, produce more HA than other prostate cancer cell lines which are less aggressive. These three cell lines are also those initially isolated from the more aggressive cancers (metastatic to bone, bone and brain, respectively).
- Treatment with HMC in vitro has been demonstrated to decrease HAS 2 and HAS3 expression levels in prostate cancer cell lines. The exception to this is DU145, where expression increased slightly in both cases following treatment with HMC. These results have been verified and we are currently working to discover what is unique about DU145.
- Treatment with HMC in vitro has been shown to decrease HA production as measured by ELISA in all of the prostate cancer cell lines that produced detectable levels of HA. Levels of HA in DU145 were reduced but the result was not significant. This has now been verified.
- Treatment with HMC in vitro resulted in a significant reduction in cell growth over time in all of the cell lines examined. This is consistent with anti-tumorigenic behavior. Interestingly, HMC significantly reduced the in vitro growth of DU145 cells despite our findings that it increased expression of HAS2 and HAS3.
- The 3g/kg dose of HMC was well-tolerated by mice and had no observed side effects. However, we have found that with the acidic form of HMC is difficult to get into solution. As a result, we have begun to use the HMC salt solution at a 1mg/kg/day dose.

- All of our data has been repeated with the HMC salt solution and it indeed is as effective in vitro as the acidic form of HMC.
- Bioavailability of the acidic form of HMC has been an issue. We have repeated all of Task 1 with the HMC salt solution and found that it does indeed act similarly in vitro.
- In vivo evaluation of the HMC salt solution on the growth of prostate cancer cells in vivo has been completed. HMC has significant effects on the growth of both PC-3 and VCaP tumors, and thus may be an excellent treatment for prostate cancer.
- In vivo evaluation of HMC for the prevention and treatment of metastases, particularly bone metastases is currently underway utilizing both PC-3 and VCaP cells. Preliminary evidence indicates that HMC will be effective on bone metastases in a subset of tumors that have an upregulated HA pathway.

#### **REPORTABLE OUTCOMES:**

May 2009 Research Presentation to Department of Hematology/Oncology, University of Texas Health Science Center at San Antonio

August 2009 Research Presentation to Department of Medicine, Division of Endocrinology, University of Texas Health Science Center at San Antonio

November 2010 Poster Presentation, Cancer Prevention and Research Institute of Texas Annual Meeting, Austin, TX (copy of poster attached, Appendix 1)

March 2011 Poster Presentation, Department of Defense Prostate Cancer Research Program, Innovative Minds in Prostate Cancer Today (IMPACT) Meeting, Orlando Florida (copy of abstract attached, Appendix 2)

July 2011 Manuscript in Preparation

#### **CONCLUSIONS:**

Hyaluronic acid (HA) levels and HAS2 and HAS3 expression levels are elevated in prostate cancer cell lines that are more aggressive in in vivo models of tumor growth and metastasis. Our data indicates that HMC is capable of decreasing HA levels and HAS2 and HAS3 expression in vitro. Furthermore, HMC significantly decreases the growth of prostate cancer cells in vitro, indicating that it may represent a viable option for prostate cancer patients. In vivo testing indicates that there are no serious side effects in mouse models. In vivo analysis of the effects of HMC on tumor growth indicates that it may be a viable treatment in at least a subset of patients with elevated HA levels. All of our data to this point has been repeated with the HMC salt solution and it appears

to be a valid alternative to the acidic form of HMC. We have also eliminated DuCaP cells from in vivo analysis because of difficulty with the cells forming tumors in mice. Unfortunately, the MicroPET scanner we had planned to use is broken and it is unclear when it will be repaired. As a result, we are currently looking for alternative imaging methods. Our experiments this year were also hampered some by the retirement of Mr. Barry Grubbs, the senior research technician on the project, following some health issues. We have recently found a replacement for Mr. Grubbs and things are moving along nicely again. We are currently completing evaluation of HMC for the ability to prevent/treat metastases particularly to bone in prostate cancer models.

These results are a first step demonstrating the potential of HMC in the treatment of prostate cancer. HMC represents a unique agent since it is already widely used in Europe for the treatment of digestive complaints with few, if any side effects. Thus, if our in vivo studies demonstrate it is a viable treatment option, it could be fast-tracked for patient use as an alternative therapy.

**REFERENCES .**

None

**APPENDICES:**

None

## Introduction

Prostate cancer is the most common non-cutaneous malignancy in American men. Despite advances in the early diagnosis and treatment of prostate cancer, many patients eventually relapse with advanced prostate cancer. Many of these patients will develop skeletal metastases, a debilitating and devastating complication. Unfortunately, treatment options for patients with advanced disease, especially those with bone metastases are limited. Thus, there is an urgent need for therapeutics aimed at preventing and treating advanced prostate cancer.

Both hyaluronan synthase (HAS) and hyaluronic acid (HA) are upregulated in metastatic prostate cancer cells. 7-Hydroxy-4-Methyl Coumarin (HMC) is an inhibitor of hyaluronan synthase. It is commonly available in herbal supplements and, up to now, has been utilized mainly for digestion complaints. We propose that it may be efficacious in the prevention and treatment of prostate cancer. Our hypothesis is that HA is utilized by prostate cancer cells to facilitate growth and metastasis. Thus, reducing the production of HA should reduce the growth and metastatic potential of prostate cancer cells making HA an ideal target for preventing and treating metastatic disease. We set out to determine whether HAS expression and HA production in prostate cancer cells correlates with increased growth both in vitro and in vivo using both real-time PCR and protein expression assays. We also examined whether modulation of HAS expression by HMC inhibited tumor cell growth in vitro and in vivo.

## Materials and Methods

**Quantitative Expression Analysis.** Levels of HAS1, HAS2 and HAS3 expression in prostate cancer cell lines were assayed by Taqman Gene Expression primer and probe sets for each (Applied Biosystems, Foster City, CA).

**Quantitation of HA synthesis.** HA synthesis was quantitated in both cell culture supernatants and in serum collected from mice using a competitive binding assay specific for HA per manufacturer's instructions (Biotech Trading Partners, Encinitas, CA).

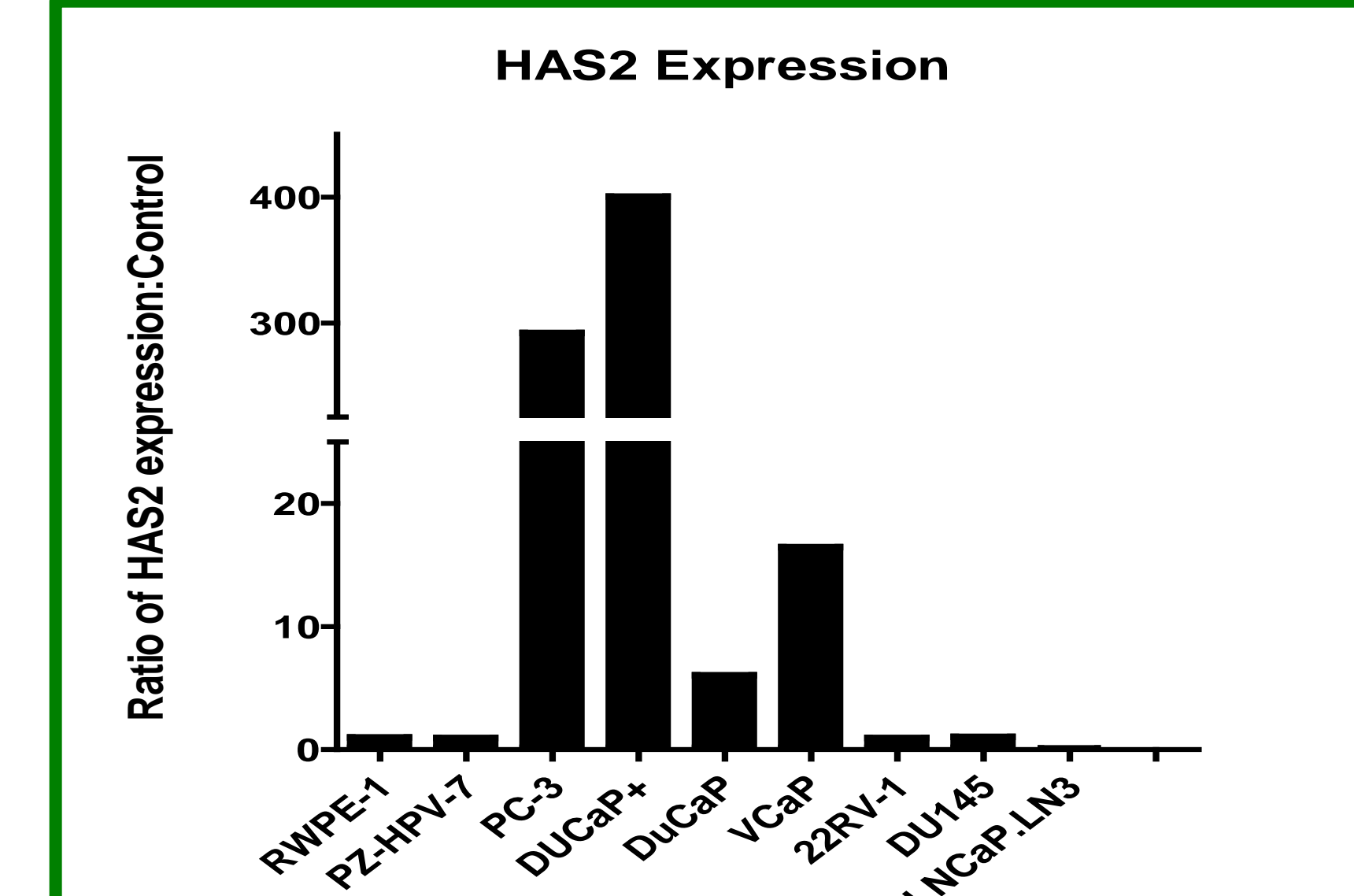
**Doubling Time Analysis.** Cells were plated at 1X10<sup>4</sup> cells/ml in 24 well plates at 1ml/well containing media without selective agents. Three wells will be harvested and counted with a hemacytometer daily for 4-5 days.

**In vivo tumorigenicity (subcutaneous growth).** 5X10<sup>5</sup> cells in a 100µl volume were inoculated into 5 to 7 week old male athymic nude mice. Tumor volumes were monitored every other day using caliper measurements.

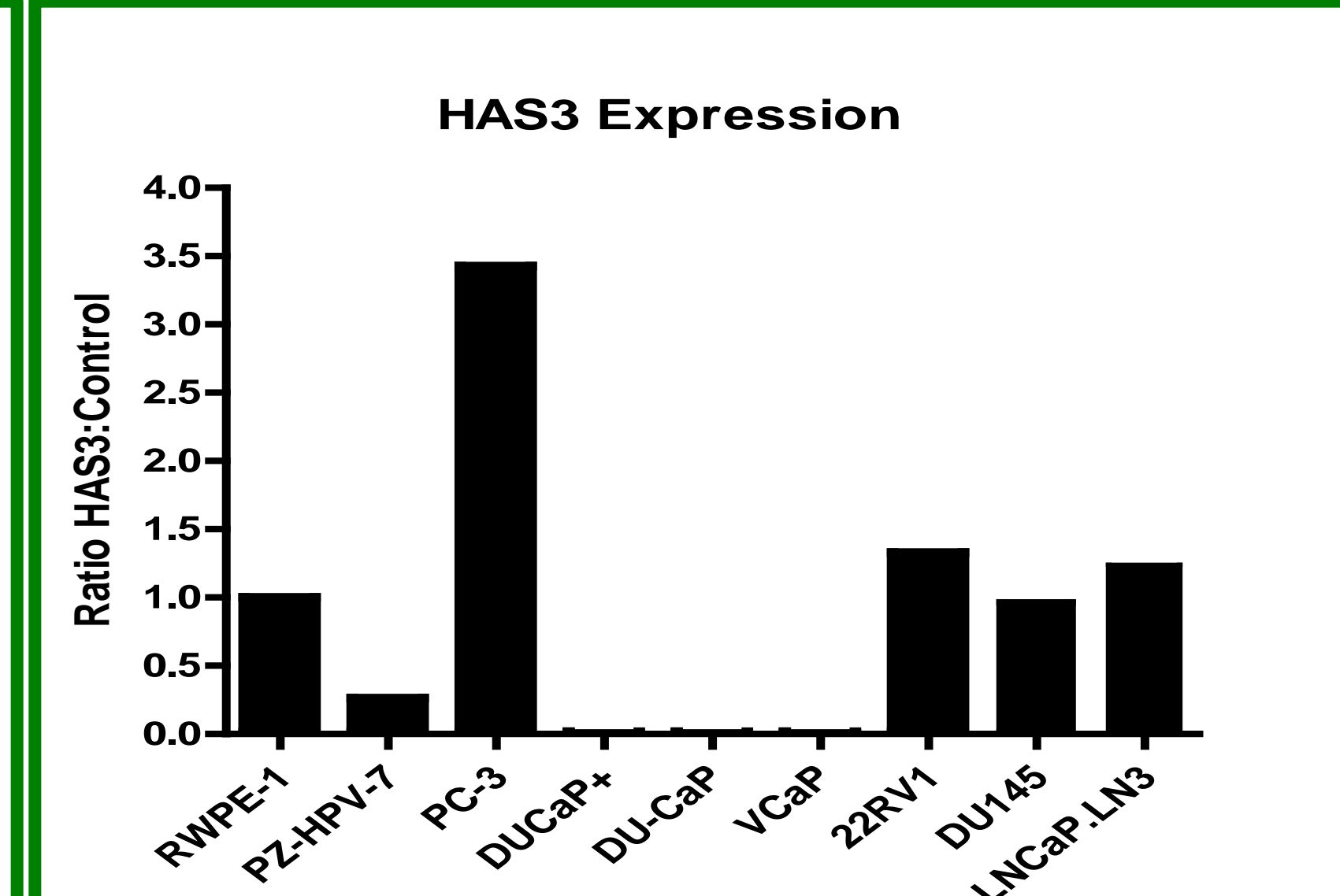
## Acknowledgements

This work was supported by a DOD Prostate Cancer Research Program Grant (W81XWH-08-1-0287) and pilot funds from the Cancer Therapy & Research Center at the University of Texas Health Science Center at San Antonio (CA054174).

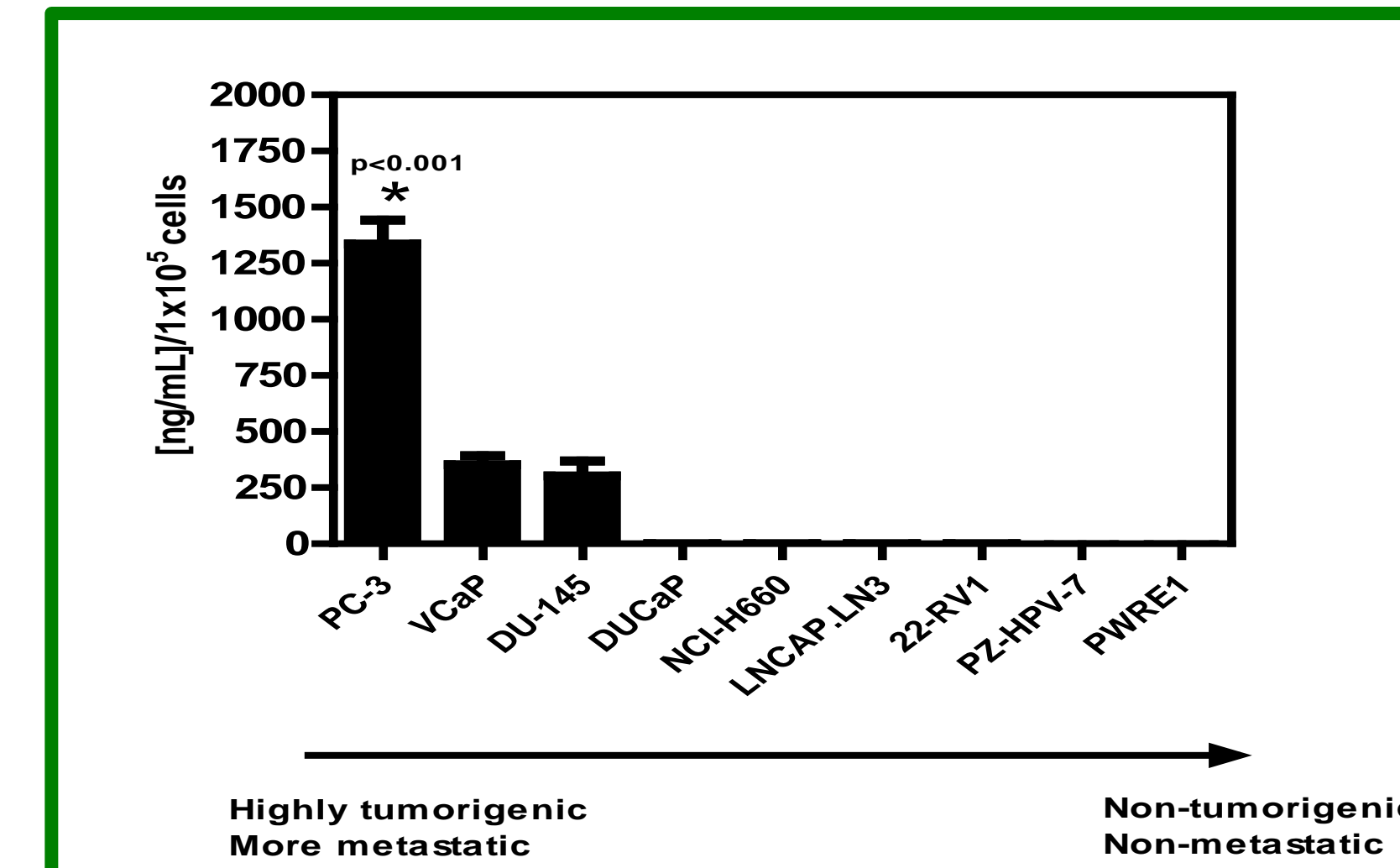
## Results



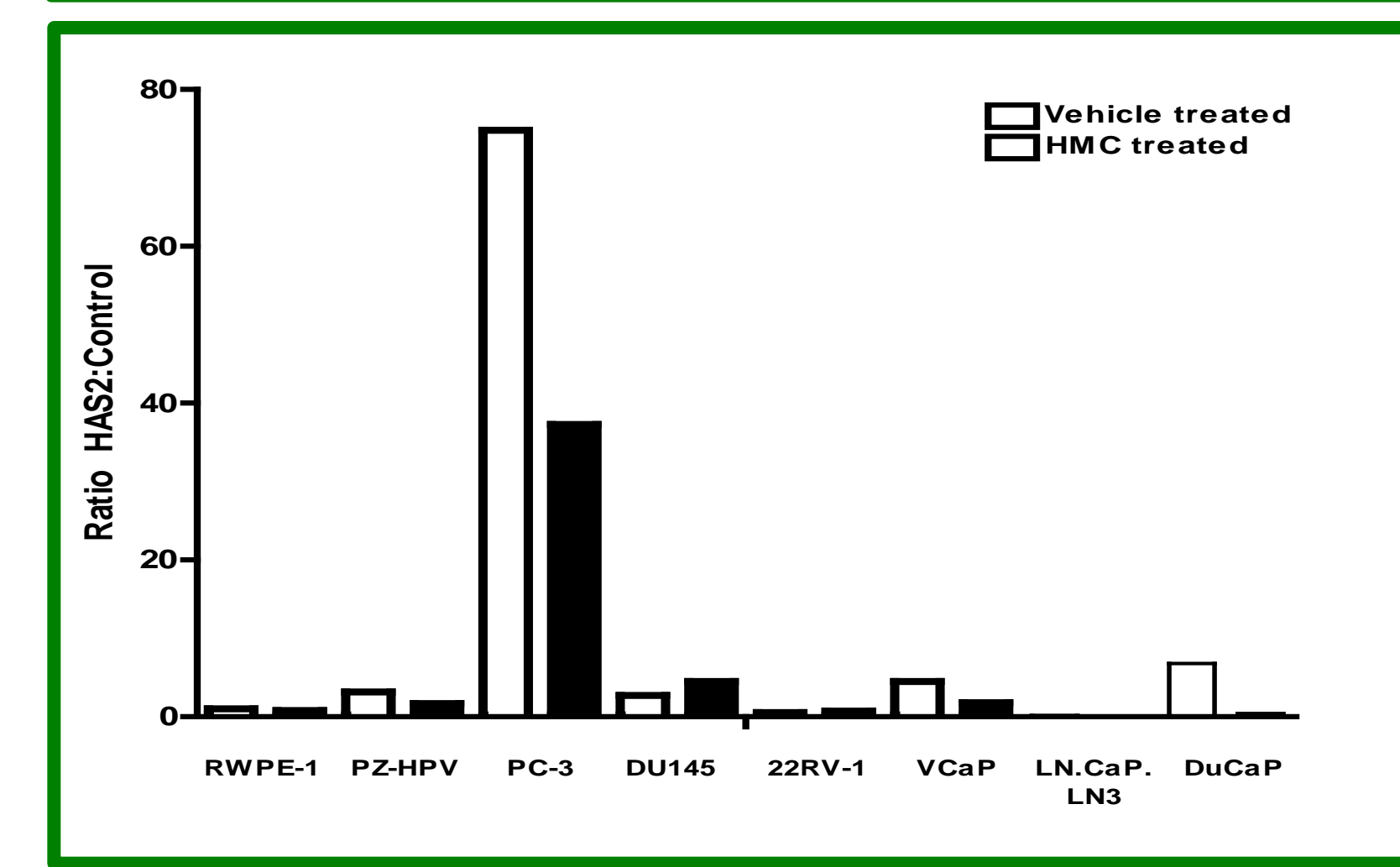
**Figure 1. Quantitative Analysis of HAS2 Expression levels by Real-Time PCR.** HAS2 expression is reported relative to expression in the RWPE-1 prostate epithelial cell line. Note that all of the prostate cancer cell lines except for LNCaP-LN3 express HAS2 at higher levels than prostate epithelial cell lines, RWPE-1 and PZ-HPV-7. DuCaP+ indicates DuCaP cells harvested with a feeder layer. DuCaP indicates only the isolated DuCaP cells.



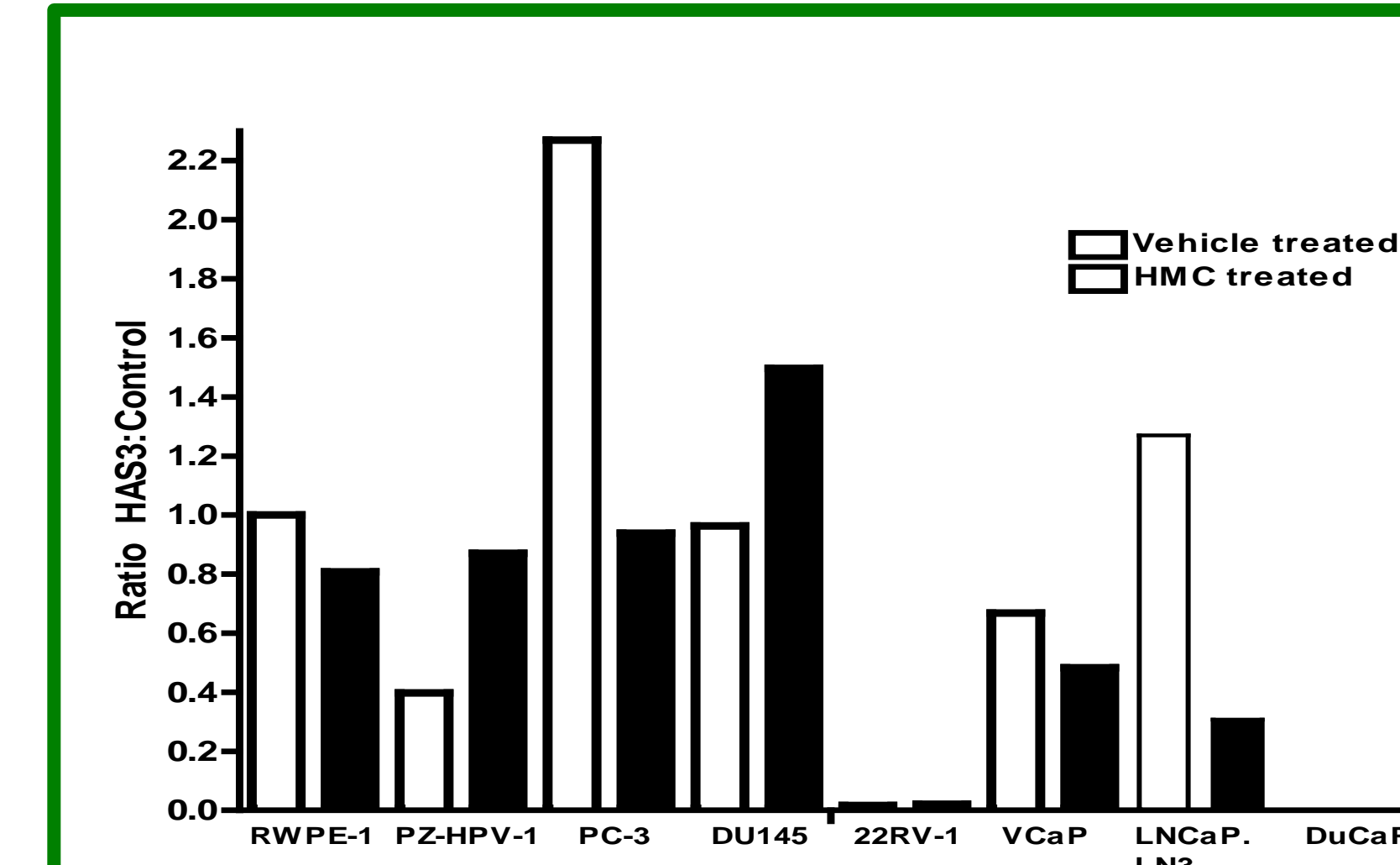
**Figure 2. Quantitative Analysis of HAS3 Expression levels by Real-Time PCR.** HAS3 expression is reported relative to expression in the RWPE-1 prostate epithelial cell line. Note that not all of the prostate cancer cell lines express HAS3. DuCaP+ indicates DuCaP cells harvested with a feeder layer. DuCaP indicates only the isolated DuCaP cells.



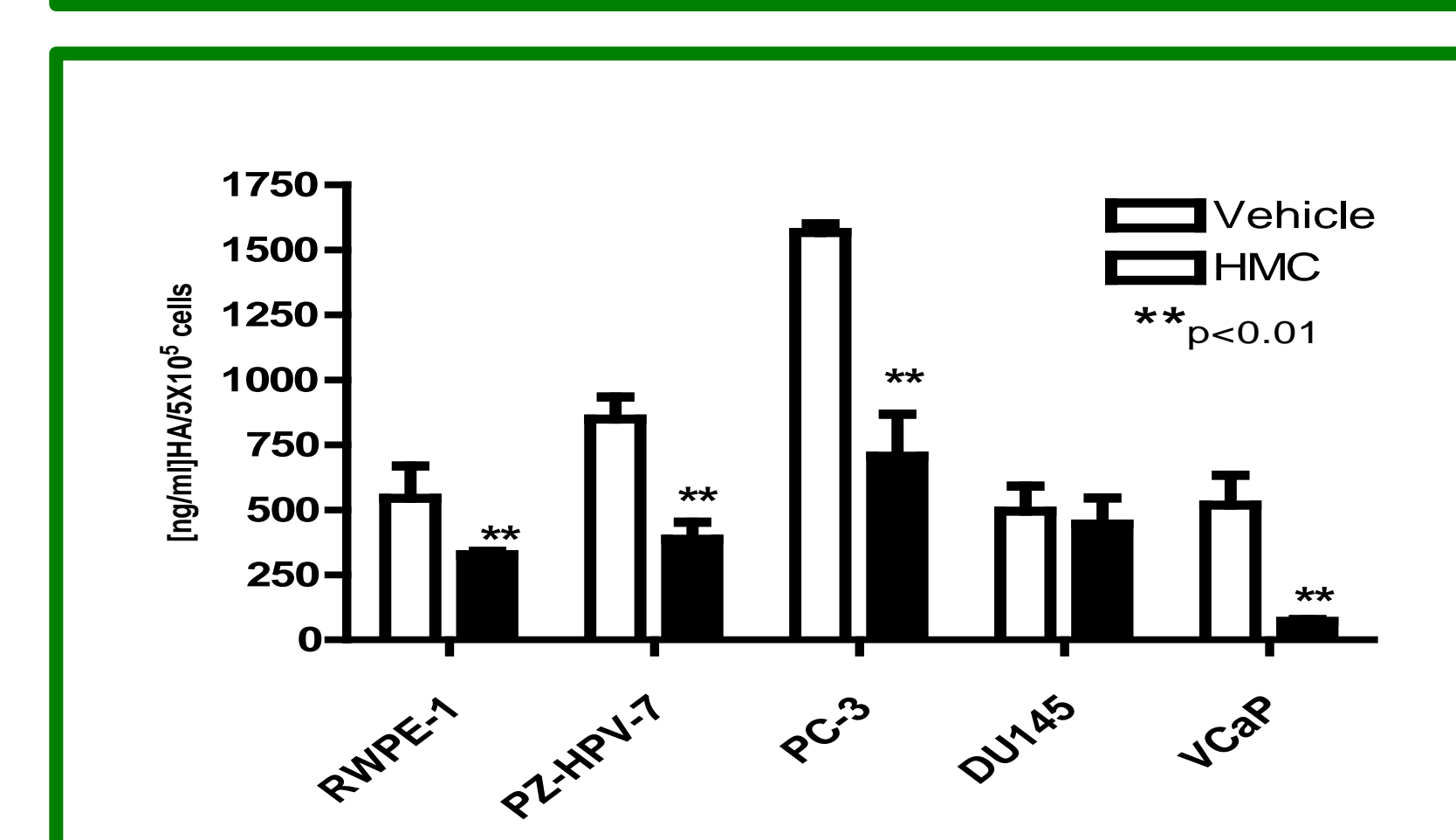
**Figure 3. HA synthesis by prostate cancer cell lines.** Note that the metastatic prostate cancer cell lines, PC-3, VCaP and DU-145 make HA. PC-3 cells make significantly more HA than other prostatic cancer cell lines and the prostate epithelial cell lines, PZ-HPV-7 and RWPE-1. The other prostate cancer cell lines make very low levels or undetectable levels of HA. These results correlate with in vivo tumorigenicity and metastatic potential.



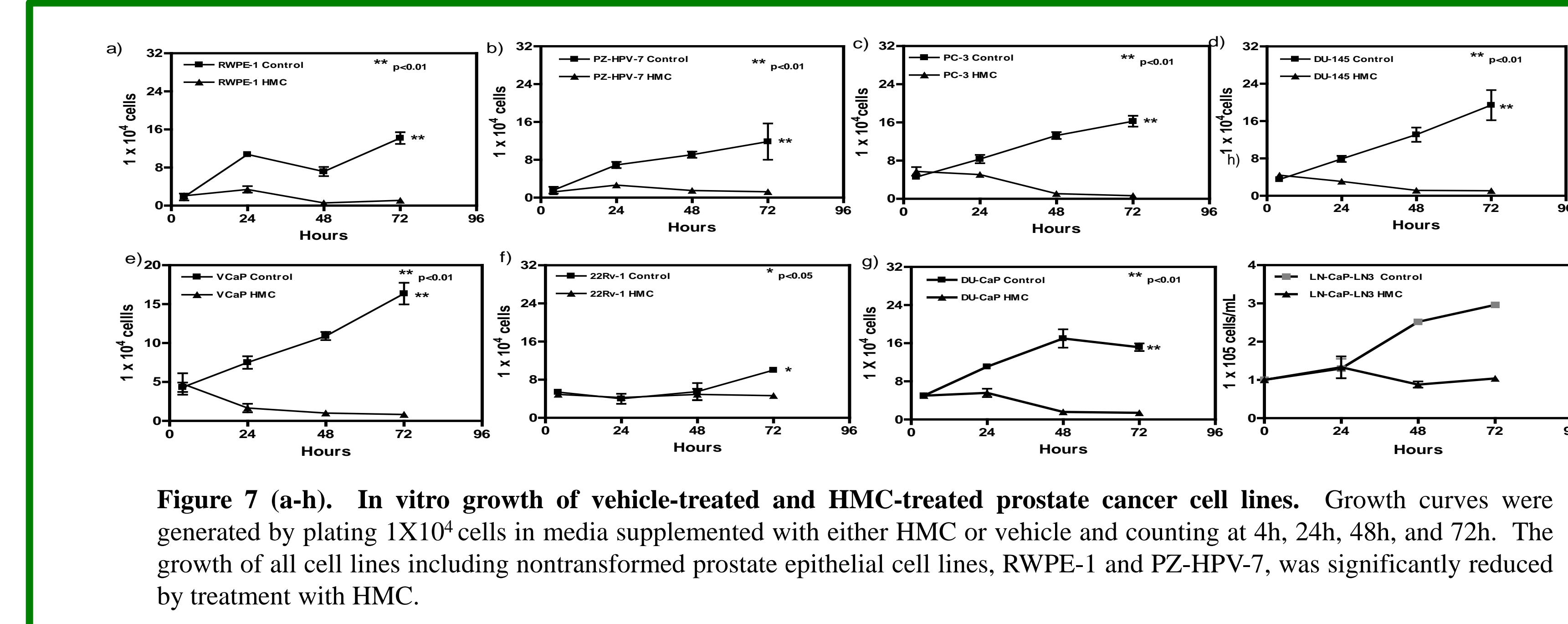
**Figure 4. Quantitative Analysis of HAS2 Expression levels by Real-Time PCR.** HAS2 expression is reported relative to expression in the vehicle treated RWPE-1 prostate epithelial cell line. HMC treatment reduced HAS2 expression levels in all of the cell lines except DU145.



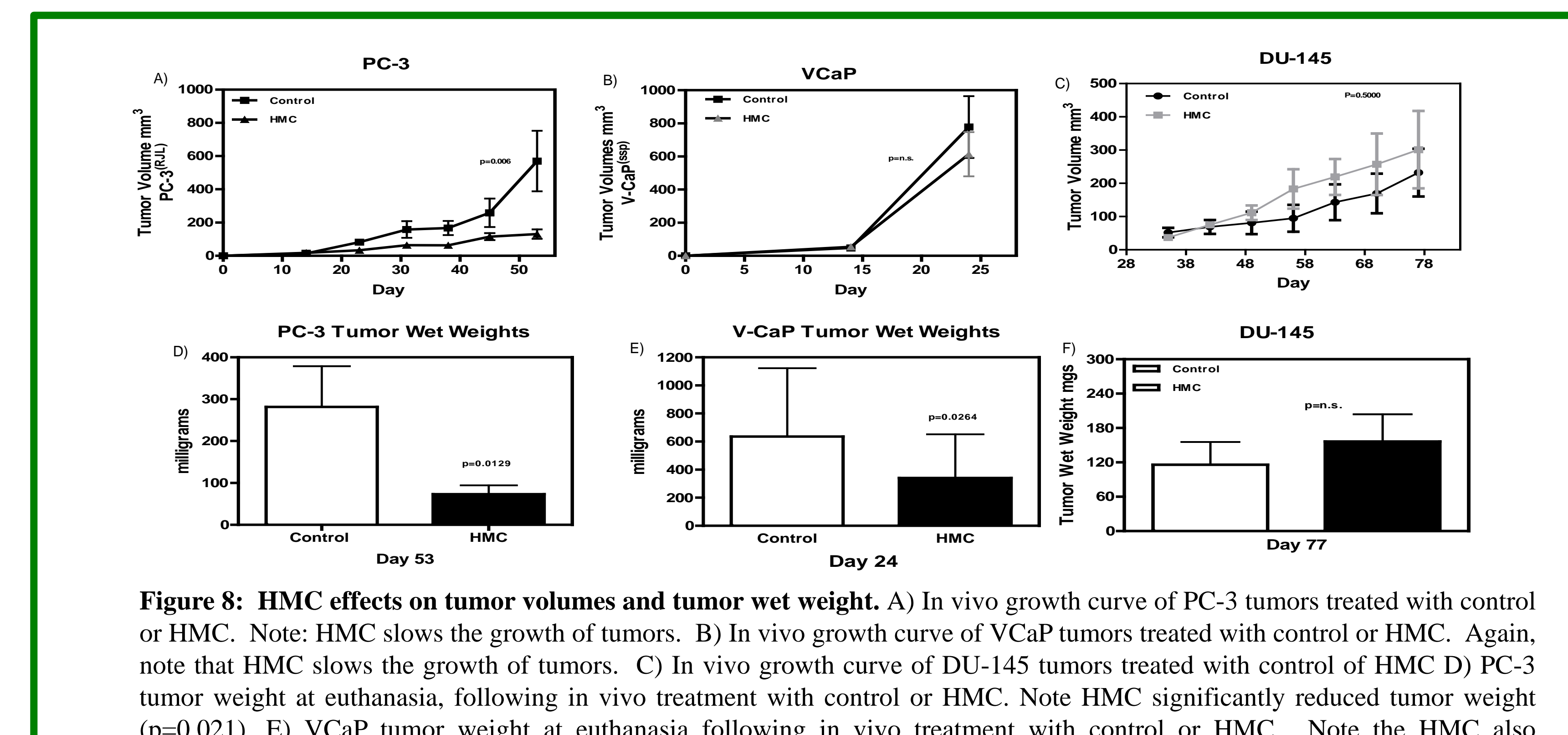
**Figure 5. Quantitative Analysis of HAS3 Expression levels by Real-Time PCR.** HAS3 expression is reported relative to expression in the vehicle-treated RWPE-1 prostate epithelial cell line. HMC treatment reduced HAS3 expression levels in all of the cell lines except DU145 and the prostate epithelial cell line PZ-HPV-7. We are currently investigating the reasoning for this. Note that DuCaP cells do not express HAS3.



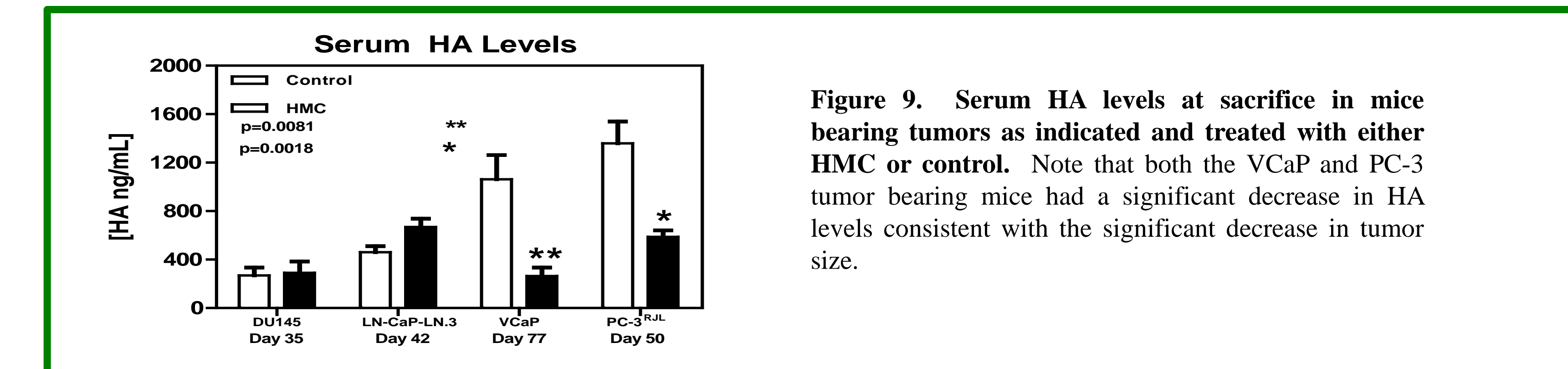
**Figure 6. HA synthesis by prostate cancer cell lines.** Note that the metastatic prostate cancer cell lines, PC-3, VCaP and DU145 make HA and thus were included in this experiment. HA production was significantly reduced in both PC-3 and VCaP but not in DU145 cells, consistent with the results of HAS2 and HAS3 expression analysis (Figures 4 and 5). The other prostate cancer cell lines make very low levels or undetectable levels of HA by this assay and thus were not included here.



**Figure 7 (a-h). In vitro growth of vehicle-treated and HMC-treated prostate cancer cell lines.** Growth curves were generated by plating 1X10<sup>4</sup> cells in media supplemented with either HMC or vehicle and counting at 4h, 24h, 48h, and 72h. The growth of all cell lines including nontransformed prostate epithelial cell lines, RWPE-1 and PZ-HPV-7, was significantly reduced by treatment with HMC.



**Figure 8: HMC effects on tumor volumes and tumor wet weight.** A) In vivo growth curve of PC-3 tumors treated with control or HMC. Note: HMC slows the growth of tumors. B) In vivo growth curve of VCaP tumors treated with control or HMC. Again, note that HMC slows the growth of tumors. C) In vivo growth curve of DU-145 tumors treated with control or HMC. D) PC-3 tumor weight at euthanasia, following in vivo treatment with control or HMC. Note HMC significantly reduced tumor weight (p=0.021). E) VCaP tumor weight at euthanasia following in vivo treatment with control or HMC. Note the HMC also significantly reduced the tumor weight (p=0.021). F) DU-145 tumor weight at euthanasia, consistent with the other experiments, no difference was detected.



**Figure 9. Serum HA levels at sacrifice in mice bearing tumors as indicated and treated with either HMC or control.** Note that both the VCaP and PC-3 tumor bearing mice had a significant decrease in HA levels consistent with the significant decrease in tumor size.

## Conclusions

- HAS2 expression levels and HA production
  - ↑↑ in PC-3, DuCaP and VCaP prostate cancer cell lines.
  - correlate with *in vivo* tumorigenicity and metastatic potential.
- Cells with a more aggressive *in vivo* phenotype, express more HAS2 and produce more HA.
- HMC :
  - ↓↓ *in vitro* HAS2 and HAS3 expression levels in prostate cancer cell lines.
  - ↓↓ *in vitro* production of HA in all of the cell lines that produced detectable levels of HA.
  - ↓↓ *in vitro* growth over time in all of the cell lines examined.
  - ↓↓ growth of PC-3 and VCaP prostate cancer tumors
- Modulation of HA may be a viable option for augmenting the current standard of care in prostate cancer patients.

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August 5, 2010

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Innovations in Cancer Prevention and Research Conference**

RE: Abstract entitled, "Hyaluronic Acid as a Target for Intervention in Prostate Cancer"

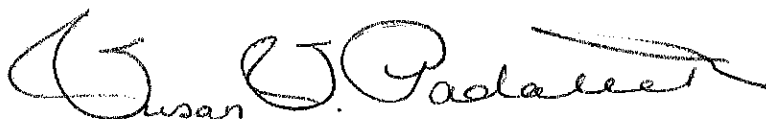
To Whom It May Concern,

We would like to submit the above referenced abstract for the "Innovations in Cancer Prevention and Research Conference." The abstract should be considered for a poster only presentation under the topic area of Cancer Treatment. I will be the presenting author. My contact information is as follows:

Susan Padalecki, Ph.D.  
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Thank you for your consideration of the attached abstract. Please feel free to contact me if you need any additional information.

Sincerely,



Susan S. Padalecki  
Assistant Professor  
Departments of Urology and Cellular & Structural Biology

## “Hyaluronic Acid as a Therapeutic Target for Prostate Cancer”

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<sup>1</sup>Department of Pediatrics, <sup>2</sup>Cellular and Structural Biology, and <sup>3</sup>Urology, <sup>4</sup>Cancer Therapy and Research Center, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229

**INTRODUCTION:** Both hyaluronan synthase (HAS) and hyaluronic acid (HA) are upregulated in metastatic prostate cancer. 7-Hydroxy-4-Methyl Coumarin (HMC) is an inhibitor of HAS. It is commonly available in herbal supplements and, up to now, has been utilized mainly in Europe for digestive complaints. We hypothesize that HA is utilized by prostate cancer to facilitate growth and metastasis and that it may be efficacious in the prevention and treatment of prostate cancer.

**AIMS AND METHODS:** We set out to determine whether HAS expression and HA production in prostate cancer cells correlates with increased growth both *in vitro* and *in vivo* using both real-time PCR and protein expression assays. We also examined whether modulation of HAS expression by HMC inhibited tumor cell growth *in vitro* and *in vivo*.

**RESULTS AND CONCLUSIONS:** HA production was shown to directly correlate with *in vivo* metastatic potential in seven prostate cancer cell lines. However, of the three HAS enzymes, only expression of HAS2 correlated with the metastatic potential of the cell lines. *In vitro*, HA levels can be modulated using HMC and tumor cell growth is reduced by HMC treatment in every cell line examined regardless of the level of HA produced. *In vivo* tumor growth has also been reduced by HMC treatment but only with PC-3 and VCaP prostate cancer cell lines. These results indicate that HMC may be a viable treatment option for patients with HA-producing prostate cancer. Additional studies are underway to investigate HMC affects on prostate cancer bone metastases.