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13. ABSTRACT ***(Maximum 200 Words)***

Prostate-Specific Membrane Antigen (PSMA) is highly expressed in virtually 100% of prostate cancers and is expressed on the cell surface which makes it a useful target for both clinical and therapeutic strategies. However, there have been reports of PSMA expression in other, normal tissues, which could weaken its potential clinical use. We have shown that another gene very similar to PSMA existed, and the initial aim of this project was to clone this "PSMA-Like" gene. We cloned the PSMA-like gene, have shown that it is expressed in kidney and liver in humans, and that it has the same NAALADase activity that PSMA does. The gene is 97% identical to PSMA. The PSMA-like gene is not expressed in the prostate, nor is it expressed in prostate cancer or in tumor-associated neovasculature. We can now tailor anti-PSMA therapies to avoid tissues expressing the PSMA-like gene (and subsequent patient toxicity). The second aim of this project was to isolate the most active portions of the PSMA enhancer for future therapeutic (gene therapy) use. We have developed a construct which has prostate specificity, driving expression of a "suicide" gene, which in future could be developed for use in therapeutic approaches.

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D. O'Kane 7-13-00

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Introduction

While PSMA appears to be an ideal prostate cancer marker and potential theraputic target, there have been reports of PSMA expression in non-prostatic tissues, including brain, kidney and liver. Such expression of PSMA could weaken the potential of the gene as a prostate cancer marker or at least produce confusing and conflicting data. Prior to the onset of this project, we had reason to believe from our own data that the non-prostatic expression of PSMA was in fact due to expression of another highly similar, but distinct gene. We had shown the existence of this gene, which we termed the PSMA-Like gene, on chromosome 11q14, while the PSMA gene is found on chromosome 11p11.2. Therefore the first objective of this project was to characterize the differences between the non-prostatic and prostatic forms of PSMA at the nucleic acid level, the protein level and functional level, which is essential for the future utility of PSMA both as a clinical marker and therapeutic target. The second aim of this project was to define the minimal region(s) of the PSMA- regulatory control regions for future use in gene therapy constructs, both by analyzing deletion constructs of the gene regulatory regions and by comparison with the PSMA-Like gene regulatory regions.

Specific Aim 1. *To Clone and sequence the "PSMA-Like" gene and comparatively analyze tissue expression of the PSMA and PSMA-Like genes.*

Genomic Sequencing and Mapping of the PSMA-Like Gene.

In the original statement of work we were going to screen genomic libraries for the PSMA-like gene. However, although several libraries were screened, none contained the gene. Next, based on the intronic sequence of PSMA, we designed primers that would amplify every one of the 19 exons of the PSMA gene. We then used these primers to amplify DNA from somatic cell hybrids containing human chromosome 11q, the location of the PSMA-Like gene – but not containing chromosome 11p (the location of the PSMA gene). We were able to amplify 18 exons of the PSMA-Like gene in this manner, and directly sequence the PCR products to determine the sequence of the PSMA-Like gene. Using this information, we were able to design primers that *specifically* amplified the PSMA-Like gene, even amongst a background that contains the PSMA gene. These primers were next used against the NIGMS hybrid panel to precisely map the PSMA-Like gene. The gene was found to map to chromosome 11q 14.3, at the SCZD2 locus (Schizophrenia Disorder Type II). We were unable however, to amplify a region in the PSMA-Like gene that corresponded to the promoter region and exon one of the PSMA gene. Interestingly, exon one of the PSMA gene contains the transmembrane domain region and encodes the intracellular region of the PSMA protein; the region that binds the antibody that is the constituent of the imaging agent ProstaScint, Cyt-356. By designing one more set of primers, we were actually able to determine that subsequent to the duplication event that formed the PSMA and PSMA-like genes, a deletion occurred that removed exon one of the PSMA gene from the PSMA-Like gene, and the active portion of the promoter region although some further sequences upstream are still present in PSMA-Like.

cDNA Sequence of the PSMA-Like gene.

Comparison of the genomic sequence of the exons of the PSMA-Like and the PSMA genes revealed that there was 98% identity. To determine which tissues the two genes were expressed in, I designed a set of primers that spanned a region in which there was one base-pair difference in the sequence of the genes that could be identified by a simple restriction-enzyme digestion. One enzyme would digest a RT-PCR product from the PSMA gene, and another enzyme would digest product from the PSMA-Like gene – but not from the PSMA gene, thereby distinguishing the two genes at the mRNA level. Analysis of a number of different tissues revealed that both the PSMA and PSMA-Like genes are expressed in the liver and kidney. Next, I cloned the entire cDNA of the PSMA-Like gene from a liver cDNA library. All clones obtained (>10) began transcription in what is known as the 5^{th} intron of the PSMA gene, and then expressed exons corresponding to 6-19 of the PSMA gene. This is most likely because the promoter region originally used by this gene is no longer present, thus a new promoter (probably found in the 5^{th} intron) is used. The expressed portion of PSMA-Like is 1,999 nucleotides, theoretically encoding a cytosolic protein of 443 amino acids which is approximately 49 kD in size before glycosylation.

Next, we examined other tissues for expression of the genes, and found that both normal prostate, prostate cancer and endothelial cells from tumor neovasculature expressed the PSMA gene and not the PSMA-Like gene. We also detected expression of PSMA in human brain via several different techniques.

Functional Analysis of PSMA-Like Protein[1]

Next, we made cell lines stably expressing PSMA-Like protein to see if it also exhibited NAALADase and folate hydrolase enzymatic activities like PSMA. Experiments showed that in fact PSMA-Like does encode NAALADase activity in what appears (in a semi-quantitative assay) to be significant levels. We will be testing shortly for folate hydrolase activity, and determining the exact kinetics of both enzymes.

Specific Aim 2. *Define the minimal promoter/enhancer regions of the PSMA and PSMA-Like genes and specific elements responsible for activity.*

We first cloned the regions in the PSMA-Like gene corresponding the promoter and enhancer regions in the PSMA gene to carry out a comparative analysis of the ability of each region to regulate gene activity. We found that there was a deletion in the PSMA-Like gene relative to the PSMA gene (remembering that both genes are derived from a gene duplication event that occurred 22 MYA), that encompasses the active promoter region in PSMA. The enhancer region of PSMA however, was also part of the duplication process and a corresponding region is found in the PSMA-Like gene. The two "enhancers" are approximately 95% identical in gene sequence. However, in two individual assays so far, the PSMA-Like

[1] This portion of work was carried out at the Lerner Research Institute, at the Cleveland Clinic Foundation.

enhancer is not active, suggesting that the minor differences between the two enhancers could indicate the binding sites of prostate-specific enhancing factors. Analysis of the sequence does not reveal binding sites for any currently known enhancer binding proteins. We are currently confirming these results and sequence differences.

We also made a number of deletion constructs of the PSMA-enhancer region, and used these to drive luciferase reporter gene expression in combination with either the PSMA or SV-40 viral promoters. We found that the enhancer seems to contain both positive and negative regulatory elements, and that a 1,648 base pair enhancer region in combination with the PSMA promoter had the highest and most specific expression (see attached paper submitted). We next replaced the reporter gene with the bacterial gene encoding cytosine deaminase. Cell lines derived from different human tissues were transfected with the construct and treated with the non-toxic prodrug 5-fluorocytosine. Prostate cell lines were killed by the drug, and cell lines that do not normally express PSMA were protected from cytotoxicity. We are hoping that this model can serve as the basis for future gene therapy strategies against prostate cancer.

Conclusions

We have cloned and significantly characterized the closest known homolog of PSMA, the PSMA-Like gene. The gene is functional, and expressed in liver and kidney. The PSMA gene itself is expressed in prostate, prostate cancer and tumor neovasculature. While PSMA mRNA seems to be expressed in the brain, we have not detected significant expression of PSMA protein using immunohistochemistry. Further, most treatments targeting PSMA are constituted of compounds that will not cross the blood-brain barrier, although it will be important to keep this in mind in the development of future therapies. We are still determining the exact amount of PSMA relative to PSMA-Like present in the liver and kidney, however in the liver it appears that the predominant form is PSMA-Like. Because PSMA-Like is a cytosolic protein, therapies targeted against PSMA that are activated at the cell surface (ie. Prodrugs such as methotrexate-tri-glutamate or antibodies linked to cytotoxic compounds) will not affect cells expressing PSMA-Like. Furthermore, antibodies that target protein sequences encoded by the first five exons of PSMA will not bind to the PSMA-Like protein. In addition, clinical tests such as RT-PCR for PSMA can use the same technique I have described here to determine if "non-specific" expression seen is due to PSMA or PSMA-Like expression.

In the second part of this project, we have compared the regulatory regions of the PSMA and PSMA-like genes, carried out deletion analyses of the PSMA-enhancer and determined the regions important for activity. We subsequently carried out *in vitro* "gene therapy" experiments and demonstrated prostate-specific toxicity of our construct. The remaining term of this project will be devoted to finishing the enzymatic and kinetic analysis of the PSMA-Like gene, and isolating the prostate-specific enhancer binding proteins responsible for activity of the PSMA enhancer.

Appendix

Key Research Accomplishments

- I have cloned the closest known homolog of PSMA, and called it the PSMA-Like gene
- I have shown that the PSMA-Like gene is indeed expressed, and shown the presence of PSMA-Like mRNA in human liver and kidney tissue
- I have developed a method to distinguish the PSMA and PSMA-Like genes, which are 97% identical, and shown that the form expressed in prostate cancer and tumor-associated neovasculature is in fact PSMA and not PSMA-Like
- I have identified antibodies, which based on the predicted protein sequence of the PSMA-Like gene, will not bind to PSMA-Like protein and therefore can be used for strategic targeting of PSMA and prostate cancer
- I have shown that the region in the PSMA-Like gene that is homologous to the PSMA enhancer region is 95% identical in sequence, however it is unable to drive reporter gene expression. Therefore the 5% difference between the two genes in this region must be crucial to regulation of PSMA expression
- I have determined using deletion constructs of the PSMA enhancer several regions responsible for positive and negative regulatory activity and developed a prostate-specific vector expressing the "suicide" gene cytosine deaminase which can be used in future for prostate cancer gene therapy

Reportable Outcomes

Manuscripts / Book chapters

1. **O'Keefe DS,** Bacich DJ, Heston WDW. Prostate-Specific Membrane Antigen. *in press* in "Prostate Cancer in the 21st Century", Eds. Leland Chung, John Issaacs.
2. Gong MC, Chang, SS, Watt, F, **O'Keefe, D.S.,** Bacich, D.J., Uchida, A., Bander, NH., Reuter, VE., Gaudin, PB., Molloy, PL., Sadelain, M. and Heston, W.D.W. An Overview of Evolving Strategies Incorporating Prostate-Specific Membrane Antigen (PSMA) as a Target for Therapy. Molecular Urology *(in press).*
3. **O'Keefe, D.S.,** Uchida, A., Bacich, D.J., Watt, F.B., Molloy P.L. and Heston, W.D.W. Prostate-Specific Suicide Gene Therapy using the Prostate-Specific Membrane Antigen (PSMA) Enhancer. The Prostate *(in press).*
4. **O'Keefe,D.S.**, Bacich,D.J. and Heston,W.D.W. Expression Profile of Prostate-Specific Membrane Antigen (PSMA) versus a Prostate-Specific Membrane Antigen-Like Gene in Normal Tissues, Prostate Cancer and Tumor Associated-Vasculature *(in preparation).*
5. **O'Keefe,D.S.,** Bacich,D.J. and Heston,W.D.W. Cloning and Characterization of a novel glutamate-preferring peptidase that maps to the SCZDII locus: a candidate gene for Schizophrenia? *(in preparation).*

Abstracts and Presentations

1. **O'Keefe, D.S.,** Bacich, D.J., and Heston, W.D.W. Characterization of the Prostate-Specific Membrane Antigen-Like Gene. Abstract 313, Keystone Symposia on Advances in Human Breast and Prostate Cancer, Lake Tahoe, NV., March 2000.
2. Balaji, K.C., Bacich, D.J., **O'Keefe, D.S.**, and Heston, W.D.W. Differential Gene Expression between Androgen Dependent and Independent Prostate Cancer Cell Lines. Abstract 1978, 91 St. AACR Annual Meeting, San Francisco, April 2000.*

3. Uchida, A., **O'Keefe, D.S.**, Bacich, D.J., Watt, F., Molloy, P.L. and Heston, W.D.W. Prostate-Specific Suicide Gene Therapy Using the Newly Discovered Prostate-Specific Membrane Antigen (PSMA) Enhancer. Abstract 2413, 91st. AACR Annual Meeting, San Francisco, April 2000.*
4. Balaji, K.C., Bacich, D.J., **O'Keefe, D.S.** and Heston, W.D.W. Combination Gene Therapy Using Antisense Strategy For Advanced Prostate Cancer. Abstract 2414, 91 St. AACR Annual Meeting, San Francisco, April 2000.*
5. Bacich, D.J., **O'Keefe, D.S.,** Watt, F.B., Molloy, P.L. and Heston, W.D.W. Prostate-Specific Membrane Antigen (PSMA) Promoter and Enhancer Driven Green Fluorescent Protein (GFP) Expression in Transgenic Mice. Abstract 122, 91 St. AACR Annual Meeting, San Francisco, April 2000.
6. **O'Keefe, D.S.**, Bacich, D.J. and Heston, W.D.W. Cloning and Characterization of the Prostate-Specific Membrane Antigen-Like Gene. Abstract 251, 91 St. AACR Annual Meeting, San Francisco, April 2000.
7. Richter, F., **O'Keefe, D.S.,** Bacich, D.J., Uchida, A., Bisognia, M. and Heston, W.D.W. Breast Cancer Cell Line Stimulates PSMA Expression in Microvascular Endothelial Cell Lines. Abstract 5051, 91 St. AACR Annual Meeting, San Francisco, April 2000.*
8. Richter, F., **O'Keefe, D.S.**, Bacich, D.J., Uchida, A., Bisogna, M., Heston, W.D.W. Co-incubation of Microvascular Endothelial Cells with a Breast Cancer Cell Line Stimulates PSMA-Expression *in vitro*. Abstract 127, American Urological Association Annual Meeting, Atlanta, GA, May 2000.*

Cell lines developed

I have developed two cell lines, both consisting of PC-3 cells (available from ATCC), one of which has been stably transfected with the PSMA-Like gene, and one which has been transfected with an empty vector (for use as a negative control).

Other training accomplishments

As part of my training, I have been helping to train Clinical Research Fellows from the Department of Urology at Memorial Sloan-Kettering Cancer Center, as is evidenced by the abstracts and presentations listed with an asterisk above. To my regret, the fellows did not consult me when submitting abstracts for conferences as I had at this point moved to Cleveland. As such, they did not include recognition of USAMRC funding in their acknowledgements. I apologise for this and will do my best to make sure this does not occur again in the future.

Please find attached three copies of each of the above-listed manuscripts and abstracts.

313 Characterization of the Prostate-Specific Membrane Antigen-*Like* Gene.

D.S. O'Keefe, D.J. Bacich and W.D.W. Heston. *Departments of Cancer Biology and Urology, The Cleveland Clinic Foundation, Cleveland, OH 44195.*

Prostate-Specific Membrane Antigen (PSMA) is a 100kD type II transmembrane protein with folate hydrolase and NAALADase activity. PSMA is highly expressed in prostate cancer and the vasculature of most solid tumors, and is currently the target of a number of diagnostic and therapeutic strategies. However, northern analyses using probes directed against PSMA have shown expression of PSMA or a similar gene in some non-prostatic tissues, including brain. We recently determined that a gene homologous to PSMA exists on chromosome 11q14.3. Although the major coding regions of the two genes are 97% identical, we were able to examine the mRNA tissue distribution pattern of the two genes using assays which specifically distinguish between them. The "PSM-like" gene is expressed in a number of tissues, with the highest expression seen in the kidney and liver, and barely detectable expression in the prostate. This is not suprising, as we have also shown that the two genes utilize entirely different promoters and transcription start sites. In addition, the PSMA-like cDNA includes a novel region not found in PSMA transcripts. As such, we can now generate antibodies that specifically target PSMA, avoiding tissues that express the PSMA-like gene. These efforts should assist in development of clinical and therapeutic strategies which specifically target PSMA expressed on prostatic cells, as opposed to a "PSMA-like" gene expressed in non-prostatic tissues.

This work was supported in part by a grant from the A.F.U.D./A.U.A. Research Scholar Program & the C.R. Bard Foundation (D.S.O'K.) & grant PC990017 from the U.S. Army (D.S.O'K.), & NIH grant DK/CA47650 (W.D.W.H.).

314 Alterations in cellular biology in prostate cancer is similar in black and white men

EO Olapade-Olaopa, O Ogunbiyi, EH MacKay, DK Moscatello, OB Shittu, AJ Wong, TR Terry, and FK Habib. Leicester General Hosp., Leicester UK, Univ. College Hosp. Ibadan, Nigeria, Kimmel Cancer Inst., Philadelphia, USA, and Western General Hosp. Edinburgh. UK.

Introduction: Prostate cancer (CaP) is more common and more aggressive in blacks, and racial differences in the expression of molecular factors of the disease have been suggested as a possible explanation for this observation. We have recently described alterations in cellular pathways that attend malignant transformation of the prostate in specimens from (UK) white men. These are the expression of a variant epidermal growth factor receptor (EGFRvIII) by CaP epithelium (Olapade-Olaopa *et al. Br J Cancer* 1999 [In press]), and the loss of androgen receptor (hAR) expression in the nuclei of adjacent stroma (Olapade-Olaopa *et al. Clin. Cancer Res.*, 5: 569-576, 1999). In this present study we have evaluated the presence of these mechanisms in prostatic specimens from black men.
Methods: Paraffin-sections from 36 benign prostatic hyperplastic (BPH) glands (19 white and 17 black) and 71 CaP glands (38 white and 33 black) were immunostained with anti-EGFRvIII antibody. 26 BPH (17 white and 9 black) and 50 CaP (32 white and 18 black) were also stained for hAR.
Results: EGFRvIII expression was seen in BPH and CaP glands, but staining was much stronger and more widespread in CaP sections in both races. There was no statistical significant difference between mean EGFRvIII scores in black and white specimens (BPH [p = 0.85], CaP [p = 0.92]). In addition, there was similar heterogeneous hAR expression in epithelial cells in BPH and CaP glands in both groups [p = 0.49]. However, hAR was not expressed by the stromal cells surrounding malignant glands in 3/18 UK (17%) and 4/32 African (12.5%) CaP sections (UK vs African [p = 0.4]). In contrast there was hAR-positivity of stromal cells surrounding BPH glands in BPH sections (CaP stroma vs BPH stroma [p = <0.0001])
Conclusions: Alterations in EGFR and androgen receptor expression in stromal and epithelial cells in CaP are similar in black and white men. Although these changes represent potential mechanisms for the development of hormone resistant disease, they do not account for differences in epidemiology of the disease between the two races. The more aggressive biology of prostate cancer observed in black men may thus be due to changes in other unidentified genetic or environmental factors.

315 Common Somatic Mutations in the Human Steroid 5α-Reductase Type 2 (*SRD5A2*) Gene in Prostate Cancer

Juergen K. V. Reichardt and Abebe Akalu
Institute for Genetic Medicine and Department of Biochemistry & Molecular Biology, USC Keck School of Medicine, Los Angeles, CA 90033

Prostate cancer is known to be an androgen-dependent disease. Thus, somatic mutations in androgen metabolic genes may play a role in prostate cancer progression. We have, therefore, systemtically analyzed the human prostatic (or type 2) steroid 5α-reductase enzyme encoded by the *SRD5A2* gene by automated DNA sequencing. This enzyme catalyzes the irreversible conversion of testosterone to dihydrotestosterone (DHT), the most active androgen in the prostate.

We have sequenced the entire protein coding region of this locus in 30 microdissected prostate adenocarcinomas. We identified a total of 18 *de novo* amino acid substitutions in 13 of these tumors. We also identified 6 additional polymorphic substitutions. Three of the missense substitutions and one polymorphic change are recurrent suggesting that they may play an important role in prostate cancer progression. Similar analyses of the matched constitutional ("germline") DNA were performed to verify that the somatic mutations in fact occurred *de novo*.

In summary, 60 % of the tumors examined had somatic mutations in the prostatic steroid 5α-reductase coding region. These data, therefore, support the proposition that the *SRD5A2* locus is a common target of somatic mutations in prostate cancer. This gene may be an important target in disease progression.

316 Progression to androgen independence can be delayed by inhibiting the expression of TRPM-2, an anti-apoptotic gene.

P. S. Rennie, H. Miyake, C. Nelson, M. E. Gleave, The Prostate Centre, Vancouver General Hospital and the Department of Surgery, University of British Columbia, Vancouver, B.C., Canada, V6H 3Z6

The goal of this study was to determine whether blocking TRPM-2 gene expression could delay progression to androgen independence. Northern analyses of TRPM-2 expression in Shionogi tumors of mice pretreated with and without CA^{2+} channel blockers (CCBs) prior to castration revealed that CCBs inhibited castration-induced apoptosis, tumor regression, and TRPM-2 gene upregulation; illustrating that TRPM-2 is not directly androgen-repressed, but is regulated by apoptotic stimuli. To determine the functional role of TRPM-2 overexpression in vivo, nude mice bearing control or TRPM-2 transfected LNCaP tumors were castrated, and changes in tumor volume and serum PSA levels were measured. Mice bearing Shionogi tumors were castrated, treated i.p. with antisense TRPM-2 oligonucleotides (ODNs) or 2-base mismatch control daily for 15 days, and time to androgen independent (AI) recurrence was determined. Systemic administration of antisense TRPM-2 ODN resulted in more rapid onset of apoptosis and a significant delay of emergence of AI recurrent tumors. In mice with TRPM-2 overexpressing LNCaP tumors, tumor volume and serum PSA levels increased 2-3 times faster after castration compared to mice bearing control LNCaP tumors. Collectively, these findings illustrate that TRPM-2 is an anti-apoptotic, rather than an androgen-repressed, gene that confers resistance to androgen withdrawal when overexpressed, and that reduction of TRPM-2 gene expression by antisense TRPM-2 ODN can enhance castration-induced apoptosis and delay progression to androgen-independence.

Grant Acknowledgment: NCIC/Terry Fox Program Grant

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[127] CO-INCUBATION OF MICROVASCULAR ENDOTHELIAL CELLS WITH BREAST CANCER CELL LINE STIMULATES PSMA-EXPRESSION IN VITRO.

Frank Richter, Denise S. O'Keefe, Dean J. Bacich, Atsushi Uchida, Maria Bisogna, Warren D. W. Heston New York, NY

INTRODUCTION: Immunhistochemistry of tissue sections from several malignomas revealed the presence of PSMA within tumor neovasculature, but not within benign vascular endothelial cells. In-vitro investigation of cultured vascular endothelial cell lines should determine, if and under what condition these cell lines express PSMA.
METHODS: Five primary vascular endothelial cell lines (HUVEC, HMVEC-L, UtMVEC-Myo, HMVEC-dAd, HMVEC-dNeo) were cultured in a medium containing VEGF, FGF and EGF. RT-PCR was used to search for expression of PSMAmRNA. Western blots using the antibodies J591 (extracellular PSMA-domain) and CYT351 (intracellular PSMA-domain) were used to show PSMA-protein expression.
RESULTS: RT-PCR with RNA isolated from vascular endothelial cells did not demonstrate PSMA expression. However, when microvascular endothelial cells were co-incubated with the breast cancer cell line MCF-7, RT-PCR showed PSMAmRNA-expression. Western blots demonstrated the expression of PSMA protein in co-incubated microvascular endothelial cells. The expression of PSMA could be suppressed by co-culturing MCF-7 with microvascular endothelial cells within the same medium, but separated by a semi-permeable membrane on transwell plates.
CONCLUSIONS: Malignant cells are able to stimulate microvascular endothelial cells to express PSMA. Direct cell-cell interaction and the presence of growth factors seem to be necessary to achieve endothelial cell stimulation.
Supported by: NIH, NIDDKD/NCI47650, and CaP CURE

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genic lesion often present in HIV infected patients. HHV-8 genome encodes a viral Bcl-2 homologue and Bcl-2 expression has been found in AIDS-related KS-derived as well as in cells derived from iatrogenic and sporadic KS, indicating the importance of bcl-2 in the pathogenesis of KS. Our purpose was to explore bcl-2 expression and modulation in our KS-derived cell line and its possible effects in the angiogenic process. We transfected KS-IMM cells with a construct containing a bcl-2 cDNA gene or the vector alone (neo). Different clones from transfected cells were isolated and grown; no difference in the growth curve was found between neo and bcl-2 transfected clones in vitro. Selected clones were injected subcutaneously in nude mice. Tumors arised 28 days after injection. The bcl-2 expressing clone KSB45 showed an average tumor volume five times larger than the corrisponding tumors caused by neo clones. The histological analysis on clone 45 showed an enhancement of formation of new blood vessels in the tumors grown from clone KSB45 injection. Our study shows that Bcl-2 favours angiogenesis without affecting cell survival.

#5051 BREAST CANCER CELL LINE STIMULATES PSMA-EXPRESSION IN MICROVASCULAR ENDOTHELIAL CELLS. Frank Richter, Denise S O'Keefe, Dean J Bacich, Atsushi Uchida, Maria Bisognia, and Warren D W Heston,

INTRODUCTION: Immunhistochemistry of tissue sections from several malignomas revealed the presence of PSMA within tumor-neovasculature, but not within benign vascular endothelial cells. In vitro- investigation of cultured vascular endothelial cell lines should determine, if and under what condition these cell lines express PSMA. METHODS: Primary vascular endothelial cell lines (HUVEC, HMVEC-L, UtMVEC-Myo, HMVEC-dAd, HMVEC-dNeo) were cultured in a medium containing VEGF, FGF and EGF. RT-PCR was used to search for expression of PSMA-RNA, Western blots using the antibodies J591 (extracellular PSMA-domaine) and CYT351 (intracellular PSMA-domaine) were used to show PSMA-protein expression. RESULTS: RT-PCR with RNA isolated from vascular endothelial cells did not demonstrate PSMA-expression. However, when microvascular endothelial cells were coincubated with the breast cancer cell line MCF-7, RT-PCR showed PSMA-RNA-expression. Western blots demonstrated the expression of PSMA-protein in coincubated microvascular endothelial cells. CONCLUSIONS: Malignant cells are able to stimulate microvascular endothelial cells to express PSMA. Direct cell-cell interaction and the presence of growth factors seem to be necessary to achieve endothelial cell stimulation.

#5052 PROSTATE ADENOCARCINOMA CELLS RELEASE THE PRO-INFLAMMATORY POLYPEPTIDE EMAP-II IN RESPONSE TO STRESS. J Clifford Murray, Georgina Barnett, Maarten P Tas, Anne-Mette Jakobsen, and James Carmichael, *CRC Dept of Clin Oncology, Nottingham Univ, Nottingham, United Kingdom*

Endothelial-monocyte activating polypeptide-II (EMAP-II) is a novel molecule with pleiotropic activities toward endothelial cells, monocyte/macrophages and neutrophils. Based upon the cDNA sequence, EMAP-II is predicted to occur intra-cellularly as a 34kDa precursor, which is cleaved and released from the cell in a 20kDa form by an unknown mechanism. The amino acid sequence at the cleavage site suggests a caspase-like enzyme may be involved. Furthermore, it has been shown that apoptosis is a stimulus for the release of EMAP-II by murine cells. We studied the effects of chemical and physiological stresses on the expression of EMAP-II in two human prostate adenocarcinoma cell lines, LNCaP and DU145, using western blotting and ELISA with polyclonal antibodies raised against recombinant human EMAP-II. We detected 34kDa EMAP-II intra-cellularly in both cell types. We then treated the cells with chemical agents known to induce apoptotic or necrotic cell death. EMAP-II was processed and released in response to potent inducers of apoptosis, such as ionomycin, thapsigargin and camptothecin. Antimycin-A and saponin, which induce necrosis, also stimulated release. Hypoxia, which induces primarily necrotic cell death in these cell lines, was also a very powerful stimulus for EMAP-II processing and release. We conclude that EMAP-II may become biologically available and active following a variety of stresses. The release of EMAP-II may impact on the physiology of solid tumors and their response to treatment.

#5053 THE METASTASIS-ASSOCIATED MTS1(S100A4) PROTEIN IS AN ANGIOGENIC FACTOR. Noona Ambartsumian, J. Klingelhofer, M. Grigorian, Y. Cao, R. Cao, M. Kriajevska, V. Berezin, E. Bock, and E. Lukanidin, *Danish Cancer Society, Copenhagen, Denmark, Karolinska Inst, Stolkholm, Sweden, and Panum Institute, Univ of Copenhagen, Copenhagen, Denmark*

Neovascularization of blood vessels is important for rapid growth and metastasis of solid tumours. The involvement of Mts1(S100A4) Ca^{++}-binding protein in tumour progression and metastasis has been demonstrated *in vitro* and *in vivo* using *mts1 (S100A4)* hybrid transgenic mice. However the mechanism by which *mts1(S100A4)* promote metastasis remains unknown. Here we demonstrate that *mts1(S100A4)* is able to act as an angiogenic factor *in vivo* and *in vitro.* High incidence of hemangiomas benign tumours of vascular origin, was observed in aged transgenic mice ubiquitously expressing the *mts1(S100A4)* gene. Furthermore, the amount of Mts1 (S100A4) protein in serum increased with ageing. Purified Mtsl(S100A4) protein stimulated neovascularization of mouse corneas *in vivo* and increased endothelial cell motility *in vitro.* Since tumour cells *in vitro* release the Mts1(S100A4) and since transgenic mouse tumours expressing Mts1(S100A4) protein are characterised by enhanced vascular density the obtained data allow us to conclude that the mechanism of action of *mts1(S100A4)* gene in tumour progression is stimulation of angiogenesis.

#5054 CXCR2 CHEMOKINE RECEPTOR EXPRESSION AND CELLULAR LOCALIZATION ON HUMAN ENDOTHELIAL CELLS. Seth Berman, Eugene Maxwell, Mike Malkowski, Rich Bond, Bimal DasMahapatra, Jonathan Pachter, and Lydia Armstrong, *Schering-Plough Res Inst, Kenilworth, NJ*

IL8 and related ELRCXC chemokines stimulate the migration of leukocytes from the blood toward sites of inflammation. Studies suggest that the chemokine receptor CXCR2 which is present on leukocytes is also present on endothelial cells, thereby playing a role in endothelial cell migration, proliferation, and angiogenesis. CXCR1 and CXCR4 chemokine receptor expression on human endothelial cells has been documented (Finn et. al. 1999) but only the mRNA for the CXCR2 chemokine receptor in human endothelial cells has been observed. In this study, CXCR2 receptor expression was observed on human umbilical vein endothelial cells (HUVEC) and dermal microvascular endothelial cells using western analysis of membrane fractions. To show localization of CXCR2 we used flow cytometric analysis and confocal microscopy. While localization of CXCR2 by immunofluorescence microscopy appeared membrane associated, there were also significant levels of intracellular CXCR2 in HUVECs. Using flow cytometry, surface expression of CXCR2 on HUVECs was compared to 293 cells transfected with CXCR2. Using various antibodies which recognize either an extracellular domain or intracellular domain of CXCR2, we demonstrated that cell surface expression of CXCR2 on HUVECs is relatively low compared to the transfected cells. However, when cells were first permeabilized with triton-X then assayed for CXCR2, our results show significant amounts of CXCR2 present on the inside of the cell. Migration towards IL8 was time and concentration dependent which suggests that CXCR2 is functional on HUVECs. These data demonstrate that CXCR2 is present in vascular endothelial cells but the level of surface expression may be regulated by angiogenic stimulators such as IL8.

#5055 EXTRACELLULAR MATRIX PROTEIN 1 (ECM1) IS A NOVEL ANGIOGENIC FACTOR EXPRESSED BY BREAST CANCER TUMOR CELLS. Zeqiu Han, Jian Ni, Patrick Smits, Charles B Underhill, Bin Xie, Ningfei Lui, Przenko Tylzanowski, David Parmelee, Ping Feng, Ivan Ding, Feng Gao, and Reiner Gentz, *Human Genome Sci, Inc, Rockville, MD, Lombardi Cancer Ctr, Georgetown Univ, Wanhington, DC, Rochester Univ Med Ctr, Rochester, NY, Univ of Leuven, Leuven, Belgium, and Universiteitsplein1, Wilrijk, Belgium*

In this study, ECM1, a newly described secretory glycoprotein, was found to promote angiogenesis. This was initially suggested by *in situ* hybridization studies of the ECM1 message in mouse embryos revealed that it was associated with blood vessels and its distribution was similar to flk-1, a recognized marker for endothelium. Furthermore, ECM1 could bind to heparin and hyaluronan, a property shared by other angiogenic factors. In importantly, highly-purified recombinant ECM1 stimulated the proliferation of cultured endothelial cells and promoted blood vessel formation in the chorioallantoic membrane of chicken embryos. Immunohistochemical staining with specific antibodies indicated that ECM1 was expressed by the human breast cancer cell lines MDA435 and LCC15, both of which are highly tumorigenic. The staining of tissue sections from patients with breast cancer revealed that ECM1 was present in a significant proportion of primary and secondary tumors. Collectively, the results of this study suggest that ECM1 is an angiogenic factor that may promote tumor progression.

IMMUNOLOGY/PRECLINICAL AND CLINICAL 16: Tumor Vaccines: Novel Antigens and Vectors I

#5056 DNA VACCINATION OF BRAIN TUMOR BEARING MICE WITH AUTOLOGOUS TUMOR CELLS, PRODUCING VARIOUS CYTOKINES. G. Safrany, K. Lumniczky, B. Szende, Egon J Hidvegi, and H. Hamada, *Cancer Institute, Tokyo, Japan, National Res Institute for Radiobiology and Radiohygiene, Budapest, Hungary, and Semmelweis Univ Med Sch, Budapest, Hungary*

To develop adjuvant therapy for glioma patients vaccination by autologous cancer cells, producing various cytokines was studied and the combination of the DNA vaccination with irradiation of tumor. The induced immunological processes were also investigated. Glioma 261 tumor cells were grown in vitro and transduced with adenoviral vectors containing various mouse cytokine genes. Mice were transplanted with glioma 261 cells intracranially (i.c.), vaccinated with cytokine gene transduced autologous, irradiated tumor cells and survival followed. About 20–40% of glioma bearing mice were cured by vaccines producing IL-2, IL-4, IL-12, GM-CSF, resp. The IL-6, IL-7, TNF_α, LIF, LT proved to be ineffective. Combination of IL-4 and GM-CSF vaccination has not potentiated the effect of either cytokine alone. However, combination of vaccination with X-irradiation of i.c. growing tumor substantially improved survival (70–100%). Vaccination induced specific activation of cytotoxic lymphocytes against glioma 261 tumor,

protection from FdUrd-induced cytotoxicity in hTS.HA adenovirus-infected cells correlated with protection from FdUrd-induced TS inhibition, as determined by an in situ TS assay, there was no difference in basal in situ TS activity between the hTS.HA-expressing and control cells, suggesting the hTS.HA protein may be catalytically inactive in the intact cell. Further studies will characterize the activity of pADloxP.hTS.HA in vivo.

#2410 SYNERGISTIC EFFECTS OF TEMOZOLOMIDE TREATMENT AND HERPES-SIMPLEX-VIRUS THYMIDINE KINASE (HSV-TK)/GANCICLOVIR GENE THERAPY IN MALIGNANT GLIOMA. Carsten Fels, T.-C. Chou, C. Schaefer, C. M Kramm, and N. G Rainov, *H -Heine-University, Duesseldorf, Germany, Martin-Luther Univ, Halle, Germany, and Memorial Sloan-Kettering Cancer Ctr, New York, NY*

Gene therapy for glioma with the HSV-TK/Ganciclovir (GCV) system is in the stage of clinical trials, but still needs improvement to achieve higher efficacy. Temozolomide (TMZ) is an alkylating drug proven to be efficient in glioma, and is well tolerated in high doses. The purpose of this study was to determine whether combining HSV-TK/GCV with TMZ would result in enhanced antumor effects in glioma. U87 glioblastoma (GBM) cells were transfected with *HSV-tk* cDNA. HSV-TK cells and control cells were treated with different concentrations of GCV, TMZ, or both for 4 days. Viability was accessed by MTT-assay. The multiple-drug effect analysis method of Chou-Talalay was used to measure the interactions between the two drugs. IC_{50} for GCV was 511 μM for control U87 cells and 14.3 μM for U87-TK cells, resulting in 35.7-fold increase of toxicity in HSV-TK expressing cells. TMZ had an IC_{50} of 22.7 μM in control cells and 3.75 μM in U87-TK cells, resulting in 6-fold increase in HSV-TK expressing cells. GCV and HSV-TK/GCV actions were synergistic in both control and U87-TK cells. In control cells, the combination index CI was below 1 for all *Fa* (affected cell fraction) values, indicating synergism at all effect levels. In U87-TK cells, CI was below 1 for *Fa* higher than 0.4, indicating synergism at higher effect levels. In conclusion, these data demonstrate for the first time that HSV-TK-expressing GBM cells are significantly more sensitive against TMZ than control cells. Furthermore, HSV-TK/GCV gene therapy of GBM in culture is synergistically enhanced by TMZ treatment. These findings seem to be of clinical importance and are currently verified in an animal model of human GBM.

#2411 THE ROLE OF MAP KINASES IN GCV-MEDIATED TUMOR CELL DEATH IN THE HSV-TK SYSTEM. Katharine Whartenby, J W Darnowski, S. M Freeman, and P. Calabresi, *Brown Univ, Providence, RI, and Rhode Island Hosp, Providence, RI*

The mechanism of action of tumor cell killing by ganciclovir (GCV) in the Herpes Simplex Virus thymidine kinase (HSV-TK) system is unclear. Our previous studies showed that interferon alpha (IFN) could synergistically enhance the killing of tumor cells, and caused disruption in a number of biochemical and metabolic parameters. We have been investigating a nuclear target for these agents to better understand their mechanism. Our present studies show that intracellular pools of cyclic GMP are significantly increased after exposure to GCV and IFN and that preventing the increase in cGMP decreased their cytotoxicity. Because cGMP is known to regulate activity of the MAP kinases, which are important in cell growth, differentiation, and survival, we investigated the effect of GCV and IFN on the activity of Erk 1 and Erk 2. Preliminary data show that Erk1/2 activity is decreased in HSVTK expressing cells exposed to GCV with and without IFN. Current studies are assessing the effects on JUN kinase and p38 to determine whether these MAP kinases are increased. Understanding the target for these agents may allow for a means to improve their efficacy as anti-cancer agents. (supported by the TJ Martell Foundation).

#2412 USE OF MULTICELLULAR LAYER CULTURES TO QUANTIFY AND OPTIMISE BYSTANDER EFFECTS RESULTING FROM PRODRUG ACTIVATION IN TUMOURS. William R Wilson, Susan M Pullen, Alison Hogg, Kevin O Hicks, Frederik B Pruijn, and William A Denny, *The Univ of Auckland, Auckland, New Zealand*

The efficacy of GDEPT (gene-directed enzyme-prodrug therapy) and other prodrug activation strategies depends critically on the bystander effect(BE)resulting from diffusion of cytotoxic metabolites from enzyme-expressing cells. This study quantifies BE due to activation of dinitrobenzamides (CB 1954 and analogs) by the *E. coli* nitroreductase NTR, and tests three hypotheses: (a) that BE efficiency is higher in 3D cultures (multicellular layers, MCL) than 2D (monolayer) cultures; (b) that BE in MCL is predictive of BE in tumors; (c) that prodrugs with more lipophilic metabolites will have larger BE. BE was quantified by clonogenic assay using monolayer and MCL cultures (V79 and WiDr backgrounds) or xenografts (WiDr) comprising mixtures of NTR transfectants ("activators") and NTR-ve cells ("targets"). Both activators and targets carried selectable markers, allowing determination of prodrug concentration (C_{10}) or dose (D_{10}) for 10% survival of each cell line in the mixtures. BE, quantified as C_{10} or D_{10} activators/targets, was much greater in stirred MCL than either stirred or unstirred monolayers. The magnitude of the BE in MCL showed a close correlation with that in tumors, with larger BE for nitrogen mustard analogs of CB 1954 and lower BE for analogs with basic or polyhydroxy sidechains. Structure-activity relationships for BE are consistent with metabolite lipophilicity *and* reactivity as being important determinants. MCL provide a tissue culture model for the tumour microenvironment (3D cell contact, tissue-like diffusion barriers, a stirred interface that simulates the effects of blood flow in washing out metabolites) in which bystander effects resulting from prodrug activation can be quantified and optimised.

#2413 PROSTATE-SPECIFIC SUICIDE GENE THERAPY USING THE NEWLY DISCOVERED PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) ENHANCER. Atsushi Uchida, Denise S O'Keefe, Dean J Bacich, Fujiko Watt, Peter L Molloy, and Warren D W Heston, *CSIRO Div of Molecular Sci, North Ryde, Australi, and The Cleveland Clin Fdn, Clevland, OH*

PSMA is expressed by both benign and malignant prostatic epithelium and up-regulated by androgen deprivation. Therefore, PSMA is an attractive therapeutic target for advanced prostate cancer. Recently, the enhancer driving prostate-specific expression of the PSMA gene was loned by Watt *et al.* (unpublished data). We then subcloned a number of enhancer-deletion constructs and tested them for maximum activity. We subsequently cloned the most active region of the enhancer into a plasmid containing the PSMA promoter driving expression of the suicide gene cytosine deaminase (CD) from *E.coli*. Specificity of this technique was examined *in vitro* using the prostate cancer cell line C4-2 and breast cancer cell line MCF-7. The cells were transiently transfected and the IC50 of 5-fluorcytosine (5-FC) and 5-fluorouracil (5-FU) of the cells was determined by the MTS assay. Toxicity of 5-FC on each transfected cell line was compared to that with parental cell line. The IC50 of 5-FU and 5-FC were 5–10 uM and 10–20 mM, respectively on untransfected C4-2 as well as MCF-7. Enhanced toxicity of 5-FC in tranfected C4-2 cells was shown down to an IC50 of 300 uM in a dose dependent manner. On the other hand, MCF-7, which does not express PSMA, was not significantly sensitized by transfection. Transfection efficiency was 10% and 15%, respectively. A significant cytotoxicity was shown specifically in PSMA expressing cells, due to a bystander effect. We are currently using this construct *in vivo* against the C4-2 and LNCaP prostate tumor models, with the ultimate aim of developing a treatment for hormone refractory prostate cancer. This work was in part by NIH grant DK/CA47650 (W.D.W.H.).

#2414 COMBINATION GENE THERAPY USING ANTISENSE STRATEGY FOR ADVANCED PROSTATE CANCER. K. C Balaji, D Bacich, D O'Keefe, and W D Heston, *Memorial Sloan-Kettering Cancer Ctr, New York, NY, and Southern Illinois Univ Sch of Med, Springfield, IL*

Chemotherapy is of limited value in advanced prostate cancer and therefore there is a critical need to develop newer therapeutic strategies. Several distinct genetic abnormalities recently described in advanced prostate cancer can be used as targets for antisense treatment. Others and we have demonstrated that antisense to two of the commonly upregulated protooncogenes in advanced prostate cancer, c-myc and Bcl-2, independently inhibited growth and viability. We studied whether combining antisense molecules against both c-myc and Bcl-2 will be more effective in controlling prostate cancer cell growth than either of them alone. A 1-kb antisense sequence against the translation initiation site of c-myc or Bcl-2 was cloned into a pCR 3.1 plasmid (GIBCOBRL Inc.). The androgen independent metastatic prostate cancer C4-2 cells were transfected with the plasmids using Lipofectamine Plus®. The MTS viability assay and flow cytometry were used to study the effects of treatment. The absorbency at 490 nm is shown in the figure. The c-myc antisense reduced cell viability of C4-2 cells in vitro by about 30% compared to the controls, including sense control. While the Bcl-2 antisense caused a similar decrease of about 30% viability, the Bcl-2 sense caused a similar decrease as well, suggesting a non-specific response. However, the combination of antisense to c-myc and Bcl-2 significantly reduced the viability by 70% compared to sense and plasmid only controls. Flow cytometry did not show any difference in the cell cycle phases between the treatment groups suggesting increased cell death as the primary effect of treatment. In conclusion, our data suggests that combination of c-myc and Bcl-2 antisense is more effective in decreasing viability of C4-2 prostate cancer cells *in vitro*, compared to either of the antisense alone or no treatment.

#2415 REGRESSIONS OF ESTABLISHED BREAST CARCINOMA XENOGRAFTS USING CARBOXYPEPTIDASE G2 SUICIDE GENE THERAPY AND THE PRODRUG CMDA ARE DUE TO A BYSTANDER EFFECT. Caroline J Springer, Robert A Spooner, Stephen M Stribbling, Frank Friedlos, Janet Martin, Lawrence C Davies, and Richard M Marais, *Institute of Cancer Res, London, United Kingdom,*

The role of the bystander effect in suicide gene therapy was examined with carboxypeptidase G2 (CPG2) and CMDA in the treatment of a human breast carcinoma xenograft, MDA MB 361. Cells expressing stably an enzymatically-active surface-tethered bacterial CPG2 (stCPG2(Q)3) were mixed with control β-galactosidase (lacZ)-expressing cells to give stCPG2(Q)3: lacZ ratios respectively of: group 1, 0:100; group 2, 10:90; group 3, 50:50; and group 4, 100:0. Four days after injection into nude mice, the prodrug 4-[2-chloroethyl)(2-mesyloxethyl) amino]benzoyl-L-glutamic acid (CMDA) was administered. Tumor growth delay correlated well with the levels of stCPG2(Q)3 expression: group 1, 0 day; group 2, 10 days; group 3, 16 days; and group 4, 90 days delay. Similarly, the number of cures was strongly correlated to the level of stCPG2(Q)3 expression: group 1, 0% cured; group 2, 17% cured; group 3, 50% cured and group 4, 67% cured. There was a good correlation between CPG2 activity in the tumors and the numbers of cures. Immunohistochemical staining showed that the majority of cells from groups 2 and 3 were apoptotic whilst those from group 1 were not, indicating a

issues we have used SAGE (Serial Analysis of Gene Expression) to obtain global gene expression profiles of three ovarian tumors, four cell lines, a pool of cell lines, one cystadenoma, and normal human ovarian surface epithelium (HOSE). Sequencing 12,091 clones from six of the SAGE libraries generated a total of 215,499 transcripts. This corresponds to 56,696 genes identified, 18,266 of which are represented by at least two tags. Comparing the SAGE-generated expression profiles between ovarian cancer and HOSE has provided us with an abundance of candidates to be pursued for use as clinical markers. To confirm the SAGE comparisons, fifteen differentially expressed genes were further characterized by Northern blot analysis and immunohistochemistry. Interestingly, genes implicated in the immune response, proliferation regulation, and protein folding were identified, many of which encode membrane localized or secreted proteins. In depth analyses of these genes should permit the identification of reliable serological and molecular genetic markers to aid diagnosis, increase our knowledge of the biology of ovarian cancer, and eventually lead to mechanism-based therapies.

#1976 COMBINED SAGE AND ARRAY TECHNOLOGIES IDENTIFY GENES DIFFERENTIALLY EXPRESSED IN BREAST CANCER. Saraswati V Sukumar, Mariana Nacht, Anne T Ferguson, and Stephen L Madden, *Genzyme Molecular Oncology, Framingham, MA, and Johns Hopkins Oncology Center, Baltimore, MD*

We demonstrate the strength of combining two approaches, Serial Analysis of Gene Expression (SAGE) and DNA arrays, to help elucidate pathways in breast cancer progression by finding genes consistently expressed at different levels in primary breast cancers, metastatic breast cancersm, and normal mammary epithelial cells. SAGE profiles of 21PT and 21MT, two well characterized breast tumor cell lines, were compared with SAGE profiles of normal breast epithelial cells to identify differentially expressed genes. A subset of these candidates was then placed on an array and screened with clinical breast tumor samples to find genes and ESTs that are consistenly expressed at different levels in diseased and normal tissues. In adition to finding the predicted overexpression of known breast cancer markers HER-2/neu and MUC-1, the powerful coupling of SAGE and DNA arrays resulted in the identification of genes and potential pathways not previouly implicated in breast cancer. Moreover, these techniques also generated information about the differences and similarities of expression profiles in primary and metastatic breast tumors. Thus, combining SAGE and custom array technology allowed for the rapid identification and validation of the clinical relevance of many genes potentially involved in breast cancer progression. These differentially expressed genes may be useful as tumor markers, prognostic indicators, and may be suitable targets for various forms of therapeutic intervention.

#1977 GENOMIC HYBRIDIZATION TO CDNA MICROARRAYS IDENTIFIES A NOVEL AMPLIFIED GENE, *ZNF133*, IN NEUROBLASTOMA. Mervi Anneli Heiskanen, Michael L Bittner, Yidong Chen, Javed Khan, Kononen Juha, Guido Sautter, Olli P Kallioniemi, Jeffrey M Trent, and Paul S Meltzer, *National Human Genome Res Institute, Bethesda, MD, and Univ of Basel, Basel, Switzerland*

We have developed a gene amplification screening technique that is based on the hybridization of total genomic DNA on cDNA microarrays. In addition to the identification of gene amplifications, this approach also makes it possible to carry out gene expression analysis in parallel on identical cDNA microarrays. In a hybridization of a NGP neuroblastoma cell line DNA on a 1400 element cDNA microarray we identified a novel amplified zinc finger gene, *ZNF133*, that has been mapped to 20p11.2. Amplification was confirmed by fluorescent *in situ* hybridization on NGP interphase nuclei. Hybridization of cDNA from this same cell line on an identical microarray showed that *ZNF133* is also highly overexpressed. Amplification or overexpression of this gene have not been previously reported, but the amplification of this chromosomal region has been detected by comparative genomic hybridization in several different malignancies. We are now in the process of evaluating the frequency of *ZNF133* amplification in tumor tissues by hybrization on tissue microarrays. This example demonstrates the power of microarray technologies for the discovery of genetic abnormalities in tumor cells and for studying these events in a large series of primary tumors.

#1978 DIFFERENTIAL GENE EXRPESSSION BETWEEN ANDROGEN DEPENDENT AND INDPENDENT PROSTATE CANCER CELL LINES. K. C Balaji, D Bacich, D O'Keefe, and W D Heston, *Memorial Sloan-Kettering Cancer Ctr, New York, NY, and Southern Illinois Univ Sch of Med, Springfiled, IL*

Progression to androgen independence is the main cause of death in prostate cancer and currently there is no effective treatment for this disease. Therefore, there is an urgent need to further understand the progression to androgen independence and to develop newer therapeutic strategies. We used the LNCaP and C4-2 cells for our study because similar to androgen independent metastatic prostate cancer in humans, the C4-2 cells are derived from androgen dependent LNCaP cells, proliferate rapidly with or without androgens, produce PSA and are highly metastatic. We isolated mRNA from both these cell lines and studied the differential expression of over 7000 genes using gene microarray chip (Genomesystems Inc. St. Louis, MO). The top five most differentially expressed genes are shown below. Our data highlights several potential candidate genes for further investigation. While some of these are known to play an important role in tumor growth, many are novel to the field of prostate cancer.

Genes ↓ in C4-2 cells	ratio	Genes ↑ in C4-2 cells	ratio
Drosiphila fat facet related protein	7.1	Dipeptidylpeptidase 1V	6
Protein C Kinase (mu)	6	HS mRNA for KIAA0610	6
Homo sapien embryonic lung protein	5.9	Angiopoietin 1	5.6
3-Oxoacid CoA Transferase	4.7	EST (Incyte PD: 3117642)	5
Clathrin, Heavy chain polypeptide	4.5	EST (Incyte PD: 220566)	4.9

#1979 HUMAN PAPILLOMAVIRUS (HPV) TYPE 16 ONCOPROTEINS E6 AND E7 DOWN-REGULATE GENES RESPONSIVE TO TGF-β, INTERFERONS AND NF-κB IN HUMAN KERATINOCYTES. Matthias Nees, J Geoghegan, L Miller, and C D Woodworth, *National Cancer Inst, Bethesda, MD, and NCI Array Facility, Advanced Technology Ctr, Gaithersburg, MD*

High risk HPV-types such as HPV-16 are found in more than 90% of invasive cervical carcinomas. The viral oncogenes E6 and E7 functionally inactivate the tumor suppressor proteins p53 and pRb, respectively, and alter differentiation and growth of cervical keratinocytes. Using the cDNA microarray technology (NCI Oncochip), we identified over 90 cellular genes that were significantly altered by E6 and/or E7 in differentiating keratinocytes (factor >2.5). Most could be grouped in 4 categories: **1.** Genes that are regulated by transforming growth factor beta (TGF-β), including TGF-β2. **2.** Interferons (IFN)-α and -β and IFN-regulated genes. **3.** Genes regulated by the transcription factor NF-κB. **4.** S-phase genes. Treatment of cells containing HPV-16 E6 and E7 with recombinant TGF-β2 or IFN-α or -β restored expression of genes in category 1 and 2, respectively. Treatment of cells with tumor necrosis factor alpha (TNFα), which activates NF-κB, restored expression of genes in categories 3 and some of category 2. A subset of S-phase genes was suppressed by recombinant TGF-β2. We are currently investigating the status of NF-κB activity in cells expressing HPV-16 E6 and E7. Our experiments confirmed the existence of a limited number of master regulatory factors, which are altered in expression or function by HPV oncogenes. Our large-scale analysis of cellular gene expression reveals common mechanisms of gene regulation that are targeted by HPV E6 and E7 oncogenes and might play a role in early stages of cervical carcinogenesis.

#1980 ANDROGEN-INDEPENDENT PROSTATE CANCER GROWTH ANALYZED BY GENE EXPRESSION PROFILING USING CDNA AND TISSUE MICROARRAYS: FROM GENE DISCOVERY TO MOLECULAR PATHOLOGY. Spyro Mousses, Lukas Bubendorf, Juha Kononen, Michael L Bittner, Yidong Chen, Niels Willi, Thomas G Pretlow, Guido Sauter, and Kallioniemi P Olli, *Case Western Reserve Univ, Cleveland, OH, NIH NHGRI, Bethesda, MD, and Univ of Basel, Basel, Switzerland*

Although prostate cancers initially respond to androgen depletion, most of them will eventually progress to become androgen independent. Here, we applied fluorescence based cDNA microarrays containing 6048 transcripts (including 4032 known genes and 2016 ESTs) to identify the gene expression changes involved in this process. Four hormone-sensitive human prostate cancer xenografts (CWR22), 5 xenografts at various stages of regression after castration, and 6 hormone-refractory xenografts (CWR22R) were studied. Pathways and critical genes involved in the tumor regression and relapse were identified by bioinformatics, such as global analysis by multidimensional scaling, and hierarchical gene clustering. In addition, we developed a novel gene clustering algorithm, based on defined profile templates, to identify expression profiles that reflect tumor response to treatment. Several perturbations of functional pathways were implicated by expression changes including increased signal transduction (by MAP-KAP, NFκB, and PKC), steroid receptor deregulation (by HSP-like chaperones), and increased angiogenesis (by decrease of thrombospondin and increased angiogenic factors). Finally, we constructed a tumor tissue microarray (Nat. Med, 4: 844, 1998) with 580 clinical prostate specimens at various stages of progression. Tissue microarrays have helped us to quickly translate alterations identified in the CWR22 model to hundreds of clinical specimens *in vivo*. For example, IGFBP2 and HSP27 proteins were found to be preferentially induced in androgen independent recurrent cancers, and that may contribute to androgen-deprivation therapy failure in patients.

#1981 LINKING GENE EXPRESSION PATTERNS TO THERAPEUTIC GROUPS IN BREAST CANCER. Katherine J Martin, Brian M Kritzman, Laura M Price, Alan Mackay, Michael J O'Hare, Carolyn M Kaelin, George L Mutter, Arthur B Pardee, and Ruth Sager, *Brigham & Women's Hosp, Boston, MA, Dana Farber Cancer Institute, Boston, MA, and LICR/UCL, London, United Kingdom*

A major objective of current cancer research is to develop a detailed molecular characterization or "fingerprint" of tumor cells that is linked to clinical information. This has the potential to help patients by very accurately classifying tumor subtypes, which will improve clinicians' ability to distinguish prognostic groups and predict effective therapies. Toward this end, we have identified approximately one-quarter of all the genes differentially expressed in a breast cancer cell line compared to sorted normal breast epithelial cells using differential display. The cancer cells under-expressed many genes involved in cell adhesion, communication, and maintenance of cell shape, while they over-expressed many synthetic and metabolic enzymes important for cell proliferation. High-density, membrane-

therefore, is to identify prognostic markers that accurately predict outcome at the preinvasive phase. To this end, we applied suppression subtractive hybridization to compare the gene expression between two samples of prostate tumors (pT2; Gleason score 7) obtained from patients who present disease recurrence (n=3), and disease free survival (n=3) during five years of follow-up. Two normalized, reversely subtracted libraries were thus obtained and is presently undergoing characterization. Results to date include 13 individual, unique clones of which 5 have been identified as known genes, including a metalloproteinase, a DNA binding protein of unknown function (chromosome 6), a DEAD box protein (17q23), a homeobox protein (15q14) and a BAC clone of chromosome 16 (16p11.2). Furthermore, the same cohort was screened using the Clontech Atlas Cancer array to examine the differences in expression levels of known genes. Increased gene expression associated with neovascularization, including VEGFR and VEGF was detected in this complimentary study. These results suggest that understanding the patterns of gene expression in early CaP lesions will be of prognostic value and will serve as the basis for the design of future CaP specific gene chips. PCP was supported by a grant from AUA/AFUD.

#247 SPECTRAL KARYOTYPING (SKY) DERIVED MARKERS AS NOVEL TOOLS FOR PROSTATE TUMOR PROFILING AND IMPROVED CLINICO-PATHOLOGICAL EVALUATION. Meena Augustus, Wei Zhang, Tammy Lawrence, Denise Young, Frank Avallone, Fathollah K Mostofi, Judd W Moul, Isabell A Sesterhenn, Thomas Ried, and Shiv Srivastava, *AFIP, Washington, DC, Nci/Nih, Bethesda, MD, Usuhs, Bethesda, MD, and Usuhs-Cpdr, Bethesda, MD*

Rearranged genomes and cytogenetic aberrations are hallmarks of tumorigenic conversion and progression of human cancer. Only recently, SKY is helping to identify chromosome changes of relevance to prostate tumorigenesis. However, multiple, histologically distinct tumors in a given prostate, pose significant problems for accurate prognostication. SKY markers as probes for molecular-cytogenetic tumor profiling could significantly enhance our understanding of prostate tumor heterogeneity, clinical outcome and disease progression. Spectral karyotypes were established for 10 primary prostate cancers, both before and after immortalization with HPV-E6/E7 genes. In situ hybridization using probes for selected chromosomes was performed on whole mount paraffin embedded sections of the prostates from where cells in culture were derived for SKY analysis. Consistent cytogenetic changes involving chromosomes 8, 12, 18 and 20 in the early stages/lower grades to late stage events/higher grades, involving chromosomes 6q, 8q, 10q, 11, 16, 17 and 19 and other occult, cryptic rearrangements were accurately defined by SKY. Recurring sites of chromosomal aberrations seen in one of the many tumor biopsies obtained from a malignant prostate, need not reflect the stage of progression of the disease per se. Tumor heterogeneity is known to complicate the assessment of clinical outcome. Molecular tumor profiling using stage and progression specific markers as was done in this study will help enhance prognostication and management of the patient with prostate cancer.

#248 HUMAN GLANDULAR KALLIKREIN (HK2) AND PROSTATE SPECIFIC ANTIGEN (PSA) IN PROSTATE AND BREAST CANCER. Angeliki Magklara, A. Scorilas, W. J Catalona, and E. P Diamandis, *Mount Sinai Hosp, Toronto, ON, Canada, Univ of Toronto, Toronto, ON, Canada, and Washington Univ Sch of Medicine, St. Louis, MO*

Human glandular kallikrein (hK2) is a serine protease that shares many biochemical and structural properties with prostate specific antigen (PSA). Recent studies indicate that hK2 may be a novel marker for prostate cancer, supplementing me the well established clinical value of PSA and that both kallikreins may play an important role in the physiology of normal and malignant breast tissue. We analyzed 210 serum samples from men with histologically confirmed BPH or CaP with total PSA in the "grey zone" (4–10 ug/L). Statistical analysis showed that the hk2/free PSA ratio (AUC=0.69, p<0.0001) was stronger predictor of CaP than the free/total PSA ratio (AUC=0.64, p<0.001). At the level of 95% specificity, the hK2/free PSA ratio identified 30% of patients who had cancer. Our data suggest that hK2 in combination with free and total PSA can enhance the biochemical detection of prostate cancer in patients with moderately elevated total PSA levels. We also investigated the steroid hormone regulation of hK2 and PSA in several breast cancer cell lines. BT-474 cells produce more hK2 than PSA, whereas the situation is reversed in T-47D cells. From all steroids tested, mibolerone was the most potent stimulator for both kallikreins followed by norgestrel. MFM-223, an androgen responsive cell line devoid of other steroid hormone receptors, was also capable of producing hK2 and PSA but at much lower amounts. MCF-7, ZR-75-1, MDA-MB-435 and BT-20 cell lines failed to produce any protein. Our data suggest that the expression of the hK2 gene in breast cancer cell lines is mainly under the control of androgens and progestins, similarly to PSA. These cell lines could be an important tool in the investigation of the molecular mechanisms that control the expression of the hK2 and PSA genes in-vitro and in vivo facilitating a better understanding of the pathophysiology of steroid hormone-dependent breast tumors.

#249 DISEASE-ASSOCIATED MOLECULAR FORMS OF PROSTATE-SPECIFIC ANTIGEN (PSA) IN THE DISCRIMINATION OF PROSTATE CANCER FROM BENIGN DISEASE. Stephen D Mikolajczyk, T J Wang, L S Millar, H G Rittenhouse, L S Marks, W Song, T M Wheeler, and K S Slawin, *Hybritech Inc , a subsidiary of Beckman Coulter, Inc, San Diego, CA*

The most widely used serum marker for the early detection of prostate cancer (PCa) is prostate-specific antigen, PSA, which is comprised of multiple molecular forms. These include PSA complexed to alpha1-wantichymotrypsin, and several noncomplexed forms of enzymatically inactive, or "free" PSA. Changes in the percentage of free PSA in the serum have been shown to correlate with disease state, which suggests that identification of specific sub-populations of free PSA may improve the diagnostic value of PSA measurements. We have previously reported that proPSA is found in PCa serum, and we now show that proPSA is significantly elevated in prostate tumor compared to benign tissue (median 3% and 0% of total PSA, respectively; p<0.0026). We have also identified a degraded form of PSA called BPSA that is elevated in benign prostatic hyperplasia (BPH) tissues compared to tumor. Our early studies of patient serum using BPSA-specific mAbs have shown significantly elevated levels of BPSA in BPH serum compared to both normal and PCa serum (p<0.007). Together, proPSA and BPSA may be complementary as serum markers to aid in the discrimination of PCa from benign disease.

#250 SUB-CLONING AND EXPRESSION OF TRUNCATED PROSTATE SPECIFIC MEMBRANE ANTIGEN. Nathaniel J Buffington, and Thomas D Schmittgen, *Washington State Univ, Pullman, WA*

Prostate specific membrane antigen (PSMA) is a transmembrane glycoprotein that is highly specific to the plasma membrane of prostate cancer cells. Its expression is upregulated with androgen deprivation and with increasing tumor stage and grade. These characteristics make the antigen an excellent choice for a diagnostic. The object of the current study is to express the extracellular truncate of human PSMA in eukaryotic cells. The part of the gene coding for amino acids 44-750 or the extracellular portion of PSMA was amplified by RT-PCR from LNCaP cells. In order to generate enough of the gene for further analysis, the truncated version of PSMA (tPSMA) was subcloned into the pCR2.1 TOPO TA vector. The truncated gene was then inserted into the pBlueBacHis2 vector. Sf9 insect cells are currently being used to express tPSMA using this baculovirus expression system. The gene has also been inserted into the pIZ expression vector, which allows for eukaryotic protein expression without the use of baculovirus. Polyclonal antibodies to tPSMA are being generated in rabbits. For this purpose, an octamer peptide epitope of tPSMA was synthesized and linked to hemocyanin using glutaraldehyde. After expressing and purifying the recombinant protein in both expression systems, it will be characterized for its enzymatic activity, glycosylation status, and binding affinity for PSMA antibody by Western blotting. Support: CA81396.

#251 CLONING AND CHARACTERIZATION OF THE PROSTATE-SPECIFIC MEMBRANE ANTIGEN-*LIKE* GENE. Denise S O'Keefe, D. J Bacich, and W.D. W Heston, *The Cleveland Clin Fdn, Clevland, OH*

Prostate-Specific Membrane Antigen (PSMA) is a 100kD type II transmembrane protein with folate hydrolase and NAALADase activity. PSMA is highly expressed in prostate cancer and the vasculature of most solid tumors, and is currently the target of a number of diagnostic and therapeutic strategies. However, RNase protection assays and northern analyses using probes directed against PSMA have shown expression of PSMA or a similar gene in some non-prostatic tissues, including brain. We recently mapped the gene encoding PSMA to 11p11.2, and determined that a gene homologous but not identical to PSMA exists on chromosome 11q14.3. Although the major coding regions of the two genes are 97% identical, we were able to examine the mRNA tissue distribution pattern of the two genes using assays which specifically distinguish between them by exploiting single base coding differences. The "PSMlike" gene is expressed in a number of tissues, with the highest expression seen in the kidney and liver, and barely detectable expression in the prostate. This is not suprising, as we have also shown that the two genes utilize entirely different promoters and transcription start sites. In addition, the PSMA-like cDNA includes a novel exon which is not expressed in PSMA mRNA transcripts. As such, we are now able to generate antibodies that specifically target PSMA, avoiding tissues that express the PSMA-like gene. These efforts should assist in development of clinical and therapeutic strategies which specifically target PSMA expressed on prostatic cells, as opposed to a "PSMA-like" gene expressed in non-prostatic tissues. This work was supported in part by a grant from the A.F.U.D./A.U.A. Research Scholar Program & the C.R. Bard Foundation (D.S.O'K.) & grant PC990017 from the U.S. Army (D.S.O'K.), & NIH grant DK/CA47650 (W.D.W.H.).

#252 OVEREXPRESSION OF HYPOXIA-INDUCIBLE FACTOR 1 IN HIGH-GRADE PROSTATIC INTRAEPITHELIAL NEOPLASIA. Colleen F Hanrahan, A M De Marzo, H Zhong, E Laughner, V L Marchi, G L Semenza, and J W Simons, *Johns Hopkins Univ Sch of Medicine, Baltimore, MD*

Hypoxia-inducible factor (HIF-1), a heterodimer composed of α and β subunits, is a basic-helix-loop-helix (bHLH)-PAS transcription factor, that transactivates genes encoding EPO, transferrin, ET-1, iNOS, HO-1, VEGF, IGF-2 and IGFBP-1, -2 and -3, and 13 different glucose transporters and glycolytic enzymes in response to hypoxia. Most of these proteins are implicated in tumor progression. In this study, HIF-1 expression was analyzed by immunohistochemistry in high-grade Prostate Intraepithelial Neoplasia (PIN) which is considered the precursor of a majority invasive prostate carcinoma. HIF-1α was overexpressed in 11 of 14 high-grade PIN lesions identified in a total 10 human prostate biopsies relative to the respective normal epithelium, stromal cells and benign prostatic hyperplasia.

this downregulation is at the transcriptional level. We have cloned the human LAMA3a promoter. Comparison of the human sequence with the murine sequence showed 77% sequence identity. Using this promoter which extends from -1410 to -6 from the initiator codon, we created a series of clones to enable functional analysis of the promoter. These clones include a series of mutants of the LAMA3a promoter inserted upstream from a luciferase reporter gene. We find that the basal promoter extends from -589 to -112 and that -1410 to -589 has modest enhancer activity. We also tested the promoter both in the parental MCF10A cells and cells overexpressing p300 and show that this promoter has decreased activity in cells overexpressing p300. Computer analysis of the promoter using transcription factor databases identifies several potential transcription factor binding sequences. Some of these transcription factors are known to interact with p300 such as AP1, glucocorticoid receptor, c-jun, and AP2 that suggest areas to target for studies on how p300 affects the transcription of this gene.

#121 MECHANISM CONTROLLING GENE EXPRESSION OF FOLATE RECEPTOR β. Bridget Kathleen Dillon, *Med Coll of Ohio, Toledo, OH*

In normal hematopoiesis, folate receptor β (FR-β) expression is restricted to the myelomonocytic lineage, predominantly expressed in mature neutrophils and upregulated in activated monocytes and macrophages. FR-β is upregulated in more than half of all myeloid leukemias. To understand the mechanism for tissue specific expression of FR-β its promoter is being characterized. The proximal promoter of FR-β contains a Sp1 element and two distinct downstream ets binding sites termed EBSI and EBSII. Functional Characterization of the promoter by mutagenesis and EMSA indicate that Sp1 is crucial for basal activity of this TATA-less promoter. EBSII plays a major role in promoting transcription of FR-β, where as EBSI mediates its repression. Regulation of promoter activity mediated by EBSII may occur either by active recruitment of Sp1 or by precluding the binding of a repressor to EBSI. Futhermore EBSI may work in collusion with a novel upstream repressor element. The significance of these control mechanisms in cell-type specific expression of FR-β will be discussed.

MOLECULAR BIOLOGY 4: Gene Regulation in Prostate and Breast Cancer

#122 PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSMA) PROMOTER AND ENHANCER DRIVEN GREEN FLUORESCENT PROTEIN (GFP) EXPRESSION IN TRANSGENIC MICE. Dean J Bacich, D. S O'Keefe, F. B Watt, P. L Molloy, and W.D. W Heston, *CSIRP Div of Molecular Sci, North Ride, Australia, and The Cleveland Clin Fdn, Cleveland, OH*

Prostate-Specific Membrane Antigen (PSMA) is a 100kD type II transmembrane protein with folate hydrolase and NAALADase activity. PSMA is highly expressed normal prostatic epithelium and is upregulated in prostate cancer. In addition PSMA is expressed in the neovasculature of most solid tumors, but not the vasculature of normal tissues. It's unique expression pattern has made it the target of numerous therapeutic strategies for not only prostate cancer but all solid tumors. The identification of the regulatory elements that control its expression in the prostate and/or tumor vasculature is required for the development of gene therapy approaches utilizing PSMA unique expression pattern. Recently, Watt et al., identified an enhancer form the PSMA gene that appears to drive expression specifically in prostatic cell lines. We generated transgenic mice which have this PSMA promoter and enhancer construct driving the expression of Green Fluorescence Protein. We now can identify the organs and cell types that this PSMA promoter/enhancer drives expression in by fluorescence microscopy. The expression pattern of this promoter and enhancer is also being examined in normal murine neovasculature, and in tumor neovasculature. In addition these mice have been crossed with the prostate cancer producing TRAMP mice, to further characterize the enhancer's expression profile in vivo, and the effects that various treatments, such as androgen ablation, has on expression. This work was supported in part by a grant from the NIH grant DK/CA47650.

#123 CREATING CHIMERIC TRANSCRIPTIONAL REGULATORY ELEMENTS THAT GREATLY AUGMENT THE ACTIVITY AND SPECIFICITY OVER THE NATURAL PROSTATE SPECIFIC PSA ENHANCER. Lily Wu, Michael Carey, Jamie Matherly, Arie S Belldegrun, and Jean deKernion, *UCLA Sch of Medicine, Los Angeles, CA*

The goal of this study is to improve the transcriptional activity and specificity of the prostate specific PSA enhancer/promoter such that efficient prostate targeted gene expression can be achieved for use in gene therapy. The PSA enhancer and promoter (PSE) can confer exquisite prostate-specific expression of linked reporter gene, with strong androgen stimulation in *in vitro* transfection of PSA producing prostate cells. However, when inserted into adenovectors the native PSA enhancer/promoter activity is low. Detailed analysis of the enhancer minimal core (-4326 to 3935) clearly established the functional importance of AR binding in this region. Moreover, the cooperative and synergistic binding of AR to the multiple AREs contribute greatly to its androgen responsive activity. We created an artificial regulatory element composed of 4 tandem copies of ARE (ARE4). Insertion of this element into a PSE construct, consisting of -5322 to -2875 and 541 to +11 of PSA gene, increased activity and androgen induction greater than 10-fold in transfections and luciferase assay. Further modification of the enhancer such as duplication of the minimal core and further deleting enhancer sequence from 3935 to 2875 (a construct designated PSEΔB+ARE+core) resulted in 15 to 20 fold augmentation of transcriptional activity in the presence of androgen in LNCaP cells. Moreover, androgen induction is 1746 fold compared to 429 fold of the PSE construct. This construct exhibited >100-fold higher expression in LNCaP cells over non-permissive cells. The prostate specificity is retained even in Hela cells engineered to constitutively express excess AR since expression in this setting is still 20-25 lower than in LNCaP cells.

#124 PROSTATE-SPECIFIC ANTIGEN UPSTREAM ENHANCER IS A TARGET FOR GATA TRANSCRIPTION FACTORS. Carlos Perez-Stable, and B. A Roos, *Miami VA Med Center/GRECC, Miami, FL, and Univ of Miami Sch of Medicine/SCCC, Miami, Fl*

The prostate-specific antigen (PSA) gene is regulated by a far upstream DNA enhancer region containing an androgen-response element (ARE) and by a proximal promoter region containing two AREs. We have investigated DNA elements and transcription factors other than ARE and androgen receptor (AR) that are important in the regulation of the PSA gene. Multiple (7) GATA DNA elements flanking the PSA enhancer ARE are targets for GATA-2 and -3 transcription factors and are essential for optimal androgen induction of transfected PSA enhancer/promoter plasmids in the LNCaP prostate cancer cell line (PSA positive). Four of these GATA sites overlap recently identified low affinity AREs. We have further analyzed other regions in the PSA enhancer and promoter by using electrophoretic mobility shift assays, site-directed mutagenesis, and plasmid transfections into LNCaP, androgen-independent prostate cancer cell lines and the MCF-7 breast cancer cell line. Our results are summarized as follows: 1) PSA enhancer ARE contains overlapping GATA and octamer sites that compete with AR for binding; 2) cotransfection of GATA-2 expression plasmid in LNCaP reduced PSA enhancer/promoter activity 40-fold; 3) cotransfection of AR expression plasmid in MCF-7 (high levels of GATA-3) increased PSA enhancer/promoter activity 150-fold and activated endogenous PSA gene transcription. We propose a model in which the balance of AR, GATA, and possibly octamer factors are important in the regulation of the PSA upstream enhancer.

#125 PROSTATE CANCER PROGRESSION CORRELATES WITH UPaR UPREGULATION. Jareer N Kassis, David A Jones, and Alan Wells, *Huntsman Cancer Institute, Salt Lake City, UT, and Univ of Pittsburgh, Pittsburgh, PA*

The mechanisms by which tumors progress to invasion and metastasis are just now being elucidated, and have centered around autocrine signaling by receptors such as the EGF receptor as well as the key steps of invasion: recognition of the extracellular matrix, proteolytic rearrangement of the ECM, and migration through the ECM. Previous investigations determined a pivotal role for phospholipase C-γ in ligand-induced migration of tumor cells of the breast and prostate, and subsequent studies showed marked inhibition of tumor cell invasion by inhibition of PLCγ both in vitro and in vivo. However, the changes in the cell's proteome that follow activation of PLCγ and are essential for tumor progression are unknown. We hypothesized that PLCγ activation results in specific gene expression alterations that establish the invasive phenotype. Using cDNA microarray technology, we compared the transcriptional profiles of 9216 human genes in DU-145 prostate carcinoma cells that have been engineered to possess different degrees of invasiveness. All three DU-145 sublines form large tumors in vivo, but only those that activate PLCγ are invasive. We have identified 8 transcripts (including 4 novel genes) that inversely change with invasiveness, suggestive of tumor suppressors, and 11 genes (4 novel) that are increased in parallel with invasive potential, implying the role of tumor promoters. The most markedly increased transcript is that of the urokinase receptor (UPaR), which shows an 8-fold increase from the non-invasive c'973 DU-145 cells to the highly invasive WT DU-145 cells, suggesting that UPaR contributes to the invasive phenotype of these cells. The causal linkage of this transcriptional change as well as the operative step in invasion promoted by UPaR signaling are being determined.

#126 INDUCTION OF CELLULAR MORPHOLOGY CHANGES IN DU145 PROSTATE CANCER CELL LINE ASSOCIATED WITH HOXC8 DECREASED EXPRESSION. Y Alami, N Clausse, D Marechal, and V Castronovo, *Univ of Liege, Liege, Belgium*

Homeobox-containing genes (HOX genes) encode a family of conserved transcription factors that are involved in the control of normal development during embryogenesis. Aberrant expression of HOX genes has been associated with both morphological abnormalities and oncogenesis. We have recently observed that HOXC8 mRNA expression is increased in high score prostate adenocarcinoma. To understand the functional role of HOXC8 in prostate cancer, we have downregulated its expression through the use of specific antisense oligonucleotide added to the culture medium of DU145 cells. A reduction of HOXC8 expression was obtained with 10μM of oligo after 72 hr. No effect was detectable using HOXC8-scrambled oligonuleotide as control. We observed that antisense oligonucleotide treatment induced a change in the appearance of DU145 cells: protuberances resulting in more spindle and less rounded shape and a crown cells

TITLE PAGE

Prostate-Specific Suicide Gene Therapy using the Prostate-Specific Membrane Antigen (PSMA) Promoter and Enhancer.

Running Title: Gene Therapy using the PSMA Promoter/Enhancer.

Authors: Denise S. O'Keefe, Atsushi Uchida, Dean J. Bacich, Fujiko B. Watt, Anna Martorana, Peter L. Molloy and Warren D.W. Heston.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest with any of the authors of this paper.

ABSTRACT

Background

Prostate-Specific Membrane Antigen (PSMA) is abundantly expressed in virtually 100% of prostate cancers and metastases. In addition, unlike Prostate-Specific Antigen (PSA), PSMA is up-regulated under conditions of androgen deprivation. Therefore, PSMA is an attractive therapeutic target for advanced prostate cancer. Recently, both the promoter and the enhancer driving prostate-specific expression of the PSMA gene were cloned. We describe here our analysis of the PSMA enhancer for the most active region(s) and present a way of using the enhancer in combination with the E. *coli* cytosine deaminase gene for suicide-driven gene therapy that converts the non-toxic prodrug 5-Fluorocytosine (5-FC) into the cytotoxic drug 5-Fluorouracil (5-FU) in prostate cancer cells.

Methods

Deletion constructs of the full-length PSMA enhancer were subcloned into a luciferase reporter vector containing either the PSMA or SV-40 promoter. The most active portion of the enhancer was then determined via luciferase activity in the C4-2 cell line. We then replaced the luciferase gene with the E. *coli* cytosine deaminase gene in the subclone that showed the most luciferase activity. The specificity of this technique was examined *in vitro* using the prostate cancer cell line LNCaP, its androgen

independent derivative C4-2, and a number of non-prostatic cell lines. The toxicity of 5-FC and 5-FU on transiently transfected cell lines was then compared.

Results

The enhancer region originally isolated from the PSMA gene was approximately 2kb. Deletion constructs revealed that at least two distinct regions seem to contribute to expression of the gene in prostate cancer cells, and therefore the best construct for prostate-specific expression was determined to be 1648 bp long. The IC50 of 5-FC was similar in all cell lines tested (>10mM). However, transfection with the 1648nt PSMA enhancer and the PSMA promoter to drive the cytosine deaminase gene enhanced toxicity in a dose-dependent manner more than 50-fold, while cells that do not express the PSMA gene were not significantly sensitized by transfection.

Conclusions

Suicide gene therapy using the PSMA enhancer may be of benefit to patients who have undergone androgen ablation therapy and are suffering a relapse of disease.

Keywords

Enhancer elements, promoter regions, gene therapy, prodrugs, flucytosine, PSMA

Introduction

Prostate cancer is the second leading cause of cancer death (1). Although the most recent data suggests that the incidence of death from this disease is on the decline due to the advent of the PSA test and subsequent early intervention, once a patient becomes hormone refractory there are few treatment choices available (1). One approach currently being considered is prostate-specific gene therapy followed by prodrug administration. The prodrug 5-fluorocytosine (5-FC) is converted by the bacterial enzyme cytosine deaminase (CD) to 5-fluorouracil (5-FU) (2; 3). 5-FU has already been used for treatment of metastatic prostate cancer, while 5-FC is used in the treatment of fungal infections and is non-toxic to humans. PSMA is highly expressed in prostate cancer and normal prostate (4-6) , and more recently, expression of PSMA had been observed in tumor-associated neovasculature of nearly all solid tumors (7-10).

Expression of PSMA or a "PSMA-Like" gene has also been seen in some other tissues including kidney, liver and brain, although at a much lower level than in the prostate or prostatic carcinoma as determined by northern analyses (6; Bacich et *al.*, unpublished observations). Furthermore, expression of PSMA is upregulated under conditions of androgen deprivation, which makes it a useful marker for patients that have undergone hormonal ablation (6). Accordingly, PSMA is currently being used as a therapeutic and clinical target in a number of strategies against prostate cancer. Recently, our group cloned the PSMA promoter and an enhancer from intron 3 of the PSMA gene that drives prostate-specific expression of reporter genes (11-13). To further our aim of generating a way of targeting prostate cancer and cancer metastases using gene therapy,

we have designed a genetic construct that places expression of the bacterial CD gene under the regulatory control of the PSMA promoter and enhancer.

Materials and Methods

Cell Culture

The prostate cancer cell line LNCaP, the breast cancer cell line MCF-7, colorectal cancer line HCT8 and lung cancer cell line H157 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The androgen independent subline of LNCaP, C4-2, was purchased from UroCor Laboratories (Oklahoma City, OK). LNCaP and HCT8 cells were grown in RPMI 1640 (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (FBS). C4-2 and MCF-7 were grown in DMEM (Life Technologies, Rockville, MD) containing 10% FBS. H157 cells were grown in RPMI 1640 with 2mM L-glutamine adjusted to contain 1.5g/l sodium bicarbonate, 4.5g/l glucose, 10mM HEPES, 1.0mM sodium pyruvate with 10% FBS. All cells were grown in the absence of antibiotics.

Cloning of the Enhancer Deletion Constructs

The promoter of the PSMA gene was cloned into the pGL3-Basic vector (Promega, Madison, WI) as previously described to form pGL3-B-PSM (11). Deletion constructs of the PSMA enhancer were produced using primers with an artifically incorporated *Bam*HI restriction endonuclease recognition site. The following primer sets were used to amplify genomic DNA NA1944 (Coriell Cell Repositories, Camden, NJ). This DNA was derived from a somatic cell hybrid containing retaining human 11pter>cen translocated to a hamster chromosome, and therefore does not contain the

PSMA-Like gene. PCR was carried out under standard conditions. Following PCR, the resultant products were digested with *Bam*HI and subject to gel electrophoresis followed by gel purification. The gel purified products were cloned into pGL3-B-PSM or pGL3-promoter vector (containing the SV-40 promoter and no enhancer; Promega, Madison, WI), which had been digested with *Bam*HI and treated with alkaline phosphatase. The primer sets used were:

S 14704 5' CGCGGATCCGCCTTCTAAAATGAGTTGGG 3'

With each of the following primers:

AS 15205 5' CGCGGATCCCAACATAGTGGAACCACGTC 3' (501 bp)

AS 15573 5' CGCGGATCCTGAGAAAAGATTGCCAACGC 3' (869 bp)

AS 15994 5' CGCGGATCCATTAGGTTCCAAAGGAAGCC 3' (1290 bp)

AS 16352 5' CGCGGATCCGGCTACTACATAAGTATAAGTC 3' (1648 bp)

AS 16617 5' CGCGGATCCATGACACCAAAGCTTTAGGG 3' (1913 bp)

The artifically incorporated *Bam*HI restriction sites are underlined.

Analysis of the PSMA enhancer constructs

Activity of the PSMA enhancer constructs was determined using luciferase as a reporter gene following transient transfection. C4-2 and MCF-7 cells were used as positive and negative cells respectively to determine activity as tissue specificity of the entire enhancer region has already been shown (13). The day before transfection, 50,000

C4-2 and 75,000 MCF-7 cells were plated into 24-well plates. Cells were then transfected with the 400ng of each enhancer construct, using Lipofectamine Plus Reagent (Life Technologies, Rockville, MD), according to the manufacturer's instructions, with the exception that the DNA/Lipofectamine complexes were allowed to remain on the cells overnight. Approximately 40 hours after transfection, the cells were harvested and analysed for reporter activity using the Dual Luciferase Assay Kit (Promega, Madison, WI). All experiments were carried out in triplicate and each well was co-transfected with 100ng of pBIND (Promega, Madison, WI), which is a plasmid carrying the *Renilla* Luciferase gene under the control of the SV-40 promoter and enhancer, to control for transfection efficiency.

Cloning of the Cytosine Deaminase Gene Construct

The plasmid pCD containing the cytosine deaminase gene from E.*coli* was kindly provided by Dr. I. Yoshimura (Department of Urology, National Defence Medical College, Saitama, Japan). The gene was modified to possess a eukaryotic translation initiation signal. The luciferase gene in the enhancer construct showing the most reporter gene activity, pGL3-B-PSM-Enh1648, was removed by restriction enzyme digestion with *Xba*I and replaced with the CD gene from pCD (the 1.5kb CD gene from pCD was excised using *Xba*I and *Spe*I digestion). *Xba*I digestion actually removes some of the PSMA promoter sequence, but this removal of this region has no effect on promoter activity (O'Keefe et *al.*, unpublished observations). Subsequent clones were sequenced to determine the orientation of the CD gene with respect to the PSMA promoter. The

clones used for the following experiments were named pPSM/CD-sense and pPSM/CD-antisense where sense and antisense refers only to the direction of the CD gene.

Cytotoxicity / proliferation inhibition assay

Initially, cytotoxicity of 5-FC and 5-FU in non-transfected cell lines was examined. Cells (2.5 x 10^3 C4-2, H157, HCT8 and 4.0 x 10^4 LNCaP and MCF-7 cells) were plated in triplicate for each time point, in 24-well plates. The cells were then exposed 24 hours later to various concentrations of 5-FC (Sigma, St. Louis, MO) or 5-FU (Sigma, St. Louis, MO). Relative cell numbers were assayed after 3 days using the MTS assay (Cell Titer 96 AQ_{ueous}, Promega, Madison, WI) and the inhibitory concentration (IC50) determined by figuring the concentration at which the cell number was 50% of the number of control (untreated) cells grown for the same length of time.

Cytotoxicity of 5-FC on transiently transfected cells

The cytoxicity of 5-FC on cells transiently transfected with pPSM/CD-sense or pPSM/CD-antisense or liposome only was determined. Cells were plated in 24-well plates and transfected as described above. Transfection efficiency was determined by *in situ* X-gal staining replica wells transfected with pSV-β-galactosidase (Promega, Madison, WI). Two days after the initiation of transfection, cells were exposed to various concentrations of 5-FC for three days, and cell viability measured.

Enzymatic activity of cytosine deaminase in the transfected cells

The enzymatic activity in the cell lysate was measured with spectrophotometry (14). H157, LNCaP and C4-2 cells were plated in a 100mm dish and pre-incubated for 24-48 hours. At 50% confluency, the cells were transfected with pPSM/CD-sense, pPSM/CD-antisense or pSV-β-galactosidase. Two days after the initiation of transfection, the cells were collected and preserved at –20°C. On the initial day of the assay, 110μl of PBS was added to each cell pellet. Pellets were then sonicated and centrifuged at 14000 x *g* at 4°C. Ten μl of each supernatant was used to measure the protein concentration using the BCA protein assay (Pierce, Rockford, IL). The remaining 100μl of cell lysate was added to 900μl of 3mM 5-FC in PBS. The reaction mixtures were incubated at 37°C. At various time points, 50 μl of each aliquot was removed and added to 950 μl of 0.1M HCl. The concentration of 5-FC was measured by determining the absorbance at 290nm (A290) and 255nm (A255). The concentration of 5-FC was calculated as follows:

5-FC (mM) = 0.119xA290-0.025xA255 (14)

The enzymatic unit of cytosine deaminase was defined as nmol of 5-FC catalyzed per minute per mg of protein from whole cell supernatant.

Results

PSMA Promoter and Promoter/Enhancer driven GFP expression in various cell lines

Green Fluorescent Protein (GFP) expression was used to compare the activity of the PSMA promoter with that of the PSMA promoter and PSMA enhancer, that was identified by Watt et al., (13). These constructs were transiently transfected into LNCaP, C4-2, PC-3 and NIH-3T3 cell lines, and the cells examined for green fluorescence (see figure 1). Minimal GFP expression was seen in LNCaP, C4-2 and NIH-3T3 cells with the promoter alone, though PC-3 cells had moderately high levels of GFP expression with the PSMA promoter alone. Addition of the PSMA enhancer to the construct resulted in significantly increased levels of GFP expression in the LNCaP and C4-2 cell lines, although there appeared to be no enhancement of GFP expression in the PC-3 and NIH-3T3 cells over that seen with the PSMA promoter alone.

Analysis of the PSMA Enhancer

Reporter plasmids carrying various portions of the PSMA enhancer and driven by either the PSMA promoter or the viral SV-40 promoter were analysed for luciferase expression in the prostatic cell line C4-2 and the breast cancer cell line, MCF-7. The entire enhancer sequence is shown in Figure 2a, and the constructs indicated by the primer positioning so that all deletions were made progressively from the 3' end of the enhancer (see Figure 2b). All the enhancer deletion constructs were tested with the enhancer placed in both orientations (downstream of the luciferase reporter gene), with respect to the PSMA or SV-40 promoter. All results were normalized for transfection efficiency, and the mean and standard deviation of the experiments in triplicate expressed

as a percentage of the pGL3-Control (SV-40 promoter and enhancer). All the enhancer deletions were able to drive prostate-specific expression of luciferase in both orientations, although with varying amounts of activity (see figure 3). The most active constructs were the pGL3-B-PSMA1648 and pGL3-B-PSMA1290 plasmids, which in the antisense orientation showed approximately 20-fold expression over the pGL3-control vector, and 200-fold over the PSMA promoter alone in C4-2 cells. Conversely, when the same constructs were transfected into breast cancer MCF-7 cells, the enhancer provided no additional activity over the PSMA-promoter alone. When the enhancer constructs were driven by the SV-40 promoter, the activity of each deletion was consistent with that seen in combination with the PSMA promoter, although overall activity of the two best constructs pGL3-B-PSMA1648 and pGL3-B-PSMA1290, was increased to about 25 fold of that of the pGL3 control vector in C4-2 cells. However, when the SV-40 promoter was tested with the enhancer deletions in MCF-7 cells, these same two constructs showed up to 20% of the activity of the pGL3-control. This suggests that there may be some elements of the PSMA promoter itself that contribute to prostate-specific expression. Given these results, we next took the pGL3-B-PSMA1648 plasmid and replaced the luciferase reporter gene with the E.*coli* CD gene to form pPSM/CD (sense or antisense, dependent on the orientation of the CD gene relative to the PSMA promoter).

Cytotoxicity / Proliferation Inhibition of 5-FU and 5-FC

In order to determine the sensitivity of each cell line to 5-FU, cytotoxicity / proliferation inhibition assays were performed (see figure 4 and Table 1). The IC50 of 5-FU for all the cell lines was similar, ranging between 1 and 10uM according to several

independent experiments. Compared to 5-FU, 5-FC is much less toxic for all cell lines. IC50 of 5-FC for all cell lines was over 10mM (more than 1000 times that of 5-FU).

Cytotoxicity / proliferation inhibition in transiently transfected cells

Transient transfection of LNCaP and C4-2 cells with pPSMA/CD-sense resulted in sensitization against 5-FC. In C4-2 cells, the 5-FC at 200, 300 and 400 uM inhibited the cell growth to 67, 39 and 38% repectively compared to the control (non-transfected cells without 5-FC). The IC50 of 5-FC on transfected C4-2 was between 200 and 300 uM.

Compared to the IC50 of non-transfected cells (>10mM), C4-2 was sensitized nearly 40 fold by transfection with pPSMA/CD-sense. It should be noted that the transfection efficiency of C4-2 cells was only 8% as determined by X-gal staining, and exposure to 5-FC was only 3 days. The enhanced cytotoxic effect of 5-FC was also noted in LNCaP cells when transfected with the pPSMA/CD-Sense plasmid. However, the sensitization in LNCaP cells (IC50 1-5mM), was not as great as that in C4-2 cells. This could be because LNCaP cells are less sensitive to 5-FU treatment (see Table 1), probably due to genetic differences between the two cell lines. Another cause could be because the recovery period of LNCaP after transfection is much longer than that of C4-2. Therefore there is less chance of LNCaP cells incorporating 5-FU as their cell cycle is longer. In H157, HCT8 and MCF-7 cells, there was no significant difference in cell numbers between transfected with pPSMA/CD sense and antisense constructs,

demonstrating the specificity of the PSMA promoter/enhancer construct for prostate cells.

Cytosine Deaminase Enzymatic Activity in Transfected Cells

The cytosine deaminase activity in C4-2 cell lysate was dependent on transfection efficiency. When 5% and 1% of C4-2 and 5% of LNCaP cells in a 100mm dish were transfected with pPSM/CD-Sense plasmid, the lysate showed an enzymatic activity of 2.4, 1.6 and 0.7nmol/min/mg protein, respectively (data not shown). The enzymatic activity in the lysate of cells transfected with pPSM/CD-antisense plasmid, as well as H157 transfected with the pPSM/CD sense construct, did not show any detectable enzymatic activity of cytosine deaminase.

Discussion

Gene therapy is in the process of becoming an acceptable treatment method for patients for whom there is no other effective treatment. Control of drug activation using prodrug therapy in combination with gene therapy is theoretically a way of staging a maximal attack on the targeted tumor, while controlling cytotoxicity to non-cancerous tissue. To date, the two promoter/enhancers most often considered for use in prostate-specific therapy have been those of the rat probasin gene, and the human PSA gene. Both of these promoter/enhancers are positively regulated by androgens, and therefore would be of little use to patients who are undergoing androgen ablative therapy (15; 16). However, the PSMA gene is up-regulated in the absence of androgens and highly expressed in virtually all tumors and their metastases examined so far. As such, we have chosen to use regulatory elements from the PSMA gene to develop a construct that could be used to control expression of a "suicide gene" in a gene therapy approach against this disease. We recently cloned the promoter of the PSMA gene, which is able by itself to drive prostate-specific expression of a reporter gene; albeit at relatively low levels (11). More recently, we isolated a prostate-specific enhancer from the third intron of the gene, which is upregulated in the absence of androgens (13). Addition of the full-length enhancer to the PSMA promoter in a reporter construct results in approximately a 100-fold increase in expression. This data demonstrates that the enhancer retains full activity when positioned downstream of the gene; previous results were obtained with the enhancer upstream of the promoter (13). As the full-length enhancer had not been previously tested in C4-2 cells, we initially compared its ability to drive the GFP reporter gene in a number of cell lines. While the enhancer clearly shows a significant increase in

activity over the PSMA promoter alone in LNCaP and C4-2 cells, we were surprised to see more activity of the PSMA promoter alone in PC-3 cells than in either LNCaP or C4-2. PC-3 cells do not express PSMA, however expression of the PSMA-promoter would suggest that while the transcription factors necessary to drive the promoter's expression are available in PC-3 cells, there is some kind of defect in the PSMA promoter in these cells. Such a defect might be due to homozygous deletion or methylation of the PC-3 PSMA promoter. In addition, the enhancer did not seem to significantly increase expression over promoter alone in PC-3 cells. This may be due to a number of reasons, including that the factors involved in up-regulation via the enhancer are not present in PC-3 cells, and the fact that fluorescence microscopy is less quantitative than luciferase assays.

Interestingly, analysis of the deletion constructs revealed that there appears to be at least two distinct positive-regulatory regions within the enhancer region, as the smallest construct (501 bps) still exhibited more than a 50-fold increase in expression over the promoter alone. However, the most active constructs were clearly the 1648 and 1290 bp fragments, which showed a 175 to more than 200 fold increase in expression over the PSMA promoter alone. As this is more than the entire enhancer, it would suggest that there may be a negative regulator between nt 1648 and 1913 of the originally defined enhancer region. The identity of either of the positively- or negatively regulating elements is unclear, as there are no consensus enhancer elements present in the PSMA enhancer sequence. There also appeared to be an effect of the orientation of the enhancer with respect to the promoter. While all the constructs showed significantly increased activity over both the SV-40 control and the PSMA-promoter alone, the constructs with

the enhancer in the anti-sense orientation with respect to their orientation in the native PSMA gene seemed to have significantly more activity, and the reason for this is unknown.

We tested the most active PSMA-promoter enhancer/ CD gene construct *in vitro* using a transient transfection approach because it probably most resembles the principal problem with genetic therapy at the present time; delivery of the vector to the target tissue. One advantage of the cytosine deaminase / 5-FC therapy approach is the bystander effect in which cells close to the actual cell producing the engineered enzyme (in this case CD), are affected by the converted prodrug and destroyed. All results in this study were normalized for transfection efficiency so as a logical comparison could be made between the different cell lines. However, even with the average transfection efficiency of C4-2 cells of around 8%, significant conversion of 5-FC to 5-FU and subsequent cell death was seen. Transfection with the suicide construct sensitized C4-2 cells to 5-FC nearly 40-fold, with an IC50 of between 200-300uM. The usual oral dose of 5-FC for fungal treatment in humans is 37.5mg per kg body-weight every six hours, which results in peak serum concentrations of 540-620 uM in adults. The specificity of this method was shown by the lack of sensitization to 5-FC by transfection in the breast, colon and lung derived cell lines. However, it still remains to be determined if this enhancer is active in other tissues *in vivo* where PSMA is reportedly expressed. Other enhancers could be responsible for this non-prostatic expression, or this expression might be due to activation of another gene such as the PSMA-Like gene. Furthermore, it will be important to determine if this PSMA enhancer is responsible for the expression of PSMA-protein seen in tumor neovasculature (10), as this would provide a much more

wide use for gene therapy constructs utilizing the PSMA regulatory control regions described here. We are currently attempting to answer both of these questions, in part by generating a transgenic mouse model carrying the human PSMA promoter and full-length enhancer driving expression of green fluorescent protein.

Conclusions

We have identified the PSMA promoter and enhancer regulatory regions responsible for the strong expression of PSMA in prostate tumor cells such as LNCaP and C4-2. Futhermore, we have demonstrated that this PSMA promoter/enhancer construct is capable of selectively driving expression of therapeutic genes and are currently exploring which gene or genes would be most useful and ways to increase the effectiveness of vector delivery to the tumor or metastatic site. Suicide gene therapy for prostate cancer using the PSMA promoter and enhancer holds promise for the treatment of prostate cancer.

Figure Legends:

Figure 1: (a) the PSMA promoter alone, or (b) the PSMA promoter and enhancer driving GFP expression in LNCaP, C4-2 and PC-3 (prostatic cell lines) and in NIH 3T3 cells (used as a negative control). Addition of the PSMA enhancer to the PSMA promoter clearly results in a large increase in GFP reporter expression in LNCaP (200X magnification, 30 second exposure) and C4-2 (400X magnification, 30 second exposure) cells, while no expression is seen in NIH-3T3 (400X magnification , 60 second exposure) cells. Note the fluoresence of the promoter alone in PC-3 (400X magnification , 30 second exposure) cells, which do not express PSMA.

Figure 2: (a) PSMA Enhancer sequence; the grey arrows indicate positioning of primers used to create the deletion constructs. Numbering refers to Genbank accession number AF007544. (b) Pictorial representation of the PSMA enhancer region of the deletion constructs.

Figure 3: Luciferase activity after normalisation for transfection efficiency, expressed as a percentage of the positive control (pGL3-control), where the control was set at 100% activity. The enhancer deletions are indicated; "s" refers to the enhancer in the sense orientation with respect to the promoter; "as" refers to the antisense orientation. Addition of the PSMA enhancer to either the PSMA promoter or the SV40 promoter results in a significant (up to 300 fold) increase in reporter gene expression over the pGL3 control vector in the prostate cancer cell lines C4-2 and LNCaP, while minimal

expression is seen in the breast cancer cell line MCF-7. The most active deletions are the antisense 1648 and 1290 bps constructs respectively.

Figure 4: Determination of the Inhibitory Concentration 50 (IC50) of 5-Fluorocytosine (5-FC) and 5-Fluorouracil (5-FU) in non-transfected cells, and cells transfected with both sense and antisense Cytosine Deaminase driven by the PSMA promoter and the 1648 bp enhancer from the PSMA gene. The dotted line dropped from the 50% horizontal indicates the IC50. It can be seen that transfection with the sense Cytosine Deaminase construct sensitizes LNCaP and C4-2 cells significantly to 5-FC treatment, while the non-prostatic cell lines H-157, HCT-8 and MCF-7 are not sensitized to 5-FC, demonstrating the specificity of this approach for prostate cancer.

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Table 1

Cell line	Tissue Origin	PSMA Expression	IC50 5-FU (μM)	IC50 5-FC parental (μM)	IC50 5-FC PSMA/CD (μM)	Ratio IC50 of 5-FC (parental) / (PSMA/CD	Transfection Efficiency (%)
C4-2	**prostate**	**+**	**1-5**	**15000**	**200-300**	**>50**	**8**
LNCaP	**prostate**	**+**	**1-10**	**15000**	**1000-2000**	**10**	**5**
H-157	lung	-	1-10	>20000	>10000	<2	10
HCT-8	colon	-	1-5	20000	>15000	<2	6
MCF-7	breast	-	1-5	10000	>10000	<2	15

Table 1 Comparison of sensitivity to 5-fluorouracil (5-FU) and 5-fluorocytosine (5-FC) in each cell line. The inhibitory concentration 50% (IC50) of 5-FU and 5-FC in all cell lines was very similar. However, when cells were transfected with the pPSMA/CD-Sense plasmid, C4-2 and LNCaP cells were sensitized to 5-FC, 50 and 10 fold more respectively than the parental non-transfected cells, whereas the other non-prostatic cells were not significantly sensitized. Note that the sensitization was achieved with only 8% transfection effciency in C4-2 cells.

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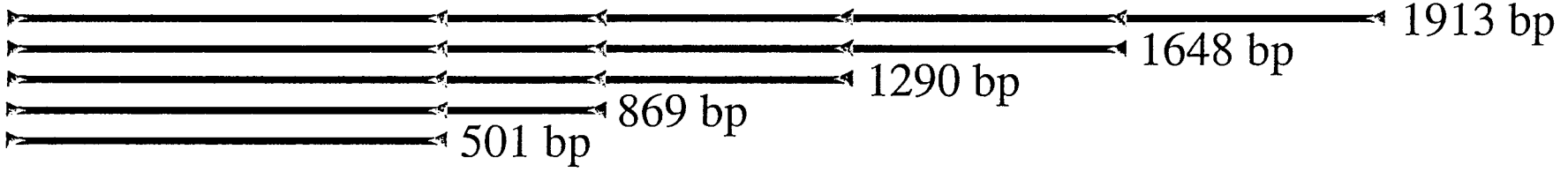
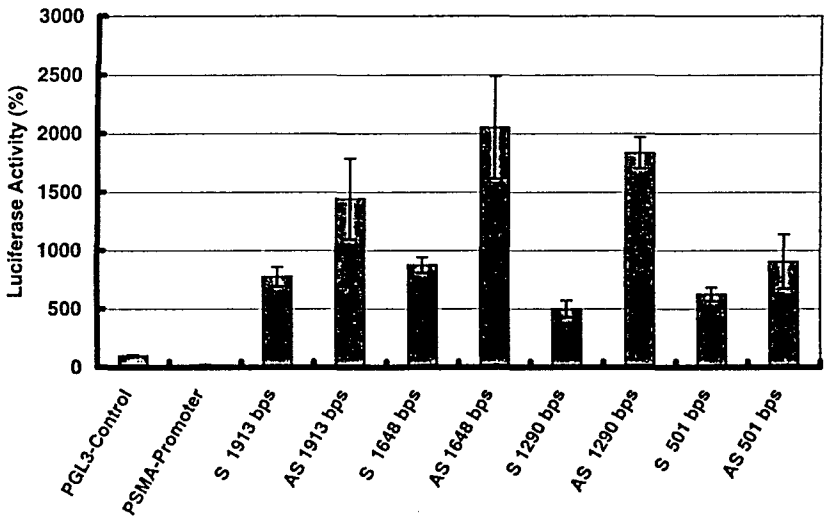
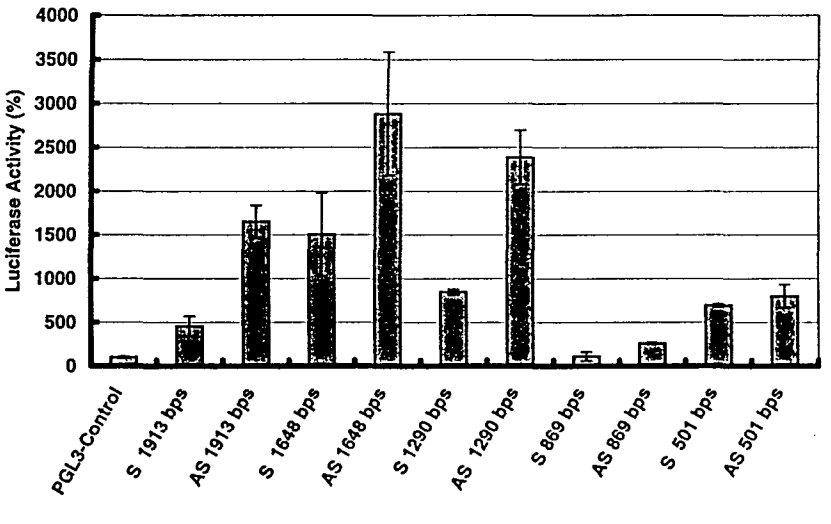


PSMA Promoter and Enhancer Activity in C4-2 Cells



SV40 Promoter and PSMA Enhancer Activity in C4-2 Cells



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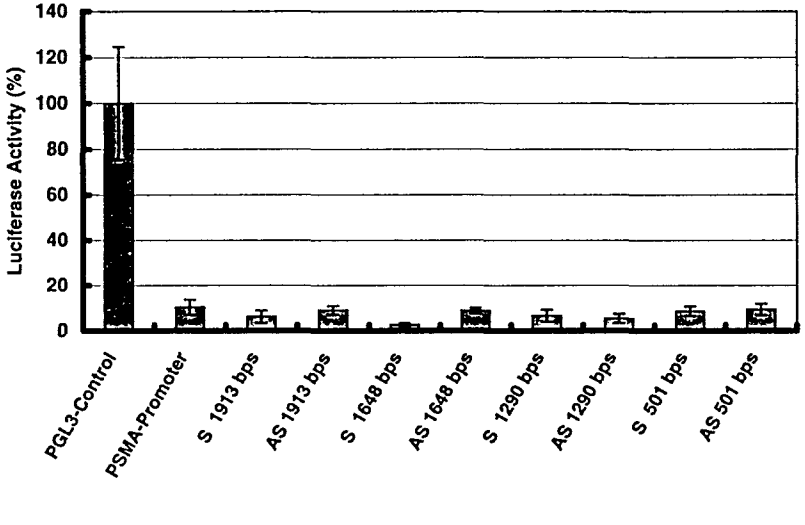
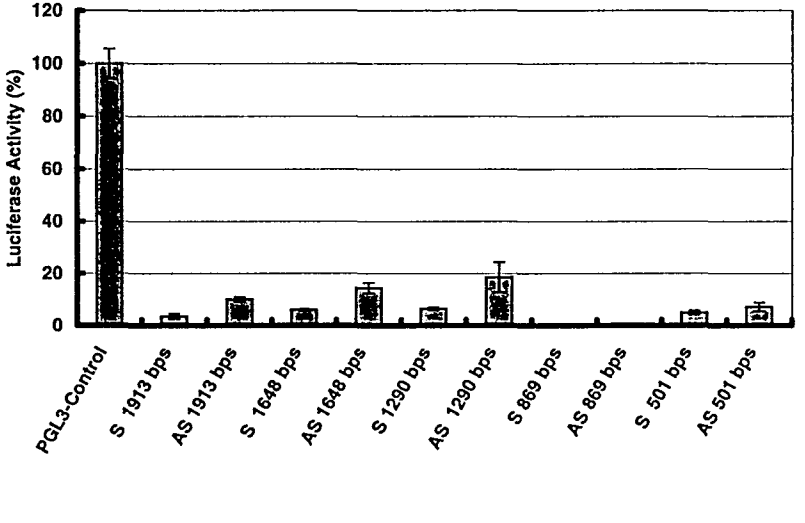
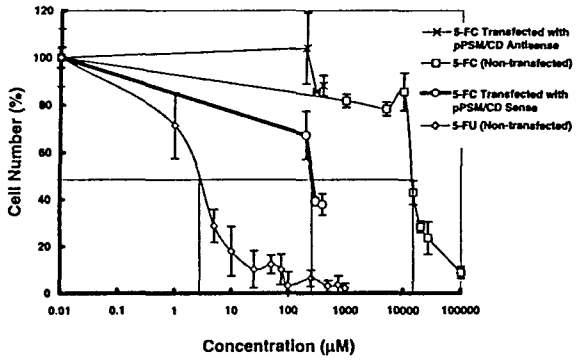


SV40 Promoter and PSMA Enhancer Activity in MCF-7 Cells



C4-2 Cells



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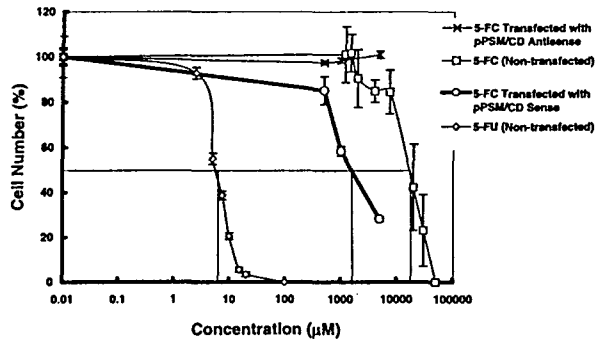
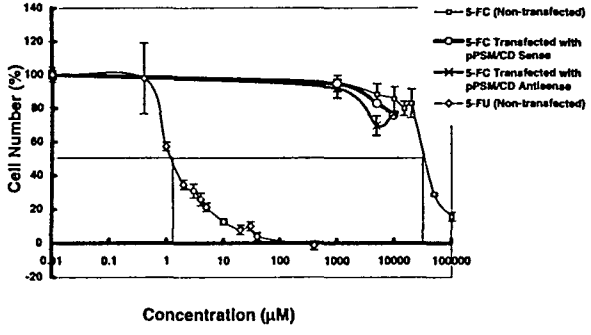


H-157 Cells



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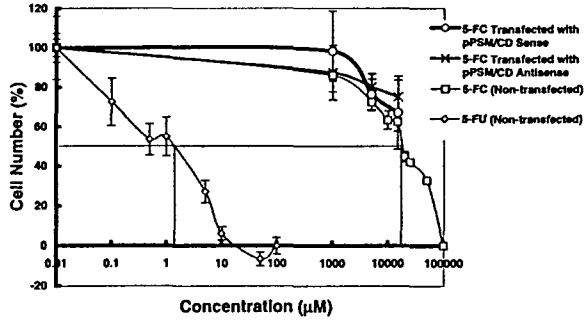

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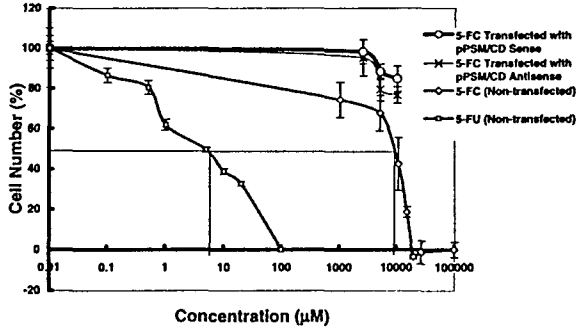

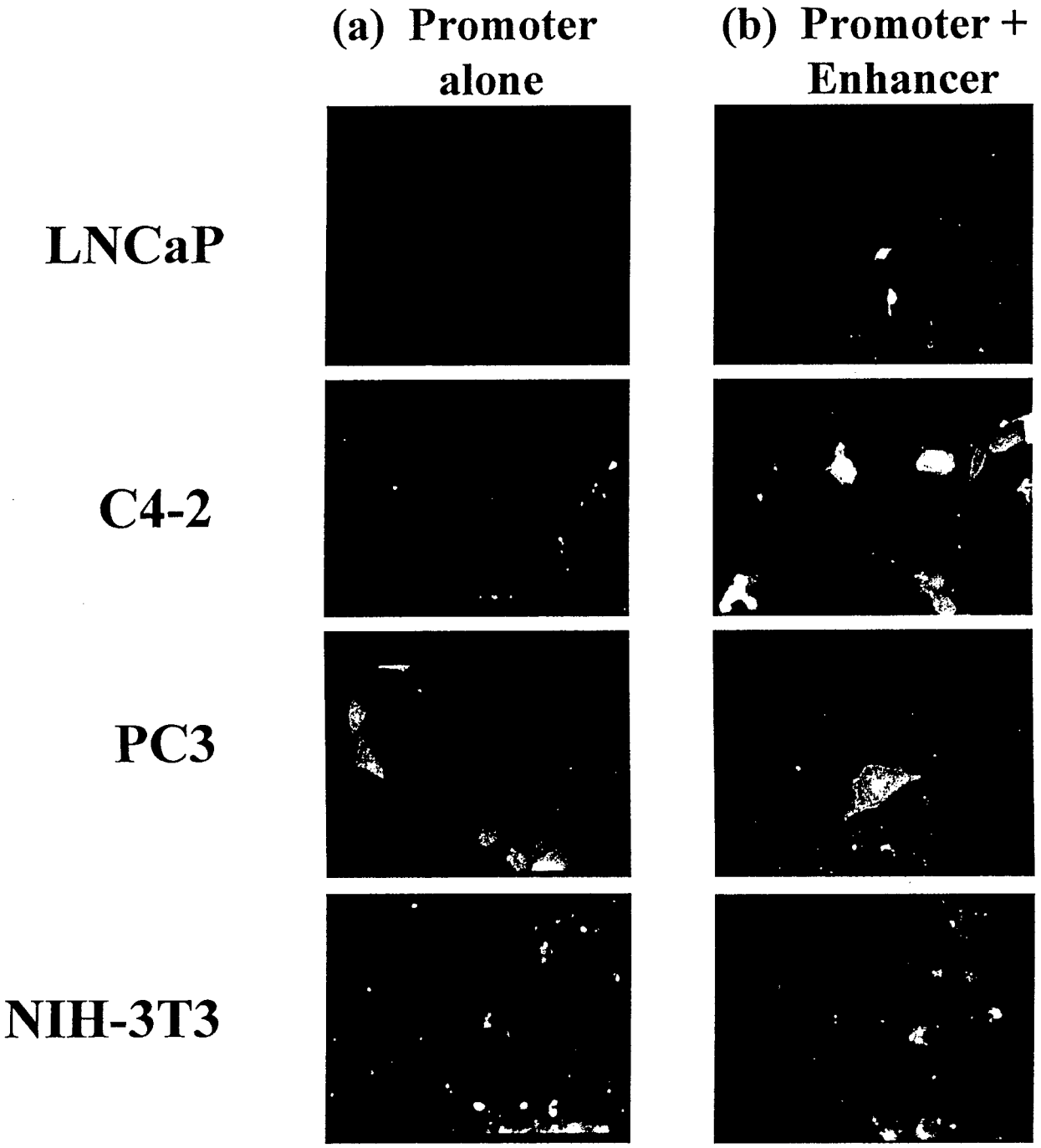
(a) Promoter alone
(b) Promoter + Enhancer
LNCaP
C4-2
PC3
NIH-3T3

CHAPTER 11

Prostate-Specific Membrane Antigen

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The molecular basis of prostate carcinoma has always been less understood than that of other cancers, despite its high incidence in the population. One of the reasons for this is that the molecular pathways leading to prostate cancer do not seem to parallel that of other cancers, and until recently there has been a lack of markers for this tumor. As such, one of the most exciting recent findings in prostate cancer was the discovery of Prostate-Specific Membrane Antigen (PSMA). PSMA is a glutamate carboxypeptidase, and switches from a cytosolically located protein in normal prostate, to a membrane-bound protein in prostatic carcinoma. Additionally, the majority of PSMA expression appears to be restricted to the prostate with some expression seen in the brain, salivary glands and small intestine. Intriguingly, our group recently found that PSMA is expressed in the endothelial cells of the neovasculature of almost all solid tumors examined. The membrane-bound nature of this protein, and the limited sites of expression as well as expression in tumor-associated neovasculature, makes PSMA an ideal marker and therapeutic target for clinical studies and treatment of not only prostate cancer, but other solid tumors as we progress into the next millenium.

The Discovery of PSMA

The antigen itself was discovered by Horoszewicz et al.,*(28)* who isolated LNCaP cell membranes and immunized mice with the mixture, producing the antibody known as 7E11C5.3. The LNCaP cell line is derived from a Lymph Node metastasis from a Carcinoma of the Prostate, and is considered the most relevant of the few prostatic cell lines available, because it retains expression of Prostate-Specific Antigen (PSA), Prostatic Acid Phosphatase (PAP) and the androgen receptor, among other characteristics typical of human prostate cancers *in vivo (29,30,23,25)*. Characterization of the antibody revealed that it specifically bound epithelial cells of normal prostate, benign prostatic hypertrophy (BPH) and prostatic carcinoma specimens, making PSMA an attractive prostate-specific marker *(28)*. The rights to the 7E11C5.3 antibody were then bought by a biotechnology company called Cytogen Corporation (Princeton, New Jersey).

Cytogen modified the antibody such that it could be labelled with ^{111}Indium while retaining its specificity, and renamed it Cyt-356. The radio-labelled antibody was then administered to nude mice carrying tumors established from the LNCaP cell line. After three days, 30% of the injected dose had localized to the LNCaP xenograft, with no significant amounts found in other tissues *(42)*. This was despite the fact that the same investigators had noted that immunohistochemical staining using this modified antibody against normal human tissues showed weak reactivity with cardiac muscle, proximal kidney tubules and sweat glands. There was also strong binding to a subset of skeletal muscle cells. A phase I clinical study using radio-labeled Cyt-356 as an imaging agent for metastatic deposits was then carried out in patients with prostate cancer and known distant metastases *(78)*. No adverse affects of the agent were noted in the patients, and the results showed promise for use of the immunoconjugate as an imaging agent. At this stage it was clear that a deeper understanding of the molecular basis and function of PSMA expression in prostate cancer was needed.

Cloning and Expression Pattern of Prostate-Specific Membrane Antigen

The complementary (cDNA) sequence encoding Prostate-Specific Membrane Antigen was cloned in 1993 using a classic text-book approach *(33)*. The monoclonal antibody Cyt-356 was used to immunoprecipitate PSMA from LNCaP cell membranes, the protein was then electrophoresed on, and isolated from a polyacrylamide gel. PSMA was then subject to proteolytic digestion and the subsequent peptide fragments microsequenced to determine their amino-acid composition. Based on the amino-acid sequence, degenerate oligonucleotide primers were designed that could theoretically amplify the PSMA cDNA sequence from LNCaP reverse-transcribed mRNA. The resultant PCR product was cloned and used to probe a LNCaP cDNA library and isolate the full-length PSMA transcript of 2,653 nucleotides (the sequence can be found in Genbank under the accession number M99487).

Translation of the cDNA sequence predicted that the protein consists of 750 amino-acids with a molecular weight of 84 kD before post-translational modifications *(33)*. It was later shown that *in vitro* translation of the PSMA cDNA sequence with and without dog pancreatic microsomal membranes

(which permit glycosylation of proteins to occur *in vitro*), produces proteins of 100 and 84 kD respectively. This is consistent with the 100 kD molecular weight of PSMA in seen in LNCaP cells *(32)*. PSMA is a type II integral membrane protein, and as such the short N-terminal of the protein is located on the cytoplasmic side of the membrane with the majority of the protein located on the extracellular side of the membrane, making it available for clinical and therapeutic targeting *(33)*. Northern analyses using the PSMA cDNA probe and ribonuclease protection assays using a probe corresponding to nucleotides 242-588 of the PSMA cDNA sequence showed no expression of PSMA mRNA in the prostatic cell lines PC-3 and DU145, and no expression in normal tissues from kidney, liver, lung, mammary gland, pancreas, placenta, skeletal muscle, spleen and testis. There was however high expression in normal prostate and prostatic carcinomas and barely detectable expression in salivary gland, whole brain and small intestine. Expression of PSMA mRNA in BPH specimens was either reduced relative to that of normal prostate, or absent all together *(32)*, which is most likely an indication of the major cell type that constitues BPH (stromal cells that do not express PSMA), rather than a biologic phenomenon. Interestingly, our group and others recently found that PSMA is expressed in the endothelial cells of neovasculature associated with almost all solid tumors, but not in normal vasculature *(40,61)*. Immunohistochemistry using five different antibodies against PSMA has confirmed this, as have *in situ* hybridization and RT-PCR results *(8,9)*. In fact, the only tumor which does not seem to consistently express PSMA in the associated vasculature is that of the prostate (2/12 prostate cancer specimens expressed PSMA in the vasculature), perhaps providing a clue as to the function of PSMA in these cells *(9)*.

As PSA expression is modulated by androgens, Israeli et al., examined the effect of various steroids on PSMA expression in LNCaP cells *(32)*. In contrast to PSA expression, PSMA is down-regulated in the presence of androgens, with the highest amount of PSMA expressed in LNCaP cells grown in charcoal-stripped (and therefore steroid-reduced) media. This finding was later supported both *in vitro* in LNCaP cells, and *in vivo* by Wright *et al.*, *(76)*, who found that in 55% (11 of 20) and 100% (4

of 4) primary and metastatic tumor specimens, PSMA expression was significantly upregulated in patients who had undergone some form of hormonal deprivation, relative to matched specimens from the patients before treatment. These findings are particularly significant, because of the implication that PSMA can be a highly useful clinical and therapeutic target for patients with recurrent disease.

Regulation of PSMA Expression: Cloning of the PSMA promoter and enhancer.

To obtain more information about the genetic regulation of PSMA expression, we set out to determine the complete sequence of the gene. A bacteriophage P1 library containing fragments of DNA from normal human lymphocytes in the order of 60-80 kb in size was screened using PCR. Two sets of oligonucleotide primers were used; one set corresponding to the 5' end of the PSMA cDNA sequence, and one set corresponding to the 3' end of the sequence. It was fortunate that we carried out the screening this way, because the gene turned out to span more than 60 kb of DNA, and we had to analyze two P1 clones that overlapped by about 5.6 kb to acquire the entire sequence *(50)*.

Comparison of the genomic and cDNA sequences of the PSMA gene revealed 19 exons ranging in size from 64 to 379 nucleotides, and 18 introns from 300 to 7,363 base pairs (fig. 1). The entire genomic sequence of the gene can be found in Genbank, under accession number AF007544. One of the most striking features of the genomic sequence was the presence of a CpG island at the 5' end of the gene. From nucelotides 2,661-2,990 of the genomic sequence, which extends from exon 1 into the first intron of the gene, the observed/expected ratio of the CpG dinucleotide was 1.85, which is significantly greater than the ratio for bulk human DNA (0.25)*(1,50)*. CpG islands are substrates for DNA methyltransferase, and the presence of a CpG island in the 5' region of the PSMA gene suggests a role for DNA methylation in the regulation of PSMA expression.

Once we had the genomic sequence of the 5' portion of the gene, we were able to clone the promoter controlling transcription of PSMA mRNA. To confirm the transcription start site indicated by the initial PSMA cDNA sequence *(33)*, we carried out 5' Rapid Amplification of cDNA Ends (5' RACE). 5' RACE is a form of PCR that uses one primer based in known cDNA sequence of the gene,

and one primer that binds to 5' ends of all mRNA transcripts. As such only one primer is specific to the gene of interest, but by using this method it is possible to determine the exact start sites of transcription, and therefore predict the sequence encompassing the promoter region of the gene. Our 5' RACE experiments confirmed the original start site (+1 of the cDNA sequence), but also showed other start sites within this region, at -195 and -235, in addition to the original start site at -262 relative to the translation initiation codon of the gene. Such heterogeneity of transcription start sites is not uncommon in genes where the promoter lacks a TATA-box, and consistent with this, the DNA sequence upstream of these start sites has no typical TATA-boxes *(50)*. We next cloned the 1244 base pair region of genomic DNA spanning the start sites and about 1 kb of the region 5', into a reporter vector to test if the region was capable of driving transcription of the firefly luciferase gene. To our surprise, not only did this region of the gene have significant activity/ milligram of cell protein relative to the strong SV-40 viral promoter/enhancer in LNCaP cells, it also appeared to be prostate-specific, in that we could not detect significant levels of luciferase in the DU145 and MCF-7 cell lines (prostate and breast carcinoma lines). These cell lines do not express PSMA and therefore presumably do not contain the appropriate trancription factors for activation of the PSMA promoter.

Interestingly, we did see luciferase expression driven by the PSMA promoter in PC-3 cells, another prostatic cell line that does not express PSMA, at levels corresponding to about 10% of that of the control SV-40 promoter/enhancer *(50)*. This led us to examine the genomic region of PC-3 DNA containing the CpG island for hypermethylation by Southern analysis with methylation-sensitive restriction enzymes. We were able to demonstrate that while DNA from normal male lymphocytes and the LNCaP cell line was not hypermethylated, DNA from PC-3 cells was at least partially hypermethylated in this region. We next treated PC-3 cells with the demethylating agent 5-azacytidine, but were unable to detect expression of PSMA after treatment with the drug (O'Keefe et al., unpublished data) and we are still investigating this phenomenon.

In further experiments to delineate the minimal promoter region of the gene, we discovered that our original estimation of the strength of the PSMA promoter was somewhat over-estimated. We had calculated the strength of the PSMA promoter relative to that of the usually strong SV-40 promoter/enhancer in the luciferase-reporter experiments. However, when we transfected various cell lines with the SV-40 promoter alone, and after adjusting for transfection efficiency compared these transfections to those with the SV-40 promoter/enhancer combination, we were surprised to find that in LNCaP cells addition of the SV-40 enhancer to the basal SV-40 promoter actually did not enhance reporter gene transcription, and in some cases repressed it. In contrast, in other cell lines such as PC-3, the SV-40 promoter/enhancer combination significantly increases reporter gene transcription (O'Keefe et al., unpublished data). Other laboratories have reported similar findings in LNCaP cells when combining the SV-40 enhancer with other basal promoters such as the PSA minimal promoter (Peter Molloy, personal communication) although the reason for this remains unclear.

Deletion constructs of the promoter region allowed us to localize the minimal promoter to between bases 461-1097. The original promoter construct had contained an Alu repeat sequence, and we also found that once this region was deleted, reporter gene expression increased (Horiguchi et al., unpublished data). Although PSMA expression is regulated by androgens *(32,76)*, there are no typical androgen response elements in the promoter region or in the entire PSMA genomic sequence. However, because there might be novel androgen response elements, we tested the deletion constructs and the original promoter construct for androgen responsiveness in LNCaP cells.

While the minimal PSMA promoter described above appeared to exhibit prostate-specificity, it could only promote basal levels of reporter gene expression *(75)*. Watt et al. *(75)* cloned the PSMA enhancer region (PSME) using an "enhancer-trap" system. The enhancer trap library was created by partial digestion of the P1 bacteriophage clones containing the PSMA genomic sequence, and subcloning the resultant fragments into a vector containing the PSMA promoter driving expression of the Green Fluorescent Protein (GFP) gene. Screening of the library for DNA fragments able to increase GFP

expression over that seen by the promoter alone was carried out in LNCaP cells and a number of other non-PSMA expressing cell lines. Using this method, a fragment of DNA that was able to increase transcription from the PSMA promoter by 250 fold was identified. When the enhancer was linked to other stronger basal promoters instead of the PSMA promoter, transcription levels were increased by at least ten-fold; in the most impressive experiment, the PSME was linked to the herpes virus thymidine kinase (TK) promoter and transcription was nearly three-fold that of the Rous-Sarcoma Virus promoter/enhancer, which in itself is a strong viral promoter. The PSME retains prostate-specificity even when linked to the TK promoter which is not prostate-restricted in expression, and the PSME also shows repression by androgens *(75)*. As such, the PSME shows excellent promise for use in gene therapy approaches targeting prostate cancer in the near future.

Alternative Splicing of the PSMA Gene

Using RT-PCR of normal prostate tissue *(64)* discovered the first reported mRNA splice-variant of the PSMA gene. The variant, PSM' (PSM-prime), transcribes from the regular PSMA promoter and uses an alternative 5' splice donor site within exon one of the gene, deleting bases 114-379 which includes the translation start codon for PSMA. Initiation of translation of the PSM' protein begins at nucleotide 427, producing a glycoprotein of about 95 kD that lacks the intracellular and transmembrane domains of PSMA. As such, PSM' is located within the cytoplasm, but still retains the enzymatic activity of PSMA *(21)*. RNase-Protection Assays differentiating PSMA from PSM' mRNA transcripts revealed that in normal prostate PSM' is the dominant isoform, while in prostate tumors and the LNCaP cell line, PSMA is more prevalent *(64,36)*. Compilation of the data to form a "tumor index" comparing the ratio of PSMA:PSM' resulted in a score of 9-11 for LNCaP cells, 3-6 for prostate carcinoma, 0.75-1.6 for BPH and 0.075-0.45 for normal prostate. Unfortunately, further analysis of the ratio of PSMA:PSM' in clinical specimens has not been reported so it remains unclear whether or not this tumor index could have a clinical impact.

Another alternative splice-form of PSMA was isolated from human brain, prostate and liver, and deletes amino-acids 657-688 of the protein *(5)*, and creates an amino-acid substitution (Asn→Lys). These amino-acids correspond to the entire 18th exon of the gene so the splicing event probably occurs by "exon skipping". We have also seen this splice form in cDNA derived from a colon tumor. Further investigation of this variant is required to determine if it retains the activity of the full-length PSMA protein, and if it exists in significant levels relative to PSMA and PSM'.

Finally, when we were carrying out 5' RACE of the PSMA gene using LNCaP cells, we discovered a number of novel transcripts. The first, which we have called PSM-C, begins transcription at the same nucleotides as the PSMA and PSM' transcripts, then uses the same splice donor site as PSM' (nt. 114), but uses an alternative splice acceptor site located within intron one. Nucleotides 3,270-3,402 of the genomic PSMA sequence are transcribed, followed by exon 2 and exon 3. Translation of this variant containing a previously unidentified exon which we have termed exon 1b, would result in a protein identical to PSM'. Another variant, PSM-D again uses the same splice donor site as PSM', and a unique splice acceptor site in intron one, including another novel exon (exon 1c) which is from nucleotides 4,289-4,389 of the genomic PSMA sequence. The putative translation of this protein reveals a new translation initiation start site located in exon 1c, followed by 42 novel amino-acids and the rest of the PSMA protein in-frame. Interestingly, there is a motif in the novel region consisting of the peptide Ala-Ala-Tyr-Ala-Cys-Thr-Gly-Cys-Leu-Ala, that is similar to that seen in the growth-factor cys-knot family of proteins. Using RT-PCR we were able to demonstrate the existence of this variant in normal prostate and LNCaP cells, however we were unable to demonstrate significant amounts of this mRNA splice variant via RNase protection assays on these tissues.

At least one other group has also found splice variants arising from novel exons in intron one of the gene; one exon continuing on from the 3' end of exon 1 for 68 nucleotides and another extending for 97 nucleotides. All three variants include exon 1 (nt 1-379), and thus would be predicted to translate into a protein with a transmembrane domain. One of the variants contains both new exons aligned in tandem,

and the three variants are expected to produce proteins between 40 and 805 amino-acids in length *(53)*. It remains to be seen if novel variants such as those described above contribute to or are functionally involved in prostate cancer, could therefore be clinically significant.

Mapping of the PSMA Gene and Identification of the PSMA-like Gene.

Chromosomal localization of the PSMA gene proved to be controversial. Initial mapping by two independent research groups using Fluorescent In Situ Hybridization (FISH) and the full-length cDNA sequence as a probe indicated two regions for the gene, 11p11-12, and 11q14 *(38,56)*. To identify the true location of the PSMA gene, Leek et al. *(38)* used PCR of somatic cell hybrids containing various regions of chromosome 11, and assigned the gene to 11p and suggested that the 11q14 locus represented a PSMA pseudogene. Rinker-Schaeffer et al. used two P1 clones containing approximately 120 kb of the PSMA gene and surrounding sequence to repeat the FISH experiment, and under conditions of high stringency, assigned the gene to 11q14 *(56)*. Later, it became apparent that FISH can be subject to artefact under conditions of high stringency when one of the regions involved is close to a centromere, which in this case, is the 11p11 locus. We therefore mapped the gene again, using a number of sets of oligonucleotide primers designed to bind both intronic and exonic sequences of the gene, and PCR against a panel of somatic cell hybrids containing various regions of chromosome 11. We found that in fact, the PSMA gene does map to 11p11, approximately 7 mb from D11S1350. We also established that the "PSMA pseudogene" sequence reported on Genbank as mapping to 11q14 (accession number HSU93599), did not exist, but instead a gene that is highly homologous to the PSMA gene resides on 11q14.3 *(50)*. Further analysis of the gene at the 11q14 locus (which we have termed the "PSMA-like" gene), revealed that exons 2-19 of the PSMA gene are duplicated on the long arm of chromosome 11, along with their corresponding introns. We have been unable to detect duplication of the promoter region, or of exon one *(50)*. This would suggest a different mechanism of regulation and therefore different tissue expression pattern of the PSMA-like gene. The exonic sequences of the two genes are highly conserved (97% identical), and we have been able to determine that the PSMA-like gene is in fact

transcribed, and expressed in kidney and liver tissue, but not in prostate samples (O'Keefe *et al.*, unpublished data). Complete characterization of the PSMA-like gene is necessary, so that clinical and therapeutic strategies targeting PSMA can be designed to avoid PSMA-like expressing tissues or targets and subsequently limit lack of specificity and unecessary toxicity.

Molecular Evolution of the PSMA Family of Proteins

Comparison of the intronic sequence differences of the PSMA and PSMA-like genes allowed us to calculate that the time in evolution at which the original gene duplicated was 22 million years ago *(50)*. This data is consistent with the report that the tyrosinase gene, which is closely linked to the PSMA-like gene on chromosome 11q14, was subject to duplication to 11p 24 million years ago *(17)*. It would therefore seem that the locus at 11q14 contained the original gene, and that a little more than 20 million years ago - after the divergence of man and rodent, but before the separation of man and chimp this locus was duplicated on 11p11. Both genes then evolved further with the PSMA gene gaining expression in the in prostate. Southern blot analysis has shown PSMA homologs exist in many species, and this is supported by the finding of homologs as evolutionarily far back as yeast and *C. Elegans* (Fig. 2; Heston et al., unpublished; *(55)*). Similarly, it is interesting to note that although PSMA homologs have been cloned in several of these species, there are no reports of PSMA expression in the prostate *(5,22,2)*. Instead, these PSMA homologs seems to be primarily expressed in the small intestine, brain and kidney of other species. In the species which is most often used as an experimental model resembling man, the mouse, there is clearly no prostatic-expression of the murine homolog of PSMA (termed MoPSM) as determined by northern analysis and RT-PCR *(2)*. Furthermore, there is only one gene in mouse, and the MoPSM gene maps to mouse chromosome 7D1-2, which is syntenic with human 11q14 *(2)*. Considering these facts, it is tantalizing to suggest that the PSMA-like gene contributes to the extra-prostatic expression currently attributed to PSMA, and that expression of PSMA in the prostate might somehow put the prostate at high risk for developing mutations and subsequent carcinogenesis.

Enzymatic Activity of PSMA and Related Genes

Recently, PSMA homologs have been cloned from mouse *(2)*, rat *(5)* and pig *(22)*, in addition to the cloning of more distantly related paralogues of PSMA in humans *(18,51)*. The cloning and comparison of these homologs and paralogs has shed considerable light on some of the activities of PSMA, and provided some insight into which amino-acid sequences appear to be crucial in these activities. PSMA has three known activities; (1) a folate poly γ glutamyl carboxypeptidase (folate hydrolase)*(54)*, (2) a NAALADase *(7)* and (3) a dipeptidyl peptidase IV *(51)*. A comparison of these three activities in the PSMA homologs and paralogs is summarized in Table 1.

The folate hydrolase activity of PSMA describes the sequential removal of γ-linked glutamates from conjugated folates and folate analogs such as methotrexate γ-glutamate and pteroylpentaglutamate as shown in Fig. 3a *(54)*. The folate hydrolase activity of PSMA is maintained in the presence of sulfhydryl reducing agents and p-hydroxymercuribenzonate, in contrast to an unrelated folate hydrolase enzyme that is located in lysosomes.

PSMA also possesses NAALADase activity, in that it is able to hydrolyze the neuropeptide N-acetyl-L-aspartyl-L-glutamate (NAAG) to form N-acetyl-L-aspartate and glutamate. This hydrolysis is of the aspartyl α linkage as shown in Fig. 3b. This activity was first reported by Robinson et al. *(57)* in 1986 in the rat brain and was demonstrated to be inhibited by quisqualate. NAALADase and its neuropeptide substraight NAAG have been implicated in the regulation of excititory signalling in the nervous system *(14,77)*. Altered activity of NAALADase has been associated with various neurolgical disorders including Schizophrenia *(73,11)*, Alzheimer's Disease and Huntington's Disease *(52)*. In addition, increased levels of NAALADase have been observed in animal models for epilepsy *(47,45,46,52)* and amylotrophic lateral sclerosis *(58,74,72)*.

Carter et al., *(7)* used antisera to purified rat NAALADase to screen a rat brain expression library resulting in the isolation of a partial 1428 nt cDNA clone that had 86% homolgy to part of the human PSMA cDNA sequence. The entire rat PSMA/NAALADase sequence was subsequently cloned by *(5)*, and when transiently transfected into PC-3 cells (which are NAALADase negative), they gained

NAALADase activity which could be inhibited by quizqualic acid. Human PSMA was also demonstrated to posses NAALADase activity *(7)*. Further characterization by Luthi-Carter et al., demonstrated that the human brain NAALADase could be immunoprecipitated with the mAb 7E11-C5 *(44)*. As this antibody binds to residues not conserved in PSMA-like (O'Keefe et al., unpublished), it suggests that human NAALADase and PSMA are derived from the same gene. In addition, Luthi-Carter amplified from human cerebellum RNA by RT-PCR a sequence identical to the LNCaP derived PSMA sequence, indicating that this RNA is presents, but in itself not proving that all of the NAALADase activity found in the brain is from PSMA.

Recently, it was shown that PSMA also has dipeptidyl peptidase IV activity, which refers to the ability to hydrolyze Glycine-Proline-7-amido-4-methylcoumarin *(51)*. This amino dipeptidyl peptidase IV activity cleaves the bond between the proline residue and amido methylcoumarin molecule, as shown in Fig. 3c. It was first reported that PSMA possesses this activity when Pangalos et al *(51)* transiently transfected COS cells with PSMA cDNA and assayed for the dipeptidyl peptidase IV activity. Although the mock-transfected COS cells had dipeptidyl peptidase IV activity, the PSMA transfected COS cells had significantly more activity. The physiolgical role of the dipeptidyl peptidase IV activity of PSMA is not clear, however it may have a role in the regulation of various biologically active peptides, including collagen, neuropeptide Y and growth hormone releasing factor *(51)*. As such it would appear that PSMA is a multi-functional enzyme, possessing both amino- and carboxy-peptidase activities as a mono- and dipeptidase.

Rawlings and Barrett predicted the secondary structure of PSMA using a number of protein prediction and protein alignment programs, editing the results so that the potential zinc ligand binding sites and other blocks of secondary structures were aligned *(55)*. They predicted PSMA is made up of 6 organizational domains with domain E (a.a. 273-587) resonsible for the catalytic activity. They were then able to assign this catylicic domain to the M28 peptidase family and predicted that Asp^{377}, Asp^{387}, Glu^{425}, Asp^{453} and His^{553} are ligands for two atoms of zinc required for catalytic activity *(55)*. Speno

et al. performed site directed mutagenesis experiments altering these residues, resulting in severely reduced NAALADase activy, and were also able to demonstrate that substitution of some amono acids near the putative zinc ligands also has a large impact on enzymic structure and/or function *(63)*.

The Role of PSMA in Prostate Carcinogenesis and Progression

PSMA is thought to be involved both in glutamatergic signaling and in folate metabolism. Although there is no evidence that the PSMA substrate NAAG is present in the prostate, our laboratory has demonstrated the existence of Glu 2/3 and Glu 4 glutamate receptors using immunohistochemistry *(26)*. This would suggest that when PSMA is expressed on the surface of the prostate epithelial cell, and especially so when the membrane bound form of PSMA is upregulated in cancer, sufficient quantities of glutamate could be released to stimulate these receptors, leading to oxidative stress and subsequent cell and DNA damage *(12)*- further enhancing the ability of the cell to mutate and the cancer to progress.

Another possible function of PSMA was recently reported by Liu *et al. (41)* who observed a di-leucine motif in the amino-terminal of the protein indicating a possible role in internalization of ligands via PSMA. Incubation of LNCaP cells with antibodies against PSMA followed by laser scanning confocal microscopy revealed that the antibodies were internalized and remained in endosomes within the cell. The endocytosis occurred via clathrin-coated pits, and was shown to occur constitutively, although it was enhanced by the presence of antibodies suggesting a role for PSMA in the internalization of as yet undefined ligand(s)*(41)*.

Our most favored theory is based on the folate hydrolase activity of PSMA and PSM' that releases the terminal gamma linked glutamates from folates. To appreciate how the folate hydrolase activity of PSMA might be involved in carcinogenesis and the progression of prostate cancer, it is first necessary to understand the role of folate in this tissue. Dietary folates are generally polygammaglutamated. However, folate can only enter the cell by passive diffusion if it has been de-glutamated (although most extracellular folate is monoglutamated). Again, within the cell, folate is polyglutamated so that it can not diffuse out of the cell. The presence of PSM' in the cell would lead de-

glutamation of the polyglutamated folate, and subsequent loss of folate from the cell . In the prostate, there is an increased need for folate relative to that of other tissues *(24)*. Folate hydrolase would be expected to deglutamate folate and thus allow it to be in a form that could easily diffuse out of the cell, putting the cell at risk of becoming folate deficient *(54)*. Folate deficiency is associated with DNA damage and carcinogenesis *(13,34)*.

Folic acid deficiency can lead to DNA damage via increased uracil incoporation resulting in single stranded DNA breaks, and decondensation of chromosomes *(37,4)*. Folate deficiency may also lead to carcinogenesis by reducing DNA methylation, which in turn has been demonstrated to lead to the overexpression of some genes including a number of oncogenes *(16,35,3)*. Folate is integral to a number of basic metabolic processes in the cell, including DNA synthesis, DNA methylation, and the formation of methionine and polyamines. The prostate is the major organ that produces polyamines, producing between 8 and 100 fold greater amounts than other polyamine producing tissues *(24)*. This high production of polyamines is a drain on the folate-methionine pathway to begin with, and as a result the prostate is at greater risk of DNA damage induced by a low folate environment.

Therefore it is our hypothesis that PSM', the cytosolic version of PSMA expressed in normal prostate epithelial cells, would be a "catalyst" of DNA damage and subsequently carcinogenesis by depleting the prostate of intracellular folate. PSMA, the membrane bound isoform highly expressed in the tumor and metastatic deposits, could be also be expected to hydrolyse poly-γ-glutamated folates, allowing them to diffuse into cells in the local microenvironment. Although poly-γ-glutamated folates are not typically considered extracellular substances, within an environment such as a prostate tumor, there is a considerable number of dead or dying cells that can liberate these polyglutamated folates. Therefore cells expressing a membrane folate hydrolase such as PSMA would have a growth and survival advantage over non-expressing cells, especially if the levels of PSM' decreased in the PSMA-expressing cells. A possible extension of this hypothesis might explain why the neovasculature of most solid tumors express PSMA, in that tumors which characteristically have an inadequate blood supply,

may be able to sequester folate from dead cells if the they could induce the endothelial cells of the vasculature to express such a folate hydrolase.

After the switch of mRNA splicing to predominantly form PSMA, the membrane bound isoform of the protein, folate uptake by the cell would be enhanced. This in turn would lead to a greater proliferation rate of the cell, and could possibly also lead to enhanced mutation rates via glutamate receptors and oxidative stress on the cell. In this case, increased expression of PSMA could assist in evolution of the tumor and tumor growth, and progression of the cancer.

To assess this theory in the laboratory, we are currently using the transgenic mouse model. This is significantly assisted by the fact that the murine prostate does not express the homolog of PSMA, MoPSM. We have created transgenic mice expressing human PSMA and/or PSM' under the control of a prostate-specific promoter, and are examining the effect of folate deficiency on the rate of DNA damage in the presence of the PSMA isoforms (Bacich et al., unpublished).

Clinical Utility of Prostate-Specific Membrane Antigen

The potential of PSMA as a marker of clinical progression was first noted during characterization of the 7E11C5.3 antibody *(28)*. Sera from 20 of 43 patients with prostate cancer appeared to carry molecules reactive with the 7E11C5.3 antibody. However, none of 30 normal blood donors or 7 patients with BPH exhibited such reactivity. The authors also reported that prostate cancer patients who tested positively were more likely to be in progression ($p<0.05$) *(28)*, although other groups have not been able to detect PSMA in the serum of any patients with metastatic disease *(69)*. The possibility that PSMA could be used as a marker of circulating prostate-cancer cells was quickly examined by a number of groups. Israeli et al. *(31)* developed a highly-sensitive technique using Reverse-transcriptase PCR with nested primer sets to amplify PSMA sequences from patient blood samples. Using similar nested, "enhanced" or radioactive PCR-based methods, the consensus appears to be that there is no correlation between PSMA-positive results and clinical stage, pathological stage or tumor grade *(6,62,49)*. There are yet other reports of PSMA-mRNA expression in normal lymphocytes,

urine and bone marrow (including specimens from female controls), as the result of "illegitimate transcription"; that is insignificant numbers of PSMA transcripts produced to have any functional effect, but that are able to be detected by sensitive PCR techniques *(39,80,15,10)*.

As with all PCR-based methods to detect circulating cancer cells, the technique needs to be standardized between laboratories. It is clear from the literature that in the hands of different researchers, there is significant variation in the test results. Furthermore, the presence of circulating cells does not appear to be directly related to metastatic potential of the primary tumor, for example, Loric *et al. (43)* have shown that patients with inflamed prostates also exhibit circulating prostate cells. However, there are new technologies available now that may be able to solve these problems. The advent of "Real-Time" PCR which allows sensitive quantitation of PCR products, and the expanding access to custom-designed "Gene Chips" should make it easier to quantitate one or a number of prostate-specific transcripts. It has already been shown that a combination of RT-PCR methods for PSMA- and PSA-expressing cells is more accurate than either technique alone *(79,20)*. In addition to combining several different markers for analysis, it might also be fruitful to examine relative amounts of the PSMA mRNA splice variants, particularly PSM' versus PSMA.

PSMA RT-PCR, in combination with PSA RT-PCR has also been used to determine the "molecular surgical margins" at radical prostatectomy, by examining five biopsy specimens from the prostatic fossa *(65)*. The results, although preliminary, are promising. The authors found a perfect correlation between a positive PCR result, and histopathological determination of positive margins or extracapsular extension. Furthermore, control biopsy specimens taken from men undergoing radical cystoprostatectomy for bladder cancer or abdominoperineal resection for rectal cancer were all negative for the test. Interestingly, in four of sixteen cases with histopathologically negative surgical margins, the molecular margins were positive *(65)*. Validation of this unique method requires larger, longer and multi-institutional studies.

At the present time clinical imaging using PSMA-directed immunoconjugates utilize the Cyt-356 antibody, commercially known as the "Prostascint Scan™". While the results are promising, the test is probably not optimally designed. In studies localizing the target epitope of Cyt-356, Troyer et al. *(71)* found that the antibody binds to the short cytoplasmic domain of the protein. As such, Cyt-356 binds efficiently to dead cells, and not viable cells *(71,40,70)*. The ability of Cyt-356 to image metastatic deposits is most likely due to necrotic cells in the tumors and therefore the sensitivity of the imaging would be expected to be enhanced by use of antibodies directed against the external domain of PSMA. A number of groups have developed such "second generation" antibodies *(40,48)* and are currently carrying out phase I trials using the antibodies as both imaging agents and as therapeutic vectors.

The Future of Clinical and Therapeutic Strategies Utilizing PSMA

In an immunotherapeutic approach, Tjoa et al. *(66)* showed that T-cell proliferation could be induced *in vitro* by autologous dendritic cells pulsed with peptides from the PSMA amino-acid sequence. Dendritic cells are professional antigen presenting cells that can induce T-cell proliferation and cytotoxicity against specific antigens. This study was followed by phase I and II clinical trials, which showed positive results, with partial responders identified in groups of patients with both metastatic and suspected local recurrent disease. Follow up of the responsive patients nearly 300 days later revealed that more than 50% of the subjects were still responding *(67,59,60,68)*. While these results indicate a promising future for immunotherapeutic strategies against prostate cancer, there are several intrinisic problems. The therapy described here is restricted to patients of major histocompatibility antigen type A2 (HLA-A2) tissue type. Furthermore, HLA antigens are down-regulated by tumor cells, and as such would not be available for immuno-targeting.

In an innovative approach to avoid these restrictions, Gong et al. *(19)* devised an immunotherapeutic method that completely circumvents the need to have MHC mediated presentation of peptides at all. An artificial T-cell receptor was generated by cloning the DNA sequence responsible for recognition of PSMA by the J-591 antibody described above *(40)*, followed by a linker region and the

zeta chain receptor, into a retroviral vector *(19)*. T-cells (CD4+ and CD8+) from prostate cancer patients were then transduced with the vector and their response to cells expressing PSMA examined. The transduced cells efficiently and specifically lysed PSMA expressing cells, and also release cytokines in response to PSMA suggesting a prolonged response might be able to be attained *(19)*. If these results are as impressive *in vivo* as they are *in vitro*, such an approach should be able to target both the primary tumor and metastatic deposits, as well as the neovasculature of other solid tumors.

Other therapeutic approaches targeting PSMA currently being investigated by our and other laboratories include the use of prodrug strategies and gene therapy. To investigate prodrug strategies against prostate cancer, NIH3T3+/- PSMA and PC-3+/- PSMA transfected cells and LNCaP cells were grown in the presence of methotrexate triglutamate *(27)* and Heston *et al.*, (unpublished). In the cells expressing PSMA, the drug was converted into its cytotoxic derivative methotrexate and cell growth inhibited. However, in the non-PSMA expressing cells, the drug was non-toxic. While these results show promise, we are currently using the LNCaP xenograft model to determine if toxicity is specifically targeted to the tumor, or affects other cells expressing the murine homologs of the genes described in Table 1.

Cloning of the PSMA promoter, and more particularly the enhancer, has made the use of gene therapy constructs carrying either therapeutic or cytotoxic genes a viable alternative to those described above. In this approach, cytotoxic genes such as the cytosine deaminase (CD) or herpes virus TK genes are linked to the PSME and a compatible promoter and the patient is treated with cytotoxic prodrugs such as 5-fluorocytosine or gancyclovir. However, the first challenge is to demonstrate prostate-specificity of the PSME in the transgenic mouse model and we are currently evaluating this aspect (Bacich et al., unpublished).

Looking forward to the 21st Century

The PSMA story has yielded several unexpected surprises so far, but we still do not know how or if expression of this gene influences the development or progression of prostate cancer. The possible

role of PSMA in the angiogenic pathway of tumors is intriguing, but also suggests we have much left to learn about this fascinating protein. The expression of PSMA in tumor-associated vasculature, as well as its high expression in virtually all prostate tumors and metastases, and particularly in hormone refractory disease for which there is currently no efficient treatment, indicates targeting of PSMA may be highly valuable as a treatment for not only prostate cancer, but several kinds of solid tumors.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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