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TITLE: Generation of Transgenic Animals Producing Ezymatically Active Prostate-Specific Antigen (PSA) in Normal and Malignant Prostate Tissue

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Prostate-specific antigen (PSA) is used extensively as a serum marker to screen for prostate cancer and is also used as a surrogate marker to assess response to therapy for prostate cancer. The physiologic role of PSA in normal prostate biology is uncertain but the protein appears to play a role in reproduction by enhancing sperm motility. PSA is a serine protease with chymotrypsin-like specificity. PSA, through its proteolytic activity, may play an important role in prostate cancer progression, invasion, angiogenesis, and/or metastasis. To date, no lab has successfully produced a mammalian cell line that makes large amounts of enzymatically active PSA. This inactivity appears to be due to lack of, or incomplete processing of, Pro-PSA to the active form, presumably due to the absence of the necessary processing protease. Currently available prostate cancer cell lines and mouse models producing enzymatically inactive PSA are not useful for developing these prodrug therapies or for understanding the role of PSA in the biology of prostate cancer. We have generated a modified PSA gene that was used to generate cell lines that produce much higher levels of total PSA and most of this PSA is enzymatically active. This PSA gene was used to generate mice that produce PSA in their prostates. These mice can be used to study role of PSA in biology of prostate cancer and to test PSA-activated prodrug therapies.

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INTRODUCTION:

Prostate-specific antigen (PSA) is used extensively as a serum marker to screen for prostate cancer and is also used as a surrogate marker to assess response to therapy for prostate cancer . The physiologic role of PSA in normal prostate biology is uncertain but the protein appears to play a role in reproduction by enhancing sperm motility. PSA is a serine protease with chymotrypsin-like specificity. PSA, through its proteolytic activity, may play an important role in prostate cancer progression, invasion, angiogenesis, and/or metastasis. The PSA protein is secreted as an inactive zymogen and a 7 amino acid "Pro" sequence must be correctly processed to generate enzymatically active PSA. To date, no lab has successfully produced a mammalian cell line that makes large amounts of enzymatically active PSA. This inactivity appears to be due to lack of, or incomplete processing of, Pro-PSA to the active form, presumably due to the absence of the necessary processing protease. Our laboratory and others are developing prodrugs that are activated by PSA. Other groups are interested in studying the role of enzymatically active PSA in prostate tumor growth, progression, angiogenesis, and metastasis. Currently available prostate cancer cell lines and mouse models producing enzymatically inactive PSA are not useful for developing these prodrug therapies or for understanding the role of PSA in the biology of prostate cancer. The underlying hypothesis of our proposal was that a modified PSA gene can be engineered that will yield a PSA protein that will be correctly processed to enzymatically active PSA both in vitro and in vivo. Through site directed mutagenesis we have removed the 21 nucleotides coding for the 7amino acid wild type Pro-PSA sequence and replaced them with the coding sequence that generates the amino acid sequence recognized by furin, a ubiquitously expressed protease. This modified PSA gene will be used to produce a transgenic mouse model that makes enzymatically active PSA in its prostate. The wild type PSA gene will also be used to make a second transgenic mouse and expression of active PSA in these two models will be compared. These cell lines and mouse models that can be used in development of PSA-activated prodrug therapies and can also be used to study role of enzymatically active PSA in biology of prostate cancer.

Body:

The goal of this Exploration: Resource Development award was to generate cell lines and transgenic animals that produce increased amounts of enzymatically active PSA compared to available PSA-producing prostate cancer models. **Task 1** was to characterize the enzymatic activity of clones from Pre-Pro PSA and Pre-Pro"Furin" PSA transfected prostate cancer cell lines.

For this task we generated two PSA constructs. One of these consisted of the wild type Pre-Pro-PSA sequence and the second consisted of a construct in which the Pro portion of the sequence was replaced with a sequence that encoded for a polypeptide recognized as a substrate for the ubiquitously expressed furin protease. These constructs were transfected into the human prostate cancer cell lines CWR22R, LNCaP and PC-3. Previously we had demonstrated that CWR22R produced low levels of PSA in vitro with less than 15% of this PSA being enzymatically active. LNCaP cells produce higher amounts of PSA, but again we have demonstrated that only ~ 15% of this PSA was active.

We have generated clones of LNCaP, and CWR22R expressing both the wild type and mutant PSA genes. PC-3 cells have also been transfected and are currently being characterized. Figure 1 shows representational data on relative production of PSA in a CWR22R clones. These results demonstrate that both wild type and mutant transfected clones produce about 10-fold higher amounts of PSA than the untransfected wild type CWR22R. In addition, the cells transfected with the mutant PSA produce about 70-fold higher amount of active PSA compared to the untransfected wild type. The cell transfected to overexpress wild type PSA generate more PSA than the untransfected wild type, but both cell types only produce a low percentage of active PSA.

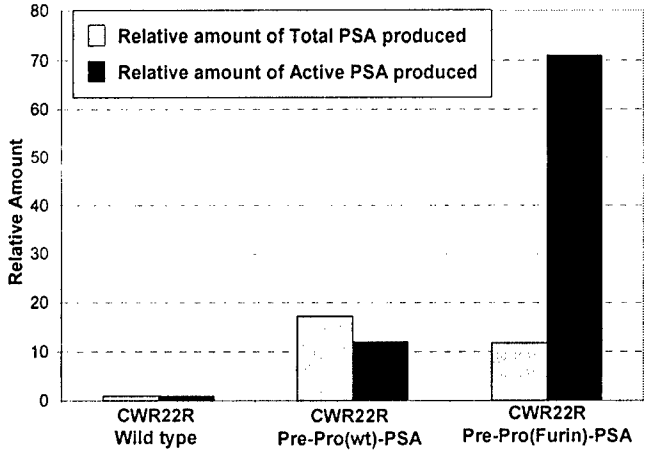

Figure 1. Relative production of total PSA and enzymatically active PSA by wild type (untransfected) CWR22R cells compared to Pre-Pro(wt)-PSA and Pre-Pro(Furin)-PSA transfected CWR22R cells. PSA produced by wild type scaled to 1 relative unit. PSA from wild type CWR22R cells was 16% active, Pre-Pro(wt)-PSA was 11% active and Pre-Pro(Furin)-PSA was 99% active compared to enzymatically active PSA purified from human seminal plasma.

Clones of both wild type and mutant transfected LNCaP and CWR22R were evaluated for effects of increased expression of active PSA on growth. In both cases no significant change in growth rates were observed between the two sets of clones (data not shown). In addition growth rate of CWR22R untransfected wild type and CWR22R mutant PSA transfected were compared in vivo when inoculated subcutaneously. These experiments demonstrated no significant difference in tumor doubling times between the two lines (data not shown). Overall these results suggest that increased production of enzymatically active PSA does not change growth rate of cells in vitro or in vivo when growing subcutaneously. Further experiments are underway in our laboratory to assess effects of enhanced production of active PSA on growth of tumors within the prostate and within the bone.

Task 2 of the award was to produce transgenic animals expressing either Pre-Pro"Furin" PSA or Pre-ProPSA gene under control of androgen regulated prostate-specific probasin promoter. To accomplish this we cut Pre-Pro PSA and Pre-Pro "furin" PSA gene out of pcDNA3.1 vector and reinserted it downstream of

$(ARR)_2$ probasin promoter in pGL2-Basic Vector. The linearized gene was provided to the Johns Hopkins Transgenic Animal Core Facility which provided us with founder animals expressing either of each construct.

The Core Facility initially provided us with animals expressing wild type PSA. Through serial crossing we have been able to generate animals that are homozygous for PSA production in the prostate, figure 2. Prostates from these animals have been isolated and total PSA produced determined from homogenates. Levels of PSA produced are in the 1-2 μg/ml range which is approximately 100-fold lower than those observed for human prostate. Greater than 95% of this PSA was in the free (i.e. uncomplexed) form. Mixing the PSA isolated from these prostates with the PSA inhibitor alpha-2-macroglobulin demonstrated that some fraction of this PSA was enzymatically active, figure 2 (white arrows). Following immunoprecipitation of PSA from these homogenates, incubation with a PSA-selective fluorescent substrate demonstrated the percent enzymatic activity to be ~ 15%.

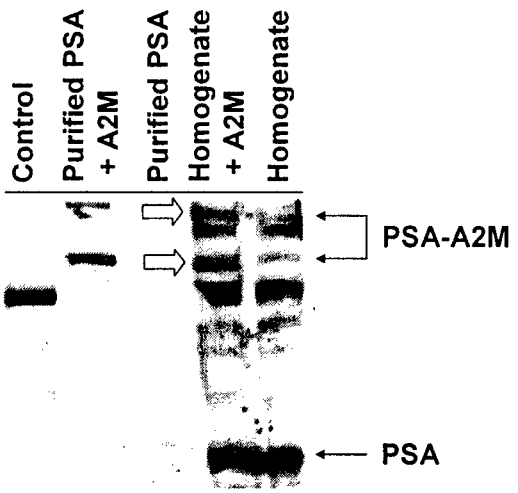

Figure 2. Western blot analysis of PSA produced by Pre-Pro(wt)-PSA mouse. Black arrows show 33 kDa PSA and PSA-A2M complex. White arrows indicate increased density of PSA-A2M band following incubation of prostate homogenate with purified human A2M.

At the same time these animals were being developed a series of Pre-Pro"Furin" PSA founder animals were also identified. These animals were crossed with wild type animals x 2 and none of ~ 20 offspring were positive for the PSA gene. These results suggested that the original founders did not have PSA in the germline. Recently a second set of founder animals were generated by the transgenic core facility. These animals have been crossed with wild type and we have identified progeny that are positive for PSA by PCR-analysis. We are currently waiting for these animals to mature and plan to characterize the amount of PSA produced as well as the percent enzymatic activity in order to confirm whether our original hypothesis will be true. Subsequently, these wild type and mutant PSA-producing animals will be characterized for effects of PSA expression on prostate morphology. Animals will also be crossed with various prostate cancer mouse models (i.e. TRAMP, c-myc, SKP-2) to determine if PSA expression in these cancers changes morphology, growth rate, and metastatic potential.

Key Accomplishments:

1. Generated PSA construct that yields cell lines that produce high levels of enzymatically active PSA.

2. Generated PSA-producing transgenic mouse that produces relatively high levels of PSA in the prostate. However, only small percentage of PSA produced by this mouse is enzymatically active

3. Generated mutant PSA-producing transgenic mouse that will potentially produce high levels of enzymatically active PSA in prostate

Reportable Outcomes:

1. Generated cell lines producing high levels of enzymatically active PSA that will be made available to prostate cancer research community.

2. Generated PSA-producing transgenic mice that will be made available to the prostate cancer research community.

3. Post-Doctoral Fellow Dr. Simon Williams awarded DOD PCRP Post-Doctoral Fellow Award based on this work.

4. Applied for DOD PCRP Idea Development Award (2/05) to support further characterization of these transgenic animals and effect of PSA-production on prostate cancer growth and metastases.

References:

1. Denmeade SR, Sokoll LJ, Chan DW, Khan SR, Isaacs JT. Concentration of enzymatically active prostate specific antigen (PSA) in the extracellular fluid of primary human prostate cancers and human prostate cancer xenograft models. Prostate. 2001 Jun 15; 48(1):1-6.

2. Denmeade SR, Lovgren J, Khan SR, Lilja H, Isaacs JT. Activation of latent protease function of pro-hk2, but not pro PSA, involves autoprocessing. Prostate. 2001 Jul 1;48(2):122-6.

3. Mhaka A, Denmeade SR, Yao W, Isaacs JT, Khan SR. A 5-fluorodeoxyuridine prodrug as targeted therapy for prostate cancer. Bioorg Med Chem Lett. 2002 Sep 2;12(17):2459-61.
4. Denmeade SR, Sokoll LJ, Dalrymple S, Rosen DM, Gady AM, Bruzek D, Ricklis RM, Isaacs JT. Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hk2, and PSMA in human prostate cancer models. Prostate. 2003 Mar 1;54(4):249-57.

5. Denmeade SR, Litvinov I, Sokoll LJ, Lilja H, Isaacs JT. Prostate-specific antigen (PSA) protein does not affect growth of prostate cancer cells in vitro or prostate cancer xenografts in vivo. Prostate. 2003 Jun 15;56(1):45-53.

6. Denmeade SR, Jakobsen CM, Janssen S, Khan SR, Garrett ES, Lilja H, Christensen SB, Isaacs JT. Prostate-specific antigen-activated thapsigargin prodrug as targeted therapy for prostate cancer. J Natl Cancer Inst. 2003 Jul 2;95(13):990-1000.

Conclusions:

A more relevant prostate cancer model would be one that produces higher amounts of enzymatically active PSA at levels that approach those produced by human prostate cancers. In the twelve months of support from this award we have generated such improved PSA-producing cell lines. In addition, we have generated transgenic animals that produce PSA in the prostate that could be used to characterize role of PSA in prostate cancer biology, develop new vaccine strategies and test PSA-activated prodrug/protoxin therapies.