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Analysis of the Role of the Wnt/B-Catenin Pathway in Prostate Development and Tumorigenesis

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We have initiated studies to determine whether dysregulation of the Wnt/B-catenin signaling pathway results in prostate disease. Our preliminary results show that prostate-specific deletion of Apc, a genetic alteration associated with increased levels of B-catenin, results in the induction of early onset prostate cancer. Analysis of older mice reveals these prostate tumors have the propensity to metastasize, at least to lymph nodes. To directly address whether B-catenin activation induces progression, we have created mice that allow us to induce expression of an oncogenic form of B-catenin in the prostate. We have also crossed the mice carrying the prostate specific deletion of Apc with mice deficient for the homeobox gene Nkx3.1. Nkx3.1-deficient mice reproducibly develop prostate hyperplasia and dysplasia, but show no signs of progression beyond this point. We are looking for synergistic effects of Wnt pathway activation and Nkx3.1 loss in terms of time of tumor onset as well as metastatic spread.
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Abstracts

Copies of Four Abstracts Submitted for Presentations
4. Introduction

In order to determine the role of dysregulated Wnt signaling in the development and progression of prostate cancer, we have or currently are generating mouse models. One model system is based on creating mouse strains that allow us to temporally and spatially control the expression of an oncogenically active β-catenin gene in the prostate using the recently developed tetracycline-inducible system. We have generated founder lines to achieve this goal and have done pilot experiments to identify lines that will be most useful for this purpose. In addition, using the cre-lox system, we have generated mice lacking the Apc gene in the prostate. Apc normally controls the level of B-catenin protein in the cytoplasm of cells. Loss of Apc leads to increased levels of the B-catenin protein and subsequent activation of downstream signaling pathways. We have found that mice lacking Apc in the prostate develop early onset prostate hyperplasia that appears to progress to a more aggressive lesion over time. Finally, we have established reagents that will allow us to determine whether dysregulation of the Wnt/β-catenin/Apc signaling cascade synergizes with other genetic pathways in prostate cancer initiation or progression.
5. Body

Rationale (taken from original grant application)

Prostate cancer causes over 40,000 deaths per year in the United States (1). Most deaths are due to the metastatic spread of prostate cancer throughout the body. Currently, the only effective therapy for advanced prostate cancer is androgen depletion by surgical or chemical castration. This often causes temporary remission of the tumor. Unfortunately, prostate cancer commonly recurs in these patients in a form that is androgen-independent. There is currently no effective treatment for androgen independent prostate cancer and there is an urgent need to develop effective therapies for this disease.

β-catenin is a protein that plays multiple roles in regulating cell growth and function (2). Normally, the cytoplasmic level and nuclear localization of β-catenin is tightly regulated. In many tumors, however, this regulation is lost, either due directly to mutations in the β-catenin gene or by mutations in genes whose protein products are necessary for this regulatory process (2). One example is colon cancer, where the vast majority of tumors display increased cytoplasmic levels and nuclear localization of β-catenin due to loss of the APC gene (3). Over 20% of advanced prostate tumors have elevated levels of β-catenin, and mutations in the β-catenin gene have been identified in prostate tumors (4, 5). β-catenin can specifically associate with the androgen receptor (AR) (6–8). This interaction alters the signaling capabilities of the AR, making it more promiscuous in its ability to be activated by steroid hormones other than androgens (9). Based on these observations, β-catenin activation represents a viable target for therapeutic intervention in advanced prostate cancers.

Objective/Hypothesis. The hypothesis underlying this proposal is that activation of B-catenin signaling contributes to the progression of prostate cancer to a malignant state.

Specific Aims We will directly test the effects of activated B-catenin on 1) prostate development and homeostasis and 2) progression of prostate cancer in a mouse model that normally develops prostate hyperplasia and dysplasia.

Study Design. We will create and analyze a transgenic mouse strain that expresses a mutated, activated version of β-catenin under the control of a modified Probasin promoter (ARR2PB) (10). We will systematically analyze these mice at various ages at the anatomical and histological level for abnormalities in prostate development and histology. Since we hypothesize that activation of β-catenin will induce progression of hyperplastic or dysplastic prostate cells, we do not expect it to influence prostate cancer initiation. To directly address whether β-catenin activation induces progression, we will cross the ARR2PB-activated β-catenin transgenic mice with mice deficient for the homeobox gene Nkx3.1 and analyze the prostate of these mice at the anatomical and histological level. Nkx3.1-deficient mice reproducibly develop prostate hyperplasia and dysplasia, but show no signs of progression beyond this point (11). By introducing expression of activated β-catenin on this background, we predict that these hyperplastic/dysplastic lesions will progress toward malignancy and may become invasive into the normal tissue around the prostate. We are assisted in these experiments
via collaboration with Dr. Wade Bushman. Dr. Bushman has extensive experience in the analysis of mouse models of prostate development and tumorigenesis (12-15), and this proposal represents the continuation of an established collaborative relationship between our laboratories.

In support of this work, a post-doctoral fellow in my laboratory has traveled to Madison, Wisconsin to spend time in the Bushman laboratory to learn more about techniques in prostate analysis. This fellow, Dr. Troy Giambernardi, devotes 50% of his effort towards this project and is funded by a grant from the American Cancer Society. A full time technician, Holli Charbonneau, will begin work this April on this project. Holli has worked on this project as an undergraduate intern for the past year.

Objective 1: To determine the effect of transgenic activation of β-catenin on prostate morphology.

Task 1. Develop a plasmid construct that directs the expression of an activated form of β-catenin under the control of the ARR2PB promoter (ARR2PB-S37A β-catenin), sequence confirm, and prepare for microinjection (Months 1-2)

Task 2. Perform pronuclear microinjection (in collaboration with Bryn Eagleson) and screen resulting offspring for the presence of the transgene (Months 3-5)

Task 3. Generate offspring from each founder line to establish strains (Months 6-10)

Mice carrying a transgene directing the expression of an activated form of B-catenin under the control of the modified probasin promoter (ARR2PB) (10, 16) were created by pronuclear microinjection. This was performed by Bryn Eagleson, director of the VARI Transgenic Core Facility. Twelve potential founders were created and nine of those transmitted the transgene through the germline. These founder strains are identified by the following numbers: 1652, 1655, 1658, 1674, 1688, 1692, 1697, 1760, and 1764. We have established breeding lines for each of these and have begun to screen the males in each line for proper expression. This work has been delayed recently because the room that these lines were being maintained in was exposed to mice that arrived from the NCI-Frederick mouse facilities that carried mouse hepatitis virus (MHV) (17). These mice were sent to numerous facilities throughout the country. Luckily, our vivarium is a shower-in, barrier facility in which each of the cages is maintained in an isolated environment. Our vivarium staff tested every cage and found that the MHV infection was contained within two cages in that room. We made the decision to sacrifice most of the animals in the room and maintain a small number of cages in an isolated room so that we could rederive the strains back into our facility in a clean manner. We have done this for three of the lines (partly based on the initial screening of these lines described below).

Task 4. Screen males from founder lines for proper expression of activated β-catenin (Months 11-14)
We have collected samples from the nine lines for analysis. We have performed Western analysis on lysates from these lines. Our preliminary analysis suggested that at least two of the lines, 1655 and 1764, expressed the TetON protein at high levels in the dorsal and ventral lobes of the prostate. Initial analysis of the other lines suggested that they did not express the proteins to the same level. We have also collected samples for immunohistochemical analysis. We have not been able to use the antibody we used for Western analysis to detect the TetON protein in formalin-fixed paraffin sections. We are in the process of evaluating in situ hybridization based approaches for cell-specific expression of the TetON protein.

Figure 1. Mice carrying a transgene directing the expression of a Tet-ON protein express the TetON protein in the prostate. Samples from a cell line not expressing TetON (HeLa), cell lines expressing the TetON protein (HeLa TetON), and tissue lysates from various transgenic mice were obtained. (AP=anterior prostate or coagulating gland; DVP=pooled Dorsal-ventral glands) 1888, 2318, and 2319 were three independent transgenic mice from the 1764 line.

![Western Analysis of Tet-on Founder with VP-16 Antibody](image)

We have also begun to evaluate the functionality of the two best strains, 1764 and 1655, by crossing these strains to strains which express various reporter genes under the control of the tetracycline responsive element (TRE). Compound transgenic mice, upon exposure to doxycycline either in the drinking water or the food, should express the gene under the control of the TRE. We have obtained a strain of mice that expresses both the Wnt1 oncogene and the luciferase reporter (18). This strain was generously provided by Dr. Lewis Chodosh (University of Pennsylvania). We have crossed this strain with the 1764 strain and generated compound transgenic male mice. We have exposed these mice to doxycycline for three months and then taken samples of the prostatic lobes. We will examine these for expression of the luciferase gene by making protein lysates from them and running standard luciferase assays. We will also perform immunohistochemistry on these samples for the Wnt1 protein.

In addition to crossing the 1764 strain to the Wnt1-luciferase strain, we have also crossed it to a strain that expresses an oncogenic version of K-ras. We chose this because we had
access to this strain at the beginning of the year and knew the strain worked in other contexts (for example, modeling lung cancer (19)). Induction of K-ras in compound transgenic mice resulted in prostatic dysplasia. This further supports the functionality of the 1764 strain for prostate cancer modeling in the mouse.

**Task 5. Generate increased numbers of mice for analysis (Months 14-19)**

**Task 6. Analyze mice at the desired ages for prostate morphology at the gross anatomical and histological level (Months 20-36)**

We have begun to cross the 1764 strain to a strain of mice that expresses an activated, oncogenic version of β-catenin (S37A β-catenin) under the control of the tetracycline responsive element (TRE-β-catenin). Analysis of this mouse strain in other contexts revealed that β-catenin could be induced with doxycycline treatment. We are generating the relevant mice on which to evaluate the effect of oncogenic β-catenin on prostate growth. We have generated compound transgenic mice for the 1764 strain and the TRE-β-catenin strain. Preliminary analysis has not shown any detectable phenotypic abnormalities, although we have only looked at mice in which β-catenin was ectopically expressed for less than four months.

We have also pursued an alternative approach to alter Wnt signaling specifically in the prostate. This was done by creating mice in which the Apc gene was specifically inactivated in prostate tissue via the use of the cre/lox system. In this approach, mice are genetically engineered which contain genes in which genetic regions required for function are flanked by loxP sites or “floxed.” These floxed alleles retain normal function until they are exposed to cre recombinase. Upon exposure to cre, the genetic elements between the loxP sites are excised, leading to inactivation of the specific gene (20). We have created mice carrying a floxed allele of Apc via generation of chimeric mice (and subsequent breeding) using embryonic stem cells obtained from Dr. Tetsuo Noda (Japan) (21). Mice expressing cre in a prostate specific manner (Probasin-cre or PB-cre) were obtained from Dr. Pradip Roy-Burman (University of Southern California) (22). Through several rounds of mating, we have created mice that are homozygous for the floxed allele of Apc and also carry the PB-cre transgene (PB-cre; Apc-flox/flox). Our analysis has shown that such mice develop enlarged prostates associated with prostatic hyperplasia as early as three months of age. We have allowed a cohort of such animals to age and found via analysis of animals at 7 months of age that these mice continue to progress to more aggressive states of hyperplasia displaying widespread prostatic intraepithelial neoplasia (PIN) and areas of microinvasion. We have also noted the presence of enlarged lymph nodes in these animals and are currently assessing whether these represent areas of metastasis. During the next two years of this study, we will examine whether androgen deprivation (via surgical castration) can inhibit the development of these tumors and whether the tumors ever become androgen independent. We are also going to examine whether these prostate tumors are capable of metastasizing to other organs in the body such as the lung, liver, or bone.
Objective 2: To examine the effect of transgenic activation of β-catenin on inducing prostate cancer in the Nkx3.1-deficient mouse.

Task 1. Order Nkx3.1-deficient mice from the Mouse Models of Human Cancer Consortium repository and establish a colony in the Van Andel Institute mouse facility. (Sometime within the first 12-14 months)

Task 2. After identifying which ARR2PB-S37A β-catenin transgenic strains exhibit the desired expression patterns of β-catenin (Objective 1, Task 4), breed these strain(s) to the Nkx3.1-deficient mice. It will require two generations of crosses to generate ARR2PB-S37A β-catenin transgenic mice with varying Nkx3.1 genetic status. (Months 14-19).

Task 3. Analyze mice at the desired ages for prostate morphology at the gross anatomical and histological level (Months 20-36)

We have obtained Nkx3.1-deficient mice (11) from the MMHCC repository in Frederick, Maryland and successfully rederived them into our barrier facility at VARI. We have then begun to cross them with both the PB-cre;Apc-flox/flox mice and the PB1764;TRE-β-catenin mouse strains to eventually generate mice that have alterations in Wnt signaling and are deficient for Nkx3.1. In each case we will evaluate whether dysregulation of these two pathways results in synergistic effects on prostate tumor progression and metastasis. Finally, we are also generating mice that are deficient for both the Apc and Pten genes in
the prostate by creating mice of the following genotype: *PB-cre;Apc-flox/flox;Pten-flox/flox*. Given that prostate-specific deletion of *Pten* leads to prostate cancer in the mouse that metastasizes to the lung (23), and that our first year of work on this grant has shown that mice lacking *Apc* also develop prostate cancer, we are interested in determining whether there may be a synergistic effect of loss of these two genes. Given the role of Wnt signaling in bone development (24), we are especially keen to determine whether a mouse model of prostate cancer that metastasizes to the bone can be developed in any of these contexts.

6. Key Research Accomplishments

During our first year of work, we have produced what, in my opinion, are several key research accomplishments. These are described in the previous section and can be summarized as the following:

1. We have created and completed initial characterization of a strain of mice that expresses the TetON protein specifically in the prostate. This strain (PB1764) will not only allow us to progress in our experiments for the completion of the work proposed for this grant, but should also be an important resource for others in the field of prostate cancer to conditionally express other genes in a prostate-specific manner.

2. During our characterization of the PB1764 strain, we showed that ectopic expression of an activated K-ras gene leads to the development of prostatic dysplasia. This result was obtained by doing a small pilot control experiment for the work to eventually dysregulate the Wnt signaling pathway in the prostate, but is interesting in and of itself. This is due to the fact that several publications have linked ras activation to some prostate cancers. Perhaps most interesting is that ras mutations are associated more commonly with prostate cancer in Japanese populations relative to American populations (25). Thus, further work on this observation may reveal insights into genetic background differences and the development of prostate cancer.

3. We have also developed a mouse model for dysregulated Wnt signaling in the prostate. In this model, loss of *Apc* leads to the development of early onset prostate cancer. Furthermore, this model appears to develop a metastatic version of the disease.
7. Reportable Outcomes

A. Abstracts (see attached information in the Appendix)

B. Presentations

The abstracts included in the Appendix were all submitted as poster presentations for the indicated meetings. In addition, I have been asked to present an oral presentation at the Michigan Prostate Colloquium Meeting on May 1, 2004.

C. Animal Models

As can be determined by reading the body of this report, the vast majority of work in support of this grant award is focused on the generation of mouse models for human prostate cancer. A summary of these is provided below in this section.

1. We have created a mouse in which the TetON protein is expressed specifically in the prostate. This mouse strain is useful for not only some of our studies outlined in this report, but should also be useful to the field in general as it allows for prostate specific expression of any transgene that is controlled by a tetracycline responsive element.

2. In a pilot study to determine the functionality of the Probasin-TetON strain, we have shown that mice that express activated K-ras specifically in the prostate develop prostatic dysplasia.

3. We have also created mice that lack the Apc gene specifically in the prostate and shown that they develop early onset prostate cancer which progresses to a metastatic state.

4. Other models of Wnt signaling dysregulation in the prostate are currently being developed based on the strains outlined above.
8. Conclusions

While we are still in the early stages of this work, we can already conclude based on our mouse models that alterations in the Wnt signaling pathway lead to early onset prostate cancer. We have also established that expression of oncogenic K-ras specifically in the prostate leads to prostatic dysplasia.

In terms of the "so-what" factor, we believe these observations are important scientific products. In the past, human prostate tumors had been shown to contain mutations in genes of the Wnt signaling pathway. However, it was not clear if these mutations had anything to do with the initiation or progression of the tumor. Two other recent reports have suggested that alterations in the Wnt pathway can induce changes in the prostate (26, 27). However, if our initial observations suggesting that we have created a model system in which invasive prostate tumors develop as a result of inactivating Apc, it would be the first demonstration of induction of invasive prostate cancer by dysregulation of the Wnt pathway. We believe this observation has important implications in examining patients with human prostate cancer and in developing treatments to inhibit progression of the disease. In addition, the system we have developed to allow for inducible gene expression in the prostate could have applications for others in the field and should be a valuable resource. In the context of characterizing this strain, we performed a pilot study using an inducible k-ras gene. The observation that ectopic expression of K-ras induces prostatic dysplasia in this study could have important implications in understanding normal prostate growth.

9. References

Appendix Material (Abstracts submitted for presentations at meetings)

1. 2004 Student Poster Session on Capitol Hill, Washington, DC (April 2004)

Developing a Mouse Model for Prostate Cancer.

Troy A. Giambernardi¹, James C. Goolsby¹, Jose A. Toro¹, James H. Resau¹, Aubie Shaw², Wade Bushman², and Bart O. Williams¹

¹Van Andel Research Institute, Grand Rapids, MI, USA, ²University of Wisconsin-Madison, Madison, WI, USA.

Prostate cancer is diagnosed in over 200,000 American men annually and causes approximately 42,000 deaths per year. However, its etiology is poorly understood. Prostate carcinogenesis is a multi-step process involving progression from small latent carcinoma, to large, higher grade, metastasizing carcinoma. Recent data suggest that a variety of pathogenetic pathways exist. We describe our initial studies on two different genes known to be dysregulated in prostate cancer: K-ras and B-catenin. K-ras mutations result in constitutive activation of numerous downstream signaling cascades associated with unregulated proliferation and impaired differentiation. Activating K-ras mutations are present in a variable percentage of prostate carcinomas. The clinical significance of K-ras mutations is unclear, although they are associated with lower median patient survival times. To investigate the role of activated K-ras in prostate carcinogenesis, we developed a mouse model where oncogenic K-ras expression can specifically be induced in the prostate. Our data indicates over-expression of mutant K-ras in prostate epithelial cells causes dysplasia. Wnt signaling plays key roles in development, and alterations in the Wnt pathway are commonly associated with human tumors. Several groups have identified activating point mutations in subsets of prostate carcinomas. These mutations occur focally within the tumor, suggesting that B-catenin mutations contribute to tumor progression. We have created transgenic mice where expression in a mutated B-catenin gene can be induced by exposure to doxycycline and are in the initial stages of examining them.
Assessing the Role of Wnt Signaling in Prostate Development and Tumorigenesis.

Troy A. Giambemardi¹, Holli Charbonneau¹, Pradip Roy-Burman², Aubie K. Shaw³, Wade Bushman⁴, and Bart O. Williams¹.

¹Laboratory of Cell Signaling and Carcinogenesis, Van Andel Research Institute, Grand Rapids, MI, USA, 49503; ²University of Southern California, Los Angeles, CA; ³Division of Urology and McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI.

Prostate cancer causes over 42,000 deaths per year in the United States. Most deaths are due to the metastatic spread of prostate cancer throughout the body. Currently, the only effective therapy for advanced prostate cancer is androgen depletion by surgical or chemical castration. This often causes temporary remission of the tumor. Unfortunately, prostate cancer commonly recurs in these patients in a form that is androgen-independent. There is currently no effective treatment for androgen independent prostate cancer and there is an urgent need to develop effective therapies for this disease. β-catenin plays multiple roles in regulating cell growth and function. Normally, the cytoplasmic levels and nuclear localization of β-catenin is tightly regulated. In many tumors, however, this regulation is lost, either due directly to mutations in the β-catenin gene or by mutations in genes whose protein products are necessary for this regulatory process. One example is colon cancer, where the vast majority of tumors display increased cytoplasmic levels and nuclear localization of β-catenin due to loss of the APC gene. Over 20% of advanced prostate tumors have elevated levels of β-catenin, and mutations in the β-catenin gene have been identified in prostate tumors. β-catenin can specifically associate with the androgen receptor (AR). This interaction alters the signaling capabilities of the AR, making it more promiscuous in its ability to be activated by steroid hormones other than androgens. Based on these observations, β-catenin activation represents a viable target for therapeutic intervention in advanced prostate cancers.

To gain insight into the role of Wnt signaling in prostate development and tumorigenesis, we are pursuing a variety of related approaches. First, we are characterizing mice that carry mutations in both the Lrp5 and Lrp6 genes. We have found synergistic effects of the mutations in several tissue types, and specifically found that the development of prostate branching and morphogenesis is altered in mice carrying compound mutations in the two genes. To further address the role of the canonical Wnt signaling pathway in prostate morphogenesis, we are creating mice that carry a prostate-specific deletion of the B-catenin gene.

We are also pursuing experiments to address the effect of inappropriate activation of the canonical Wnt signaling pathway on the prostate. We have created mice carrying a prostate-specific deletion in the Apc gene and found that such mice develop early onset prostate cancer. Characterization of these tumors is ongoing. We are also developing tetracycline inducible systems to temporally regulate the activation of the pathway.
Basic and Clinical Advances in Prostate Cancer Research
4th Symposium of Michigan Prostate Research Colloquium
Van Andel Research Institute, Grand Rapids, MI
May 1, 2004
Abstract Deadline: April 10, 2004

DEVELOPING A TETRACYCLINE INDUCIBLE SYSTEM FOR MODELING GENETIC CHANGES IN PROSTATE CANCER.
Troy A. Giamberardi¹, Holli Charbonneau¹, JC Goolsby², James Resau², Aubie K. Shaw³, Wade Bushman³, and Bart O. Williams¹.
¹Laboratory of Cell Signaling and Carcinogenesis and ²Analytical, Cellular and Molecular Microscopy Laboratory, Van Andel Research Institute, Grand Rapids, MI ³Division of Urology and McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI

Prostate cancer is diagnosed in over 200,000 American men annually and causes approximately 42,000 deaths per year. However, its etiology is poorly understood. Prostate carcinogenesis is a multi-step process involving progression from small latent carcinoma, to large, higher grade, metastasizing carcinoma. Recent data suggest that a variety of pathogenetic pathways exist. We describe our initial studies on two different genes known to be dysregulated in prostate cancer: K-ras and B-catenin.

K-ras mutations result in constitutive activation of numerous downstream signaling cascades associated with unregulated proliferation and impaired differentiation. Activating K-ras mutations are present in a variable percentage of prostate carcinomas. The clinical significance of K-ras mutations is unclear, although they are associated with lower median patient survival times. To investigate the role of activated K-ras in prostate carcinogenesis, we developed a mouse model where oncogenic K-ras expression can specifically be induced in the prostate. Our data indicates over-expression of mutant K-ras in prostate epithelial cells causes dysplasia.

Wnt signaling plays key roles in development, and alterations in the Wnt pathway are commonly associated with human tumors. Several groups have identified activating point mutations in subsets of prostate carcinomas. These mutations occur focally within the tumor, suggesting that B-catenin mutations contribute to tumor progression. We have created transgenic mice where expression in a mutated B-catenin gene can be induced by exposure to doxycycline and are in the initial stages of examining them.
Basic and Clinical Advances in Prostate Cancer Research
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ASSESSING THE ROLE OF WNT SIGNALING IN PROSTATE DEVELOPMENT AND TUMORIGENESIS.
Troy A. Giambardelli¹, Holli Charbonneau¹, Pradip Roy-Burman², Aubie K. Shaw³, Wade Bushman³, and Bart O. Williams⁴.
¹Laboratory of Cell Signaling and Carcinogenesis, Van Andel Research Institute, Grand Rapids, MI, USA, 49503; ²University of Southern California, Los Angeles, CA; ³Division of Urology and McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI.

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