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Resubmitted Progress Report
PCRP Prostate Cancer Training Award #W81XWH-07-1-0039
PI: Robin Tharakan, Ph.D.

Introduction:

The PCRP Prostate Cancer Training Award has supported my training for the past 12 months. During this time, I have worked towards attaining a better understanding of (a) the biology of prostate cancer, (b) the biology of specific growth factor signaling pathways, (c) the principle and application of cutting edge techniques in modern molecular and cellular biology, and (d) the principle and application of genetically engineered mouse (GEM) models, with the intent of becoming uniquely positioned to investigate the role of specific factor signaling in the initiation, progression and metastasis of spontaneous autochthonous prostate cancer and the emergence of the castration resistant phenotype. In addition to this rigorous technical training, I have continued to attend weekly lab meetings, division wide Cancer Biology Seminar series, and the Pacific Northwest SPORE in Prostate Cancer meetings.

Body:

Specific aim 1: Determine the influence of enforced expression of the proto-oncogene, *Wnt3*, on epithelial cells of the mouse prostate.

After I assumed responsibility for this project I immediately immersed myself in studying the biology of the epithelial and stromal compartments of the mouse prostate. To this end I decided to create assays that would allow me to measure quantitatively changes in expression of various markers that were intricately associated with epithelial and stromal differentiation. This was deemed necessary to elucidate the phenotypic consequence of deregulated growth factor expression in the transgenic models as previously described by Dr. Houghtaling. Of note, I established a quantitative RT-PCR (QPCR) based methodology to measure levels of mRNA transcripts encoding E-cadherin, N-cadherin, cytokeratin-5, cytokeratin-8, cytokeratin-14, cytokeratin-18, cytokeratin-19, synaptophysin, chromogranin A, Slug, Snail, Smooth muscle actin, vimentin, androgen receptor, insulin like growth factor-1, Ki-67 and ZEB 1 and 2. I am currently expanding the assay to include various specific targets in the Wnt/TCF signaling pathway. As the mice previously generated by Dr. Houghtaling become older I will perform detailed analysis of the histology and use the RT-PCR methods to measure specific changes in gene expression as a function of time. QPCR analysis on Wnt 3 IRES-Luc mice are presented in Figures 1A and 1B. I noted changes in the mRNA levels of several genes of Wnt3 IRES-Luc positive mice compared to non transgenic littermates. The Wnt3 transgenic mouse had over 40% increase in mRNA levels compared to non transgenic littermate controls (Figure 1A). This is not unexpected as this mouse was engineered to over express the Wnt3 gene. Cytokeratin 18 was the only tested cytokeratin with a marked 42% increase in mRNA levels compared to littermate controls (Figure 1A). Both Vimentin and Calponin also showed increased expression, 19% and 25 % respectively in Wnt3 IRES-Luc mice compared to non transgenic controls (Figure 1A). All the E-box transcription factors except Zeb2 showed an increase in mRNA levels with Slug having almost 20% more expression compared to control (Figure 1B). We also noted decreased expression cyclin D1 and cytokeratin 5 in the Wnt3-IRES-Luc mice compared to controls. We continue to test the hypothesis that inappropriate expression of *Wnt3* and other members of the Wnt family (specifically *Wnt4*) will be able to cause the transformation of the mouse prostate. Notably, we are also interested in how misregulation of the Snail E-box repressor during transformation facilitates the epithelial-mesenchymal transition and we hope to eventually cross a line of Snail^{LoxP} mice with the mice misexpressing *Wnt3* (or *Wnt4*) to test the hypothesis that deregulated Slug or Snail expression facilitates a more aggressive metastatic disease.

Specific aim 2: Determine the influence of enforced *Wnt3* expression during tumor progression and metastasis in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model.

We are still waiting to select the lines with the highest spatially restricted expression of *Wnt* prior to crossing to TRAMP.

Specific aim 3: Develop lines of reporter mice to detect activation of the Wnt/ β -catenin pathway *in vivo* during normal development and cancer progression.

These studies are ongoing and we are refining our imaging technology.

Methods:

Mice

Strains and breeding. All animal studies were conducted in accordance with institutional guidelines for humane animal treatment. Mice were maintained at 22°C in a 12-h light and dark cycle with ad libitum access to water and food. Mice homozygous for the Wnt3 IRES-Luc mice transgene were maintained in a pure FVB/NJ background.

RNA preparation and PCR analysis

Total RNA was extracted from separate lobes of the mouse prostate using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. Briefly, mouse prostates were immediately dissected from euthanized mice. The ventral, anterior, dorsal and lateral prostate lobes were separated and placed in RNA later stabilization buffer (Qiagen). Samples were then homogenized and RNA extracted, purified and resuspended in RNase free water. Purified RNA was then quantitated on NanoDrop UV-Vis spectrophotometer (Thermo Fisher, Waltham, MA). Total RNA (1 μ g) was reverse transcribed using an SuperScript II First-Strand synthesis kit. (Invitrogen, Carlsbad, CA). Briefly, following manufacturer's instructions RNA was denatured at 72°C for 10 min then 200 U for MMLV reverse transcriptase enzyme were used along with oligo dT primers in a final reaction of 20 μ l. Freshly reverse transcribed cDNA (1 μ l) was used for real-time RT-PCR with mouse-specific primers for genes as shown in Table 1. DNA synthesis was monitored via the fluorescent dye SYBR Green present in HotStart-IT SYBR Green qPCR Master Mix, (USB Coroporation, Cleveland OH), using the Bio-Rad real-time DNA amplification system (Bio-Rad, Hercules, CA). The following protocol was used; a 95°C denaturation step for 5 min followed by 40 cycles with denaturation for 1 min at 95°C, annealing for 1 min at 58 C, and extension for 1 min sec at 72°C. Gene expression was normalized to mouse ribosomal protein gene S16 or mouse beta-actin. PCR products were subjected to a melting curve analysis, and the data were analyzed and quantified with the MyiQ analysis software (Bio-Rad). The relative quantitative value for each target gene compared with the calibrator for the target was expressed as comparative Ct ($2^{-\Delta C_t - C_c}$) method (Ct and Cc are the mean threshold cycle differences after normalizing to 16 S). Real-time RT-PCR experiments were performed in triplicate and the percent coefficient of variation for the set value was less than 0.1. PCR efficiency with given primers was between 95 and 105%. Relative percent gene expression level changes in Wnt 3 IRES-Luc positive mice were then compared to negative control littermate gene expression.

Key Research Accomplishments: Created novel real time quantitative PCR based method to measure specific mRNA transcripts related to epithelial differentiation and detect epithelial to mesenchymal transitions.

Reportable Outcomes: Quantitative analysis of Wnt3 prostate mRNA.

Conclusions:

The results of this work should support or refute the hypothesis that enforced expression of growth factor signaling pathways such as Wnt is sufficient to drive tumorigenesis in the mouse prostate. We are now prepared to measure the quantitative increase in the GU weight to support the central hypothesis that deregulated Wnt signaling promotes prostate cancer in the mouse. Furthermore, we feel that cooperation between the *Wnt* and E-box repressors like Slug and Snail will facilitate more aggressive metastatic disease and increase the frequency or size of osteoblastic bone metastases. Our current QPCR data suggests that Wnt3 may have a role in regulating Slug and other genes related to cell morphology. Our future studies with the Wnt3 mice bred with our TRAMP mice may reveal the role of Wnt3 regulation in prostate cancer progression.

References: none

Appendices: none

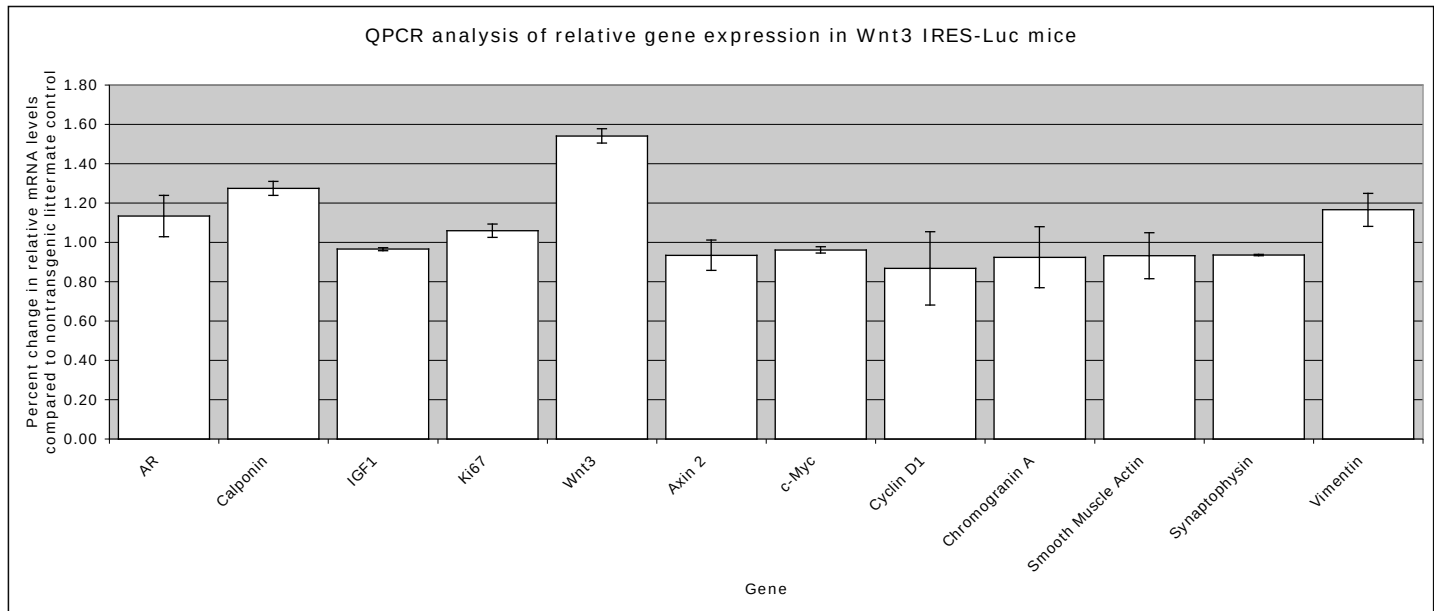
Supporting Data:

Table 1. Real Time PCR Primer Sets

Gene	Primer Sequence
Androgen Receptor	Forward 5' AACAGCAGCAGGAGGTAATCTCCGA 3' Reverse 5' GGCGTAACCTCCCTTGAAAGAGGAA 3'
Axin 2	Forward 5' CTCCTTGGAGGCAAGAGC Reverse 5' GGCCACGCAGCACCGCTG
Beta-Actin	Forward 5' GTGGGCCGCCCTAGGCACCAA Reverse 5' CTCTTTGATGTCACGCACGATTTC
Calponin	Forward 5' GCACATTTTAACCGAGGTCC Reverse 5' TGACCTTCTTCACAGAACCC
Chromogranin A	Forward 5' CAGCAGCTTTGAGGATGAACT Reverse 5' CTTGGTTAGGCTCTGGAAAGG
c-Myc	Forward 5' TCTCCACTCACCAGCACAACTACG Reverse 5' ATCTGCTTCAGGACCCT
Cyclin D1	Forward 5' CTGGCCATGAACTACCTGGA Reverse 5' ATCCGCCTCTGGCATTCTTG
Cytokeratin 5	Forward 5' TCAAGAAGCAGTGTGCCAAC Reverse 5' TCCAGCAGCTTCCTGTTAGGT
Cytokeratin 8	Forward 5' ACATCAACAACCTCCGCCG Reverse 5' TCCATAGACAGCACACAGACG
Cytokeratin14	Forward 5' AAGGGCTCTTGTGGTATCGGTG Reverse 5' CCAAACTGCTGCTGCTGAAG
Cytokeratin 18	Forward 5' ATGCCCCCAAATCTCAGGAC Reverse 5' CCAAGTCAATCTCCAAGGTCTGG
Cytokeratin 19	Forward 5' TTCAGTACGCATTGGGTCAG Reverse 5' GCCAGGGTCCCCTAAACT

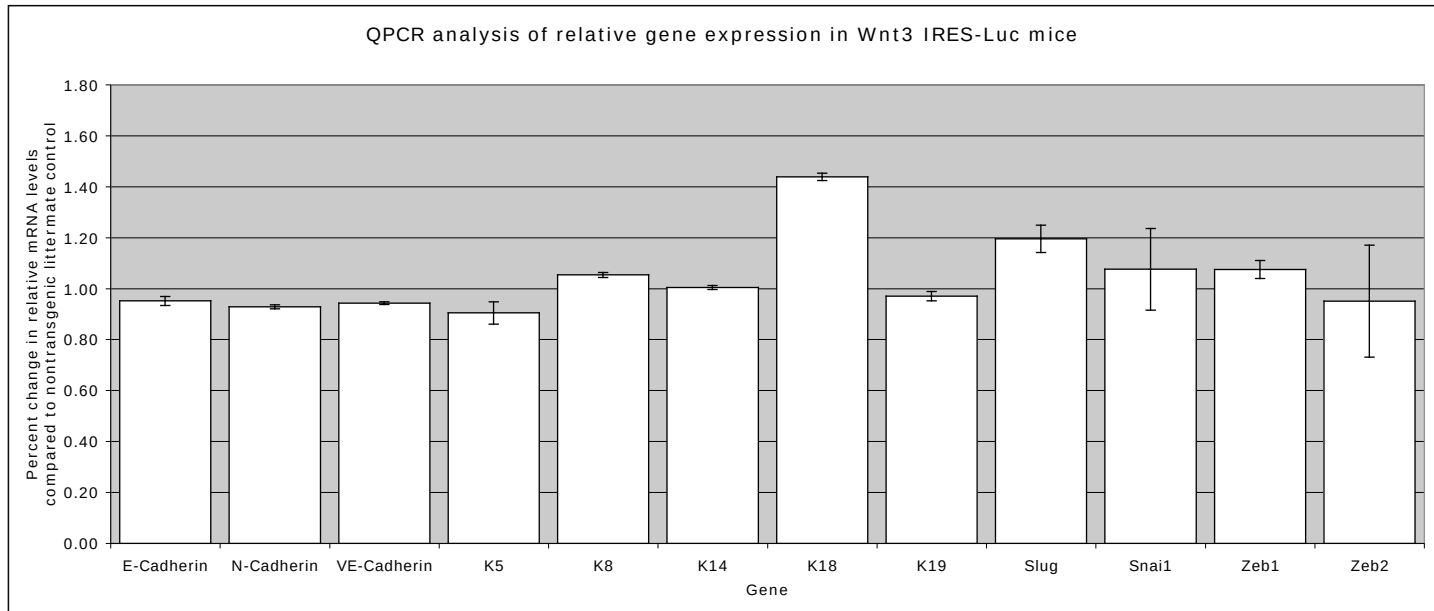
E-cadherin	Forward 5' GACAACGCTCCTGTCTTCAAC Reverse 5' TCTGTGACAACAACGAACTGC
IGF I	Forward 5' TCGTCTTCACACCTCTTCTACCTGG Reverse 5' TGCTTTTGTAGGCTTCAGTGGGGCA
IRS I	Forward 5' AAGCCCAAGAGTATGCATAAGCGCT Reverse 5' AGCGTCTGATATTCATCAGCTGCAG
Ki67	Forward 5' ACAGGCTCCGTACTTTCCAAT Reverse 5' ACCGGAGTCTTCTTCTCAAGG
N-cadherin	Forward 5' ATTGTCTGATCCTGCCAACTG Reverse 5' CAGTGTTCTGTCCCCTCAT
S16	Forward 5' AGGAGCGATTTGCTGGTGTGGA Reverse 5' GCTACCAGGCCTTTGAGATGGA
Slug	Forward 5' AGATCTGTGGCAAGGCTTTCT Reverse 5' GGAGCAGTTTTTGCCTGGTA
Smooth Muscle Actin	Forward 5' CGATAGAACACGGCATCATC Reverse 5' CATCAGGCAGTTCGTAGCTC
Snai1	Forward 5' CAACTATAGCGAGCTGCAGGA Reverse 5' ACTTGGGGTACCAGGAGAGAGT
Synaptophysin	Forward 5' GTGGTTATCAACCCGATTACG Reverse 5' CAGGCCTTCTCTTGAGCTCTT
Vimentin	Forward 5' GTTTCOAAGCCTGACCTCACTG Reverse 5' CTCTTCCATCTCACGCATCTGG
Wnt 3	Forward 5' AAATTGGGTAGCCAGCGAGGAC Reverse 5' ACTTCAGGGTGAGTTTGGGGAC
VE-cadherin	Forward 5' GTTCACCTTCTGTGAGGAGATGG Reverse 5' ATCTCCAGCGCACTCTTGCTAT
Zeb 1	Forward 5' GGAAACCGCAAGTTCAAGTG Reverse 5' CACCACACCCTGAGGAGAAC
Zeb 2	Forward 5' AGCTCGAGAGGCATATGGTG Reverse 5' TGTTTCTCATTCCGGCCATT

Figure 1A.



Quantitative real-time PCR analysis detecting mRNA levels of Wnt regulated and other target genes detected in the ventral prostate of 12 week Wnt3 IRES-Luc mice. Samples were done in triplicate and compared to non transgenic littermate mRNA levels that were set to one.

Figure 1B.



Quantitative real-time PCR analysis detecting mRNA levels of cadherin, cytokeratins and E-box transcription factor related genes detected in the ventral prostate of 12 week Wnt3 IRES-Luc mice. Samples were done in triplicate and compared to non transgenic littermate mRNA levels that were set to one.