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ROLE OF RUNX2 IN PROSTATE CANCER PROGRESSION

A Major Qualifying Project Report

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ABSTRACT

Prostate cancer (PC) that results from metastasis of the tumor primarily to the skeleton is among the leading causes of death for men. Involvement of the bone related transcription factor, Runx2, has been implicated in the progression of PC, however the levels of Runx2 and its targets have not been analyzed in PC. In this project, two transgenic mouse models for PC, the LADY and the TRAMP, were used to analyze Runx2 expression. Cell lines derived from both mice models showed increased expression of Runx2 from sexual maturity to the advancement stages, but an absence in normal prostate. Tumors were isolated from the mouse prostate at different time points (6wk, 12wk, 16wk, and 21wk). The tissues were analyzed for protein and gene expression and by histology for cellular properties. In both models, we found Runx2 in stromal and epithelial cells of the early PIN (prostatic intraepithelial neoplasia) lesion and activation of Runx target genes known to promote bone metastasis in the prostate by week 16. These results suggest that Runx2 may be a significant protein for consideration as a potential new therapy for prostate cancer.

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BACKGROUND

Prostate Cancer

Prostate cancer (PC) is among the leading causes of death for men, especially over the age of 75 (NIH.gov, 2010). The prostate is one of the organs of the male reproductive system. It is wrapped around the urethra, which explains the manifestation of symptoms of prostate cancer, which include trouble urinating, blood in the urine, and pain in the lower back or pelvic bones once the cancer has spread. There are two diagnostic tests for prostate cancer, a prostate biopsy, and screening for prostate prostate-specific antigen (PSA) (NIH.gov, 2010). PSA is a protein that dissolves the coagulation of seminal fluid, keeping seminal fluid liquid. Although PSA is used as a diagnostic test for prostate cancer, it is more accurately a marker of an enlarged prostate (Kim, 2011).

The current treatments for prostate cancer include surgery, radiation therapy, and/or hormone therapy (NIH.gov, 2010). In hormone replacement therapy, also termed androgen ablation therapy, testosterone levels are lowered using medications like Lupron or Zoladex to inhibit the production of testosterone in the testes (KnowCancer.com, 2009). Lowering testosterone levels usually slows tumor growth because initially the tumor growth is dependent on the presence of testosterone. However, after an initial response to this treatment, many cancers become androgen-independent, meaning the tumor continues to grow in the absence of testosterone (Sartor, 2005). Because androgen ablation therapy is not 100% successful, there is a need for new therapies to treat prostate cancer. Over the past decade, antibodies or drugs have been designed that inhibit proteins involved in PC growth and/or cell survival, but these drugs have shown limited

success. Once PC tumors become aggressive, there is a very high frequency of metastasis to the lung, and especially to bone.

Prostate Cancer Mouse Models

Two mouse models have been used to study prostate cancer. The first is known as the LADY mouse. This model uses the probasin (epithelial) promoter to drive the expression of the late T antigen, which has a mutation that removes the expression of the small T antigen, which encodes tumor suppressor proteins. Thus, in this model, the late T antigen is expressed in epithelial cells, causing tumor formation of the prostate. However, metastasis is not common in this model (Gipp et al., 2007).

The TRAMP mouse uses the probasin promoter to drive expression of the SV40 T antigen to cause orthotropic prostate tumors (Greenberg et al., 1995; Gingrich et al., 1996). Many stages of prostate cancer have been observed in the TRAMP model. This model is reliable, and adenocarcinomas (AC) were found in 100% of TRAMP mice between 10 and 20 weeks of age (Sharma, and Schreiber-Agus, 1999). Adenocarcinomas are advanced lesions occurring after the first stage PIN (prostatic intraepithelial neoplasia) lesion (**Figure-1**). AC lesions are found in human PC's, and therefore the TRAMP mouse is a relevant model for human PC.

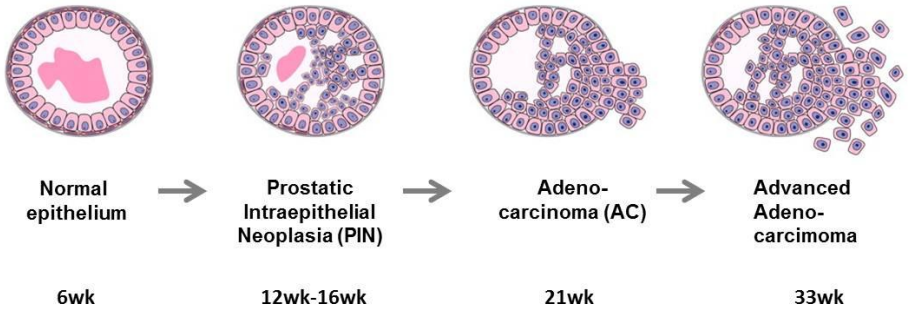

Figure-1: Advanced Stages of Mouse Prostate Cancer.
(Adapted from: Abate-Shen et al, Genes & Dev, 2000)

Prostate Cancer Progression and Runx2

The Runx proteins are transcription factors involved in hematopoiesis (Runx1), osteogenesis (Runx2), and tumor suppression (Runx3). Mutations in the Runx proteins are also involved in cancer development.

Runx2 is a transcription factor with a well characterized function in bone morphogenesis (Akech et al., 2010). However, Runx2 is also expressed in a variety of tissues other than bone, and functions in several processes related to tumor formation (**Figure-2**). Runx2 has been shown to up-regulate several genes related to tumorigenesis, including TGFR, VEGF, MMP, Integrins, and IHH (Pratap et al., 2008, Sterling et al., 2011).

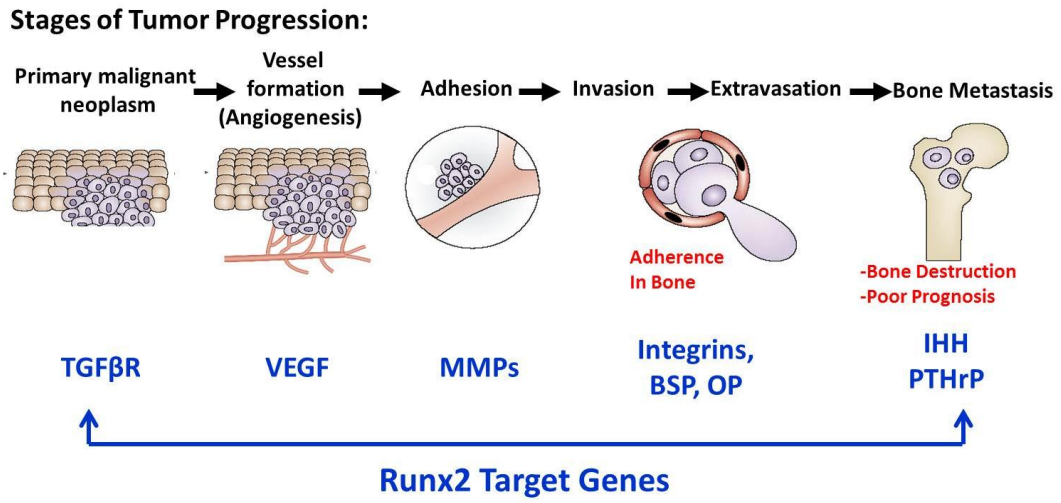

Figure-2: Various Stages of Tumor Progression and Runx2 Target Genes. Shown are the main stages of tumor formation, from left to right and the Runx2-related gene product (blue) that helps regulate each process. (Adapted from Dasgupta et al., 2012)

Each advancing state of cancer involves a Runx2 target gene. At the start of tumor progression, the receptor gene for TGF-β (TGFR) is present on the cell surface, which allows for cell proliferation. Angiogenesis, the formation of blood vessels surrounding the tumor, is supported by vascular endothelial growth factor (VEGF). MMPs are Runx2 target genes present when cells become invasive. Integrins, bone sialoprotein (BSP), and osteopontin (OP) all play a role in extravasation, the movement of cancer cells present within the primary tumor, initially preventing their metastasis to other places in the body, such as the bone.

With respect to PC, the final stage of PC is its metastasis of the cancer to the bone. At this point, Indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHrP) are detected. These factors are known to be required for bone resorption

(osteolysis) and for endochondral ossification, a process of bone formation (Rivron et al., 2012).

Runx2 has been analyzed in metastatic PC3 cells, a human prostate cancer cell line (Akech et al., 2010). The expression of Runx2 mRNA and protein was found to be higher in PC3 cells compared to non-metastatic cells lines. PC3 cells have also been injected into SCID mice bones to make a mouse model of metastatic bone disease. SCID mice, which lack an immune system, can accept human cells when injected in the bone, and showed increased osteolysis of the bone that correlated with high levels of Runx2. Runx2 involvement in prostate cancer is also linked with tumor metastases because Runx2 activates the expression of many genes that affect tumor growth (Akech et al., 2010). Because Runx2 target genes are known to be involved at different stages of tumor growth, discussed above, a future goal is to inhibit Runx2 expression/activity to help retard tumor progression.

PROJECT PURPOSE

Previous studies (discussed in the Background) have shown that transcription factor Runx2 controls the expression of a variety of genes related to cancer progression, including initial tumor growth, vessel formation, tumor invasion, tumor extravasation, and metastasis to the bone. With respect to prostate cancer (PC), *in vitro* studies have shown that Runx2 is highly expressed in metastatic PC lines. We postulate that Runx2 is likely to be expressed in the prostate gland prior to its metastasis to bone, and helps activate genes that facilitate metastatic bone disease. Specifically, this project will 1) examine *in vivo* Runx 2 expression (mRNA and protein) in mouse PC models (LADY and TRAMP), 2) identify the cells expressing Runx2 (including, normal stromal and epithelial cells, and cancer cells such as PIN and adenocarcinoma, and 3) determine whether a knockdown of Runx2 in TRAMP cells reduces their tumor metastatic properties.

METHODS

Cells and Reagents

TRAMP-C2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg/ml bovine insulin and 10 nM dehydro-isoandrosterone (androgen), 90%; fetal bovine serum, 5%; Nu-Serum IV, 5%. TRAMP-C2 cells were grown in 5% carbon dioxide (CO_2) at 37°C.

Neotag1 cells were grown in (DMEM) containing 2.5% heat-inactivated fetal bovine serum, 1% antibiotic-antimycotic (Gibco, Grand Island, NY), 50 µg/ml gentamicin (Gibco), 4 µg/ml bovine pituitary extract (Hammond Cell Tech, Windsor, CA, USA), 1% insulin-transferrin-selenium-X (Gibco), 50 ng/ml cholera toxin (Sigma, St. Louis, MO), 10 ng/ml EGF (Sigma) at 37°C in an atmosphere containing 5% CO2. The cells were maintained with 10^{-8} M dihydrotestosterone (DHT, androgen).

Mice

All animal handling was done following IACUC (Institutional Animal Care and Use Committee) approved protocols. RNA samples and cells isolated from the LADY mouse model were obtained from our collaborator Dr. S. Kasper (University of Cincinnati). The TRAMP mice were obtained from Jackson Laboratories, and bred as follows to obtain tumors:

Female		Male
Tg TRAMP	X	C57BL/6 WT

The offspring Tg-TRAMP males developed tumors by 6 weeks. Tumors were collected at 6, 12, 16, and 21 weeks. Beyond this stage, tumors are very advanced and prolonged metastasizing to lung that causes death by 26-33 weeks.

TRAMP mice were killed using a humane double kill method of overdose of isofluorine followed by cervical dislocation. Mouse prostate, lung, and lymph node tissues were obtained from mice by Dr. Jacqueline Akech with assistance from Catherine King. The entire organ was excised and allocated to use for RNA isolation, protein isolation, or histology. Organs used for RNA were placed in Trizol (Invitrogen, Carlsbad, CA), homogenized, and frozen for later use. Organs used for protein were placed in RIPA buffer, homogenized, and stored for later use. Organs used for histology were placed in fixative in preparation for tissue processing and embedding in paraffin blocks.

Mouse Genotyping for Tumor Positive Mice After Breeding

Tail genomic DNA was isolated using a Lambda Biotech genomic DNA isolation kit (Lambda Biotech, St. Louis, MO). The samples were digested with solution A and proteinase-K. Following digestion, the samples were mixed with solution B, and were incubated on ice for 10 min, then centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to new tubes, and the DNA was precipitated with 100% ethanol, then the tubes were centrifuged for 5 min at full speed in a microcentrifuge. The supernatant was then discarded, and the DNA pellet was washed with 70% ethanol, air dried, and then re-suspended in TE buffer. Gene specific primers specific for the transgene were obtained from Jackson Laboratories (Bar Harbor, Maine) (see **Table I** below).

Table-I: Primers Used for Mouse Genotyping.

Primer	Sequence 5'-3'	Primer Type
oIMR7084	GCG CTG CTG ACT TTC TAA ACA TAA G	Transgene
oIMR7085	GAG TCT ACG TTA AGT TTT GAT GTG T	Transgene
oIMR7338	CTA GGC CAC AGA ATT GAA AGA TCT	Internal Positive Control
oIMR7339	GTA GGT GGA ATT TCT AGC ATC ATC C	Internal Positive Control

The PCR cycle was 94°C for 3 min, 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min, repeat steps 2-4 for 35 cycles, then 72°C for 2 min, and 10°C hold. The PCR product was run on a 1.5% agarose gel (Figure 3). When the transgene is present, an upper band of 650 kb is present. The lower band represents the positive internal control (324 kb), and is present in all DNA samples (TRAMP + and TRAMP -).

Immunohistochemistry

Prostate tissues acquired from TRAMP mice, and their age matched littermates at 6, 12, 16, 21 weeks, were obtained using previously described methods (see Animals section). In other cases, 33 week old prostate tissues were obtained from our collaborator Lucia Languino (University of Massachusetts) to represent late stage prostate cancer. Hematoxylin and Eosin staining of tissues was performed initially followed by immunohistochemistry to detect Runx2 protein in prostate tumors, essentially in accordance with Akech et al (2010) with a few exceptions. Mouse prostate, lung, and lymph node tissues were provided in paraffin sections by the histology core of the Stein-Lian lab. The slides were dipped into xylene solution twice, 10 minutes each for

deparaffination. To rehydrate the tissue, the slides were placed in 99% ethanol three times, 10 minutes each, then in 96% ethanol twice for 10 minutes each. The slides were then placed in endogenous peroxidase blocking solution (1% BSA, 0.2% gelatin, 0.05% saponin in PBS) for 30 min, then rinsed in 96% ethanol before being placed in 70% ethanol for 10 min. The slides were then briefly rinsed with distilled water, and then briefly dipped in 1X Dako Cytomation Target Retrieval solution (pH 6.0) followed by antigen retrieval using pressure cooker method for approximately 20 min. Tissue samples were allowed to cool down, and primary antibody Runx2 (mouse monoclonal) at a dilution of 1:100 was added and placed at 4°C overnight. The slides were then washed (0.1% BSA, 0.2% gelatin, 0.05% saponin in PBS) 3 times, 10 min each. Then secondary antibody (goat-anti-mouse) was added at a 1:500 dilution for 1 hour. The slides were washed again and then received DAB with detection for 10 min. The slides were then rinsed in PBS, then in distilled water. The slides received Hemotoxylin for 2 min, and then were rinsed with running water for 20 min. Dehydration of the tissue was done by placing the slides in ethanol (70%, 96%, 99%) in order of increasing percentage for 10 min each. Then they were dipped twice in Xylene for 10 min. Finally the slides were mounted.

Immunoblotting for Runx2 (Western Blot)

Protein samples, both cellular and tissue, were harvested in RIPA buffer and 2xSDS sample buffer supplemented with protease inhibitors (Complete, EDTA-free, Roche Diagnostics, Mannheim, Germany) and MG132 (Calbiochem, City, State). For protein determination, the Biorad DC protein assay reagent was used (Biorad, Hercules,

CA). 50 μg of prostate proteins were separated on a 10% acrylamide gel using SDS-PAGE. Then the protein was transferred to a PDVF membrane by using 10 volts of electricity for 30 min via semi-dry transfer. The membrane was then blocked in 5% milk for 1 hour, shaking at room temperature. The membrane was incubated in Runx2 antibody (made by the Stein-Lian lab) at 1:1000 dilution, shaking at 4°C overnight. The next day, the membrane was washed 3 times with PBS-T buffer, for 10 min each wash. Tubulin mouse monoclonal antibodies (Sigma, St. Louis, MO) at 1:10,000 dilution was used as the internal control to ensure equal protein loading. The membrane was incubated in secondary peroxidase labeled goat-anti-mouse antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA), at a dilution of 1:5000 for 1 hour with shaking at room temperature. The membrane was washed 3 times in PBS-T. The signal was detected by chemi-luminescence (ECL) (Perkin Elmer, Waltham, MA) on Kodak film (Kodak, Rochester, NY).

RT-PCR

Total RNA was isolated using Trizol (Invitrogen) and subjected to DNase I digestion to eliminate residual DNA using Zymo DNA-free RNA kit (Zymoresearch, Irvine, CA). cDNA preparation was performed using the Superscript II First Strand synthesis cDNA kit (Invitrogen). PCR was performed as described above. Gene expression was monitored using real-time primer pairs (**Table 2**) with SYBR Green detection (Applied Biosystems, City, State) and analyzed using the ΔΔCT method.

Table 2- RT-PCR Primer Sequences

Primer	Sequence 5'-3'
NKX3.1 F	ATGCTTAGGGTAGCGGAGC
NKX3.1 R	TGCGGATTGCCTGAGTGTC
AR F	CTGGGAAGGGTCTACCCAC
AR R	GGTGCTATGTTAGCGGCCTC
Runx2 F	CGG CCC TCC CTG AAC TCT
Runx2 R	TGC CTG CCT GGG ATC TGT A
Runx1 F	AGATTCAACCTCAGGTTTGTC
Runx1 R	CGGATTTGTAAAGACGGTGATG
GAPDH F	AGGTCGGTGTGAACGGATTTG
GAPDH R	TGTAGACCATGTAGTTGAGGTCA
OC F	CTGACAAAGCCTTCATGTCCAA
OC R	GCGCCGGAGTCTGTTCACTA
OP F	TTTGCTTTTGCCTGTTTGC
OP R	CAGTCACTTTCACCGGGAGG

Runx2 Knockdown: Generation of Stable TRAMP cells Expressing Runx2 shRNA

TRAMP-C2 cells were plated $1x10^6$ per 6-well plate, then grown at 37°C in 5% CO_2. At 40% confluence, the cells were infected with 100 μl of lenti-virus expressing short hairpins indicated as control empty vector (EV-shRNA) or specific for Runx2 knockdown (Runx2-shRNA) in complete medium (see materials and methods) containing 8 μg/ml polybrene.

UT	Ev-sh	Runx2-sh
UT	EV-sh	Runx2-sh

The plates were spun for 90 min at 37°C at 2400 RPM, then incubated at 37°C in 5% CO_2. After 24 hours of incubation, the media was changed, then the cells were used for a scratch assay, and harvested for RNA and protein isolations. The effectiveness of the Runx2 knockdown was monitored by western blot (see above)

Scratch (Wound Healing Assay)

In vitro wound healing assays were performed with TRAMP-C2 cells expressing RUNX2-shRNA or EV-shRNA. Cells were grown until 80% confluence and scratched with a 200 µl pipette tip to create a cell free area ('wound'), this was considered the 0 hr time point. Cells were washed to remove unattached cells, and incubated for 24 hr and examined by light microscopy.

RESULTS

Because previous studies showed that transcription factor Runx2 controls the expression of a variety of genes related to cancer progression, and that Runx2 is highly expressed *in vitro* in metastatic PC lines, we investigated whether Runx2 is expressed in the prostate gland just prior to its metastasis to bone. We began by determining the genotype of each mouse. Then tissue, protein, and RNA were analyzed at several time points across prostate cancer progression to determine where Runx2 expression was increased compared to wild type controls.

Mouse Genotyping and Histology

C57BL/6 Tg-TRAMP males were created by breeding Tg females to WT males, then the DNA of the offspring was analyzed for the presence of the transgene by PCR (**Figure-3**). Seven positive transgenic mice and 6 negatives were identified (denoted by + or – respectively in the figure). Mice expressing the probasin transgene (PB) in TRAMP positive showed expression at 650 bp (Figure 3), while the internal control (Casein) was expressed at a slightly lower molecular weight at 324 bp. Previously analyzed samples that showed + and – PB expression (lanes 15 and 16 respectively) were used as controls for accurate genotyping. In addition, distilled H_2O used in each reaction mix was used as readout for the possibility of reagent contamination during sample preparation and PCR amplification (lane 17). Primers for both the transgene and the internal control were used simultaneously in the PCR reaction using standardized conditions. Results indicate that correct primers were used to establish PB expression,

establishing TRAMP+ mice in the population (**Figure 3**). However, in some cases positive expression of PB transgene was also observed in transgenic female mice (**Figure 3, lane 8**). These mice were not used in subsequent expression experiments for Runx2 or target gene expression analysis, but were used for further breeding and generation of transgenic TRAMP+ve male mice as indicated in the methods section. Our results show that the genotype of TRAMP mice could be used successfully to distinguish between TRAMP + and TRAMP – mice. All TRAMP - mice within each genotyping experiment were aged alongside the TRAMP +ve males for the 6, 12, 16, and 21 week time points.

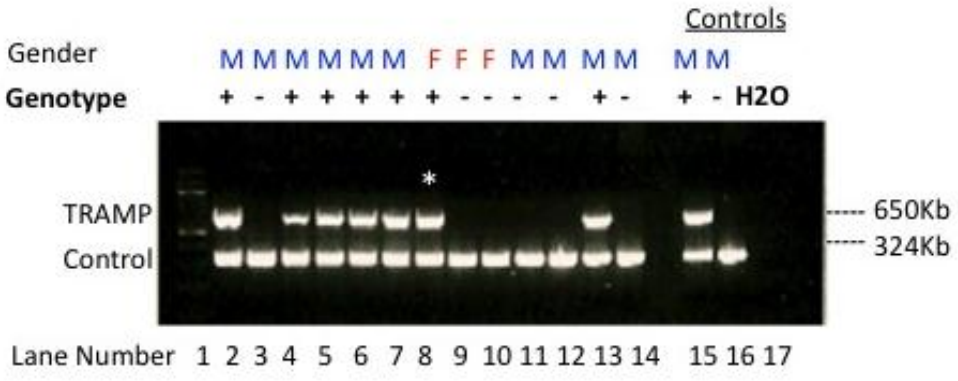

Figure-3: TRAMP PCR Mouse Genotyping. Figure shows amplified DNA of the probasin transgene (650 bp, top band) and the internal control (324 bp, lower band). Tramp+ and TRAMP- male genotype, and gender (in blue) are indicated. TRAMP+ female genotype is indicated as an asterix. Controls used (lane 15, 16, and 17) are TRAMP + male, TRAMP - male and H_20 respectively. Molecular weight marker used to determine the size of migrated DNA is indicated (lane 1).

Once the TRAMP genotype was verified, the prostate tissue was examined at 6, 12, 16, 21, and 33 weeks by hematoxylin and eosin staining for the presence of PC lesions to verify the genotyping results (**Figure-4**). The histology confirmed that mice at 6 weeks showed early stages of PC (prostatic intraepithelial neoplasia, PIN), hyperplasia

at 16 weeks, neoplasia at 21 weeks, and distal metastases at 33 weeks, while control mice lacked all of the above.

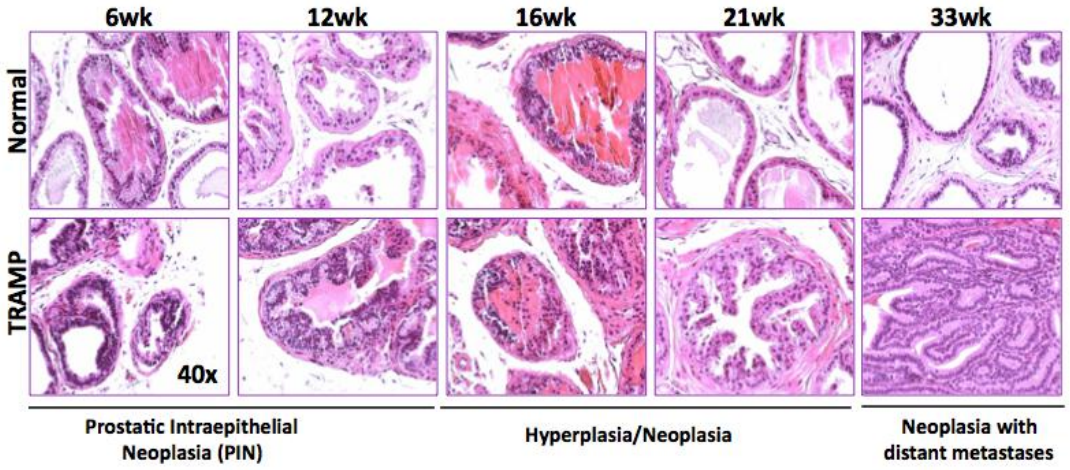

Figure-4: Hematoxylin and Eosin Histology Staining of Prostate Tissue in TRAMP Mice at Various Ages. The H&E staining showed PIN type lesions at 6 weeks, hyperplasia at 16 weeks, neoplasia at 21 weeks, and distal metastases at 33 weeks.

Analysis of Runx2 Protein and mRNA in Prostate Tissue

Previous reports showed that Runx2 is highly expressed in aggressive advanced stage tissues in human prostate cancer tissue, as well as in PC3 human prostate cancer cells (Akech et al., 2010). Therefore, the presence of Runx2 in tissues from advanced late stage prostate cancer was determined by immunohistochemistry (IHC) in 33 week old TRAMP mouse prostate tissue compared to age-matched WT tissue (**Figure 5**). Results revealed that Runx2 was expressed in advanced adenocarcinoma, suggesting a role of Runx2 in the aggressiveness of tumors in the TRAMP transgenic mouse model.

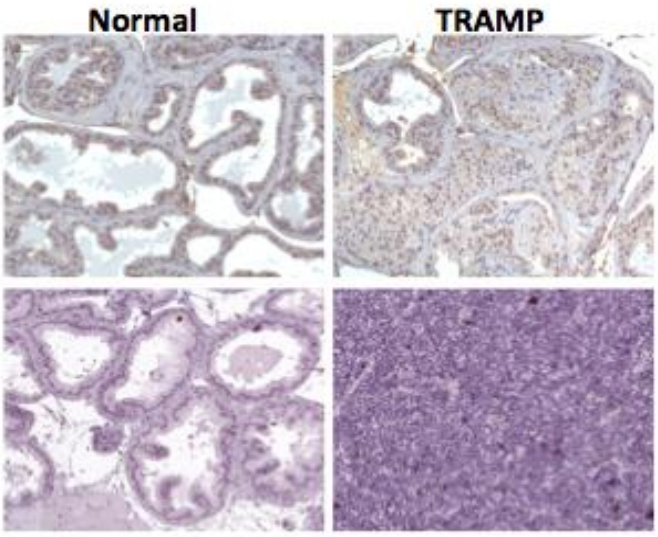

Figure-5: IHC Analysis of Runx2 Protein Levels in TRAMP versus WT Mice. Upper row denotes IHC for Runx2 protein. Lower row denotes H&E staining. The TRAMP mouse was analyzed at 33 weeks, an advanced stage of PC.

In order to verify whether Runx2 expression is increased with tumor progression, western blotting was performed on prostate tissue of TRAMP transgenic mice compared to their age-matched WT littermates. The expression profile (**Figure-6**) showed that by western blot, Runx2 expression did not change in the earlier stages of prostate cancer, however a gradual increase was observed at 16 wks and a probable decline to basal levels (compared to wild type mice) at 21 wks. These data suggests that Runx2 expression is specifically increased at the onset of adenocarcinoma, and that the expression pattern may be dynamic depending on the stage of prostate cancer progression.

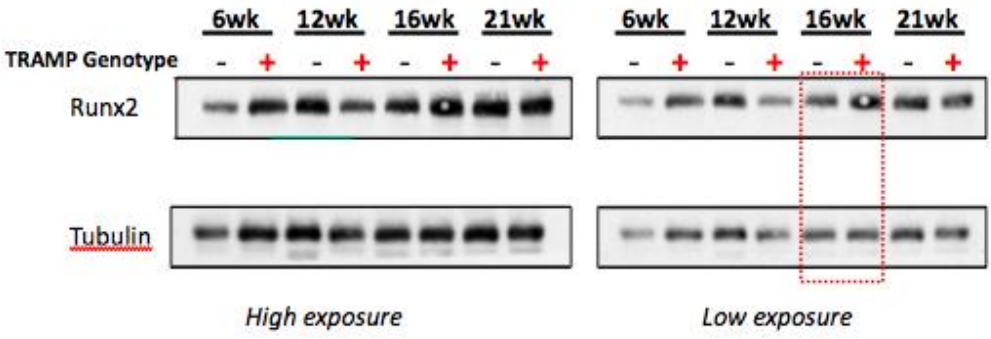

Figure-6: Western Blot Analysis of Runx2 Protein Levels in TRAMP versus WT Mice. Western blots for Runx2 or tubulin were performed for WT or TRAMP mice at various ages. The left panel represents a longer exposure of the right panel.

A comparison of Runx2 expression in tumor progression was also performed in prostate tissues from the LADY mouse model as previously described (Kasper et al., 1998). In the LADY mouse, adenocarcinoma is observed at 10 wk time point, and advanced tumors are seen between 11 and 15 weeks. RNA samples obtained from prostate tissues of LADY transgenic mice at 2-15 wks compared to their age matched littermates were analyzed by standard RT-PCR The results indicated that Runx2 mRNA was expressed throughout disease progression compared to wild type controls **(Figure 7).** Expression was increased with disease progression, but peaked at 15 wks.

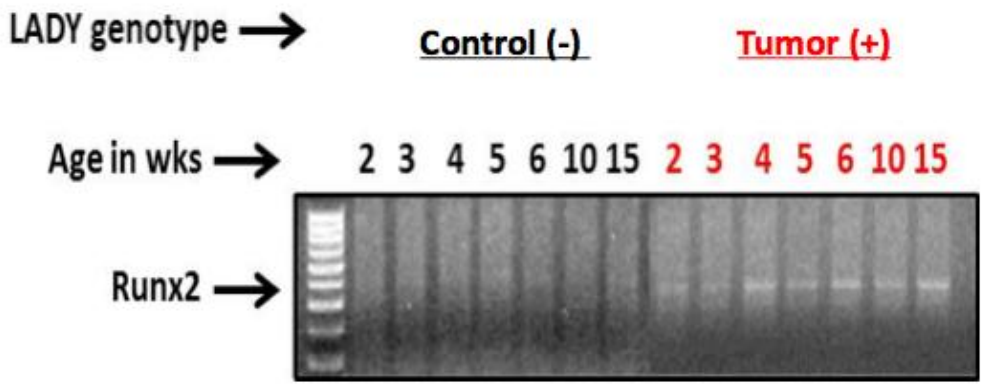

Figure 7: Expression of Runx2 mRNA in Various Stages of Tumor Progression in the LADY Mouse. RT-PCR showing Runx2 expression in mice with prostate cancer, normal prostate tissues (left side) do not show Runx2 mRNA expression.

In order to verify Runx2 relative expression in the two transgenic mouse models, real time qRT-PCR was performed in tissues from adenocarcinomas of wild type controls in the LADY (15 wks) and TRAMP (21 wks). The results indicated that Runx2 mRNA expression is increased several-fold compared to controls in advanced prostate cancer (**Figure 8**).

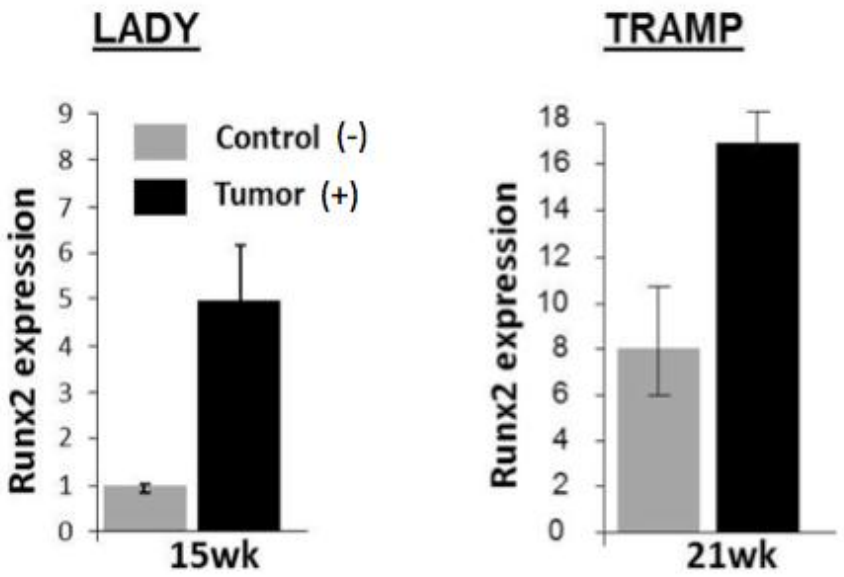

Figure 8: Expression of Runx2 mRNA in Various Stages of Tumor Progression in the LADY and TRAMP Mouse Models. Runx2 expression was analyzed by real time qRT-PCR, and is increased compared to control wild type mice across prostate cancer progression. The histobars represent the average of 3 determinations, and the error bars denote one standard deviation.

In the TRAMP transgenic mouse model, tumor progression and the role of Runx2 in the regulation of target gene was examined. RNA isolated from the prostate tissues of TRAMP mice and their age-matched littermates were used to determine the relative mRNA values across prostate cancer progression (**Figure 9**). GAPDH was used as the internal control for each sample. The results show that Runx2 mRNA is increased when compared with age-matched controls and it increases across prostate cancer progression at 6, 16, and 21 wk time points to represent PIN lesions, and early and late stage adenocarcinoma, respectively (**Figure 9**). At 12 weeks, the control and experimental

values were statistically different, and therefore were not analyzed for subsequent mRNA values.

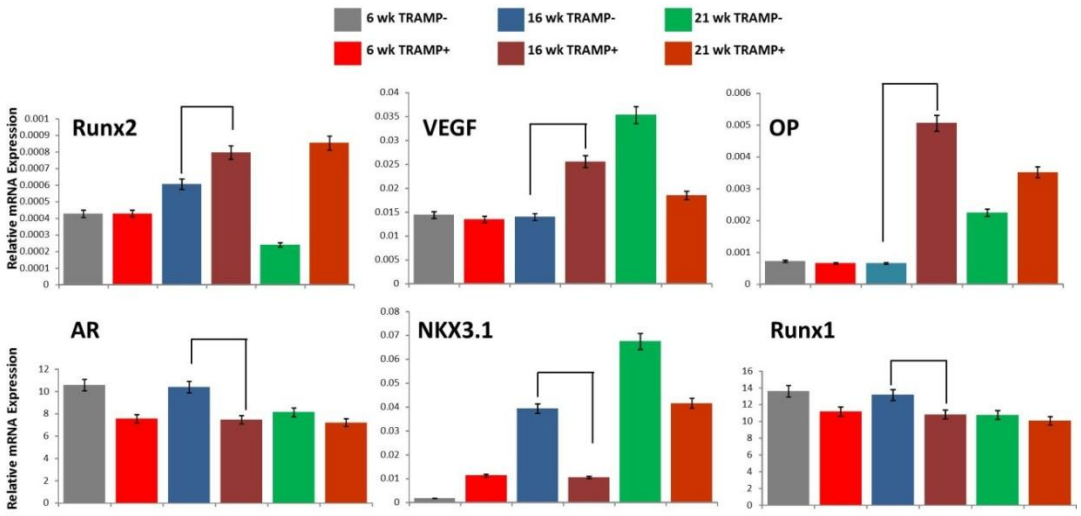

Figure 9. Transcriptional Regulation of Runx2 During Prostate Cancer Progression in the TRAMP Mouse Model. Figure shows the results of real time qRT-PCR analysis for six target genes (Runx2, VEGF, OP, AR, NKX3.1, and Runx1). VEGF is a protein involved in tumor vascularization. Osteopontin (OP) is a marker of bone metastasis. Note that expression of the androgen receptor (AR), which regulates normal prostate function and NKX3.1 a prostate tumor suppressor is lost as tumor progresses in the TRAMP mouse prostate tumors. Runx1, which regulates hematopoiesis also decreased.

We then wanted to determine whether knock down of Runx2 expression in both TRAMP-C2 and Neotag1 cells indicated a role in transcriptional control. Runx2 expression was knocked down by shRNA by lentiviral delivery. First, the efficiency of the lentivirus was determined by examining the infected cells for GFP expression present in the lentiviral vectors for EV-shRNA and Runx2-shRNA. The results revealed successful infection of lentivirus (**Figure 10**, top left panel). Expression of Runx2 and the angiogenesis target gene, VEGF were analyzed by real time qRT-PCR. Suprisingly, we observed an increased and not a decreased expression in Runx2 in both cells lines.

Expression of VEGF did not show any change or was statistically insignificant, in addition protein expression by western blotting in both TRAMP-C2 and Neotag1 cells showed no difference (data not shown). We attribute these observations possibly to a lack of specificity of the short hairpins for targeting mouse Runx2. Interestingly, when we performed functional studies to determine the defined role of Runx2 in tumor metastasis using TRAMP-C2 as a cellular model, we observed retarded migration in a wound healing (scratch) assay (**Figure 10**, lower left panel) in Runx2-shRNA compared to the EV-shRNA group. We propose that utilization of a mouse specific Runx2-shRNA is essential in these studies for better interpretation, as well as more extensive study with Runx2 protein.

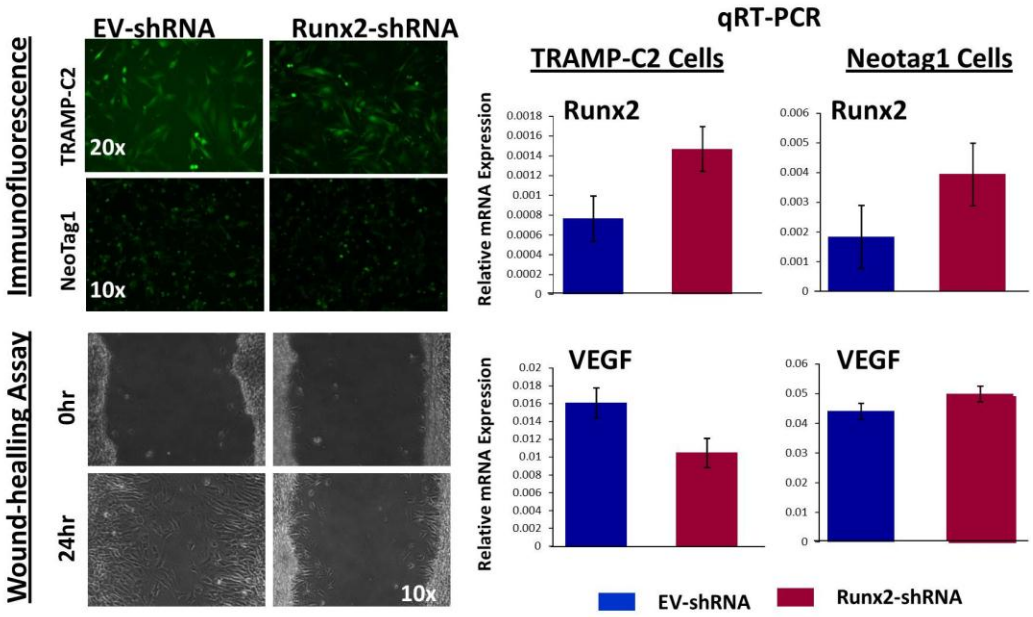

Figure 10. Functional Role of Runx2 in Prostate Cancer. Immuno-fluorescence shows successful incorporation of lentivirus (encoding Runx2 shRNA) into TRAMP-C2 and NeoTag1 cells (Top left). Wound healing assay shows slowed growth of Runx2-shRNA infected cells across scratch after 24 hours compared to EV-shRNA infected cells (Bottom left). qRT-PCR determined an unexpected increase in Runx2 in Runx2-shRNA infected TRAMP-C2 and NeoTag1 cells (Top right). VEGF levels showed no change in Runx2-shRNA infected cells (Bottom right).

DISCUSSION

The results of this project showed a dynamic expression of Runx2 in the TRAMP mouse prostate tissue throughout the progression of prostate cancer. qRT-PCR and western blot results showed a distinct increase in Runx2 mRNA and protein expression at 16 wks in TRAMP + mice compared to age-matched littermates (Figures 9 and 6, respectively). Analysis of Runx2 and Runx2 target genes across the progression of prostate cancer support the findings that 16 wk is a critical time point, because this is when expression of metastatic genes increase in TRAMP + mice compared to age matched littermates (Figure 9). Immunohistochemistry confirms increased expression in Runx2 in a 33 wk old TRAMP + mouse compared to a 33 wk old wild type mouse (Figure 5). Figure 8 shows mRNA expression of Runx2 in advanced prostate cancer in both the LADY and TRAMP mouse models. The results show an increase in Runx2 in both models compared to wild type controls. Figure 7 clearly shows the presence of Runx2 in the LADY prostate cancer model compared to wild type mice, which showed no Runx2 expression. Prostate cancer cell lines were also analyzed, but require further testing (Figure 10). From figure 10, Runx2 knock down was not observed in mRNA, but slowed growth can be seen in the wound-healing assay, which is indicative that Runx2 plays a role in migration and metastasis. Further analysis is needed for conclusive results.

These results indicate that a potential therapy, which targets Runx2, would aim to reduce the Runx2 increase seen at the 16 week time point, and would therefore disturb the normal progression of prostate cancer.

Future projects might include repeating lentiviral infection of Runx2sh-RNA knockdown experiments, Runx2 over-expression experiments, and further analysis of Runx2 target genes involved in metastasis and cancer progression. One reason the knock down did not work could have been that the sh-RNA was targeting human Runx2 and was used in mouse cell lines (TRAMP-C2 and NeoTag1). Another reason could be that the sh-RNA is not specific and is not targeting the correct area of gene expression. To correct this, an inducible system could be used when infecting the cells to ensure lentiviral incorporation and sh-RNA success. Another option would be to create a clone of Runx2-shRNA which targets mouse Runx2. This would increase the probability of Runx2 knock down in mouse cell lines. Once the Runx2-shRNA has been successfully knocked down in prostate cancer cell lines, the knock down could be introduced into prostate cancer mouse models (TRAMP and LADY). The effects would be characterized and used to discovery new therapies for prostate cancer. The over-expression experiments would include analysis of mRNA, and protein in cell lines to provide information on the function of Runx2. Runx2 target genes and cancer markers should be analyzed (both mRNA and protein) across prostate cancer progression in TRAMP mice to correlate the expression of Runx2 and the progression of prostate cancer in *vivo.*

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