


8-2011

Significance of Increased Tissue Transglutaminase in Hormone Refractory Prostate Cancer

Amy L. Han

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations

 Part of the [Biology Commons](#), [Laboratory and Basic Science Research Commons](#), and the [Molecular Biology Commons](#)

Recommended Citation

Han, Amy L., "Significance of Increased Tissue Transglutaminase in Hormone Refractory Prostate Cancer" (2011). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 160.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/160

This Thesis (MS) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digitalcommons@library.tmc.edu.

**SIGNIFICANCE OF INCREASED TISSUE TRANSGLUTAMINASE IN
HORMONE REFRACTORY PROSTATE CANCER**

By

Amy Lee Han, Bachelor of Arts

APPROVED:

Kapil Mehta, Ph.D., Supervisory Professor

Michelle C. Barton, Ph.D.

Sankar N. Maity, Ph.D.

Nora M. Navone, M.D., Ph.D.

Zahid H. Siddik, Ph.D.

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

**SIGNIFICANCE OF INCREASED TISSUE TRANSGLUTAMINASE IN
HORMONE REFRACTORY PROSTATE CANCER**

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Amy Lee Han, B.A.
Houston, Texas

August, 2011

ACKNOWLEDGEMENTS

I would like to truly thank my advisor, Dr. Kapil Mehta, who guided and supported me throughout my Master's degree pursuit. He has taken me into his lab and shared his vast knowledge with me, paving a way for me to learn and develop critical thinking and experimental skills necessary to be a successful researcher in the scientific community. In addition, thank you to all the former and current members of Dr. Mehta's laboratory who were there to teach and assist me, passing down all their experiences and wisdom to me. I will take everything that I have acquired from this lab and apply them in my future studies.

I would also like to thank all my committee members: Dr. Michelle C. Barton, Dr. Sankar N. Maity, Dr. Nora M. Navone, and Dr. Zahid H. Siddik, whose expertise, guidance, and support made it possible for me to develop and complete my research project. I appreciate all the time and effort they spent on providing me input and suggestions to make my research better. In addition, special thanks to Drs. A. Multani and S. Pathak for the chromosomal analysis and Drs. B. Aggarwal and B. Sung for the EMSA result.

I would like to thank my family and friends for their love and support. I truly wouldn't have been able to do this without their assistance. I want to thank my parents for making it possible for me to pursue a study in biomedical research. Their continual love and encouragement allowed me to push myself to work harder. I want to thank my sister for always being there for me and visiting me during my studies. I also want to thank my close friends Soo Yeon Lee and Hyojin Cho for their wonderful friendship to me and for walking with me every step of the way. Lastly, but not least, I would like to thank God for placing me here with all these great people and providing me with all that I need.

SIGNIFICANCE OF INCREASED TISSUE TRANSGLUTAMINASE IN HORMONE REFRACTORY PROSTATE CANCER

Publication No. _____

Amy Lee Han, B.A.

Supervisory Professor: Kapil Mehta, Ph.D.

The progression of hormone responsive to hormone refractory prostate cancer poses a major clinical challenge in the successful treatment of prostate cancer. The hormone refractory prostate cancer cells exhibit resistance not only to castrate levels of testosterone, but also to other therapeutic modalities and hence become lethal. Currently, there is no effective treatment available for managing this cancer. These observations underscore the urgency to investigate mechanism(s) that contribute to the progression of hormone-responsive to hormone-refractory prostate cancer and to target them for improved clinical outcomes.

Tissue transglutaminase (TG2) is a multifunctional pro-inflammatory protein involved in diverse physiological processes such as inflammation, tissue repair, and wound healing. Its expression is also implicated in pathological conditions such as cancer and fibrosis. Interestingly, we found that the androgen-independent prostate cancer cell lines, which lacked androgen receptor (AR) expression, contained high basal levels of tissue transglutaminase. Inversely, the cell lines that expressed androgen receptor lacked transglutaminase expression. This attracted our attention to investigate the possible role this protein may play in the progression of prostate cancer, especially in view of recent observations that its expression is linked with increased invasion, metastasis, and drug resistance in multiple cancer cell types. The results we obtained were rather surprising and revealed that stable expression of tissue transglutaminase in androgen-sensitive LNCaP prostate cancer cells rendered these cells independent of androgen for growth and survival by silencing the AR expression. The AR silencing in

TG2 expressing cells (TG2-infected LNCaP and PC-3 cells) was due to TG2-induced activation of the inflammatory nuclear transcription factor- κ B (NF- κ B). Thus, TG2 induced NF- κ B was found to directly bind to the AR promoter. Importantly, TG2 protein was specifically recruited to the AR promoter in complex with the p65 subunit of NF- κ B. Moreover, TG2 expressing LNCaP and PC-3 cells exhibited epithelial-to-mesenchymal transition, as evidenced by gain of mesenchymal (such as fibronectin, vimentin, etc.) and loss of epithelial markers (such as E-cadherin, β -catenin). Taken together, these results suggested a new function for TG2 and revealed a novel mechanism that is responsible for the progression of prostate cancer to the aggressive hormone-refractory phenotype.

TABLE OF CONTENTS

| | <u>PAGE #</u> |
|-------------------------------------------------|---------------|
| APPROVAL PAGE..... | i |
| TITLE PAGE..... | ii |
| ACKNOWLEDGEMENTS..... | iii |
| ABSTRACT..... | iv |
| TABLE OF CONTENTS..... | vi |
| LIST OF FIGURES..... | vii |
| LIST OF TABLES..... | ix |
| | |
| CHAPTER I: BACKGROUND AND INTRODUCTION..... | 1 |
| | |
| CHAPTER II: MATERIALS AND METHODS..... | 13 |
| | |
| CHAPTER III: RESULTS | |
| Specific Aim 1..... | 26 |
| Specific Aim 2..... | 42 |
| | |
| CHAPTER IV: DISCUSSION & FUTURE DIRECTIONS..... | 65 |
| | |
| REFERENCES..... | 79 |
| | |
| VITA..... | 97 |

LIST OF FIGURES

| <u>TITLE</u> | <u>Page #</u> |
|----------------------------------------------------------------------------------------------------------------------------------------|---------------|
| FIGURE 1. Schematic representation of androgen receptor (AR) gene..... | 3 |
| FIGURE 2. Schematic representation of tissue transglutaminase (TG2) structure and functions..... | 9 |
| FIGURE 3. The basal level of tissue transglutaminase (TG2) and androgen receptor (AR) expression in prostate cancer cell lines..... | 12 |
| FIGURE 4. Schematic representation for propagation of lentiviral particles..... | 15 |
| FIGURE 5. Scheme for chromatin immunoprecipitation (ChIP) assay..... | 25 |
| FIGURE 6. TG2 expression renders the LNCaP cells independent of androgen for their growth and survival..... | 28 |
| FIGURE 7. Expression of TG2 does not affect the overall proliferation rate..... | 30 |
| FIGURE 8. TG2 expression is associated with morphological changes..... | 32 |
| FIGURE 9. TG2 expression induces EMT in prostate cancer cells..... | 35 |
| FIGURE 10. TG2 expression promotes cell invasion..... | 38 |
| FIGURE 11. TG2 expression confers drug resistance..... | 41 |
| FIGURE 12. TG2 expression is associated with loss of AR expression..... | 44 |
| FIGURE 13. TG2 expression results in constitutive activation of NF- κ B in prostate cancer cells..... | 48 |
| FIGURE 14. Further validation of NF- κ B activation in TG2 expressing cells..... | 49 |
| FIGURE 15. Validation of TG2-induced NF- κ B activation using immunofluorescence assay..... | 50 |

| | |
|------------------------------------------------------------------------------------------------------------------------|----|
| FIGURE 16. TG2-induced activation of NF- κ B results in constitutive expression of downstream target genes..... | 52 |
| FIGURE 17. Downregulation of TG2 reconstitutes AR expression..... | 54 |
| FIGURE 18. Downregulation of p65 subunit of NF- κ B reconstitutes AR expression..... | 56 |
| FIGURE 19. TG2-induced NF- κ B binds to the AR promoter..... | 58 |
| FIGURE 20. TG2 binds to the AR promoter in complex with p65 subunit of NF- κ B..... | 61 |
| FIGURE 21. Karyotypic mapping of LNCaP, LNCaP E.V., and LNCaP TG2 cells..... | 63 |
| FIGURE 22. TG2-regulated pathways during progression of hormone-refractory prostate cancer..... | 78 |

LIST OF TABLES

| <u>TITLE</u> | <u>Page #</u> |
|---------------------------------------|---------------|
| TABLE 1. RT-PCR Primer Sequences..... | 19 |

Chapter I: Background and Introduction

Prostate cancer is the second leading cause of death among American men, with nearly 218,000 new cases diagnosed every year and 32,000 deaths [1]. In general, normal and early-stage prostate epithelial cells depend on androgen-mediated signaling for their growth and survival. Androgens play an important role in regulating the growth, differentiation, and survival of prostate epithelial cell [2]. The androgen receptor (AR) is a nuclear receptor functioning as a steroid-hormone activated transcription factor directly regulating gene expression [3]. The AR contains three main domains [4]: the N-terminal regulatory domain (NTD), DNA-binding domain (DBD), and the ligand-binding domain (LBD) (Figure 1). The DBD and LBD are highly ordered and conserved among different species, while the NTD is more diverse [5]. The DBDs and LBDs of other members of the nuclear receptor super-family, such as the progesterone receptor and estrogen receptor are also highly conserved. Among the nuclear receptors, the DBD contains the greatest degree in homology, with more than 51% shared characteristics [6]. The AR, located on the X chromosome, is composed of 8 exons and coded by a gene that is longer than 90 kb [7]. There are two isoforms of the AR: AR isoform-1 contains the full length AR with a molecular weight of 110 kDa and AR isoform-2 differs in the 5'-UTR and coding region by lacking the first 187 amino acids; therefore, with a distinct and shorter NTD, it has a molecular weight of 87 kDa [8]. Practically half of the AR coding sequence represents the NTD, which regulates majority of the transcriptional activity [9]. The structure of the NTD could possibly change once bound to DNA or other proteins [10], suggesting that it could mediate the recruitment and assembly of co-regulators to mediate cell and gene specific effects [11]. The DBD is cysteine-rich and composed of two zinc finger motifs [12]: The first one mediates DNA recognition by interacting with specific base pairs of the androgen response elements [13] and the second mediates the dimerization of the AR as well as stabilizes the DNA bound complex [12]. The AR homodimer is arranged in a head-to-head fashion allowing further AR stabilization for

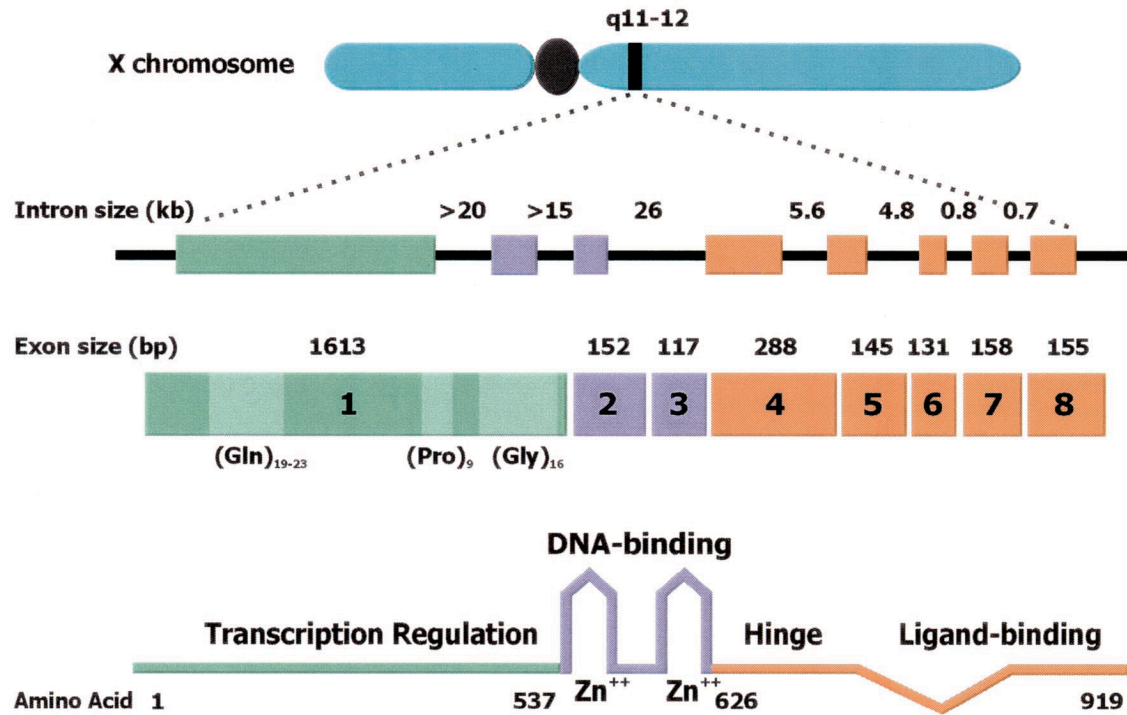


Figure 1. Schematic representation of androgen receptor (AR) gene.

The AR gene is located on chromosome Xq11-12 and is longer than 90 kb. It consists of 8 exons, which make up 3 distinct domains: N-terminal domain (NTD), DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD). The DBD contains two zinc finger motifs and a C-terminal extension, which forms part of the hinge region.

(Germann EP [7]. Reprinted with permission. © 2008 American Society of Clinical Oncology. All rights reserved.)

binding to DNA [14]. The transcriptional activation of AR is mediated by activation function 1 (AF1) and activation function 2 (AF2) [15]. While AF1 is located in the NTD, AF2 is located at the C-terminus inside the LBD. AF1 has a fairly weak transcriptional activation function whereas AF2 is the primary region that mediates the transcriptional activation. The AR also contains a small hinge region located in between the DBD and LBD [16]. A nuclear localization signal (NLS) [17] resides within this region in addition to phosphorylation [18, 19], acetylation [20], and degradation sites [21]. Lastly, the LBD facilitates the binding of the androgens [22, 23], which induces the conformational change that results in the nuclear translocation, phosphorylation, homodimer formation, interaction with DNA, and transcriptional regulation of the target gene.

Typically, in the cytoplasm, AR is bound by heat shock proteins (HSPs) [24] and is inactive. However, following its binding to the ligand androgen, it gets activated and undergoes a conformational change [25]. This change involves the dissociation of AR from HSPs, the LBD positioned in a way that prevents the dissociation of the ligand, and the NLS exposed. Thus, AR is released and targeted to the nucleus with the assistance of importins [26]. As a homodimer, the AR can then bind to specific androgen response elements, thereby activating transcriptional activity of genes needed for the growth and survival of the cells [27]. The AR specifically recognizes a 15 bp palindromic binding sequence motif in the promoter region of its target genes. This consensus sequence is composed of two hexameric half sites (5'-AGAACA-3') separated by a 3-bp spacer (5'-AGAACANNNTGTTCT-3') [28]. Prostate specific antigen (PSA) [29] and insulin-like growth factor I (IGF-I) [30] are among the specific target genes regulated by the AR.

Androgen ablation therapies (either surgical or chemical) represent the mainstay treatment for early stage prostate cancer [31]. Both castration [32] and the use of anti-androgens [33] inhibit the transcriptional activation by blocking AR signaling. Surgical castration involves the removal of the testes, the major androgen producing organ.

Chemical castration involves the use of luteinizing hormone-releasing hormone (LHRH) agonists/antagonists [34]. LHRH is required for the production of androgen by the testes. It is released by the hypothalamus, travels to the pituitary gland, which releases the luteinizing hormone that signals the testes to make androgen [35]. Anti-androgens inhibit the transcriptional activity of the AR [36, 37]. Thus, this androgen blockade helps prevent the growth and spread of prostate cancer. However, within 2-5 years of remission, the cancer returns in majority of these patients and exhibits resistance not only to androgen ablation therapies, but also to other known chemotherapies and is highly metastatic (referred to as hormone refractory or castration resistant prostate cancer: H/CRPC), resulting in patients' death [38]. Since there is no effective therapy for HRPc, understanding the mechanism(s) that contribute to the progression of hormone-responsive to hormone-refractory prostate cancer may help identify new therapeutic strategies for better clinical outcomes.

So far, three mechanisms have been linked to the development of HRPc. The first involves DNA-based alterations, such as mutations in or amplification of androgen receptor (*AR*) gene, although only a few patients fall into this category [39]. Studies have found AR mutations in HRPc that could enhance AR activation even with the low androgen levels [40] or by other steroid hormones such as progesterone [41] and glucocorticoids [42]. These mutations could also in turn allow anti-androgens to behave as agonists [43]. Some of the AR mutations are commonly found in the LBD, possibly altering the ligand binding specificity to the AR [44]. For example, a threonine to alanine amino acid change at codon 877 (T877A) renders the mutated AR to bind and be activated by progesterone, estradiol, and anti-androgens [45]. In addition, a mutation of codon 741 of the AR could potentially enable the androgen antagonist, specifically bicalutamide, to behave as an agonist. Patients who have been treated with this anti-androgen drug have shown to acquire this mutation [46, 47] as well as LNCaP cells that

have been long term cultured with bicalutamide [48]. On the other hand, the number of AR mutations found in patients who no longer respond to androgen ablation therapy are fairly low, approximately in 10% of patients with HRPc [46]. However, AR antagonists could confer selective pressures for the mutations that stimulate AR activity in addition to other mechanisms [49]. Amplification of the AR allows the prostate cancer to bypass the reduced levels of androgens by enhancing the number of androgen bound receptors. About 30% of patients show increased *AR* gene expression in tumors that have failed hormone ablation therapy while the primary tumors even within the same patients had no amplification prior to the therapy [50, 51]. High level of AR expression has the ability to compensate the reduced concentrations of androgens by maintaining enough activated AR for the continual growth and survival [52, 53]. Despite the type of DNA-based alterations, whether mutation or gene amplification, these cells still require androgen or an alternative ligand binding to promote tumor progression. These cells have instead been able to establish a lower threshold for androgens by increasing their sensitivity and adjusting to the depleted levels of androgens [54].

The second mechanism includes patients who do not have *AR* mutations or amplification, but still retain AR signaling even in the absence of androgen [39]. In these cases, alternative pathways are implicated leading to the activation of AR signaling [18]. For example, Her-2/neu-induced activation of AKT can promote AR activation by the phosphorylation of serine residues [55]. Her2/neu is a tyrosine kinase receptor from the epidermal growth factor family of receptors. Its overexpression has been implicated to play a role in promoting androgen independent growth of prostate cancer and activating AR signaling [56]. Prostate cancer xenograft models have even shown an upregulation of Her2/neu in HRPc [57, 58]. Studies have also found MAP kinases (MAPK) involved in the activation of AR signal transduction [59, 60]. MAPK signaling effectors can also activate AR signaling although only a couple have been identified and studied [38].

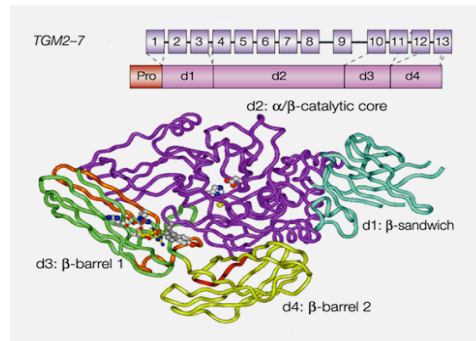
Furthermore, Aurora-A, a serine/threonine protein kinase involved in mitosis progression, was shown to phosphorylate AR at Thr-282 and Ser-293 and potentiate its transactivation [61]. In comparison with normal tissue, prostate tumors have higher Aurora-A expression [62]. All these possible alternative intracellular kinase signaling suggests that the crosstalk between the different signaling pathways can lead to the continual activation of the AR without the assistance of androgens. HRPCs tend to express AR and the *PSA* gene [63], implying that the AR signaling pathway is still functionally maintained in these cells.

The third mechanism involves a complete bypass of AR pathways as the cancer cells develop the ability to survive through alternative pathways [39]. Indeed, a recent study showed that a significant number of HRPC tumor samples from metastatic sites lacked AR expression [64]. It has been proposed that a subpopulation of the prostate cancer cells may already be independent of androgen prior to the hormone ablation therapy [65]. Since these cells would not be affected by the absence of androgens, only the androgen-dependent cells would be targeted. Thus, while the androgen-dependent cells would be abolished, the androgen-independent cells would continue to proliferate and thrive in the castrated environment. The basal, progenitor cells of the prostate are independent of androgen and their proliferation and death rates are unaffected by androgen ablation therapy [66] as is the case for advance prostate cancers, so it may be possible for the prostate tumors to adopt these prostate stem cell characteristics to resist apoptosis and proliferation [67]. In fact, one study demonstrated that the clonal expansion of androgen-independent cells at a frequency of approximately 1 per 10^5 - 10^6 androgen-dependent cells resulted in late stage androgen independence [68]. It speculated that due to the heterogeneous nature of prostate cancer cells and their varying dependence on androgen, hormone ablation therapy induces selective pressure

that changes the relative frequency of these cells allowing the androgen independent cancer cells to expand and thrive [39].

Tissue transglutaminase (TG2) is a pro-inflammatory protein belonging to the transglutaminase family of enzymes. It is a monomeric protein of 78 kDa, located on chromosome 20 and consists of 13 exons and 12 introns [69]. TG2 is structurally and functionally a complex protein implicated in multiple physiological processes such as apoptosis, wound healing, inflammation, and cell adhesion. TG2 catalyzes the cross-linking of proteins through the transamidation of γ -glutamine residues to ϵ -lysine residues in a Ca^{2+} dependent manner [70]. Besides its crosslinking activity, TG2 can bind and hydrolyze GTP and ATP [71], catalyze protein disulfide isomerase reactions [72], function as a protein kinase [73], and behave as a scaffold protein interacting directly with other proteins, such as fibronectin [74] and integrin [75]. TG2 is composed of four distinct domains: an N-terminal β -sandwich domain, a catalytic core domain, and two C-terminal β -barrel domains (Figure 2A) [70]. TG2 is predominantly an intracellular protein present in the cytosol, nucleus, and cell membrane [76]. However, there are some cases where TG2 is found secreted outside of the cell [77]. In general, under physiological conditions with low Ca^{2+} and high GTP concentrations, TG2 is kept in its inactive closed conformation state where the bound GTP keeps TG2 in its compact form, inhibiting the posttranslational crosslinking of proteins. It is in its inactive compact form that TG2 can behave as a scaffold protein participating in cell adhesion, cell survival, cell growth, invasion, and migration. It can interact with other proteins by altering their structure, function, and/or stability. However, under extreme stress or trauma, the disruption of Ca^{2+} homeostatic levels allows TG2 to be activated into its extended open conformation, exposing the catalytic transamidation site (Figure 2B). Thus, TG2 is able to crosslink intracellular proteins, leading to cell death [78]. TG2 plays an important role in wound healing [79, 80]. The cytokines and growth factors that are secreted during the initial

A



B

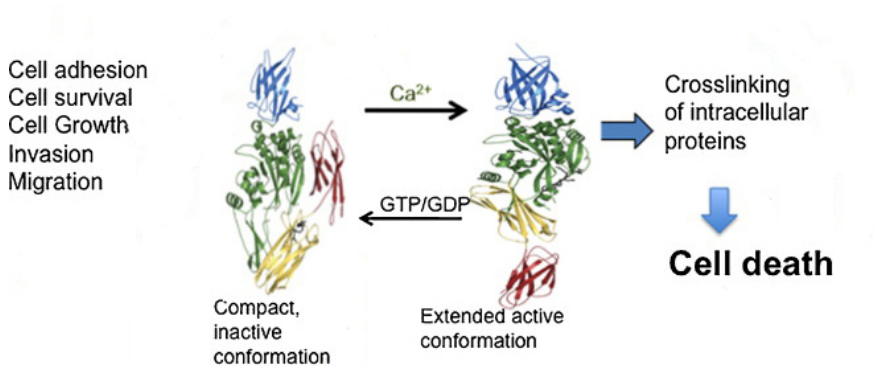


Figure 2. Schematic representation of tissue transglutaminase (TG2) structure and functions.

A) The *TG2* gene is located on chromosome 20 and consists of 13 exons, which make up 4 distinct domains: N-terminal β -sandwich domain, catalytic core domain, and 2 β -barrel domains. (Adapted from Lorand L et al. [70] by permission from Macmillan Publishers Ltd: Transglutaminases: crosslinking enzymes with pleiotropic functions, copyright 2003).

B) Under normal physiological conditions, the low Ca^{2+} and high GTP levels maintains TG2 in its compact inactive conformation where it can act as a scaffold protein participating in cell adhesion, survival, growth, invasion, and migration. However, under stressful conditions, the influx of Ca^{2+} can activate TG2 to be in its extended active conformation, allowing it to catalytically crosslink intracellular proteins leading to cell death. (Adapted from Mehta K et al. [96] copyright 2010, with permission from Elsevier).

stage of tissue injury can regulate the expression of TG2. For instance, transforming growth factor $\beta 1$ (TGF- $\beta 1$) induces TG2 expression in keratinocytes [81] and tumor necrosis factor α (TNF- α) has been seen to induce TG2 expression in liver cells [82]. At sites of injury, there is an increase in TG2 expression and activity at sites of neovascularization and in endothelial cells seeming to play a protective role [83, 84]; however, the continual TG2 expression can potentially lead to abnormal wound healing [80]. Cancer has been described as a wound that does not heal and tends to share many similarities with the inflammatory response and tissue repair that occurs upon tissue injury [85]. Inflammation has been found to assist in the progression of the tumor, promoting growth, survival, invasion, and metastasis of cancer cells [86]. TG2 has been implicated to play a role in advance-stage cancers where increased TG2 expression in cancer cells has been linked to poor patient survival [87, 88]. In fact, aberrant expression of TG2 has been linked with increased invasion [89], metastasis [87, 90], and drug resistance [87, 91] in multiple cancer cell types, including breast [92], ovarian [93], lung [94], and pancreatic [95] cancers. These characteristics also hold true for castration resistant prostate cancer where we see an increase in invasiveness, highly metastatic behavior, and drug resistance. In the present study, our findings address the significance of TG2 in hormone refractory prostate cancer. We examined whether TG2 plays a role in androgen-independent survival or growth using the loss- and gain-of-function approach. We show that aberrant expression of TG2 in prostate cancer cells promotes hormone refractoriness by silencing *AR* expression and promotes invasive phenotype by inducing epithelial-to-mesenchymal transition (EMT). Moreover, we provide a molecular mechanism by which TG2-induces the progression from androgen-dependent to androgen-independent cell growth and survival of prostate cancer cells.

Hypothesis and Specific Aims

Based on the recent findings that TG2 expression is frequently upregulated in multiple cancer cell types and its expression is associated with drug resistance and metastasis, our lab has been interested in determining the significance of TG2 in cancer development and progression. In this context, our preliminary observation that only two (PC-3 and DU-145) of the seven prostate cancer cell lines tested, showed high basal levels of TG2 expression (Figure 3, Fok J and Mehta K. Unpublished) is of significance. Inversely, the cell lines that lacked TG2 expression contained high basal expression of AR protein and transcript. These results implied that TG2 might play a role in the progression of androgen-dependent to androgen-independent prostate cancer by modulating AR expression and bypassing AR signaling for cell growth and survival.

Based on these preliminary and interesting results, we hypothesized that aberrant expression of TG2 in prostate cancer cells could promote hormone refractoriness and confer drug resistance and metastatic phenotype. To test this hypothesis, we formulated the following two specific aims:

Aim 1. To determine whether tissue transglutaminase (TG2) expression is necessary and/or sufficient in conferring androgen refractory phenotype in prostate cancer cells.

Aim 2. To determine TG2-regulated pathways that contribute to the hormone refractory phenotype in prostate cancer cells.

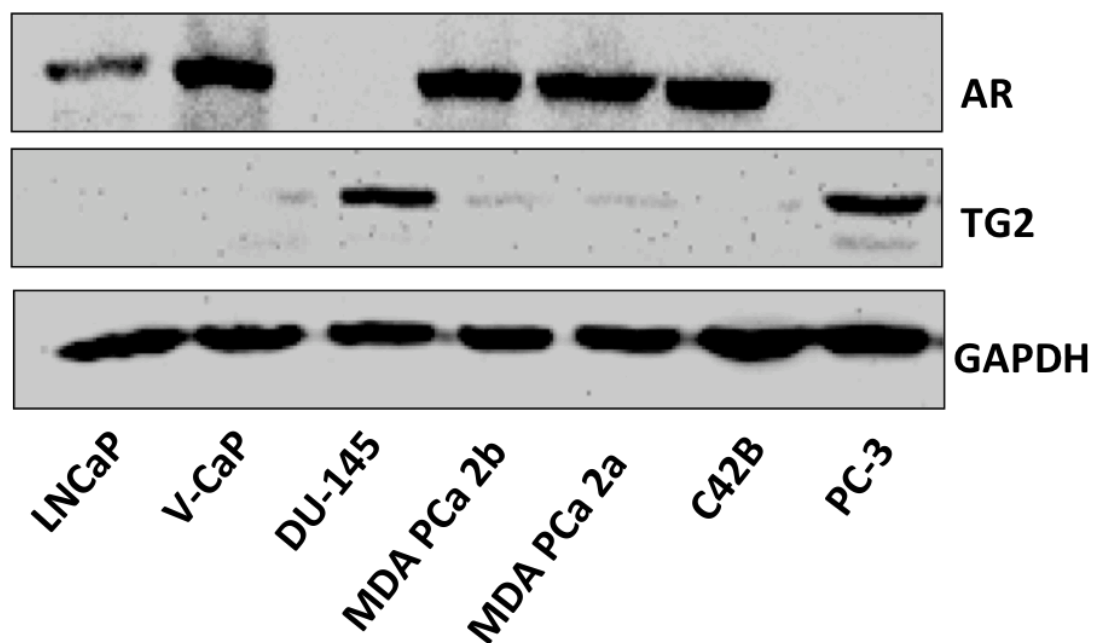


Figure 3. The basal level of tissue transglutaminase (TG2) and androgen receptor (AR) expression in prostate cancer cell lines.

TG2 and AR protein expression was determined in indicated prostate cancer cell lines using immunoblotting. Results show an inverse correlation between TG2 and AR expression. The prostate cancer cells with high AR expression, lacked TG2 expression. Conversely, the cell lines expressing high basal levels of TG2 lacked the AR expression. The TG2 expressing DU-145 and PC-3 cells are among the androgen-independent prostate cancer cell lines, suggesting that TG2 could play a role in hormone refractoriness. The membrane was reprobed with anti-GAPDH antibody to ascertain even protein loading.

Chapter II: Material and Methods

Cell Lines

The LNCaP and PC-3 cell lines were generously provided by Dr. Nora Navone (The University of Texas M.D. Anderson Cancer Center, Houston, Texas). Cells were maintained in RPMI 1640, supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and antibiotics. All media were purchased from Fisher Scientific (Pittsburgh, PA).

TG2 Lentiviral Transfection

pCDH cDNA Cloning and Expression Lentivectors (SBI System Biosciences, Mountain View, CA) was used to stably transfect LNCaP cells with TG2 (Figure 4). Briefly, HEK 293 cells were co-transfected with a lentiviral expression construct and pPACK packaging plasmid mix to create TG2 lentiviral particles. Viral particles were then collected and used to infect the LNCaP cells. Stably transfected cells were established by selection with puromycin (Invivogen, San Diego, CA) at concentrations of 1 μ g/ml.

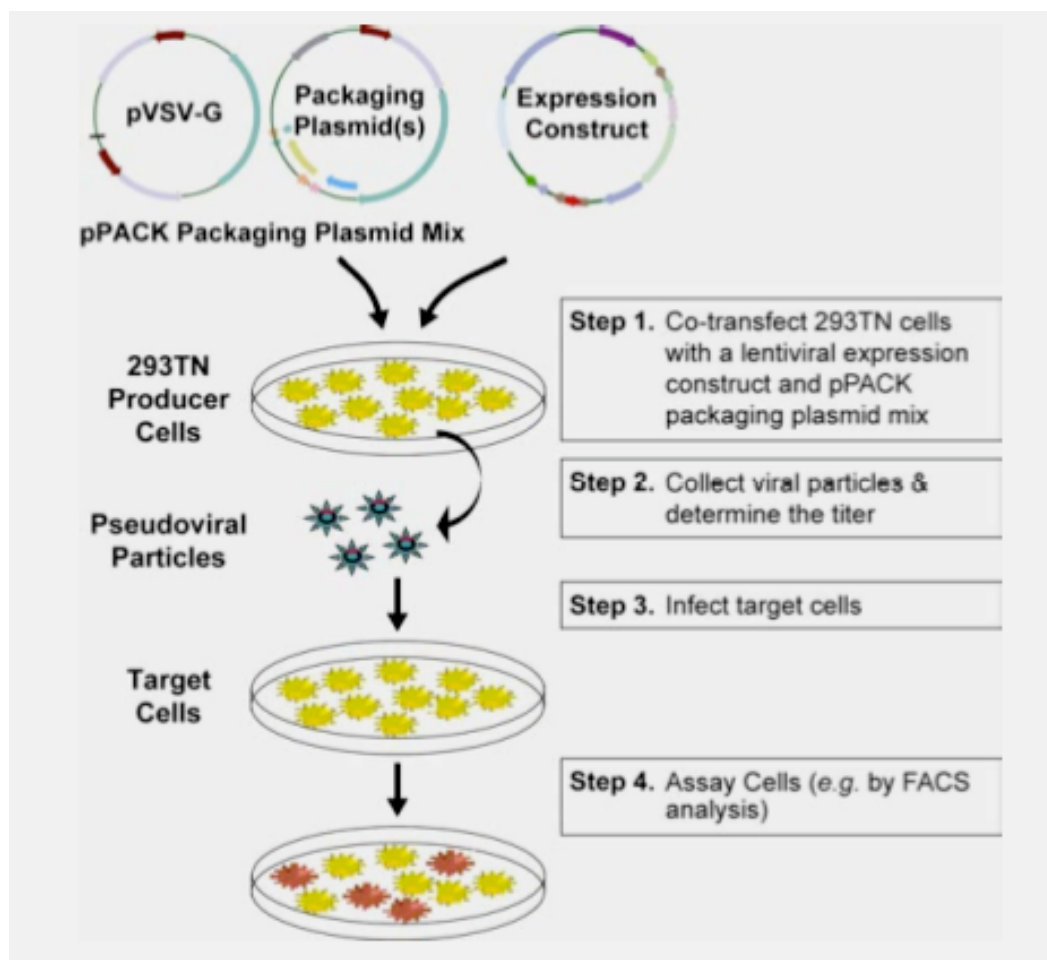


Figure 4. Schematic representation for propagation of lentiviral particles. The packaging plasmids mix and TG2 expressing vector construct are transfected into the HEK 293 cells to create packaged TG2 lenti pseudoviral particles. These TG2 pseudoviral particles can be collected from the HEK 293 medium as the over abundant production of these particles get secreted out into the cell culture medium. This medium is transferred over to the target cells (LNCaP) for infection. The selection for the successfully TG2 transfected cells is made by puromycin: Since the expression vector contains both the TG2 and puromycin resistant construct, only the TG2 transfected cells would be able to survive and withstand the presence of puromycin. (Reprinted with permission. © 2007 System Biosciences (SBI): pCDH cDNA Cloning and Expression Lentivectors User Manual).

Cell Proliferation Assay

Cells (2,000) were plated in quadruplicates (200 μ l media/well) in ninety-six well plates (2 plates). 8 wells were left empty for blank controls. 20 μ l of 5 mg/ml MTT (Thiazdyl Blue Tetrazolium Bromide, M2128-1G, Sigma Aldrich, St Louis, MO) dissolved in PBS was added to each well of one ninety-six well plate 2 hours after plating and left in the incubator (37°C, 5% CO₂) for 4 hours. Media was removed and 200 μ l of Dimethyl Sulfoxide (DMSO) (Sigma) was added to dissolve the formazan. Plate was mixed thoroughly for 5 minutes and optical density read at 570 nm. This served as the baseline to evaluate the relative cell growth. Cells of the other ninety-six well plate were kept in the incubator for 3 days in which MTT solution was added 4 hours prior to resuspending the formazan with DMSO and reading the optical density.

Cell Growth

Cells (2,000) were added in quadruplicates to ninety-six well plates and incubated overnight to allow the cells to attach to the wells. Either 0.1 nM R1881 (AR agonist, Sigma) or 50 μ M of bicalutamide (AR antagonist, Sigma) was added to the cells and left in the incubator for 48 hours. The number of viable cells remaining at the end of the treatment was determined using MTT: 20 μ l of 5 mg/ml MTT dissolved in PBS was added to each sample well. After 4 hours, media was removed and 200 μ l of DMSO was added to dissolve the formazan. Plate was mixed thoroughly for 5 minutes and optical density read at 570 nm. The untreated cells (only complete growth medium) served as the baseline to evaluate the relative number of cells.

Drug Response Assay

Cells (2,000) were plated in quadruplicates to ninety-six well plates and incubated overnight for the cells to adhere to the wells. After 24 hours, the medium was replaced with increasing concentrations of doxorubicin (0-0.1 $\mu\text{g/ml}$) (Sigma). The number of viable cells was measured 72 hours after drug treatment using MTT as mentioned previously.

Immunoblotting

Cells were grown in 100-mm dishes up to 80% confluence, washed twice with cold phosphate-buffered saline (PBS). Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL) was used to obtain nuclear protein. Otherwise, cells were lysed using NP-40 Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.5% NP-40, pH 7.5) containing 1:100 protease inhibitor cocktail (Roche Diagnostics, 11697498001, Mannheim, Germany). Cells were scraped and collected into a 1.5 ml eppendorf tube. Centrifuged at 13,000g for 10 minutes at 4°C. The supernatant was collected and measured for their protein concentration using Bio-Rad Protein Assay. 30 μg of protein was resolved in an 8-10% reducing gradient polyacrylamide gel (PAGE). Proteins were transferred onto a nitrocellulose membrane and after blocking with 5% non-fat milk or 3% BSA probed with appropriate primary antibody: anti-TG2 (Abcam, CUB7402, 1:10,000, Cambridge, MA), anti- $\alpha\beta$ -actin (mAbcam 8226, 1:5000), anti-AR (Santa Cruz, N-20, 1:1000, Santa Cruz, CA), anti-E-cadherin (Santa Cruz, sc-21791, 1:1000), anti-fibronectin (Santa Cruz, sc-71116, 1:1000), anti- β -catenin (BD Biosciences, 610153, 1:3000, San Jose, CA), anti-NF- κ B p65 (Santa Cruz, sc-109, 1:1000), and anti-I κ B α (Imgenex, IMG-127A, 1:3000, San Diego, CA). The antigen-antibody reaction was detected using the appropriate horseradish peroxidase-conjugated secondary antibody,

followed by visualization with the electrochemiluminescence detection system (Denville Scientific Inc., Metuchen, NJ).

Reverse Transcriptase-Polymerase Chain Reaction

Cells were plated on 100-mm tissue culture dishes and grown to 70% confluence, washed with PBS, and used to isolate total RNA by the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration was measured using a Nanodrop Spectrophotometer. 5 µg of total RNA was used to synthesize cDNA through SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR was then conducted with the cDNA using the specified conditions: Samples were incubated at 94°C for 5 minutes, then 35 cycles of PCR was performed under optimized conditions (denaturation at 94°C for 45 sec, refer to Table 1 for annealing temperatures for 30 sec, extension was at 72°C for 1 min 30 sec), and stored in 4°C. PCR samples were run on a 1% Agarose (Denville Scientific Inc.) gel in TBE with added ethidium bromide. Bands were viewed under UV light and images captured using Alpha Innotech FluorChem 8900.

| Gene Target | Primer Sequences (5' – 3') | Product Size (bp) | Annealing Temperature |
|-------------|--------------------------------------------------------------------|-------------------|-----------------------|
| AR | TTG GAT GGC TCC AAA TCA C GCA ATG ATA CGA TCG AGT TCC | 148 | 60°C |
| E-Cad | TCC CAT CAG CTG CCC AGA AA TGA CTC CTG TGT TCC TGT TA | 500 | 55°C |
| Vimentin | GAG AAC TTT GCC GTT GAA GC GCTTCCTGTAGGTGGCAATC | 163 | 55°C |
| ZEB1 | CTG AAG AGG ACC AGA GGC AG CCC AGA ACT GCG TCA CAT GTC | 279 | 55°C |
| ZEB2 | AAG TAC CGC CAC GAG AAG AA TTG TTT CTC ATT CGG CCA TT | 350 | 55°C |
| TG2 | TAT GGC CAG TGC TGG GTC TTC GCC GGC TCC AGG GTT AGG TTG AGC AGG | 786 | 65°C |
| GAPDH | ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA | 452 | 55°C |

Table 1. RT-PCR primer sequences.

The forward and reverse primer sequences respectively used for the RT-PCR reactions. Included are the PCR product sizes and the annealing temperature implemented to run the PCR amplification. Primers were ordered from Invitrogen.

Immunofluorescence

Cells (1000) were plated in Chamber Polystyrene Vessel slides (Fisher Scientific) and left in the incubator. Media was changed the next day. At 70% confluence, cells were washed 3 times with 1x DPBS. Cells were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 100% ice-cold methanol for 10 minutes at RT. Blocked for 1 hour in blocking solution (3% BSA, 1% Normal Animal Serum). Cells were incubated with the appropriate primary antibody (1:100) in blocking solution overnight at 4°C. Appropriate secondary antibody (Alexa Fluor 488/546 goat anti-rabbit/mouse IgG, 1:150, Invitrogen) was added for 1 hour at RT. Then stained with 0.5 µg/ml DAPI (Molecular Probes, D-1306, Invitrogen) for 5 minutes. Chambers were removed and cells were mounted on glass cover slips using anti-fade mounting media (DakoCytomation, S3023, Carpinteria, CA). Slides were left to dry and sealed with nail hardener polish. Between

every step, cells were washed three times with 1x DPBS. Images were viewed and captured using a Nikon Eclipse fluorescence microscope (Melville, NY).

Cell Invasion

Invasion was determined in vitro using Matrigel-transwell inserts. Cells were plated in 100-mm culture dishes. At 70% confluence, cell plates were rinsed with 1x PBS and serum free media was added to each plate and incubated overnight in the 37°C CO₂ incubator. Matrigel (Fisher, CB40234) was thawed on ice overnight. Transwell inserts with 8.0-µm pore size were coated with 200 µl of a 0.7 mg/ml concentration of Matrigel in cold serum-free medium and placed in the incubator for 40 minutes to solidify. Meanwhile, cells (after 24 hours in serum free media) were washed with 1x PBS then trypsinized. Cells were washed 3 times in serum-free media and pellet was subsequently resuspended in serum-free medium. Before plating cells, media was aspirated from each transwell. 500 µl (0.2 million cells) of the cell suspension was added to duplicate wells. After 72 hours of incubation, Matrigel was gently removed from wells and cells that invaded through the membrane were stained using Hema-3 manual staining system (Fisher, 22-1229-11), mounted on a glass slide, and counted using a light microscope. Five fields of cells were counted for each well, and the mean number of cells per field was calculated.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was conducted to determine NF-κB activation. Nuclear extracts were obtained from cells (1.5x10⁶ cells/ml) and incubated with ³²P end-labeled 45-mer double stranded NF-κB oligonucleotides (15 µg of protein containing 16 fM of DNA) from the HIV long terminal repeat, 5'-TTGTTACAAGGGACTTTCCG

CT**GGGGAC-TTTC**CAGGGAGGCGTGG-3' (with NF- κ B binding sites in bold) for 30 minutes at 37°C. The DNA-protein complex was run on a 6.6% native polyacrylamide gel to separate the complex from free oligonucleotides. Gel was left to dry and then visualized using a Storm 820 Phosphor Imager. Radioactive bands were quantified using Image Quant Software (GE Healthcare).

qPCR Array for NF- κ B Target Genes

Cells were plated in 6-well plates. At 70-80% confluence, RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using the RT² First Strand Kit (SABiosciences, Frederick, MD). 91 μ l of H₂O was added to each 20 μ l of cDNA synthesis reaction. The 12 RT² Profiler customized PCR Array was purchased from SABiosciences. The PCR mix for each sample (12.5 μ l RT² SYBR Green/Fluorescein qPCR Master Mix, 11.5 μ l H₂O, and 1 μ l template cDNA) would be added to each of the 12 chosen gene-specific PCR primer pair containing wells: Zeb1, Zeb2, Twist, SNAIL, COX2, I κ B, iNOS, MTA-1, HOMX1, CDKN1, CCND1, iCAM1, COPS2. The two-step cycling program for BioRad iCycler was used for 95°C, 10 min; 40 cycles of (95°C, 15 sec; and 60°C, 60 sec). The real-time thermal cycler was programmed to detect and record the SYBR Green signal from every reaction at the end of the 60°C annealing/extension step for each cycle.

Knockdown of p65 and TG2 by gene-specific siRNA

Cells (300,000) were plated into six-well plates and left to adhere overnight. Cells were transfected with 2 μ g of either NF- κ B p65 siRNA (Cell Signaling, #6535, Danvers, MA), TG2 siRNA (sense: 5'-GGGCGAACCACCUGAACAATT-3', antisense: 5'-UUGUUCAGGUGGUUCGCCCTT-3', Qiagen), or non-specific siRNAs using

oligofectamine (Invitrogen). After removing medium from the cells, 2 ml of OPTIMEM (Invitrogen) was added and cells were left in the incubator. Meanwhile, siRNA mix was prepared as follows: In one 1.5 ml tube, 5 μ l of siRNA was added to 155 μ l of OPTIMEM, in a separate 1.5 ml tube, 8 μ l oligofectamine + 32 μ l OPTIMEM and left in RT for 15 minutes. Then added the siRNA/OPTIMEM mix into the tube with oligofectamine, which was mixed and left at RT for 30 minutes. The 2 ml of OPTIMEM was removed from the cells; 800 μ l of new OPTIMEM was added, followed by the addition of the siRNA mix drop-by-drop into the well. 1 ml of normal growth media was added to the cells 24 hours after transfection and lysed 4 days after the initial transfection for immunoblotting.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin Immunoprecipitation Assay (Figure 5) was performed using the ChIP Assay Kit (Millipore, catalog #17-295, Temecula, CA) according to supplier's protocol. Cells were plated on 100-mm culture dishes to 70% confluence, treated directly with a final concentration of 1% formaldehyde (270 μ l of 37% formaldehyde into 10 ml of complete growth medium on plate), and incubated for 10 minutes at 37°C to cross link histones to DNA. Medium was aspirated from the cells and washed twice using ice cold PBS. Cells were then scraped into 15 ml tube and pelleted for 4 minutes at 2000 rpm at 4°C. Meanwhile, SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) was warmed to RT. Protease inhibitors were added to the SDS Lysis Buffer with a concentration of 50 μ l/ml. PBS removed completely and cell pellet resuspended in 200 μ l of SDS Lysis Buffer and incubated for 10 minutes on ice. DNA was sheared to lengths between 200 and 1000 base pairs through sonication conditions of six x10-second continuous pulses with 30 seconds interval between each pulse and centrifuged for 10 minutes at 13,000 rpm at 4°C. Supernatant was transferred to a new 2 ml

microcentrifuge tube. 50 μ l protease inhibitor was added per 1 ml of ChIP Dilution Buffer and used to dilute sonicated cell supernatant 10 fold (add 1800 μ l ChIP Dilution Buffer to 200 μ l of sonicated cell supernatant = total volume of 2 ml). Saved a portion of the diluted cell supernatant (~20 μ l) to use as input. 30 μ l of immunoprecipitating antibody, anti-NF- κ B p65 (Santa Cruz, sc-109) or anti-TG2 (NeoMarkers, MS-279-P1, Fremont, CA), were added to the 2 ml supernatant fraction and incubated overnight while rotating at 4°C. Next day, collected antibody/histone complex by adding 60 μ l of Protein A Agarose/Salmon Sperm DNA for one hour at 4°C with rotation. Agarose was pelleted using gentle centrifugation at 1000 rpm for 1 minute. Carefully removed supernatant, containing unbound, non-specific DNA, and washed agarose/antibody/histone complex for 4 minutes while rotating with 1 ml of each of the following buffers respectively: Low Salt Immune Complex Wash Buffer (1x), High Salt Immune Complex Wash Buffer (1x), LiCl Immune Complex Wash Buffer (1x), and TE Buffer (2x). Added 250 μ l of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃) to the pelleted complex to elute the histone complex from the antibody. Incubated at room temperature and vortexed intermittently to mix for 15 minutes. Spin down agarose by centrifugation at 1000 rpm for 2 minutes and transferred supernatant fraction to another tube. Repeated elution once more for a combined total eluates volume of 500 μ l. 20 μ l of 5 M NaCl was added to the combined elutes including the input saved the day prior and heated at 65°C for 4 hours to reverse the histone-DNA crosslinks. After the addition of 10 μ l of 0.5 M EDTA, 20 μ l 1 M Tris-HCl, pH 6.5, and 2 μ l of 10 mg/ml Proteinase K, eluates were incubated for one hour at 45°C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. 2 μ l of pellet paint co-precipitant (Novagen, Darmstadt, Germany) was used to visualize the DNA pellet and washed with 70% ethanol and air-dried. Pellet was resuspended in RNase free water. DNA was then subjected to PCR amplification using

primers as described previously [97], corresponding to the AR promoter from -38 to +246: 5'-GACCCGACTCGCAAAGTGT and 5'-CCTCCGAGTCTTTAGCAGCT as well as -760 to -460: 5'-GGGTGATTTTGCCTTTGAGA and 5'-CATGACCAAGCCAGCAGATA. Primers corresponding to exon 1 (2403 to 2647) of the AR were to use as negative control: 5'-CCTGGCACACTCTCTTCACA-3' and 5'-GGATAGGGCACTCTGCTCAC-3'. Samples were run under the following conditions: 94°C for 5 minutes, 37 cycles of PCR was performed (94°C- 45 sec, 60°C- 30 sec, 72°C- 1 min), and then stored in 4°C. PCR samples were analyzed by agarose gel electrophoresis.

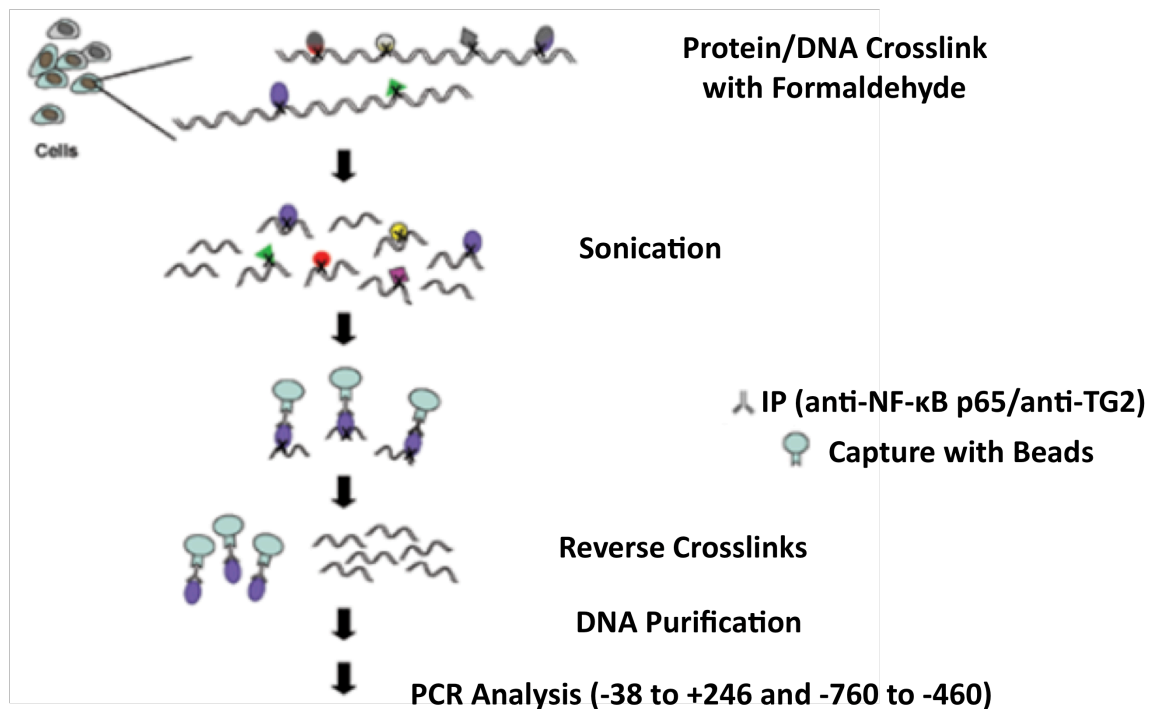


Figure 5. Scheme for chromatin immunoprecipitation (ChIP) assay.

Cells were incubated at 37 °C with formaldehyde for 10 minutes to crosslink the protein to DNA. After the cells were washed with cold PBS and lysed with SDS Lysis Buffer, they were subjected to sonication of six, 10 second long pulses to shear the DNA into fragments. These fragments were incubated overnight with anti-NF- κ B (p65) or anti-TG2 antibody. The next day, the bound antibody/histone complex would be captured with protein A agarose beads, which would be collected and washed. The histone complex will be eluted from the antibody and reverted to decrosslinking. DNA would then be extracted, purified, and subjected to PCR analysis. (Adapted with permission. © 2009 Affymetrix: USB ChIP Assay Procedure).

Chapter III: Results

(Specific Aim 1)

Overexpression of TG2 in LNCaP Cells

Multiple studies have supported that the aberrant expression of tissue transglutaminase is associated with drug resistance and metastasis in many cancer cell types. HRPCs display similar characteristics in that they are more aggressive, drug resistant, and metastatic and display high basal levels of TG2 (Figure 3). To determine the significance of TG2 expression in HRPC, we first stably transfected the LNCaP cells with TG2 using the lentiviral construct (Figure 6A). The parental LNCaP cells are androgen-dependent and have no detectable expression of TG2 but expressed high levels of AR. We first tested for the response of TG2-transfected LNCaP cells to androgen (R1881, 0.1 nM) and androgen antagonist (Bicalutamide, 50 μ M) in charcoal-stripped serum containing RPMI medium. As expected, both the control and empty vector transfected LNCaP cells showed increase in cell growth and survival when incubated in the presence of androgen (R1881). Conversely, these cells showed significant reduction in growth and survival when incubated with AR antagonist, bicalutamide (Figure 6B). Interestingly, under identical conditions the TG2-transfected LNCaP cells showed no effect on cell growth or cell survival in response to either R1881 or bicalutamide treatment. Cell growth in cultures treated with either agent was similar to the untreated cells. These results implied that TG2 expression could promote cell growth and survival in absence of androgen, and thus confer androgen-independent phenotype in LNCaP prostate cancer cells. TG2 expression by itself had no affect on cell growth, as determined by cell proliferation assay using MTT (Figure 7). The relative cell growth of control (vector-transfected or non-transfected) LNCaP cells was comparable to TG2 transfected cells as determined by MTT assay.

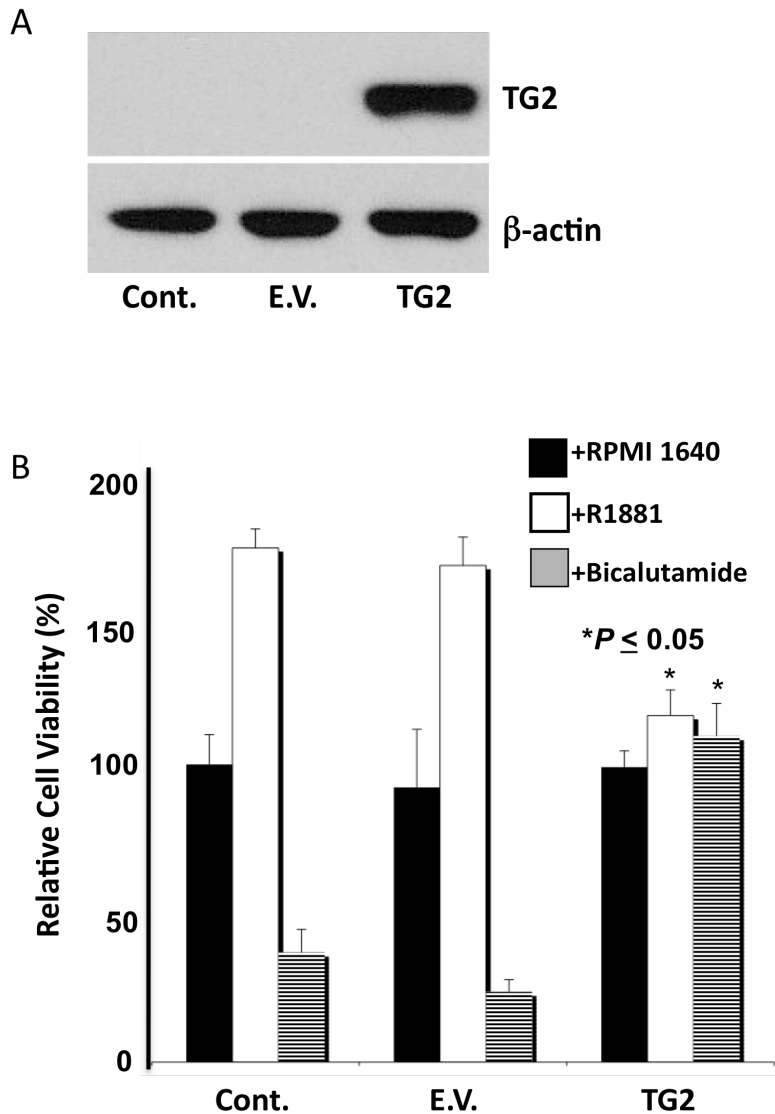


Figure 6. TG2 expression renders the LNCaP cells independent of androgen for their growth and survival.

A) Western blot analysis of the control (parental or empty vector) and TG2 transfected LNCaP cells. The LNCaP cells with stable TG2 expression were established by lentiviral infection containing TG2 construct and selection against puromycin (1 μ g/ml).

B) These cells were subjected to either androgen agonist (R1881, 0.1 nM) or antagonist (Bicalutamide, 50 μ M) to test for their response to androgen. Both control and empty vector LNCaP cells demonstrate an increase in growth upon the addition of androgen;

however, growth was inhibited by the presence of androgen antagonist. On the other hand, the LNCaP TG2 cells neither grew in response to R1881 nor displayed an inhibition of growth with bicalutamide. Results shown are means of quadruplicate values \pm standard deviation. The significance of difference (p-value) was calculated using the student's t-test and is a comparison between the response either to androgen or androgen antagonist. A p-value less than 0.05 was considered significant.

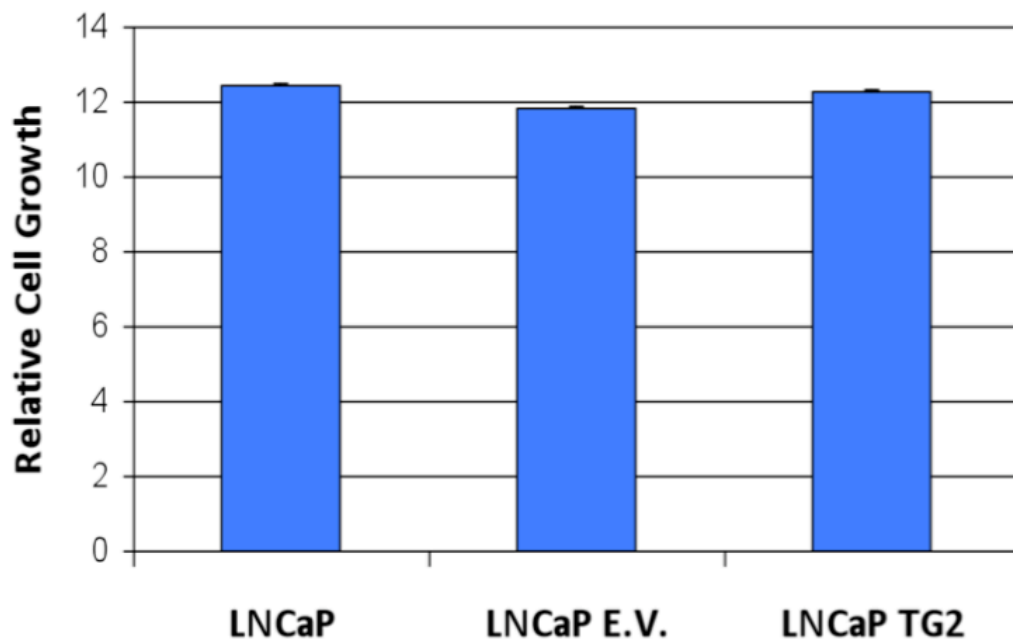


Figure 7. Expression of TG2 does not affect the overall proliferation rate.

The parental, empty vector, and TG2 transfected LNCaP cells were analyzed for their cell proliferation rate using MTT assay. This was conducted to determine whether TG2 expression had any affect on the proliferation of LNCaP cells. The cells were left in the incubator for 48 hours and the baseline was taken 6 hours after plating the cells.

Absorbance was measured at 570nm. Results indicate a fairly similar cell proliferation rate amongst the cell lines. Results shown are means of quadruplicate values \pm standard deviation from a representative experiment. Experiments were repeated at least twice displaying similar results.

TG2 Induces Changes in Morphology

TG2 expression was associated with noticeable changes in the morphology of LNCaP cells (Figure 8). The parental and empty vector-LNCaP cells grew clustered together with cell-to-cell contacts, the characteristic feature of epithelial cells. The LNCaP-TG2 cells, in contrast, grew more separated from each other, with fibroid-like appearance. These changes in the morphology indicated that TG2 expression might induce the transition of LNCaP epithelial cells to the mesenchymal state (EMT).

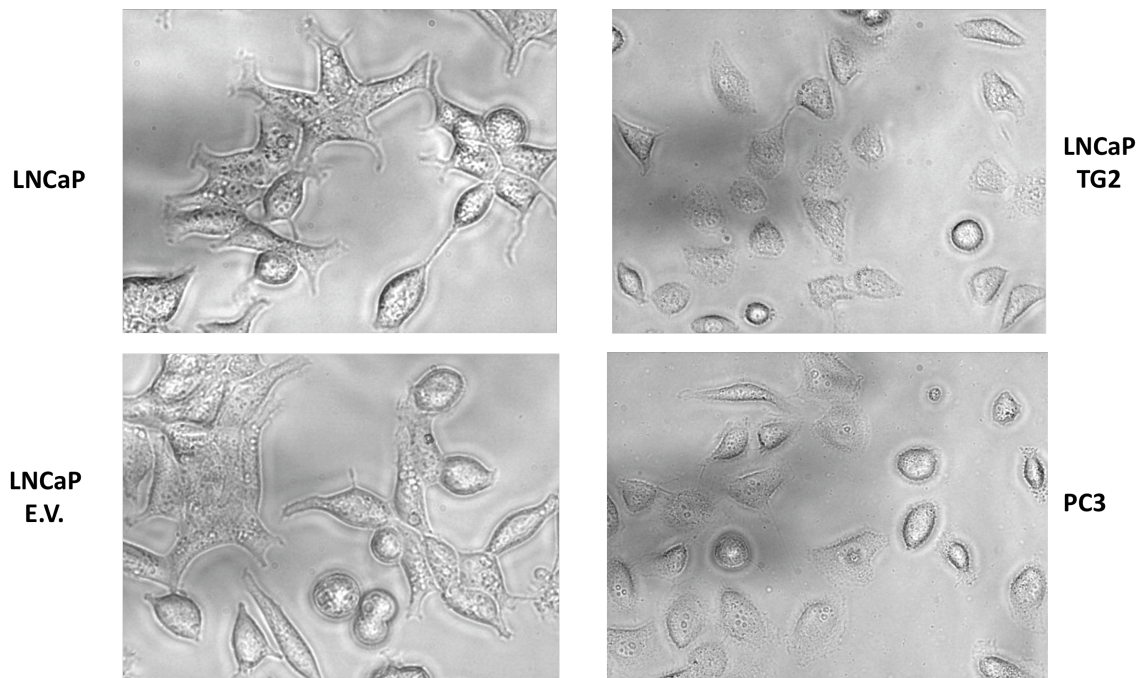


Figure 8. TG2 expression is associated with morphological changes.

Images of the prostate cancer cell lines captured using the Nikon Eclipse Microscope (20x) at 60-70% confluence. LNCaP control and empty vector cells tend to grow clustered together, displaying high cell-to-cell contact, characteristic of epithelial-like cells. TG2-transfected cells, in contrast, grow separated from each other, demonstrating loss of cell adhesion, which is indicative of mesenchymal cells. LNCaP-TG2 cells resembled PC-3 cells, which contain high endogenous basal levels of TG2. These morphological changes seem to suggest that TG2 expression may play a role in inducing the epithelial-to-mesenchymal transition (EMT).

TG2 Expression Promotes Epithelial-to-Mesenchymal Transition

To validate whether TG2-induced changes in the morphology of LNCaP cells is related to their transition into mesenchymal state, we determined various EMT-related markers in these cells. The results confirmed and were consistent to the differences seen between the cell morphology. There was a clear loss of E-cadherin in TG2 transfected cells, which is one of the primary characteristics of cells undergoing the epithelial-mesenchymal transition (Figure 9A). There is also a decrease in β -catenin protein levels, as well as a gain in fibronectin, a mesenchymal marker. The PC-3 cells were used in parallel as a control to compare TG2-induced changes in LNCaP cells. The PC-3 cells also displayed a mesenchymal phenotype with absence of E-cadherin expression and presence of fibronectin. However, the PC-3 cells displayed high expression of N-cadherin, whereas no expression of N-cadherin was evident in TG2 expressing LNCaP cells. Although the PC-3 cells display many similarities with the LNCaP TG2 cells, they do slightly deviate from each other. This suggests that TG2 expression can partially promote the EMT phenotype in prostate cancer cells. Furthermore, these distinctive EMT markers are not only seen at a protein level, but also evident at a transcriptional level (Figure 9B). We also observed gain of other key mesenchymal markers, such as vimentin, Zeb1, and Zeb2 in TG2 expressing LNCaP cells. Especially, the increase in the *Zeb1* transcriptional repressor was remarkable. The expression of EMT-related transcripts in the PC-3 cells followed similar pattern as the TG2-LNCaP cells. Immunofluorescence staining further confirmed the EMT-related changes in TG2-LNCaP cells and confirmed the loss of E-cadherin and gain in fibronectin (Figure 9C). In addition, while β -catenin expression in the LNCaP cells is membranous, β -catenin expression is nuclear in the LNCaP TG2 cells, which is indicative of the loss of E-cadherin. The androgen-independent PC-3 cells show comparable expression levels of fibronectin as well as a lack of E-cadherin expression.

Thus, these results strongly support that aberrant expression of TG2 promotes the EMT phenotype in prostate cancer cells.

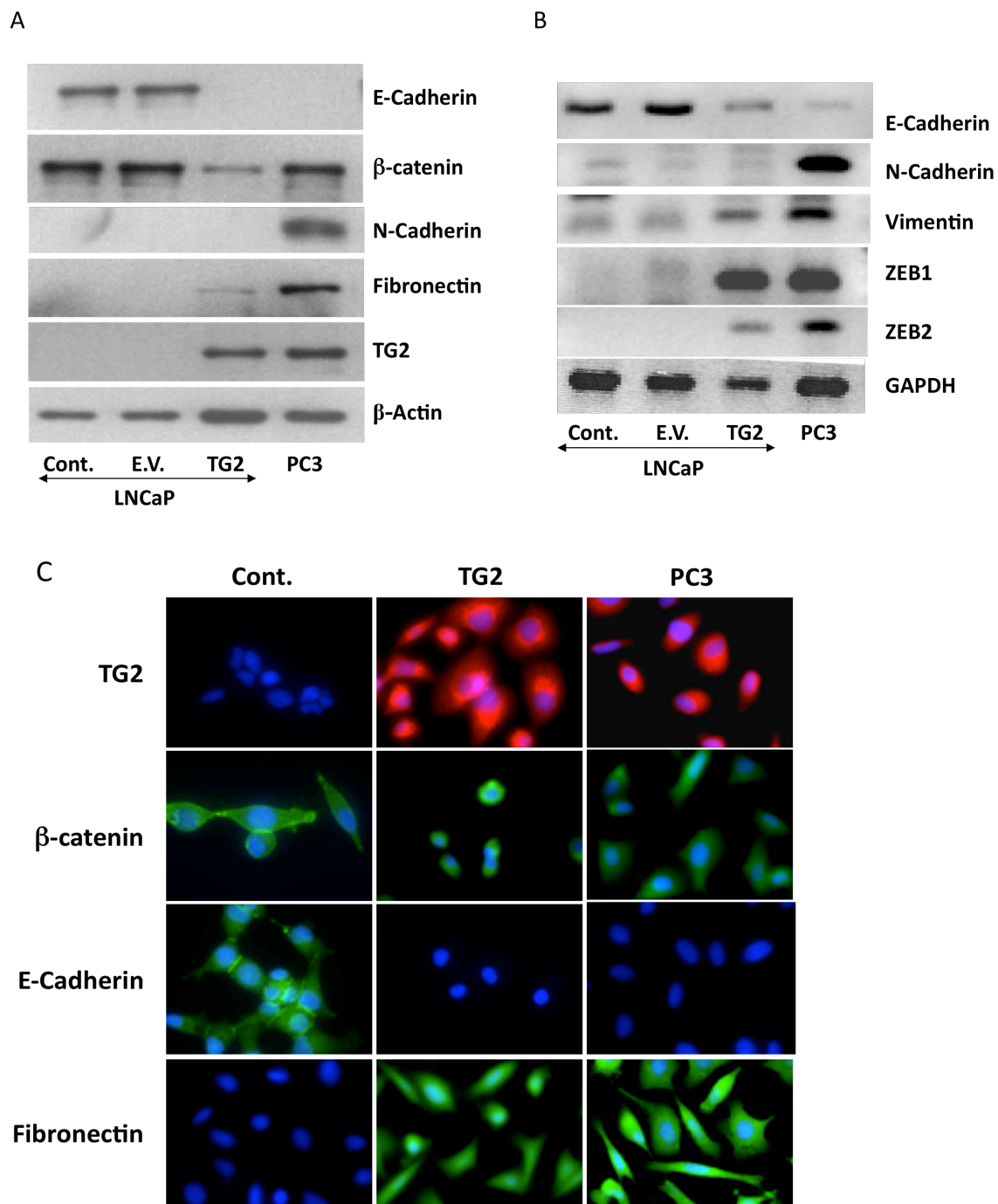


Figure 9. TG2 expression induces EMT in prostate cancer cells.

A) Protein extracts were collected from each of the individual LNCaP (control, empty vector, and TG2-transfected) and PC-3 cells to evaluate the expression of EMT markers such as E-cadherin, β -catenin, N-cadherin, and fibronectin using immunoblotting. A

complete loss of E-cadherin, decrease in β -catenin, (epithelial cell markers), and gain in fibronectin expression (mesenchymal cell marker) by TG2-infected LNCaP cells suggested acquisition of the EMT phenotype. PC-3 cells with high basal TG2 levels, displayed similar EMT-related changes, except that PC-3 cells also showed increase in N-cadherin expression (another mesenchymal marker).

B) RNA extracts were collected from each of the individual LNCaP (control, empty vector, and TG2-transfected) and PC-3 cells to evaluate the transcript levels of epithelial and mesenchymal markers. Results seem to coincide with immunoblot analysis - the loss in epithelial (*E-cadherin*) and gain in mesenchymal marker transcripts (*vimentin*, *zeb1*, *zeb2*).

C) Cells were subjected to immunofluorescence staining. Once fixed with paraformaldehyde, cells were incubated with anti-TG2, anti-E-cadherin, anti- β -catenin, or anti-fibronectin antibody. Alexa 546 anti-mouse IgG (red) or Alexa 488 anti-mouse IgG (green) was used to detect the antigen-antibody reaction. DAPI was used to stain the nuclei. Immunostaining results further supported the loss of epithelial markers (E-cadherin and β -catenin) and gain in mesenchymal marker (fibronectin) associated with TG2 expression.

TG2 Expression Promotes Cell Invasion in LNCaP Cells

EMT has also been associated with an increase in cell invasiveness. Also, TG2 expression has been found to promote invasion in many cancer cell types. Therefore, we next determined the invasive ability of TG2 expression on LNCaP cells. The invasiveness was determined using Matrigel-transwell inserts in either 10% fetal calf serum (FCS) or androgen-depleted 10% charcoal stripped serum (CSS) containing medium. Regardless of the type of serum medium used, LNCaP-TG2 expressing cells were 70-90% more invasive compared to the control or empty vector transfected LNCaP cells (Figure 10A). These results suggested that TG2-induced EMT in LNCaP cells is associated with increased invasiveness and this increased invasiveness is independent of androgen as revealed by the increased invasion of cells even in CSS medium (Figure 10B). These results indicated that TG2 expression could promote invasion in prostate cancer independent of androgen signaling.

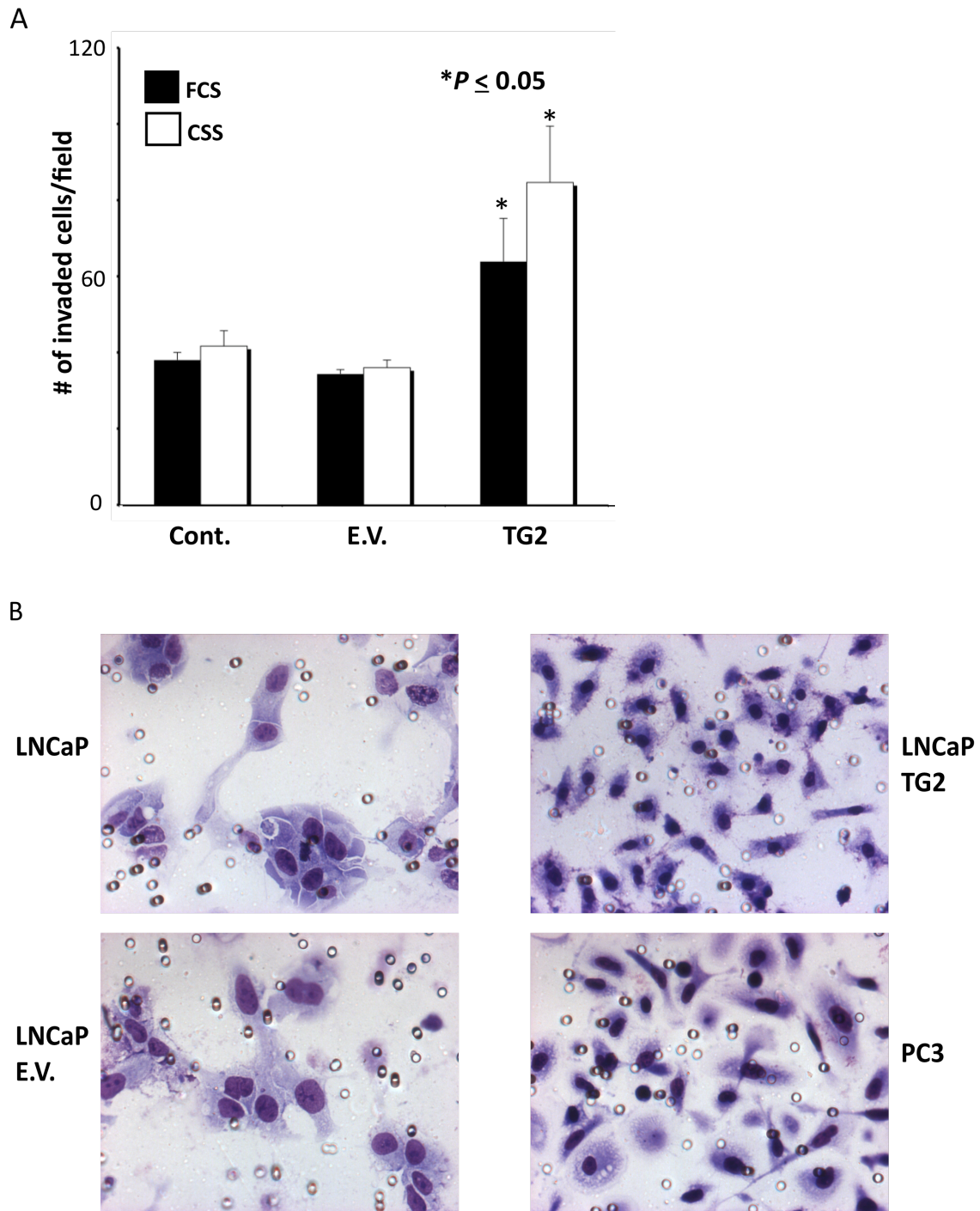


Figure 10. TG2 expression promotes cell invasion.

A) Control, empty vector, and TG2 expressing LNCaP cells were subjected to an *in vitro* Matrigel-transwell invasion assay. Cells were serum-starved for 24 hours and then seeded on top of the matrigel-transwell. The cells were placed in either 10% fetal calf

serum (FCS) or charcoal-stripped serum (CSS) medium. After 72 hours, membranes were fixed, stained, and mounted to glass slides. The number of invaded cells in 5 random microscopic fields was counted. Data shown are the mean number of cells invaded/field \pm standard deviation. LNCaP TG2 cells were significantly more invasive than the control and empty vector cells in both the FCS and CSS medium. The significance of difference (p-value) was calculated using the student's t-test and the p-value less than 0.05 was considered significant.

B) Images of the stained membranes with invaded cells through the membrane of the Matrigel-transwell inserts from a representative field. LNCaP TG2 cells show a greater number of invaded cells in comparison with the control and empty vector cells. The invasive ability of LNCaP-TG2 was comparable with that of PC-3 cells.

Tissue Transglutaminase Expression Confers Drug Resistance

An increase in drug resistance is a common feature associated with EMT. In addition, TG2 expression has been shown to confer drug resistance in multi-cancer types. Thus, we used an MTT assay to assess the drug response of the LNCaP cells. Different doses of increasing concentrations of doxorubicin (0-0.1 $\mu\text{g/ml}$) were added to the cells and the number of viable cells was measured after three days (Figure 11). Results reveal that TG2 expression is able to confer drug resistance in the LNCaP cells equivalent to the PC-3 cells, which contain high endogenous TG2 expression. These results demonstrate that TG2 expression can confer drug resistance in prostate cancer, characteristic of cells undergoing EMT and of HRPC.

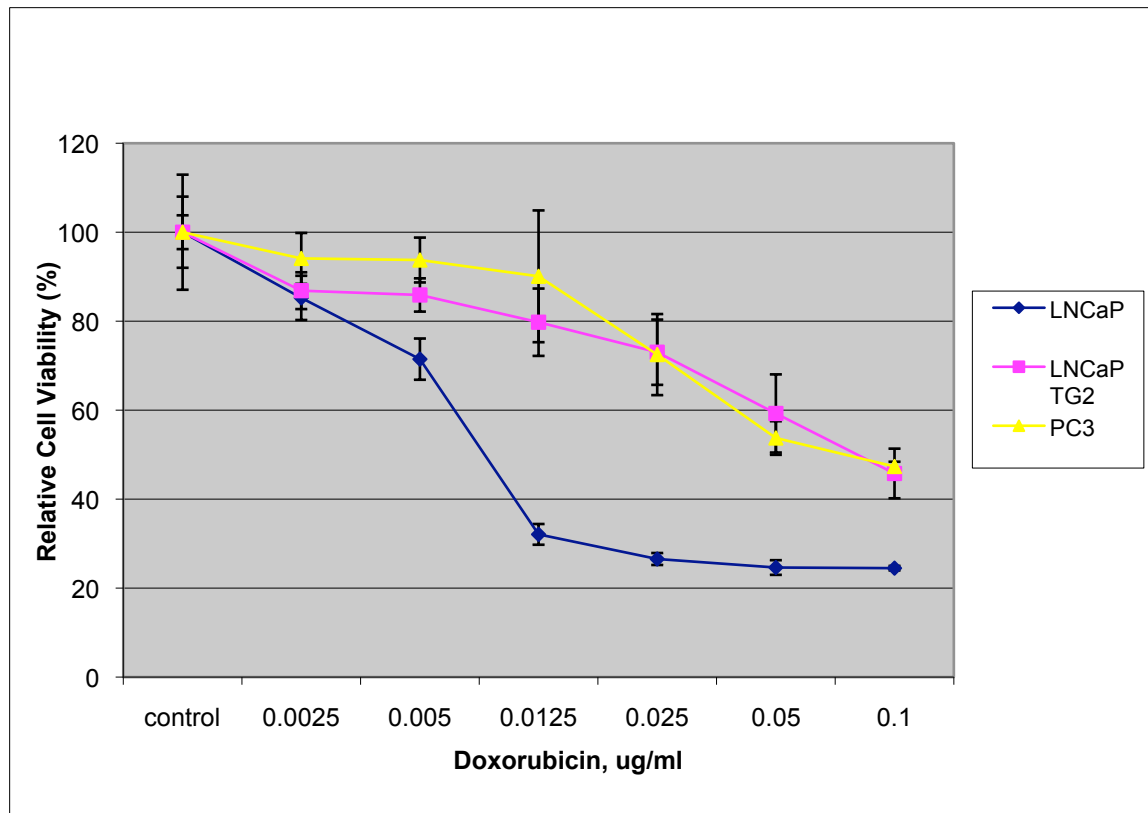


Figure 11. TG2 expression confers drug resistance.

Dose-response was conducted using MTT assay to determine the effect of doxorubicin-induced cell death in the prostate cancer cells. After overnight culture, increasing concentrations of doxorubicin (0-0.1 μ g/ml) were added to quadruplicate wells of 96-well plates containing indicated cells. The cells were incubated with the drug for 3 days and the number of viable cells remaining after the incubation was determined by MTT assay. The untreated cells served as control and baseline for comparison and the relative percent of viable cells is graphed and presented. Results are means of quadruplicate values \pm standard deviation from a representative experiment. Experiments were repeated at least twice with similar results.

Chapter III: Results
(Specific Aim 2)

TG2 Results in the Loss of Androgen Receptor Expression

As discussed in the background data, there was an inverse correlation between TG2 and AR expression: Whereas the LNCaP cells lacked TG2 expression, but had high AR expression, the PC-3 cells contained high basal levels of TG2, but lacked AR expression. Therefore, we were curious to determine the effect of TG2 on AR expression. To our surprise, we found a complete loss of the AR in the LNCaP-TG2 cells (Figure 12A). The loss in AR expression of LNCaP cells was not only at the protein level, but also at the transcript level (Figure 12B). These findings suggested that TG2-mediated silencing of the AR is occurring at the transcriptional level. The loss of AR confirmed that TG2 overexpression could bypass the AR signaling pathway completely allowing the progression of the androgen-dependent to androgen-independent prostate cancer. The loss of AR in TG2 expressing LNCaP cells was further validated by immunofluorescence staining (Figure 12C). Control and vector-infected cells showed significant staining for AR in the cytoplasm and in the nuclei while the TG2-infected cells completely lacked the AR expression.

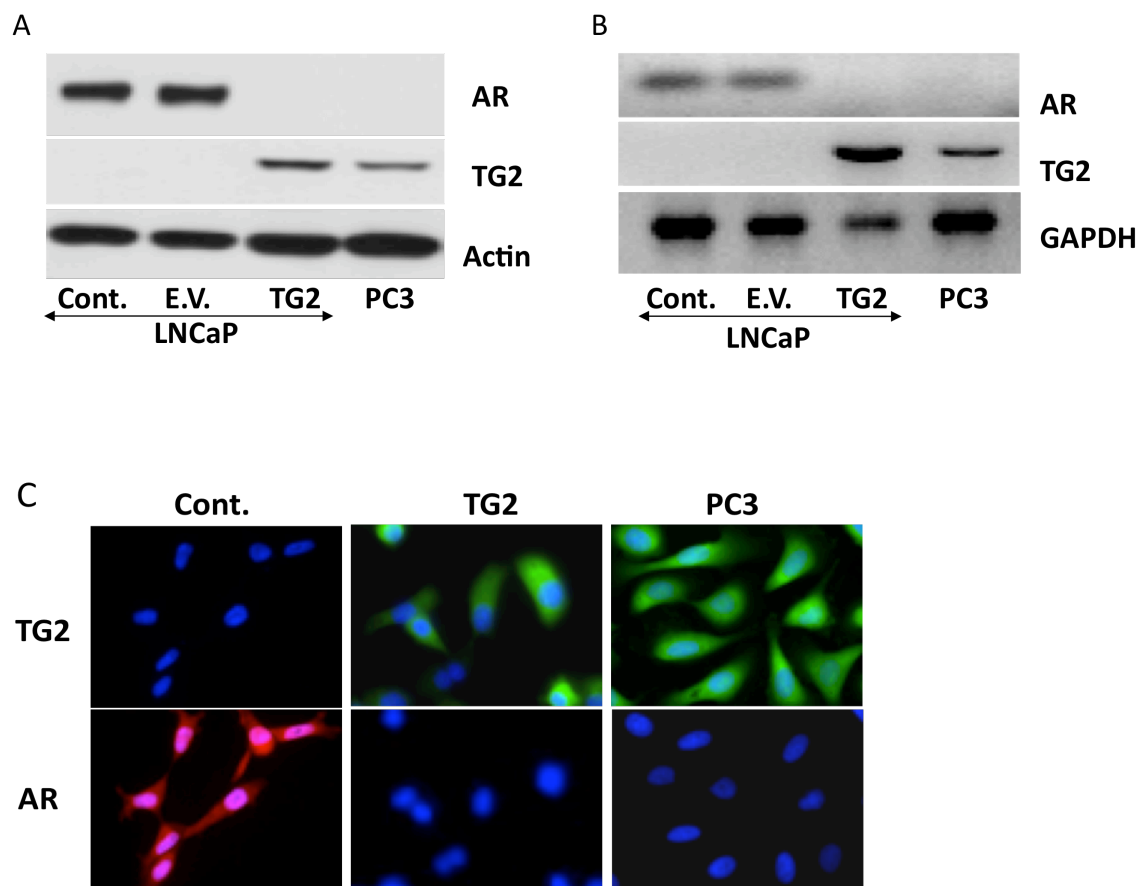


Figure 12. TG2 expression is associated with loss of androgen receptor expression.

A) Immunoblot analysis revealed a complete loss of the androgen receptor in LNCaP cells in response to TG2 expression.

B) RT-PCR results further confirmed the loss of androgen receptor in TG2 expressing LNCaP cells at transcript level.

C) Immunofluorescence staining shows high AR staining in the LNCaP cells, particularly inside the nucleus, which indicates that AR is activated in these cells. However, TG2 expression in the LNCaP cells was associated with lack of AR expression. PC-3 cells, which contain high basal levels of TG2 also lacked AR expression. Cells were fixed with paraformaldehyde and incubated with anti-TG2 and anti-AR antibody. Alexa 488 anti-

mouse IgG (green) or Alexa 546 anti-rabbit IgG (red) were used to detect the antigen-antibody reaction. DAPI was used to stain the nuclei.

TG2 Induces NF- κ B Activation in Prostate Cancer Cells

Since TG2 itself is not a transcription factor, it was unlikely that TG2 could directly silence the AR expression. So we reasoned that TG2 could affect the AR silencing either by modulating the expression of some transcription factors that then silences AR expression or it can do so by associating with some transcription factor to modulate its transcriptional activity. Indeed, TG2 expression has been linked with constitutive activation of NF- κ B, the inflammatory transcription factor that is known to regulate genes involved in EMT, drug resistance, and metastasis. In line with this observation, there have been reports documenting the high NF- κ B activation in androgen-independent cells compared to androgen-dependent prostate cancer as well as in metastatic prostate cancer versus the localized disease. Apart from these reports, NF- κ B consensus sequences have been found in the AR promoter. In fact, Ko et al. [97] recently showed that TNF α -induced activation of NF- κ B could negatively regulate AR expression. Based on these observations, we first determined if NF- κ B is indeed activated in TG2-infected LNCaP cells. Electrophoretic mobility shift assay (EMSA) to determine basal activation levels of NF- κ B revealed high NF- κ B activity in LNCaP-TG2 and in PC-3 cells (Figure 13). The vector-infected cells showed no significant NF- κ B activity. NF- κ B activity in TG2-infected cells was further validated by immunoblotting, using the nuclear and cytoplasmic protein extracts from these cells (Figure 14). While no detectable p65 subunit of NF- κ B could be observed in the nuclear extracts from vector-infected cells, a strong band for p65 was observed in TG2-infected LNCaP and PC-3 cells. The presence of p65 in nuclear extracts indicated that NF- κ B was indeed activated in the TG2 expressing cells whether TG2 expression was induced or endogenous. Immunofluorescence staining further confirmed significant translocation of p65 subunit in the nuclei of LNCaP-TG2 cells compared to the LNCaP-vector cells (Figure 15).

Similarly, PC-3 cells displayed high expression of p65 localization in the nucleus.

Transfection of PC-3 cells with TG2 shRNA to knockdown endogenous TG2 expression showed significant decrease in p65 levels in the nuclei, despite the fact that we were only able to achieve 70% knockdown of TG2 in PC-3 cells.

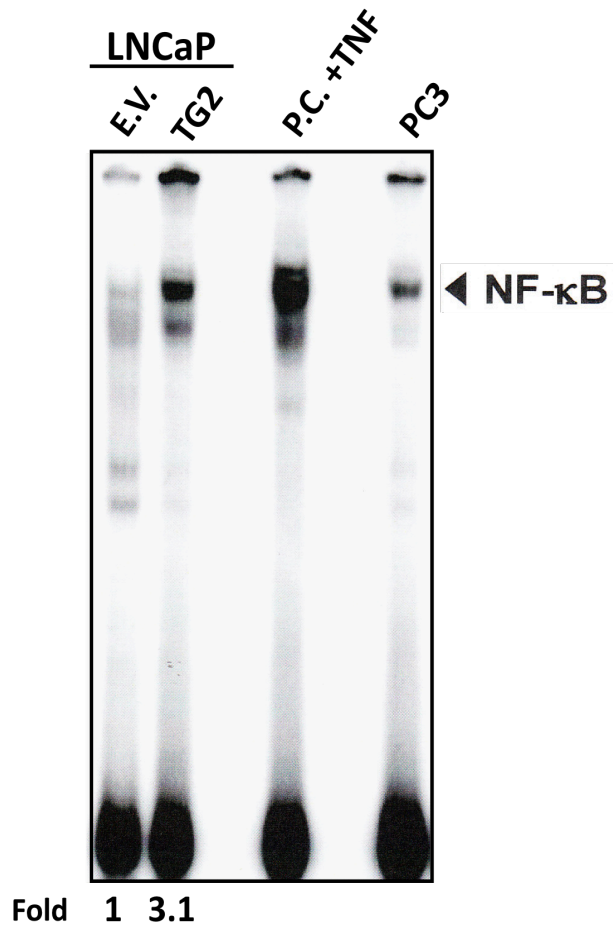


Figure 13. TG2 expression results in constitutive activation of NF-κB in prostate cancer cells.

The control (empty-vector) and TG2-transfected LNCaP cells as well as PC-3 cells (with endogenous expression of TG2) were analyzed for NF-κB activity by EMSA (in collaboration with Dr. Aggarwal's laboratory, MD Anderson Cancer Center, Houston, TX). The nuclear extracts were isolated from the cells and incubated with ³²P end-labeled double stranded NF-κB oligonucleotides. Results show NF-κB activated in the TG2 containing prostate cancer cells, whether transfected with or endogenously expressing TG2. Center lane shows the TNF-α induced NF-κB activation as positive control (P.C.). Data show a 3-fold increase in NF-κB activation in TG2-transfected LNCaP cells.

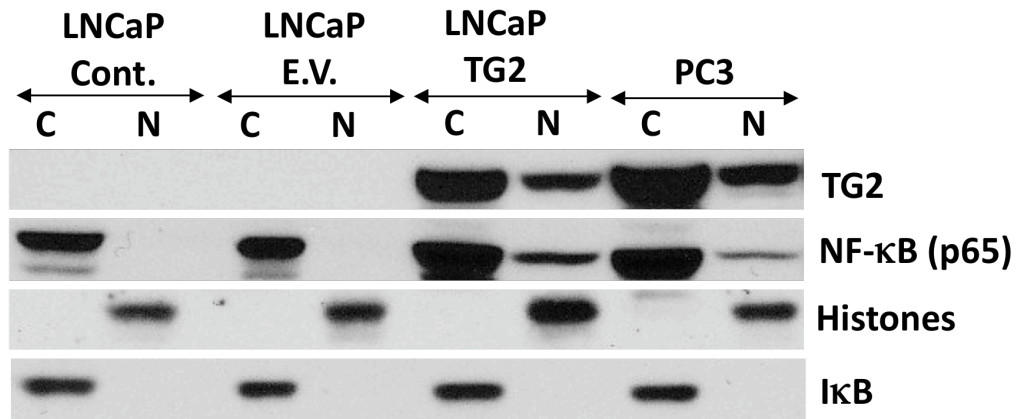


Figure 14. Further validation of NF-κB activation in TG2 expressing cells.

Nuclear and cytoplasmic protein extracts were taken from each of the individual cell lines and subjected to immunoblotting. Since inactive NF-κB is found in the cytoplasm and it is only the activated NF-κB that translocates to the nucleus, we evaluated the nuclear translocation of the p65 subunit of NF-κB in TG2 expressing cells. The results obtained consistently revealed the presence of p65 subunit inside the nucleus but only in TG2 expressing cells. This suggested that NF-κB is indeed activated in TG2 expressing cells. Importantly, TG2 translocation can also be observed inside the nuclear extracts, indicating that TG2 might translocate to the nucleus in complex with NF-κB (since TG2 lacks the nuclear location signal). Histones were used as control for the nuclear extracts, while the inhibitor of NF-κB (IκB) was used as the control for the cytoplasmic extracts.

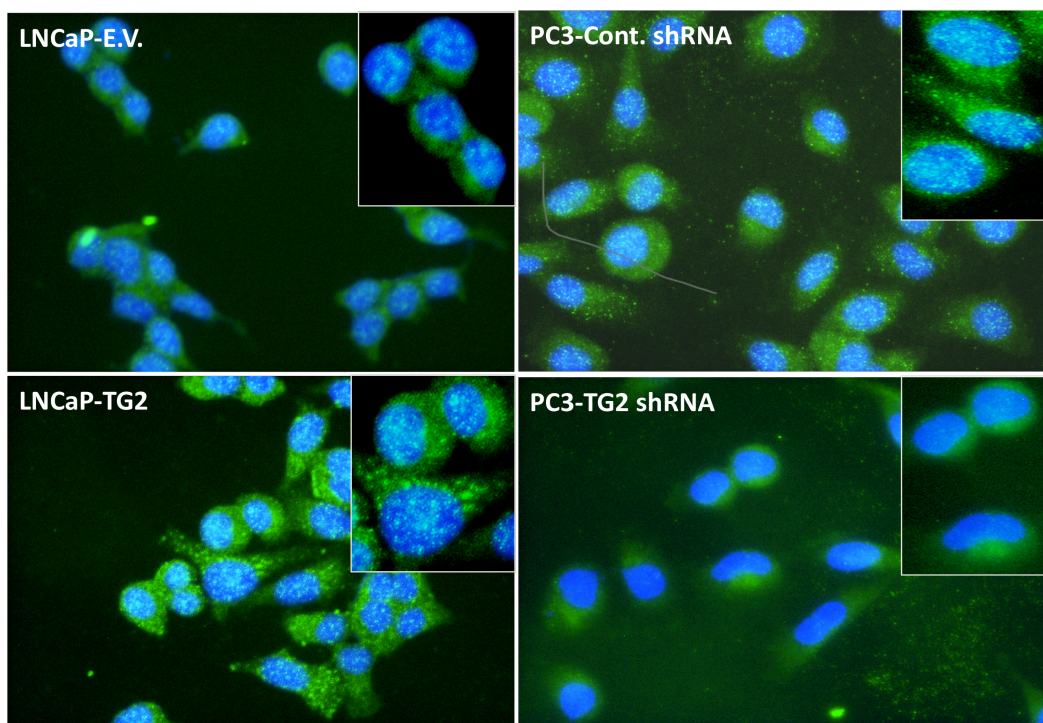


Figure 15. Validation of TG2-induced NF- κ B activation using immunofluorescence assay.

Immunofluorescence staining of p65 subunit of NF- κ B in the LNCaP empty vector, LNCaP-TG2, and PC-3 cells transfected with either control shRNA or TG2-shRNA. Cells were fixed with 3% paraformaldehyde and incubated with anti-p65 antibody. Alexa 488 anti-rabbit IgG (green) was used to detect the bound antibody. DAPI was used to stain the nuclei. The LNCaP empty-vector cells revealed some localization of p65 inside the nucleus, while LNCaP-TG2 cells relatively showed significantly higher p65 in the nucleus. Similarly, PC-3 cells transfected with control shRNA, showed high levels of p65 inside the nucleus while silencing of TG2 with TG2-specific shRNA, resulted in significant reduction in p65 in the nucleus. These results further confirmed that TG2 expression results in constitutive activation of NF- κ B.

Expression of NF- κ B Target Genes in TG2 Expressing Cells

Using quantitative PCR, we conducted a gene array for the NF- κ B target genes in the LNCaP, LNCaP TG2, and PC-3 cells (Figure 16). This gene array would not only further support whether NF- κ B is activated in these cells, but also indicate which specific target genes were affected by the TG2-induced NF- κ B activation; thereby, indicating the possible mechanism in which TG2 might be promoting HRPC. The LNCaP cells containing the empty vector demonstrate that NF- κ B was not activated in these cells, as there is no fold change in the expression in any of the 9 tested target genes. As for the LNCaP TG2 cells and the PC-3 cells, we observed a significant induction of transcript levels, particularly in *Zeb1* and *Zeb2*. There is about a 2500-fold increase in *Zeb1* and 300-fold increase in *Zeb2* transcript levels of TG2-LNCaP and PC-3 cells. These findings are consistent with the RT-PCR results obtained earlier with both the cell lines (Figure 9B). In addition, the LNCaP TG2 cells showed increase in the entire transcript levels of NF- κ B target genes tested. The PC-3 cells shared some similarities with the TG2 overexpressing LNCaP cells, but they still retained some distinctive characteristics. For example, the PC-3 cells displayed a higher transcript level of COX-2 and iNOS compared to the LNCaP TG2 cells. Overall, these results suggested that *Zeb1* and *Zeb2* could possibly play a role in the progression of HRPC in response to TG2-induced NF- κ B activation.

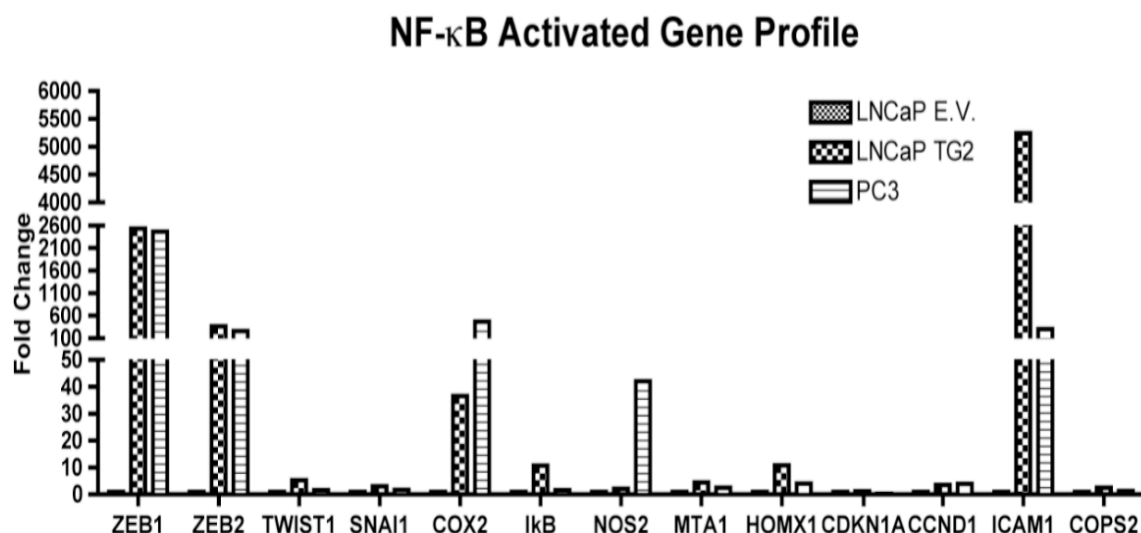


Figure 16. TG2-induced activation of NF- κ B results in constitutive expression of downstream target genes.

Empty vector and TG2-transfected LNCaP and PC-3 cells were analyzed for NF- κ B-induced target genes using quantitative PCR. After RNA extraction, cDNA was synthesized and subjected to a customized gene array kit (SABiosciences) of NF- κ B target gene profiles. SYBR Green/Fluorescein was measured at the end of every annealing/extension cycle of the qPCR samples. The histogram shows fold-increase in indicated NF- κ B target gene transcripts relative to empty vector transfected LNCaP cells. These results further supported the observation that TG2 expression results in constitutive activation of NF- κ B. Notably, both TG2-transfected LNCaP as well as PC-3 cells (with high endogenous TG2 expression), showed 2500-fold and 300-fold increase in *Zeb1* and *Zeb2* transcript levels, respectively. These results support our earlier observations that TG2 expression induces EMT in the prostate cancer cells.

Downregulation of TG2 Reconstitutes AR Expression

If TG2 expression results in the silencing of AR expression, we would expect to see that the downregulation of TG2 would restore AR expression. We used PC-3 cells, which contain high endogenous levels of TG2 and transiently transfected these cells with TG2 siRNA. After 4 days of transfection with TG2 specific siRNA, PC-3 cells were harvested and tested for AR expression using the western blot analysis (Figure 17). Unfortunately, the PC-3 cells following TG2 downregulation by siRNA appeared rather unhealthy, showing signs of autophagy and cell death. In comparison, PC-3 cells transfected with the control siRNA appeared healthy. These results indicated the possibility that PC-3 cells have become addicted to the TG2 regulated signaling for their survival and its knockdown results in spontaneous cell death. Nevertheless, transient downregulation of TG2 in PC-3 cells did reveal partial restoring in AR expression. It is possible that complete restoration of AR expression in these cells would require long-term silencing of TG2.

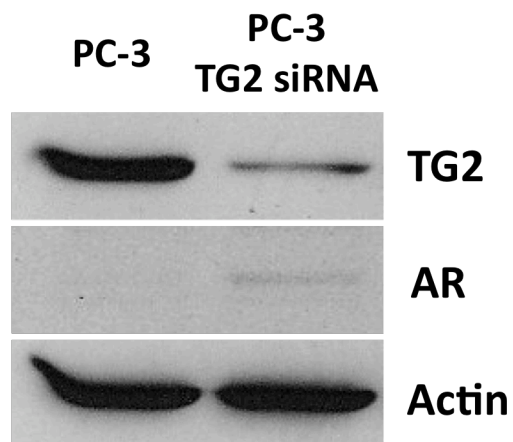


Figure 17. Downregulation of TG2 reconstitutes AR expression.

PC-3 cells were transfected with either control siRNA or TG2 siRNA. After 4 days, cells were harvested and analyzed for TG2 and AR levels by immunoblotting. We were able to achieve about 80% knockdown of TG2 and see partial re-expression of AR upon the silencing of TG2.

Downregulation of NF- κ B also Reconstitutes AR Expression

Because TG2-induced NF- κ B activation is essential for silencing the AR expression, we anticipated that the downregulation of NF- κ B, particularly p65, would reverse the silencing and allow re-expression of AR. To test this contention, we transiently transfected the PC-3 cells with p65 siRNA to see if AR expression would return (Figure 18). We obtained about a 90% knockdown of p65 and noticed that transient knockdown of p65 could partially restore AR expression in PC-3 cells. Unfortunately, since this was only after a period of 4 days, we did not see a complete return of AR expression. However, we do see a faint AR band, suggesting that AR expression could possibly fully return if the silencing of p65 can be maintained over a longer period of time.

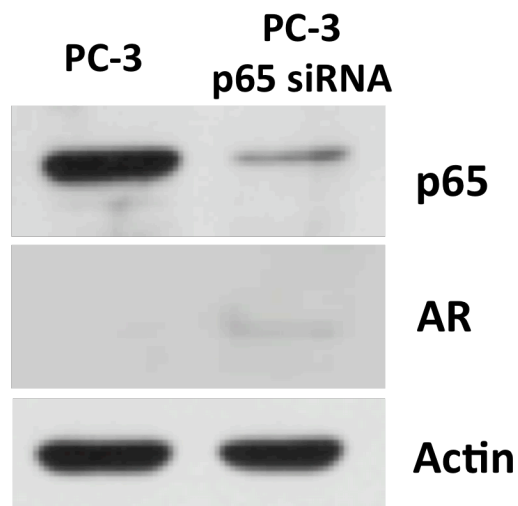


Figure 18. Downregulation of p65 subunit of NF- κ B reconstitutes AR expression.

PC-3 cells were transfected with either control siRNA or p65 siRNA. After 4 days, cells were harvested and analyzed for p65 and AR expression using immunoblotting. We were able to achieve ~90% knockdown of p65 in comparison with the PC-3 control siRNA. p65 siRNA-transfected PC-3 cells showed a partial accumulation in AR expression.

TG2 Induced NF- κ B Directly Binds to the AR Promoter

Based on the evidence presented earlier in this document that TG2 expression induces constitutive activation of NF- κ B and in conjunction with the earlier published report suggesting direct negative regulation of the AR by TNF α -induced NF- κ B, we next wanted to see if the silencing of AR by the TG2-induced NF- κ B was mediated by the similar mechanism. We conducted the ChIP assay to determine direct binding of TG2-induced NF- κ B/p65 to the promoter region of the *AR* gene. Cells extracts after protein/DNA crosslinking, were immunoprecipitated with anti-p65 or control IgG. Immunoprecipitates were tested for the NF- κ B binding consensus sequences in the AR promoter corresponding to -38 to +246 and -760 to -460 regions (Figure 19A). Results shown in Figure 19B reveal that p65 directly binds to the AR promoter, but only in TG2 expressing cells. These results clearly demonstrate that TG2-induced NF- κ B directly binds to the androgen receptor promoter at both consensus sites and thus contributes to the observed TG2-mediated silencing of the AR.

A

AR Promoter (-38 to +246)

GACCCGACTCGCAAACTGTTGC
 ATTTGCTCTCCACCTCCCAGCGC
 CCCCTCCGAGATCCCGGGGAGC
 CAGCTTGCTGGGAGAGCGGGAC
 GGTCCGGAGCAAGCCCAGAGG
 CAGAGGAGGCGACAGAGGGAAA
 AAGGGCCGAGCTAGCCGCTCCA
 GTGCTGTACAGGAGCCGAAGGG
 ACGCACCACGCCAGCCCCAGCC
 CGGCTCCAGCGACAGCCAACGC
 CTCTTGACGCGCGGCGGCTTCG
 AAGCCGCCGCCCGGAGCTGCC
 CTTTCCTCTTCGGTGAAGTTTTT
AAAAGCTGCTAAAGACTCGG

AR Promoter (-760 to -460)

GGGTGATTTTGCCTTTGAG
 AGTCTGGATGAGAAATGCA
 TGGTTAAAGGCAATTCCAGA
 CAGGAAGAAAGGCAGAGAA
 GAGGGTAGAAATGACCTCT
 GATTCTTGGGGCTGAGGGT
 TCCTAGAGCAAATGGCACA
 ATGCCACGAGGCCCGATCT
 ATCCCTATGACGGAATCTAA
 GGTTTCAGCAAGT**TATCTGCT**
GGCTTGGTCATG

B

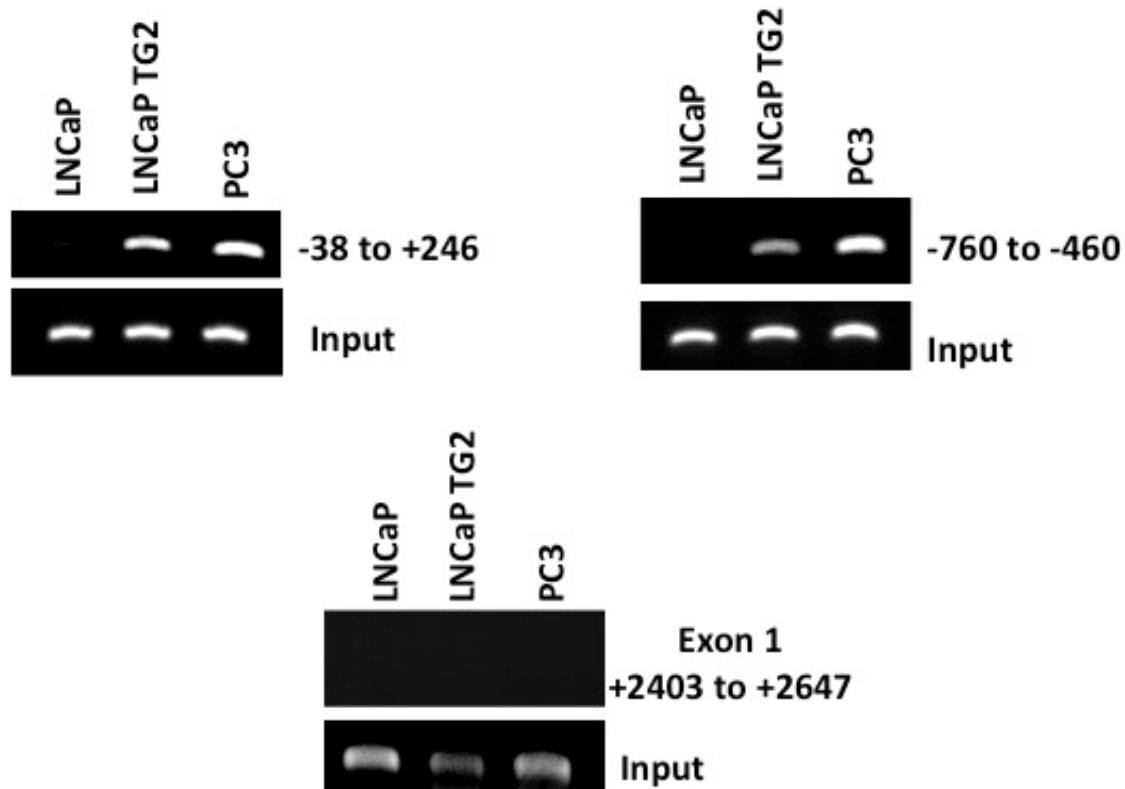


Figure 19. TG2-induced NF- κ B binds to the AR promoter.

A) Schematic representation of the *AR* promoter sequences. Primer sequences used for amplification of p65-bound region in *AR* promoter are shown in bold text. The putative NF- κ B-binding sites in *AR* promoter are underlined.

B) p65-bound region of the *AR* promoter as revealed by ChIP assay. TG2-transfected LNCaP as well as PC-3 cells revealed the binding of TG2-induced NF- κ B at -38 to +246 and -760 to -460 sites in the *AR* promoter. The primers corresponding to the first exon of the *AR* gene served as control and did not amplify the sequence.

TG2 is Recruited to the AR Promoter through NF- κ B Binding

Recently, our lab discovered TG2 and p65 interacting with each other. So, after identifying that p65 directly binds to the AR promoter in the TG2 expressing cells at both NF- κ B consensus sites, we were curious to see if TG2 was possibly bound to NF- κ B, which could then direct it to the AR promoter to induce the silencing. This time we conducted the ChIP assay, immunoprecipitating with anti-TG2 antibody to determine if TG2 was recruited to the promoter region of the *AR* gene as a complex with NF- κ B/p65. The immunoprecipitates were tested using the same primers for the NF- κ B binding consensus sequences in the AR promoter. The results reveal that TG2 is indeed recruited to the AR promoter by directly binding to NF- κ B/p65 (Figure 20). This suggested that the negative regulation of the AR is modulated by TG2's interaction with NF- κ B.

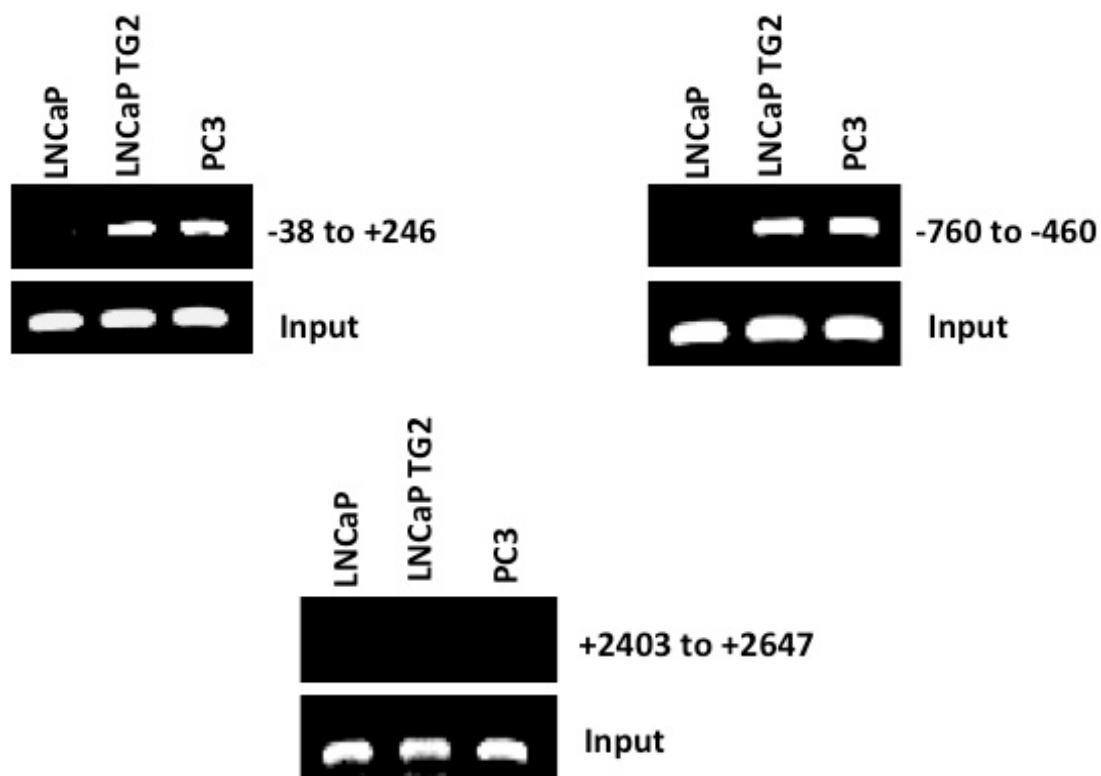
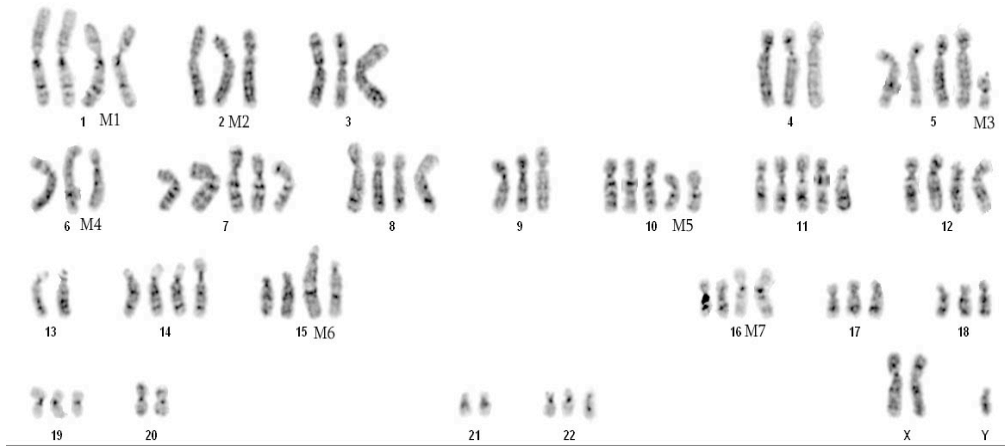


Figure 20. TG2 binds to the *AR* promoter in complex with p65 subunit of NF- κ B.

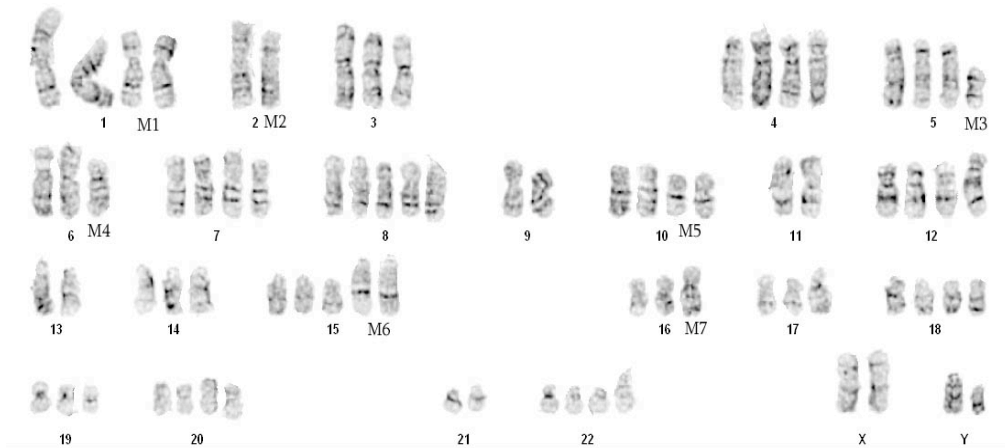
ChIP assay was performed by immunoprecipitating protein-bound DNA complexes with anti-TG2 antibody. The immunoprecipitates were subjected to PCR analysis using primers corresponding to the NF- κ B-binding site in the *AR* promoter. Only the TG2 expressing cells revealed amplification of NF- κ B binding sites. Primers corresponding to exon 1 region in the *AR* gene were used as negative control and failed to amplify any signal.

Chromosomal Analysis of the LNCaP Cell Lines

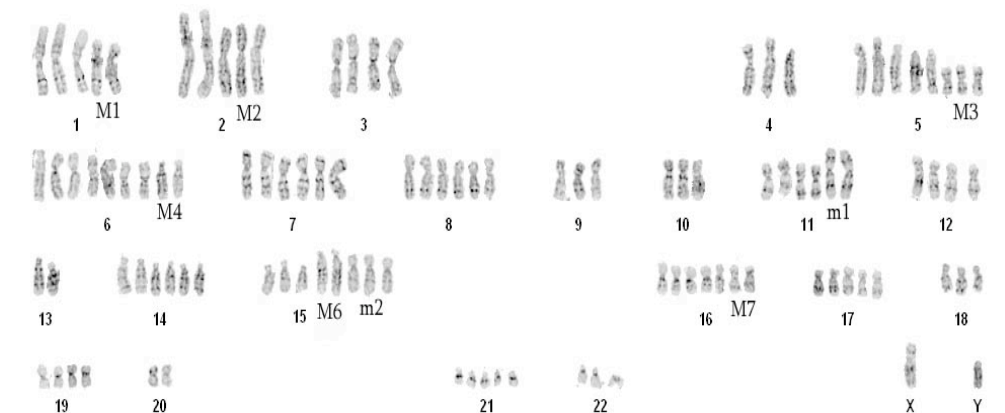
Due to significant differences in the morphology and functional phenotype, vector-infected and TG2-infected LNCaP cells were characterized for authentication by chromosomal banding analysis. The karyotypic analyses for the parental, vector-infected, and TG2-infected cells were performed in collaboration with Drs. Asha Multani and Sen Pathak (MD Anderson Cancer Center, Houston, TX). The chromosomal map for the 3 cell lines revealed that LNCaP-TG2 and vector-infected LNCaP cells were identical to the parental cell lines as suggested by the presence of seven marker chromosomes in the two cell lines. The only marker M5 on chromosome 10 (Figure 21) was missing in the TG2-infected cells. In addition, TG2 expressing LNCaP cells displayed aberrations in chromosome 11 and 15 (m1 and m2) indicating that TG2 expression may also contribute to the genomic instability of cells.



LNCaP



LNCaP E.V.



LNCaP TG2

Figure 21. Karyotypic mapping of LNCaP, LNCaP E.V., and LNCaP TG2 cells.

Chromosome mapping of the 3 LNCaP cell lines. The 7 marker chromosomes (M1-M7), characteristics of the original LNCaP cells, were retained in the LNCaP-empty vector cells. The LNCaP-TG2 cells also retained all marker chromosomes, except M5. In addition, the LNCaP TG2 cells showed some aberrations in their chromosomes, depicted by m1 and m2 label. The tentative identification of the markers are as follows: M1- del(1p), M2- del(2p), M3- iso(5p), M4- del(6p), M5- del(10q), M6-der(15), M7- 16q+, m1- der(11), and m2- der(15).

Chapter IV: Discussion and Future Directions

Specific Aim 1:

Although androgen depletion is an effective strategy for treating human prostate cancer [98], the emergence of hormone refractory prostate cancer (HRPC) cells pose a major challenge [99]. The HRPCs exhibit resistance not only to androgen ablation therapies, but are also more aggressive, highly metastatic, and resistant to conventional therapies, resulting in ultimate death of patients. Overall, prostate cancer is a very heterogeneous disease. Not only does it differ between patients, but even within the same patients [64]. Therefore, it is possible that cancer cells could use several different mechanisms whether initially to begin with or by a multistep progression to become HRPC [39]. Here we investigated the significance of tissue transglutaminase (TG2) expression in HRPC as TG2 has been found to promote invasion [89], metastasis [87, 90], and drug resistance [91] in multiple-cancer types. In our preliminary data, we observed an inverse correlation between TG2 and AR expression. Thus, HRPC cell lines (PC-3 and DU-145), which lack AR expression [100], expressed high basal levels of TG2 (Figure 3). Conversely, the androgen-dependent prostate cancer cell lines, which expressed high basal levels of AR, completely lacked TG2 expression. The C42B prostate cancer cell line with high AR expression and yet independent of androgen for their growth and survival also lacked TG2 expression. These findings suggested that TG2 could play a role in promoting HRPC through a complete bypass of the AR signaling pathway where these cells may utilize alternative pathways for their growth and survival. Therefore, our study design involved creating a stably TG2-transfected LNCaP cells to test the ability of TG2 expression to promote hormone refractoriness, metastasis, and drug resistance. We also used the PC-3 cells in conjunction with the TG2-transfected LNCaP cells to check whether the transfected or endogenous TG2 expression in the prostate cancer cells shared similar characteristics. The LNCaP cell line was originally established from a metastatic lesion of a human prostate

adenocarcinoma [101] and still retains sensitivity to androgen. The PC-3 cell line, on the other hand, was derived from a human prostatic adenocarcinoma metastatic to bone [102]. The PC-3 cells are androgen-independent with high metastatic potential. While LNCaP cells show AR expression, it has been reported that PC-3 prostate cancer line lacks AR expression [103, 104]. Both LNCaP and PC-3 are considered “classical” prostate cancer cell lines and are commonly used in the study of prostate cancer [105]. In our initial effort, we first confirmed the basal status of AR in various prostate cancer cell lines by evaluating the AR expression by immunoblotting. Using the lentiviral particles containing full length ORF for TG2 coding sequence, we established stable LNCaP cells with high TG2 expression by selection against puromycin. We were successful in establishing stable multiple TG2-transfected LNCaP subclones. The LNCaP control as well as the one transfected with the empty vector show absolutely no expression of TG2 (Figure 6A). We found that once transfected with TG2, these cells become independent of androgen for their growth and survival. Thus, neither the absence of androgen agonist nor the presence of antagonist affected their growth in culture (Figure 6B). In addition, the cell proliferation assay revealed that TG2 expression did not affect the overall growth rates of LNCaP cells, suggesting that the observed independence of TG2-expressing LNCaP cells from androgen is related to TG2-regulated mechanism (Figure 7).

Epithelial-to-mesenchymal transition (EMT) is a developmentally regulated process in which epithelial cells lose their epithelial-like characteristics and acquire the mesenchymal phenotype [106]. There are certain molecular factors that are involved in EMT and they serve as biomarkers to indicate this process: Epithelial cells are characterized by their high cell-to-cell contact due to the tight cell adhesion mediated by E-cadherin and β -catenin proteins. Mesenchymal cells show a loss in cell adhesion and instead show expression of fibronectin, vimentin, zinc finger E-box binding homeobox 1

(Zeb1), and zinc finger E-box binding homeobox 2 (Zeb2), mesenchymal markers [107]. While the epithelial cells are drug responsive, mesenchymal cells are drug resistant and display increased cell mobility [108]. Although EMT is a normal physiological process important in embryonic development [106], its reactivation in adults can initiate pathological changes such as tissue fibrosis or increased invasion and metastasis of cancer cells [109]. Immobile cancer epithelial cells in the primary tumors need to transform to the motile mesenchymal cells in order to metastasize; therefore, EMT provides a mechanism that facilitates tumor progression [110]. There has been increasing evidence in support of EMT playing a role in cancer progression as the mesenchymal cells have the mobility and invasive potential to metastasize to distant sites [111]. The downregulation of cell adhesion molecules alters the epithelial homeostasis and promotes the invasiveness of cancer cells [112]. E-cadherin is one of the primary proteins involved in adheren junctions, providing the interaction between neighboring cells [113]. Loss of E-cadherin is one of the distinct features of cells undergoing EMT [112]. In addition, β -catenin expression and localization to the nucleus is an important feature associated with EMT [114]. The presence of β -catenin in the cytoplasm helps retain the epithelial phenotype, whereas the translocation to the nucleus is associated with the loss of E-cadherin expression [115]. These traits promote the susceptibility of cancer cells to undergo EMT and to acquire invasive ability. EMT can arise from the tumor-associated stroma microenvironment [116]. Notably TGF- β , which plays a role in controlling inflammation and tumor cell death [117], is capable of inducing EMT in cells by activating a series of EMT-inducing transcription factors, such as *Zeb1* and *Zeb2* [118, 119]. These transcriptional repressors negatively regulate the expression of E-cadherin [120, 121]. Once these factors are expressed and activated, they work pleiotropically to facilitate the acquisition of a mesenchymal phenotype [107]. As mentioned earlier, TG2 expression has been associated with an increase in

invasiveness [89] and drug resistance [91, 122], both of which are also important characteristics of mesenchymal cells [112]. Based on this, we speculated that TG2 expression may play a role in inducing EMT. In fact, there have been studies in support of EMT being induced by TG2 to promote invasion and metastasis in ovarian [123] as well as breast cancer [124]. Moreover, TGF- β a well-known inducer of the EMT is a potent inducer of TG2 expression [81]. Indeed, inhibition of TG2 by siRNA rendered mammary epithelial cells unresponsive to TGF- β -induced EMT, implying that TG2 expression is an important event in TGF- β -induced EMT in cancer [124]. Moreover, recent findings support that EMT is an important step in the progression of prostate cancer as it assists in the progression to bone metastasis; the major cause of death occurring in more than 90% of prostate cancer patients [125]. As the prostate cancer commonly metastasizes to the bone, these epithelial cancer cells may need to transform into mesenchymal cells to become more motile, invasive, and drug resistant to translocate to the distant site [126]. Several studies have provided evidence of EMT playing a role in HRPC [127, 128, 129, 130, 131], where even the reversal of EMT could suppress the invasive and metastatic potential of prostate cancer cells [132]. All these reports seem to suggest and direct our attention to the potential role TG2 could play in promoting the hormone refractory prostate cancer by modulating EMT. Interestingly, one obvious difference we noticed between the LNCaP and TG2-transfected LNCaP cells was in their morphology (Figure 8). The LNCaP cells displayed high cell-cell adhesion, characteristic of epithelial cells with the cells growing clustered together, while the LNCaP TG2 cells grew separated from each other, revealing the loss of cell adhesion and portraying more of a mesenchymal phenotype. This suggested the possible role of TG2 expression in inducing the EMT in the prostate cancer cells. Earlier studies indeed support such contention and suggested that TG2 expression in ovarian and breast cancer epithelial cells induces the EMT as demonstrated by cadherin switch and

increase in invasiveness of ovarian cancer [123] as well as breast cancer [124]. These papers revealed that TG2-induced EMT is mediated at a transcriptional level through the alteration of epithelial and mesenchymal markers. One way TG2 could induce the EMT is through the increase in transcriptional repressor *Zeb1* expression. Overexpression of TG2 alone was sufficient in inducing the EMT and was associated with increase in invasiveness and resistance to chemotherapeutic drugs [124]. In this study, we found similar effect of TG2 expression in LNCaP cells. Thus, aberrant expression of TG2 in the LNCaP cells was associated with a loss in epithelial markers such as E-cadherin and β -catenin, and gain of mesenchymal markers like fibronectin, vimentin, *zeb1*, and *zeb2*. These molecular trends were seen both at the protein level as determined by immunoblotting (Figure 9A) as well as at the transcript level as determined by RT-PCR (Figure 9B). The PC-3 cells with high endogenous TG2 expression, also demonstrated similar phenotype, showing the lack of E-cadherin expression and high expression of fibronectin (Figure 9). Albeit, PC-3 cells displayed high expression of N-cadherin, which was absent in all the LNCaP cells whether transfected or not with TG2. This suggested that though TG2 transfected LNCaP cells display some parallelism with the PC-3 cells, they still have some subtle differences. In addition, there may be other pathways involved that contribute to the progression of HRPC and it is not solely based on aberrant TG2 expression. TG2-induced EMT in LNCaP cells was not only associated with morphological and molecular changes but also accompanied an increase in their invasiveness and drug resistance. Thus, LNCaP-TG2 cells were more invasive than the control or empty vector infected cells regardless of the presence or absence of androgen (Figure 10). This further validated the observation that LNCaP-TG2 cells are resistant to androgen, as their invasive ability was not influenced by its presence. The drug response assay using increasing concentrations of Doxorubicin (0-0.1 μ g/ml) revealed that TG2 expression could confer drug resistance on LNCaP cells (Figure 11). The TG2-

transfected LNCaP cells demonstrated significant resistance to doxorubicin-induced killing that was comparable to the PC-3 cells. At 0.05 $\mu\text{g/ml}$ of Doxorubicin, both the LNCaP-TG2 cells and PC-3 cells showed 50% cell death compared to the 0.009 $\mu\text{g/ml}$ concentration that caused half of the cells to die in the LNCaP-vector cells. Overall, our data suggested that TG2 expression has the ability to induce EMT as evidenced by the morphological changes, molecular markers, and the increase in invasiveness and drug resistance in the prostate cancer cells and thus may contribute to the HRPC phenotype.

Specific Aim 2:

So far, we have been able to show that TG2 expression has the ability to confer hormone refractoriness and induce EMT in prostate cancer cells and thus, promote HRPC. Next, we determined how TG2 could possibly promote the HRPC phenotype. To address this, first, we checked the status of AR expression in TG2 transfected LNCaP cells. Earlier we established an inverse relationship between TG2 and AR expression. Is TG2 expression associated with the loss of the AR? Intriguingly, we found that the answer was 'yes'. Thus, transfection of TG2 in LNCaP cells was associated with a complete loss in AR expression, both at protein and at the transcript level (Figure 12). These results explained why LNCaP-TG2 cells acquired resistance to androgen. In the absence of AR, these cells are able to bypass the AR signaling and probably rely on some alternative pathway for their growth and survival. Because TG2 is not a transcription factor, it is unlikely that it could directly silence AR expression. On the other hand, numerous studies have reported constitutive activation of NF- κ B in prostate cancer cell lines [133, 134] and in tissue samples [135, 136]. Activated NF- κ B is involved in controlling normal cellular processes, which include immune and inflammatory responses, developmental processes, growth, and survival [137]. NF- κ B is maintained in an inactive state by its inhibitor, I κ B [138]. However, once I κ B is phosphorylated by I κ B

kinase (IKK) [139], it results in the dissociation of the bound inhibitor from NF- κ B, allowing NF- κ B to be activated and translocate to the nucleus where it binds to the DNA and regulates transcription of specific target genes [140, 141]. NF- κ B has been found to be constitutively activated in many types of cancers [142]. In fact, the constitutive activation of NF- κ B has been implicated to play a role in the progression of prostate cancer [143, 144] and induction of EMT [131, 145, 146]. In addition, the overexpression of TG2 has been found to result in constitutive activation of NF- κ B [147, 148]. These evidences suggest that the overexpression of TG2 could result in the constitutive NF- κ B activation, which could then lead to the silencing of AR. There is further support to this contention: An inverse correlation between AR and the constitutive activation of NF- κ B has been reported recently. Specifically, Rajasekhar et al. [149] identified a subset of stem-like human prostate tumor-initiating cells that lacked AR expression, but exhibited increased NF- κ B activity. Most importantly, the AR promoter was found to contain NF- κ B response element. Ko et al. [97] demonstrated that NF- κ B induced by tumor necrosis factor α (TNF α) treatment of prostate cancer cells, resulted in the negative regulation of AR expression due to direct binding to the AR promoter. These studies provided compelling evidence and directed us to pursue the study of TG2-induced NF- κ B activation as the potential mechanism of AR silencing in LNCaP-TG2 cells. First, we determined whether or not NF- κ B is activated in TG2 transfected prostate cancer cells. EMSA (Figure 13), immunoblotting of nuclear and cytosolic protein fractions (Figure 14), and immunofluorescence staining (Figure 15) with anti-NF- κ B p65 antibody all revealed that forced (LNCaP-TG2) or endogenous (PC-3) TG2 expression indeed resulted in constitutive activation of NF- κ B. Similarly, the results from the qPCR of NF- κ B target gene array (Figure 16) further validated the activation of NF- κ B in TG2 expressing cells. Particularly, there was a significant increase in *Zeb1* and *Zeb2*, transcriptional

repressors known to negatively regulate E-cadherin expression and promote EMT [150, 151, 152], which is also consistent with the RT-PCR results seen earlier when studying EMT markers in prostate cancer cells (Figure 9B). Our data corresponds with previous reports of NF- κ B activation in prostate cancer cells. Gasparian AV et al. [133] had observed that the prostate cancer cell lines that lack AR expression such as PC-3 and DU-145 contained high constitutive activation of NF- κ B whereas the LNCaP cells, which are androgen sensitive, contain low NF- κ B activity levels.

If TG2 expression was directly associated with the loss of AR expression, we would expect to see the return of AR when TG2 is knocked down. In order to demonstrate this, we attempted to silence TG2 expression by transient transfection using TG2 siRNA in the PC-3. The cells were harvested four days after transfection. Downregulation of TG2 by siRNA in PC-3 cells resulted in significant cell death and induction of autophagy. The knockdown of TG2 was not complete, but close to around 80%, where we can start to see the return of AR, although the expression is very faint (Figure 17). These results suggested that PC-3 cells become dependent on TG2-regulated pathways (oncogenic addiction) for their growth and survival and inhibition of TG2 results in their spontaneous death. Nevertheless, the partial return of AR in TG2 inhibited cells do suggest that TG2 expression is indeed an important player in silencing the AR expression. Likewise, if TG2-induced NF- κ B was the contributing factor in silencing AR expression, we would expect to see the return of AR expression when NF- κ B is inhibited. Thus, we attempted to transiently transfect PC-3 cells with p65 siRNA. After a period of 4 days, we were able to obtain 90% knockdown of p65 levels and do see the partial return of the AR expression (Figure 18). But as mentioned before, complete reversal of the AR may require long-term inhibition of TG2 or its downstream NF- κ B activation. Attempts to establish a stable knockdown of TG2 or NF- κ B p65 in the

PC-3 cells using TG2 shRNA or NF- κ B p65 shRNA lentiviral particles were unsuccessful. Nevertheless, transient inhibition of TG2 or NF- κ B do support the notion that loss of AR expression in TG2 expressing cells is related to TG2-induced NF- κ B activation.

Next, we determined if NF- κ B actually binds to the NF- κ B consensus sequences of the AR promoter as reported earlier by Ko et al. [97] Using the ChIP assay, we were able to establish that TG2-induced NF- κ B does directly bind to the AR promoter at both the NF- κ B consensus binding sites, -36 to +246 and -760 to -460 (Figure 19). Although we were unable to convincingly show that TG2 and NF- κ B expression directly results in the silencing of AR from our knockdown experiments, Ko S et al. [97] were able to demonstrate a decrease in AR expression from transcriptional repression due to TNF α -induced NF- κ B as a consequence of direct binding to the AR promoter. This was illustrated in the LNCaP cell line where NF- κ B activation was induced by TNF α . As previously mentioned, TG2 is a pro-inflammatory protein; therefore, cytokines such as TNF α that are secreted during tissue injury or wound healing are able to induce TG2 gene expression [82]. Recently, our lab has established that TG2 forms a stable complex with the p65 subunit of NF- κ B. This raised the possibility of TG2 interaction with NF- κ B to potentially direct NF- κ B to translocate to the nucleus where it can directly bind to the AR promoter leading to the silencing of AR expression. Therefore, we proceeded with another ChIP assay, this time immunoprecipitating with anti-TG2 antibody. Interestingly, we found that immunoprecipitation with TG2 also resulted in the amplification of both NF- κ B consensus sites (Figure 20), indicating that TG2 is recruited to the AR promoter in complex with NF- κ B/p65. This suggests that TG2 constitutively activates NF- κ B by directly binding to it, leading to its translocation to the nucleus and binding to the AR promoter to negatively regulate AR expression. Further studies are

needed to determine whether both NF- κ B consensus sites on the AR promoter are essential for silencing the AR expression or one site being particularly dominant over the other. Also, identification of other factors recruited by TG2 and p65 complex to the promoter of the AR and their significance in silencing its expression is of interest. In addition, the exploration of histone modifications responsible for the silencing of AR would be an interesting aspect to study in order to discover how the TG2/NF- κ B complex may be modulating these effects. In conclusion, this work defined a novel function for TG2 and demonstrates, for the first time, the significance of TG2 in promoting hormone refractory phenotype in prostate cancer cells. The TG2-induced EMT conferred resistance not only to androgen depletion by silencing the AR expression but also conferred resistance to other cytotoxic drug like Doxorubicin and promoted invasiveness, important traits of HRPC (Figure 22). In view of these findings, further studies to validate the potential of TG2 as a novel therapeutic target for treating certain tumor/patient subpopulations in which TG2 expression is aberrantly upregulated is strongly warranted.

Conclusions and Future Directions

Studies reported in this manuscript provide compelling evidence supporting that TG2 expression in prostate cancer is sufficient to confer hormone refractoriness. The data obtained documented that exogenous expression of TG2 in androgen-sensitive LNCaP prostate cancer cells could render these cells independent of androgen for their growth and survival. Notably, TG2 expression, whether induced or endogenous, induced the EMT in these cells and thus contributed to their increased invasiveness and drug resistance, the important traits of hormone refractory prostate cancer cells. These results suggest a novel mechanism for androgen independence by prostate cancer cells and thus warrant future studies to determine the expression of TG2 in patient samples to establish clinical relevance of these findings. Indeed, a recent study by Shah RB et al. do support such contention and suggested that a significant number of tumor samples from advanced stage prostate patients lacked the AR expression [64].

In addition, our studies demonstrated that TG2's ability to promote androgen-independent growth and survival is due to the complete bypass of the AR signaling pathway. TG2 expression in prostate cancer cells was associated with a complete silencing of the AR expression. The data obtained revealed that this silencing of AR was mainly due to constitutive activation of the transcription factor, NF- κ B. TG2 in complex with the p65 subunit of NF- κ B was found to directly bind to the two NF- κ B binding sites in the AR promoter, which resulted in the silencing of its expression. However, it remains to be determined which of the two NF- κ B binding sites in the AR promoter is critical for TG2/NF- κ B mediated silencing of the AR expression. Similarly, future studies to determine the nature of co-repressors that are selectively recruited in response to TG2/NF- κ B binding of the AR promoter will be of interest. Previous studies have suggested a strong link between NF- κ B activation and progression of HRPC [142, 144]

and EMT [131, 145, 146] in prostate cancer cells. However, mechanisms responsible for constitutive activation of NF- κ B in HRPC cells remain largely unknown. Our data fills this important gap and suggested that aberrant expression of TG2 in prostate cancer cells results in constitutive activation of NF- κ B due to its interaction with the p65 subunit and thus, preventing its binding to the inhibitory protein I κ B α .

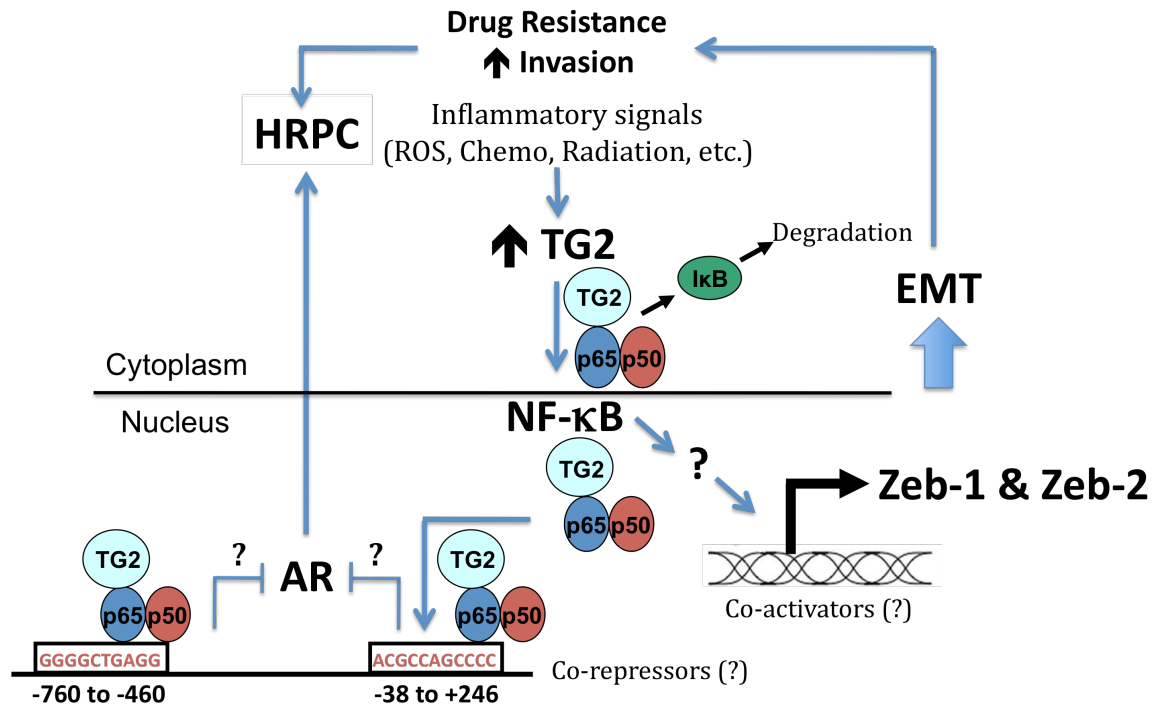


Figure 22. TG2-regulated pathways during progression of hormone-refractory prostate cancer.

Inflammatory signals can lead to the aberrant expression of TG2, which results in the constitutive activation of NF-κB due to its binding to the p65 subunit of NF-κB. This permits the translocation of TG2 to the nucleus in complex with p65. In the nucleus, TG2/p65 complex binds to the AR promoter and results in its silencing through recruitment of co-repressors. In addition, TG2-induced NF-κB could induce the expression of transcriptional repressors *Zeb1* and *Zeb2* by regulating the expression of other transcription factors such as HIF-1 α and promote the EMT state in prostate cancer epithelial cells. EMT is known to confer drug resistance and invasiveness; hence, TG2 expression may allow prostate cancer cells to bypass AR signaling for their growth and survival and induce EMT to confer drug resistance and invasiveness.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; 60:277-300.
2. Abate-Shen C and Shen MM. Molecular genetics of prostate cancer. *Genes Dev* 2000; 14: 2410-2434.
3. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev* 1995; 16:271-321.
4. Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris C, Klaassen P, van der Korput JA, Voorhorst MM, van Laar JH, Mulder E, et al. The human androgen receptor: domain structure, genomic organization and regulation of expression. *J Steroid Biochem* 1989; 34:307-10.
5. Thornton JW, Kelley DB. Evolution of the androgen receptor: structure-function implications. *Bioessays* 1998; 20:860-9.
6. Kang HY, Tsai MY, Chang C, Huang KE. Mechanisms and clinical relevance of androgens and androgen receptor actions. *Chang Gung Med J* 2003; 26:388-402.
7. Gelmann EP. Molecular Biology of the Androgen Receptor. *J Clin Oncol* 2002; 20:3001-15.
8. Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proc Natl Acad Sci USA* 1994; 91:1234-8.
9. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J Biol Chem* 1991; 266:510-8.

10. Brodie J, McEwan IJ. Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. *J Mol Endocrinol* 2005; 34:603-15.
11. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. *Int J Cancer* 2007; 120:719-33.
12. Schoenmakers E, Alen P, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, Claessens F. Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. *Biochem J* 1999; 341:515-21.
13. Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson JA, Kaptein R. Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* 1990; 249:157-60.
14. Shaffer PL, Jivan A, Dollins DE, Claessens F, Gewirth DT. Structural basis of androgen receptor binding to selective androgen response elements. *Proc Natl Acad Sci USA* 2004; 101:4758-63.
15. Jenster G, van der Korput HA, Trapman J, Brinkmann AO. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *J Biol Chem* 1995; 270:7341-6.
16. Jenster G, van der Korput JA, Trapman J, Brinkmann AO. Functional domains of the human androgen receptor. *J Steroid Biochem Mol Biol* 1992; 41:671-5.
17. Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH₂-terminal and carboxyl-terminal sequences. *J Biol Chem* 1994;269:13115-23.

18. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlage RE, Shabanowitz J, Hunt DF, Weber MJ. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J Biol Chem* 2002; 277:29304-14.
19. Zhou ZX, Kempainen JA, Wilson EM. Identification of three proline-directed phosphorylation sites in the human androgen receptor. *Mol Endocrinol* 1995; 9:605-15.
20. Fu M, Rao M, Wang C, Sakamaki T, Wang J, Di Vizio D, Zhang X, Albanese C, Balk S, Chang C, Fan S, Rosen E, Palvimo JJ, Jänne OA, Muratoglu S, Avantiaggiati ML, Pestell RG. Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. *Mol Cell Biol* 2003; 23:8563-75.
21. Tanner T, Claessens F, Haelens A. The hinge region of the androgen receptor plays a role in proteasome-mediated transcriptional activation. *Ann N Y Acad Sci* 2004; 1030:587-92.
22. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Bäsler S, Schäfer M, Egner U, Carrondo MA. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J Biol Chem* 2000; 275:26164-71.
23. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR Jr, Weinmann R, Einspahr HM. Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. *Proc Natl Acad Sci USA* 2001; 98:4904-9.

24. Sullivan WP, Vroman BT, Bauer VJ, Puri RK, Riehl RM, Pearson GR, Toft DO. Isolation of steroid receptor binding protein from chicken oviduct and production of monoclonal antibodies. *Biochemistry* 1985; 24:4214-22.
25. Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, Trapman J. Mechanisms of androgen receptor activation and function. *J Steroid Biochem Mol Biol* 1999; 69:307-13.
26. Cutress ML, Whitaker HC, Mills IG, Stewart M, Neal DE. Structural basis for the nuclear import of the human androgen receptor. *J Cell Sci* 2008; 121: 957-68.
27. Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B. The program of androgen-responsive genes in neoplastic prostate epithelium. *Proc Natl Acad Sci U S A* 2002; 99:11890-5.
28. Khorasanizadeh S, Rastinejad F. Nuclear-receptor interactions on DNA-response elements. *Trends Biochem Sci* 2001; 26:384-90.
29. Luke MC, Coffey DS. Human androgen receptor binding to the androgen response element of prostate specific antigen. *J Androl* 1994; 15:41-51.
30. Wu Y, Zhao W, Zhao J, Pan J, Wu Q, Zhang Y, Bauman WA, Cardozo CP. Identification of androgen response elements in the insulin-like growth factor I upstream promoter. *Endocrinology* 2007; 148:2984-93.
31. Eisenberger MA, Blumenstein BA, Crawford ED, Miller G, McLeod DG, Loehrer PJ, Wilding G, Sears K, Culkin DJ, Thompson IM Jr, Bueschen AJ, Lowe BA. Bilateral orchiectomy with or without flutamide for metastatic prostate cancer. *N Engl J Med* 1998; 339:10366-42.
32. Huggins C, Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol* 2002; 167: 948–51.

33. Labrie F, Dupont A, Belanger A, Cusan L, Lacourciere Y, Monfette G, Laberge JG, Emond JP, Fazekas AT, Raynaud JP, Husson JM. New hormonal therapy in prostatic carcinoma: combined treatment with an LHRH agonist and an antiandrogen. *Clin Invest Med* 1982; 5:267-75.
34. Engel JB, Schally AV. Drug Insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone. *Nat Clin Pract Endocrinol Metab* 2007; 3:157-67.
35. Schally AV, Kastin AJ, Arimura A. Hypothalamic follicle-stimulating hormone (FSH) and luteinizing hormone (LH)-regulating hormone: structure, physiology, and clinical studies. *Fertil Steril* 1971; 22:703-21.
36. Culig Z, Bartsch G, Hobisch A. Antiandrogens in prostate cancer endocrine therapy. *Curr Cancer Drug Targets* 2004; 4:455-61.
37. Powell SM, Brooke GN, Whitaker HC, Reebye V, Gamble SC, Chotai D, Dart DA, Belandia B, Bevan CL. Mechanisms of androgen receptor repression in prostate cancer. *Biochem Soc Trans* 2006; 34:1124-7.
38. Lamont KR, Tindall DJ. Minireview: alternative activation pathways for the androgen receptor in prostate cancer. *Mol Endocrinol* 2011; 25:897-907.
39. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer* 2001; 1:34-45.
40. Hakimi JM, Rondonelli RH, Schoenberg MP, Barrack ER. Androgen-receptor gene structure and function in prostate cancer. *World J Urol* 1996; 329-37.
41. Culig Z, Hobisch A, Cronauer MV, Cato AC, Hittmair A, Radmayr C, Eberle J, Bartsch G, Klocker H. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol Endocrinol* 1993; 7:1541-50.

42. Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM, Feldman D. Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat Med* 2000; 6:703-6.
43. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res* 1999; 59:2511-5.
44. Mao HL, Zhu ZQ, Chen CD. The androgen receptor in hormone-refractory prostate cancer. *Asian J Androl* 2009; 11:69-73.
45. Veldscholte J, Berrevoets C, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to anti-androgens. *J Steroid Biochem Mol Bio* 1992; 41:665-9.
46. Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J, Stadler W, Hayes DF, Kantoff PW, Vogelzang NJ, Small EJ. Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. *J Clin Oncol* 2003; 21:2673-8.
47. Yoshida T, Kinoshita H, Segawa T, Nakamura E, Inoue T, Shimizu Y, Kamoto T, Ogawa O. Antiandrogen bicalutamide promotes tumor growth in a novel androgen-dependent prostate cancer xenograft model derived from a bicalutamide-treated patient. *Cancer Res* 2005; 65:9611-6.
48. Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, Miyamoto M. Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. *Cancer Res* 2003; 63:149-53.
49. Yuan X, Balk SP. Mechanisms mediating androgen receptor reactivation after castration. *Urol Oncol* 2009; 27:36-41.

50. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet* 1995; 9:401-6.
51. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res* 1997; 57:314-9.
52. Haapala K, Kuukasjärvi T, Hyytinen E, Rantala I, Helin HJ, Koivisto PA. Androgen receptor amplification is associated with increased cell proliferation in prostate cancer. *Hum Pathol* 2007; 38:474-8.
53. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004; 10:33-9.
54. Gregory CW, Johnson RT Jr, Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res* 2001; 61:2892-8.
55. Wen Y, Hu MC, Making K, Spohn B, Bartholomeusz G, Yan DH, Hung MC. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* 2000; 60:6841-5.
56. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med* 1999; 5:280-5.
57. Mellon K, Thompson S, Charlton RG, Marsh C, Robinson M, Lane DP, Harris AL, Horne CH, Neal DE. p53, c-erbB-2 and the epidermal growth factor receptor in the benign and malignant prostate. *J Urol* 1992; 147:496-9.

58. Kuhn EJ, Kurnot RA, Sesterhenn IA, Chang EH, Moul JW. Expression of the c-erbB-2 (HER-2/neu) oncoprotein in human prostatic carcinoma. *J Urol* 1993; 150:1427-33.
59. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators' a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci U S A* 1999; 96:5458-63.
60. Gregory CW, Fei X, Ponguta LA, He B, Bill HM, French FS, Wilson EM. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. *J Biol Chem* 2004; 279:7119-30.
61. Shu SK, Liu Q, Coppola D, Cheng JQ. Phosphorylation and activation of androgen receptor by Aurora-A. *J Biol Chem* 2010; 285:33045-53.
62. Buschhorn HM, Klein RR, Chambers SM, Hardy MC, Green S, Bearss D, Nagle RB. Aurora-A overexpression in high-grade PIN lesions and prostate cancer. *Prostate* 2005; 64:341-6.
63. van der Kwast TH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CC, Mulder E, Boersma W, Trapman J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int J Cancer* 1991; 48:189-93.
64. Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M, Macvicar GR, Varambally S, Harwood J, Bismar TA, Kim R, Rubin MA, Pienta KJ. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* 2004; 64:9209-16.
65. Isaacs JT. The biology of hormone refractory prostate cancer. Why does it develop? *Urol Clin North Am* 1999; 26:263-73.

66. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* 1996; 28:251-265.
67. Bui M, Reiter RE. Stem cell genes in androgen-independent prostate cancer. *Cancer Metastasis Rev* 1998; 14:391-399.
68. Craft N, Chhor C, Tran C, Beldegrun A, DeKernion J, Witte ON, Said J, Reiter RE, Sawyers CL. Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res* 1999; 59:5030-6.
69. Mehta K. Mammalian transglutaminases: a family portrait. *Prog Exp Tumor Res* 2005; 38:1-18.
70. Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* 2003; 4:140-56.
71. Nakaoka H, Perez DM, Baek KJ, Das T, Husain A, Misono K, Im MJ, Graham RM. Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* 1994; 264:1593-6.
72. Hasegawa G, Suwa M, Ichikawa Y, Ohtsuka T, Kumagai S, Kikuchi M, Sato Y, Saito Y. A novel function of tissue-type transglutaminase: protein disulphide isomerase. *Biochem J* 2003; 373: 793-803.
73. Mishra S, Murphy LJ. Tissue transglutaminase has intrinsic kinase activity; identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. *J Biol Chem* 2004; 279:23863-8.
74. Akimov SS, Belkin AM. Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin. *Blood* 2001; 98:1567-76.

75. Akimov SS, Krylov D, Fleischman LF, Belkin AM. Tissue transglutaminase is an intergrin-binding adhesion coreceptor for fibronectin. *J Cell Biol* 2000; 148:825-38.
76. Lesort M, Attanavanich K, Zhang J, Johnson GV. Distinct nuclear localization and activity of tissue transglutaminase. *J Biol Chem* 1998; 273:11991-4.
77. Mehta K, Fok JY, Mangala LS. Tissue transglutaminase: from biological glue to cell survival cues. *Front Biosci* 2006; 11:173-85.
78. Chhabra A, Verma A, Mehta K. Tissue transglutaminase promotes or suppresses tumors depending on cell context. *Anticancer Res* 2009; 29:1909-19.
79. Verderio EA, Johnson TS, Griffin M. Transglutaminases in wound healing and inflammation. *Prog Exp Tumor Res* 2005; 38:89-114.
80. Verderio EA, Johnson TS, Griffin M. Tissue transglutaminase in normal and abnormal wound healing: review article. *Amino Acids* 2004; 26:387-404.
81. Quan G, Choi JY, Lee DS, Lee SC. TGF-beta1 upregulates transglutaminase 2 and fibronectin in dermal fibroblasts: a possible mechanism for the stabilization of tissue inflammation. *Arch Dermatol Res* 2005; 297:84-90.
82. Kuncio GS, Tsyganskaya M, Zhu J, Liu SL, Nagy L, Thomazy V, Davies PJ, Zern MA. TNF-alpha modulates expression of the tissue transglutaminase gene in liver cells. *Am J Physiol* 1998; 274:G240-5.
83. Haroon ZA, Hettasch JM, Lai TS, Dewhirst MW, Greenberg CS. Tissue transglutaminase is expressed, active, and directly involved in rat dermal wound healing and angiogenesis. *FASEB J* 1999; 13:1787-95.
84. Nardacci R, Lo Iacono O, Ciccocanti F, Falasca L, Addesso M, Amendola A, Antonucci G, Craxi A, Fimia GM, Iadevaia V, Melino G, Ruco L, Tocci G, Ippolito G, Piacentini M. Transglutaminase type II plays a protective role in hepatic injury. *Am J Pathol* 2003; 162:1293-303.

85. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; 140:883-99.
86. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; 454:436-44.
87. Mehta K, Fok J, Miller FR, Koul D, Sahin AA. Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. *Clin Cancer Res* 2004; 10:8068-76.
88. Hwang JY, Mangala LS, Fok JY, Lin YG, Merritt WM, Spannuth WA, Nick AM, Fitterman DJ, Vivas-Mejia PE, Deavers MT, Coleman RL, Lopez-Berestein G, Mehta K, Sood AK. Clinical and biological significance of tissue transglutaminase in ovarian carcinoma. *Cancer Res* 2008; 68:5849-58.
89. Mangala LS, Fok JY, Zorrilla-Calancha IR, Verma A, Mehta K. Tissue transglutaminase expression promotes cell attachment, invasion and survival in breast cancer cells. *Oncogene* 2007; 26:2459-70.
90. Verma A, Wang H, Manavathi B, Fok JY, Mann AP, Kumar R, Mehta K. Increased expression of tissue transglutaminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis. *Cancer Res* 2006; 5:1493-503.
91. Verma A, Mehta K. Tissue transglutaminase-mediated chemoresistance in cancer cells. *Drug Resist Updat* 2007; 10:144-51.
92. Kim DS, Park SS, Nam BH, Kim IH, Kim SY. Reversal of drug resistance in breast cancer cells by transglutaminase 2 inhibition and nuclear factor-kappaB inactivation. *Cancer Res* 2006; 66:10936-43.
93. Cao L, Petrusca DN, Satpathy M, Nakashatri H, Petrache I, Matei D. Tissue transglutaminase protects epithelial ovarian cancer cells from cisplatin-induced

- apoptosis by promoting cell survival signaling. *Carcinogenesis* 2008; 29:1893-900.
94. Park KS, Kim HK, Lee JH, Choi YB, Park SY, Yang SH, Kim SY, Hong KM. Transglutaminase 2 as a cisplatin resistance marker in non-small cell lung cancer. *J Cancer Res Clin Oncol* 2010; 136:493-502.
 95. Verma A, Guha S, Diagaradjane P, Kunnumakkara AB, Sanguino AM, Lopez-Berestein G, Sood AK, Aggarwal BB, Krishnan S, Gelovani JG, Mehta K. Therapeutic significance of elevated tissue transglutaminase expression in pancreatic cancer. *Clin Cancer Res* 2008; 14:2476-83.
 96. Mehta K, Kumar A, Kim HI. Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer. *Biochem Pharmacol* 2010; 80:1921-9.
 97. Ko S, Shi L, Kim S, Song CS, Chatterjee B. Interplay of nuclear-factor-kappaB and B-myb in the negative regulation of androgen receptor expression by tumor necrosis factor alpha. *Mol Endocrinol* 2008; 22: 273-86.
 98. Huggins C. Endocrine-induced regression of cancers. *Cancer Res* 1967; 27:1925-30.
 99. Daneshgari F, Crawford ED. Endocrine therapy of advanced carcinoma of the prostate. *Cancer* 1993; 71:1089-97.
 100. Tilley WD, Wilson CM, Marcelli M, McPhaul MJ. Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res* 1990; 50:5382-5386.
 101. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP. LNCaP model of human prostatic carcinoma. *Cancer Res* 1983; 43: 1809-18.

102. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 1979; 17:16-23.
103. Mitchell S, Abel P, Ware M, Stamp G, and Lalani, E.N. Phenotypic and genotypic characterization of commonly used human prostatic cell lines. *BJU Int* 2000; 85: 932-944.
104. Chlenski A, Nakashiro K, Ketels KV, Korovaitseva GI and Oyasu R. Androgen receptor expression in androgen-independent prostate cancer cell lines. *Prostate* 2001; 47: 66-75.
105. van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, Nordeen SK, Miller GJ, and Lucia, MS. Molecular characterization of human prostate carcinoma cell lines. *The Prostate* 2003; 57: 205–225.
106. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; 139:871-90.
107. Kalluri R, Weinberg R. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; 119:1420-8.
108. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* 2010; 29:4741-51.
109. López-Novoa JM, Nieto MA. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. *EMBO Mol Med* 2009; 1:303-14.
110. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* 2009; 119:1417-9.
111. Polyak K, Weinberg R. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009; 9:265-273.
112. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; 2:442-54.

113. Jeanes A, Gottardi CJ, Yap AS. Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene* 2008; 27:6930-9.
114. Kim K, Lu Z, Hay ED. Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biol Int* 2002;26:463-76.
115. Stockinger A, Eger A, Wolf J, Beug H, Folsner R. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. *J Cell Biol* 2001; 154:1185-96.
116. Tse JC, Kalluri R. Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. *J Cell Biochem* 2007; 101:816-29.
117. Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immuno Lett* 2002; 82:85-91.
118. Miyazono K. Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; 85:314-23.
119. Shirakihara T, Saitoh M, Miyazono K. Differential regulation of epithelial and mesenchymal markers by δ EF1 proteins in epithelial mesenchymal transition induced by TGF- β . *Mol Biol Cell* 2007; 18:3533-44.
120. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001; 7:1267-1278.
121. Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, Berx G, Cano A, Beug H, Foisner R. δ EF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* 2005; 24:2375-2385.

122. Antonyak MA, Miller AM, Jansen JM, Boehm JE, Balkman CE, Wakshlag JJ, Page RL, Cerione RA. Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. *J Biol Chem* 2004; 279:41461-7.
123. Shao M, Cao L, Shen C, Satpathy M, Chelladurai B, Bigsby RM, Nakshatri H, Matei D. Epithelial-to-mesenchymal transition and ovarian tumor progression induced by tissue transglutaminase. *Cancer Res* 2009; 69:9192-201.
124. Kumar A, Xu J, Brady S, Gao H, Yu D, Reuben J, Mehta K. Tissue transglutaminase promotes drug resistance and invasion by inducing mesenchymal transition in mammary epithelial cells. *PLoS ONE* 2010; 5:e313390.
125. Thobe MN, Clark RJ, Bainer RO, Prasad SM, Rinker-Schaeffer CW. From Prostate to Bone: Key Players in Prostate Cancer Bone Metastasis. *Cancer (Basel)* 2011; 3:478-493.
126. Nauseef JT, Henry MD. Epithelial-to-mesenchymal transition in prostate cancer: paradigm or puzzle? *Nat Rev Urol* 2011; doi:10.1038/nrur.2011.85.
127. Zhau HE, Odero-Marah V, Lue HW, Nomura T, Wang R, Chu G, Liu ZR, Zhou BP, Huang WC, Chung LW. Epithelial to mesenchymal transition (EMT) in human prostate cancer: lessons learned from ARCaP model. *Clin Exp Metastasis* 2008; 25:601-10.
128. Graham TK, Zhau HE, Odero-Marah VA, Osunkoya AO, Kimbro KS, Tighiouart M, Liu T, Simons JW, O'Regan RM. Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res* 2008; 68:2479-88.
129. Veveris-Lowe TL, Lawrence MG, Collard RL, Bui L, Herington AC, Nicol DL, Clements JA. Kallikrein 4 (hK4) and prostate-specific antigen (PSA) are

- associated with the loss of E-cadherin and epithelial-mesenchymal transition (EMT)-like effect in prostate cancer cells. *Endocr Relat Cancer* 2005; 12:631-43.
130. Cao J, Chiarelli C, Richman O, Zarrabi K, Kozarekar P, Zucker S. Membrane type 1 matrix metalloproteinase induces epithelial-to-mesenchymal transition in prostate cancer. *J Biol Chem* 2008; 283:6232-40.
 131. Sethi S, Macoska J, Chen W, and Sarkar FH. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *Am J Transl Res* 2010; 3:90-9.
 132. Wu K, Zeng J, Li L, Fan J, Zhang D, Xue Y, Zhu G, Yang L, Wang X, and He D. Silibinin reverses epithelial-to-mesenchymal transition in metastatic prostate cancer cells by targeting transcription factors. *Oncology Reports* 2010; 23:1545-1552.
 133. Gasparian AV, Yao YJ, Kowalczyk D, Lyakh LA, Karseladze A, Slaga TJ, Budunova IV. The role of IKK in constitutive activation of NF-kappaB transcription factor in prostate carcinoma cells. *J Cell Sci* 2002; 115:141-151.
 134. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* 1999; 18:7389-7394.
 135. Lessard L, Mes-Masson AM, Lamarre L, Wall L, Lattouf JB, Saad F. NF-kappa B nuclear localization and its prognostic significance in prostate cancer. *BJU Int* 2003; 91:417-420.
 136. Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, G  linas C, Rabson AB. Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. *Prostate* 2002; 52:183-200.
 137. Oeckinghaus A, Ghosh S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 2009; 1:a000034.

138. Brockman JA, Scherer DC, McKinsey TA, Hall SM, Qi X, Lee WY, Ballard DW. Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. *Mol Cell Biol* 1995; 15:2809-18.
139. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* 1997; 388:548-54.
140. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* 1995; 267:1485-8.
141. Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA. Phosphorylation of human I kappa B-alpha on serine 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. *EMBO* 1995; 14:2876-83.
142. Prasad S, Ravindran J, Aggarwal BB. NF-kappaB and cancer: how intimate is this relationship. *Mol Cell Biochem* 2010; 336:25-37.
143. Chen CD, Sawyers CL. NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Mol Cell Biol* 2002; 22:2862-2870.
144. Jin RJ, Lho Y, Connelly L, Wang Y, Yu X, Saint Jean L, Case TC, Ellwood-Yen K, Sawyers CL, Bhowmick NA, Blackwell TS, Yull FE, Matusik RJ. The nuclear factor-kappaB pathway controls the progression of prostate cancer to androgen-independent growth. *Cancer Res* 2008; 68:6762-9.
145. Zhang Q, Helfand BT, Jang TL, Zhu LJ, Chen L, Yang XJ, Kozlowski J, Smith N, Kundu SD, Yang G, Raji AA, Javonovic B, Pins M, Lindholm P, Guo Y, Catalona WJ, Lee C. Nuclear factor-kappaB-mediated transforming growth factor-beta-

- induced expression of vimentin is an independent predictor of biochemical recurrence after radical prostatectomy. *Clin Cancer Res* 2009; 15:3557-67.
146. Baritaki S, Chapman A, Yeung K, Spandidos DA, Palladino M, Bonavida B. Inhibition of epithelial to mesenchymal transition in metastatic prostate cancer cells by the novel proteasome inhibitor, NPI-0052: pivotal roles of Snail repression and RKIP induction. *Oncogene* 2009; 28:3573-85.
 147. Mann AP, Verma A, Sethi G, Manavathi B, Wang H, Fok JY, Kunnumakkara AB, Kumar R, Aggarwal BB, Mehta K. Overexpression of tissue transglutaminase leads to constitutive activation of nuclear factor-kappaB in cancer cells: delineation of a novel pathway. *Cancer Res* 2006; 66:8788-95.
 148. Verma A, Mehta K. Transglutaminase-mediated activation of nuclear transcription factor-kappaB in cancer cells: a new therapeutic opportunity. *Curr Cancer Drug Targets* 2007; 7:559-65.
 149. Rajasekhar VK, Studer L, Gerald W, Socci ND, Scher HI. Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF- κ B signaling. *Nat Commun* 2011; 2:162.
 150. Das S, Becker BN, Hoffmann FM, Mertz JE. Complete reversal of epithelial to mesenchymal transition requires inhibition of both ZEB expression and the Rho pathway. *BMC Cell Biol* 2009; 10:94.
 151. Korpai M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* 2008; 283:14910-4.
 152. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 2008; 22:894-907.

Vita

Amy Lee Han was born in Colorado Springs, CO on January 29, 1986. She is the daughter of Kyung Tae and Sang Sook Han. After obtaining her diploma at Grandview High School, Aurora, CO, she attended the University of Colorado at Boulder where she received her Bachelor of Arts Degree in biochemistry in May 2008. In August 2009, she entered The University of Texas Health Science Center/M.D. Anderson Cancer Center-Graduate School of Biomedical Sciences.

Permanent Address:

19560 E. Crestridge Cir.

Centennial, CO 80015