Consequences of Altering Transforming Growth Factor Beta Receptor Type III (TβRIII) Expression in the Prostate

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KEYWORDS. Prostate, stroma, TGF-β, metastasis, tumorigenesis, receptor

BRIEF. The goal of this study was to observe how changing the expression of TβRIII affects prostate cancer progression.

ABSTRACT. Prostate cancer is associated with increased expression of transforming growth factor beta (TGF-β) ligands that can potentially affect tumor cells and/or the nearby stroma. TGF-β type III receptor (TβRIII), which controls the action of the ligand TGFβ2, has established roles in regulating cell migration and the growth of blood vessels. TβRIII expression is reduced in the human prostate cancer stroma; however, little is known about the consequences of TβRIII loss in the tumor microenvironment and the role that such changes play in tumor progression. In this study, TβRIII expression was suppressed in the fibroblast line BHPrS-1 (benign human prostate stromal-1). The urogenital sinus from mice that lacked TβRIII was rescued using renal capsule grafting. The expression of proteins associated with cell specification and division—including vimentin, smooth muscle a-actin (αSMA - a smooth muscle and myofibroblast marker), p38 MAPK (mitogen-activated protein kinase), and pSmad2—were assessed in these samples. Furthermore, proliferation and associated molecules were studied. Suppression of TβRIII in fibroblasts induced changes in proliferation. In the prostate tissue lacking TβRIII, expression levels of the stromal differentiation markers vimentin and a-SMA increased. The deletion of TβRIII also decreased the relative abundance of basal epithelial cells and caused an expansion of the smooth muscle. Furthermore, there was an increase in phosphorylated-p38 MAPK in fibroblasts as well as epithelial cells, a feature noted in developing prostate cancer tissues.

INTRODUCTION.

Prostate cancer is the second leading cause of cancer-related death in men. On average, one in six men born today will be diagnosed with prostate cancer during their lifetime [1]. The three transforming growth factor-betas (TGF-β1-3) can regulate intercellular communication and many cell processes including proliferation, differentiation, migration, embryonic development, angiogenesis, and wound healing. A variety of human diseases, including cancer, are associated with changes in TGF-β signaling [2]. More specifically, prostate cancer is associated with increased expression of TGF-β ligands that can potentially elicit effects on the tumor cells and/or the nearby connective tissue (stroma). Given the overall importance of TGF-β in the regulation of proteins, a broad area of cancer research is focused on understanding it and its associated signaling mechanisms, or pathways, and corresponding functions.

TβRIII controls the action of the ligand TGF-β2, which has established roles in regulating cell migration and angiogenesis, but in general, is a poorly understood component of TGF-β action. TβRIII levels are reduced in cancerous prostate tissue compared to normal tissue; specifically, in the stroma. Little is known about the consequences of TβRIII loss in the tumor microenvironment and the role that such changes play in tumor progression.

Prostate cancer studies with model organisms have shown that modifying the signaling mechanisms of the TGF-β superfamily inhibits the growth of cells when androgen levels are depleted [3]. This causes irregular expression of TGF-β receptors. KJ Gordon and GC Blobe have shown that the expression of TβRIII in prostatic tumor tissue is reduced. When the receptor is overexpressed and xenografted in nude mice, the prostate tumor's progression slows [2]. The elimination of TβRIII in mice results in embryonic death at day 14.5 from incomplete development of the coronary arteries [4]. Such an early death among the mice does not allow for an investigation of a tumor suppressor function.

Lowered expression of the TGF-β type II receptor (TβRII) in a subpopulation of stromal cells (mimicking clinical observations) has direct effects on tumor progression. This effect mirrors fibroblasts (connective tissue cells) isolated from prostate cancer patients. These cells have an increased expression level of the stromal derived factor SDF1a, and a significant decrease in the expression of TβRIII [5]. Additionally, loss of TβRIII expression in the tumor stroma of prostate cancer patients has been observed. To better understand the role of TβRIII during development and cancer progression, a mouse model and fibroblasts lacking the expression of TβRIII were characterized.

In this study, TβRIII expression was suppressed in the fibroblast line BHPrS-1 (benign human prostate stromal-1). Understanding how TβRIII behaves in the stroma under experimental conditions may shed light on potential methods for treating and preventing prostate cancer.

MATERIALS AND METHODS.

Cells.

BHPrS-1, a normal human prostatic fibroblast cell line, was obtained from an in-house supply. Previously, the BHPrS-1 cells were immortalized using human telomerase reverse transcriptase [5]. Human prostate carcinoma-associated fibroblasts (CAFs) were also acquired from existing in-house supplies. RPMI Media 1640 (Gibco) was combined with 1% antibiotics (Life Technologies) and 5% Fetal Bovine Serum (Gibco) to maintain the cells.

Viral Infection and shRNA.

Retroviruses expressing shRNA against the human TβRIII were purchased from Origene. The HuSH pGFP-V-RS plasmid vector contains both 5' and 3' LTRs of Moloney murine leukemia virus (MMLV) that flank the puromycin marker and the U6-shRNA expression cassette. Upon transient transfection of the plasmids into a packaging cell line, replication deficient viruses can be obtained and used to infect target cells. The puromycin-N-acetyl transferase gene provides selection using the antibiotic puromycin. There is an integrated turboGFP element driven by a cMV promoter to readily verify transfection efficiency.

Immunofluorescence.

Paraffin-embedded tissue samples from TβRIII-Knockout (KO), -Heterozygous (het), and –wildtype (wt) TβRIII-null mice were used to measure protein expression of CK 14 and CK 18 (basal cell markers), αSMA and γSMA (stromal cell markers), phosphorylated-p38 MAPK, p-Akt, and β-actin (control) by single-day immunofluorescence. CK 18 was blocked with 5% goat serum in PBS, and p-Akt was blocked with 10% bovine serum albumin (BSA) in phosphate buffered saline (PBS).

Western blot analysis.

Complete protein expression level of TβRIII (Santa Cruz Biotechnology), p-p38 (Cell Signaling), and p-Akt (Cell Signaling) were measured using protein from four separate preparations of TβRIII shRNA-transduced and vector-transduced BHPrS-1 cells. Twenty micrograms of protein were run on a gel for one hour at 150 V, transferred to a nitrocellulose membrane, and blocked with 5% powdered skimmed milk in a TBST blocking buffer. Primary and secondary antibodies were added to blocking buffer before the membrane was incubated overnight at 4°C. Signals were detected with ECL (Amersham) and processed using standard methods [5].

Tissue rescue and subrenal capsule xenografts.

The urogenital sinus of embryonic lethal TβRIII-KO mice was rescued using renal capsule grafting. Urogenital sinuses from E14.5 TβRIII-null mice were obtained from a colony at the Vanderbilt University Medical Center. Heterozygous (TβRIII +/-) male and female mice were mated. At 14.5 days of gestation (plug day denoted as day 0), pregnant dams were sacrificed, and fetuses were removed. Fetuses were laid on their backs and opened along the midline from the diaphragm to the pubic symphysis. Pelvic visceral rudiments were dissected as a single unit, the urogenital sinus (UGS) isolated and grafted beneath the renal capsule of intact male athymic mouse hosts. The TβRIII status of the fetuses was determined by PCR. After 1 month of growth, fully developed prostatic ductal structures were identified grossly within grafts and harvested from the renal graft site. These were then processed for subsequent molecular and histochemical analysis as previously described.

RESULTS.

Expression of TβRIII affects graft size and invasiveness in TβRIII-null mice.

TβRIII-null mice die at embryonic day 14.5, the time when functional coronary vasculature is required for embryo viability. To study the role of loss of TβRIII during prostate development, the urogenital sinus from wt, het and KO animals were isolated and grafted under the kidney capsule of athymic mice. The TβRIII status of the null mice was determined using PCR. Neither the het nor the wt appeared invasive (Figure 1A, B). The TβRIII-het graft was larger than the TβRIII-wt, but was not invasive (Figure 1B). Gross appearance of the KO graft differed from the het and wt grafts; the KO graft was larger and seemed to be invading into the kidney (Figure 1C). Quantitation of the grafts size is shown in Fig. 1D.

Downregulation of TβRIII instigates an expansion of the stroma and decreases the presence of CK14 positive basal cells.

Protein expression was assessed using immunofluorescence in paraffin-embedded tissue sections of the TβRIII-wt, -het, and -KO grafts. Phosphorylated-p38 MAPK increased in the TβRIII-KO graft. Compared to TβRIII-wt and TβRIII-het, αSMA and γSMA expression increased in the TβRIII-KO, indicating an increase in the size of the stromal compartment relative to the epithelium. CK14 positive basal cells decreased in the TβRIII-KO (Supplement Figure 1).

Downregulation of TβRIII affects the expression of key signaling pathways molecules.

TβRIII expression was suppressed in the normal human prostatic fibroblast line BHPrS-1 using four different shRNA constructs. Efficiency of each shRNA was determined using an anti-TβRIII antibody. Expression of downstream signaling pathways including p-p38 MAPK and p-Akt were assessed. Downregulation of the TβRIII by the shRNA constructs were in a range between 20 and 50% (Figure 2). There was an increased expression of p-p38 in the shTßR3#5. Interestingly, loss of TβRIII decreased the phosphorylation of Akt.

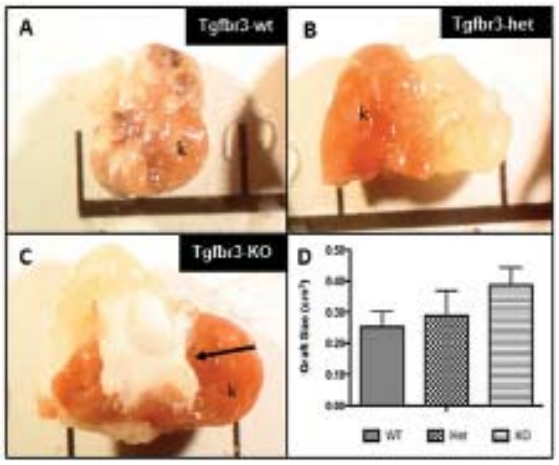

Figure 1. Urogenital sinuses from TβRIII-null mice were grafted under the renal capsule of intact male athymic mouse hosts. Gross appearance showed a relative increase in the size of the KO grafts compared to the het and wt grafts. The wt graft was smaller than the het, and neither appeared invasive. The KO showed a tendency to grow inside the renal parenchyma (arrow). D shows a quantification of the graft sizes. k=kidney.

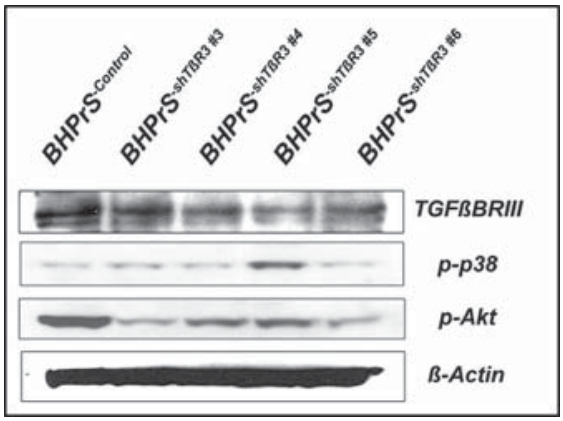

Figure 2. Western blot protein analysis of TGFβRIII, p-p38, p-Akt, and β-Actin (control) – Protein concentrations were measured in the four shRNAs (used to downregulate TβRIII in the cell line BHPrS-1) and measured in the control BHPrS-1 (without shRNA). Expression of TβRIII decreased more in shTβRIII #5 compared to the control. p-p38 increased 80% and p-Akt decreased by 55% compared to the control.

DISCUSSION.

Tumorigenesis often begins with a mutation in a cell's genetic material—such as the loss of a tumor suppressor or the gain of an oncogene—which causes uncontrolled proliferation. When the mutated cells invade into surrounding tissue through the basal lamina, the tumor is, by definition, invasive and therefore considered malignant. At this stage, the cancer has the potential to metastasize to another part of the body through the blood stream or lymphatic system. Both benign and malignant cells interact with their local microenvironment to cause changes in cell phenotype and behavior which may suppress or enhance tumor progression [6].

TGFβ ligands are critical regulators of many cellular processes. TβRIII is a membrane-bound proteoglycan that controls some aspects of TGFβ ligand signaling [7]. The role of TβRIII in prostate cancer progression and metastasis is becoming more apparent.

In many cancer types, including prostate cancer, the expression of TβRIII decreases [7]. The data presented here demonstrate that suppression of TβRIII *in vivo* can result in irregular growth of grafted tissue as well as invasion into the renal parenchyma (Figure 1C). It has been shown that restoring TβRIII in cancer cells reduces invasiveness and the ability to migrate [8]. Taken together, this supports a role of TβRIII during metastasis. Immunohistochemistry revealed a significant increase in the expression of p-p38 MAPK and an increase in the expression of αSMA and γSMA when TβRIII was downregulated *in vitro* (Supplement Figure 1). This suggests that the role of TβRIII in metastasis occurs via the p38 MAPK pathway.

Myofibroblasts are activated stromal cells that have been linked to tumorigenesis through their function of secreting growth factors, extracellular matrix (ECM) components and factors that elicit ECM remodeling; this remodeling can induce tumor progression [9]. Alpha and gamma smooth muscle actin (αSMA and γSMA) are expressed by myofibroblasts [10]. In this study, a downregulation of TβRIII caused a decrease in CK14 positive basal cells and an increase in the expression of αSMA and γSMA (Supplement Figure 1) as well as extensive growth in the tissue graft model (Figure 1). Basal epithelial cells have recently been proposed to be the source cell type for prostate cancer [11].

Stromal expansion (and morphologic changes of this type) is consistent with changes seen in pre-malignant and malignant prostatic tissue [9]. This supports a relationship between TβRIII expression and tumor progression.

Akt is responsible for many cellular processes, and mainly functions to promote growth factor associated-cell survival [12]. Akt is often overexpressed in cancer. Furthermore, phosphorylated, or activated, Akt has been shown to induce cellular transformation [12]. In this study, phosphorylated-Akt expression was inhibited by downregulation of TβRIII in stromal cells *in vitro* (Figure 2). While these data are consistent with previous work done with epithelial cells [12], this study presents unique findings for stromal alterations.

CONCLUSION.

The goal of this study was to determine whether downregulation of TβRIII expression affects graft size and downstream signaling molecules. In this study, it was hypothesized that this change in TβRIII expression would vary the expression of p-Akt and p-p38 MAPK—which are related to cancer progression and metastasis, respectively[13]. Additionally, the possibility of a change in the stromal (observed with αSMA and γSMA) and basal epithelial (CK14) compartments of the TβRIII-deficient tissue was considered. Results pointed to a relationship between TβRIII and cancer progression: as TβRIII decreases, cancer progression increases.

This concept is strongly supported by previously published literature; however, the complex underlying interactions and signaling molecules involved are unknown. For example, TβRIII could affect metastasis directly through p-p38 MAPK, or through additional gene products such as growth factors that regulate p-p38 MAPK. Further expression-level testing and analysis of other genes in the canonical and non-canonical TGF-β signaling pathways is needed to understand how TβRIII elicits its effects.

Our observations suggest that TβRIII may have a role during prostate carcinogenesis. It remains unclear, however, how these connections are fashioned. More likely than not, these relationships are due to regulation of various non-canonical downstream molecules by TβRIII. Understanding how TβRIII behaves could aid in the development of tailored treatments for patients with prostate cancer. Further research will be needed to more completely understand its indirect regulation of signaling pathways before treatments can be explored.

ACKNOWLEDGMENTS. I would like to thank everyone in the Hayward lab for their expertise and guidance throughout this project. Also, I would like to thank the School for Science and Math at Vanderbilt for giving me the opportunity to work in such a great lab. I would like to thank Joey Barnett and Joey Love for providing us with Tgfbr3-KO mice. The project described was supported by Award Number R25RR024261 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health

SUPPORTING INFORMATION.

Figure S1. Immunofluorescence After Downregulating TβRIII

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