TGF-β SIGNALING IN STROMAL CONTRIBUTION TO PROSTATE CANCER PROGRESSION

By

Mingfang Ao

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements

For the degree of

DOCTOR OF PHILOSOPHY

In

Cancer Biology

August 2006

Nashville, Tennessee

Approved:	Date:
	5·8·06
	5-8-06
	5-8-06
	5/8/06
	5/8/06

ACKNOWLEDGEMENTS

I wish to express my gratitude to the following individuals for their support and encouragement in helping me to complete my Ph.D degree. I would first like to thank my mentor, Dr. Simon W Hayward, for his guidance and training in my projects. I feel grateful for his enthusiasm in my laboratory work and his belief in me to carry out this challenging piece of research. I would like to extend my gratitude and appreciate to my thesis committee members, Dr. Carlos L. Arteaga, Dr. Stephen R. Hann, Dr. Robert J. Matusik and Dr. Albert B. Reynolds for their time and efforts. Their stimulating questions and useful suggestions have encouraged me to plan the experiments more thoroughly and to complete my project sooner and better. In addition, I am truly grateful to Dr. Neil A. Bhowmick, who shared his expertise to start my TGF-β signaling work.

I would also like to thank all the members in Hayward laboratory: Dr. Karin Williams, Dr. Ming Jiang, Dr. Xavier Stien, Dr. Kenishiro Ishii, Dr. Omar Franco, Dr. Harold Love and Mrs. Suzanne Fernandez for their precious advice and technical assistance in past few years. Also I wish to thank all the other members in The Vanderbilt Prostate Cancer Center, Dr. Robert J Matusik and his group, Dr. Susan Kasper and her group, Dr. Neil A. Bhowmick and his group, for their help and friendship.

Finally, I want to thank my family, my parents my husband and my sons for their love and support. Their daily encouragement and inspiration enabled me to pursue this goal.

TABLE OF CONENTS

Page

LIST OF FIGURES

ABBREVIATION LIST

- 5AR: 5 alpha-reductase
- AUA: American Urological Association
- AUR: Acute urinary retention
- BMPs: Bone morphogenetic proteins
- BS3: Bis (sulfosuccinimidyl) Suberate
- CAF: Carcinoma Associated Fibroblasts
- CaP: Carcinoma of the Prostate
- CK: Cytokeratin
- CXCL12: CXC-motif chemokines ligand 12
- CXCR4: CXC-motif chemokines receptor 4
- D-PBS: Dulbecco's Phosphate-Buffered Saline
- DAT□R1: Dominant Active TGF-□ Receptor
- DN-Akt: Dominant negative Akt
- ECM: Extracellular Matrix
- EMT: Epithelial to Mesenchymal Transformation

- FACS: Fluorescence Activated Cell Sorting
- HRPC: Hormone-refractory prostate cancer
- LUTS: Lower Urinary Tract Symptoms
- MAPK: Mitogen-Activated Protein Kinase
- MTOPS: Medical Therapy of Prostatic Symptoms
- MyrAkt: Myristylated Akt1
- NE: Neuroendocrine
- NPF: Normal Prostate Fibroblasts
- PI: Propidium Iodide
- PIP3: Production of phosphatidylinositol-3,4,5-triphosphate
- PI 3K/Akt: phosphatidylinositol 3-kinase/Akt
- PDK-1: Phosphoinositide Dependent Protein Kinase-1
- PSA: prostatic specific antigen
- PTEN: Phosphatase and Tensin homolog
- SDS-PAGE: SDS-polyacrylamide gel electrophoresis
- SCID: Severe Combined Immunodeficient

- SIR: Standardized Incidence Ratio SV: Seminal Vesicle
- TGF-β: Transformation Growth Factor-beta
- TβRI and TβRII: TGF-β type I and type II receptors
- UGM: Urogenital Mesenchyma
- UGS: Urogenital Sinus
- WHO: World Health Organization

CHAPTER I

INTRODUCTION

Prostate development and structure

The prostate is a male accessory sex gland located below the urinary bladder. The main function of the prostate is to provide proteins and ionic components of the ejaculate (1). The development of the prostate begins at about 10 weeks of fetal life in the human and is not determined by fetal genetic sex, but rather by exposure to androgens. Urogenital sinus (UGS) from either male or female fetuses will form functional prostatic tissue if stimulated by androgens during the appropriate developmental period (2). Initially solid epithelial buds appear and grow as solid cords into the surrounding mesenchyme. The solid cords canalize, initially in the proximal regions (adjacent to the urethra) and progress into the branching network. After birth, the gland enters a quiescent state until the circulating androgen levels increase at puberty. At this point the gland begins to grow slowly and epithelium proliferates, resulting in the increase in post pubertal prostatic wet weight. This growth phase continues until adulthood at which point the prostate becomes essentially growth quiescent (3). The prostatic rudiment contains two components: urogenital mesenchyme (UGM) and epithelium. UGM is derived from the embryonic mesoderm. Prostatic development is an androgen dependent process with ordered sequential ontogenic expression of specific markers in UGM (4, 5). The first detected mesenchymal marker is vimentin which is initially widespread through the stromal component of the prostatic rudiment. During development vimentin becomes

restricted to the interductal tissue of the prostate. Smooth muscle markers are expressed in an orderly sequence in a proximal to distal manner along the developing prostatic ducts. Expression of □-actin is followed by vinculin, myosin, desmin and laminin. Androgen receptor (AR) can be detected focally at 16 days of gestation in rat mesenchymal cells. As development progresses, expression of AR became more wide spread, and postnatally is found throughout the mesenchyme (6). AR is detected in the epithelium of UGS from day 19 of gestation. By birth AR were detectable in the epithelium of both prostatic lobes and the seminal vesicle (SV)(7).

Differentiation of prostatic epithelium occurs concurrently with maturation of the stroma. The early epithelium contains a single, apparently homogenous cell type which initially forms solid cords. These cords canalize giving rise to two distinct classes of epithelial cell basal and luminal epithelium which play distinct architectural and secretory roles (6, 7). Adult prostatic epithelium consists of flattened basal, tall columnar luminal secretory, and neuroendocrine (NE) cells. In the human prostate the secretory compartment consists of a luminal layer which expresses the AR, cytokeratin (CK) 8 and 18, and prostatic specific antigen (PSA). The majority of basal cells can be distinguished from secretory cells, because they express p63, CK5 and CK14, and generally lack expression of luminal cell markers such as CK8 and CK18. Prostatic neuroendocrine cells expressing chromogranin A are very rare and can be characterized by the lack of AR and PSA. In addition to typical basal, secretory, and NE cells, there are cells with intermediate phenotypes expressing a mixture of basal and luminal markers (CK5, CK8, CK14, CK18, and PSA(8). The stroma of the human prostate consists of a number of

different cell types. The most abundant cell type in this compartment is the smooth muscle cell, derived from the mesenchyme of the embryonic urogenital sinus (UGS). Other cell types located in stroma include fibroblasts, nerves, endothelial cells and vascular smooth muscle cells. Using the androgen insensitive testicular feminized (Tfm) mouse, Cunha's group demonstrated that an androgen responsive stroma is necessary for the development of normal prostatic epithelial architecture in the rodent (2). The developing epithelium is also necessary to induce primitive mesenchymal cells to differentiate into smooth muscle. Neither prostatic epithelium nor prostatic smooth muscle can develop in the absence of the other tissue (9). Mesenchymal-epithelial interactions then, are crucial components of prostatic development and continue to be very important in benign and malignant adult prostate (10).

PSA, a kallikrein serine protease, is secreted by the prostatic epithelial cells into the lumen of the prostate duct during the formation of seminal plasma. PSA is a 30 kDa serine protease that cleaves biological substrates in the seminal fluid, including seminogelin I, seminogelin II, and fibronectin, into small peptides (11, 12). PSA has also been shown to cleave other biological substrates, including insulin-like growth factor-binding protein-3 and laminin, indicating the potential role of PSA in the regulation of various biological functions (13). The cellular expression of PSA is under androgen regulation, and the reduction of androgen function has been shown to reduce prostate tissue expression of PSA. Nonprostatic sources of PSA have now been documented, including the milk of lactating women, amniotic fluid, and cerebrospinal fluid.

Another secretory protein of the human prostate is prostate specific acid phosphatase (PSAP). This enzyme shows a marked preference for tyrosine phosphate as a substrate. While the nature and localization of the physiological substrate are unknown, the most likely candidate is a tyrosine-phosphorylated protein on the sperm called "forward motility protein". Additional or alternative targets could be present in vaginal or uterine secretions or in cervical mucous (14). Acid phosphatase would seem to have a general function as it has been found to be present in the prostatic secretions of not only man but also monkeys, dogs and rodents. Monoclonal antibodies are commercially available for both PSA and PSAP and are used clinically as markers of prostatic disorders, both showing increased serum levels in benign prostatic hyperplasia (BPH) and especially in carcinoma of the prostate (CaP).

Lowsley, working on the developing human fetal prostate described a dorsal or posterior lobe, a median and two lateral lobes, and additionally a ventral lobe which atrophied after birth (15). In the adult human these lobes are fused and cannot be separated by dissection giving rise to a number of different views on the anatomic division of the human prostate (16, 17). The situation is further confused by the fact that in most other animals, including other primates, the various prostatic lobes are separable in varying degrees on an anatomical, histological and physiological basis.

McNeal, whose descriptions of prostatic anatomy are the most widely accepted today, divides the prostate into three anatomically separate and histologically distinct areas. These are the non-glandular fibromuscular stroma which surrounds the organ, and two

glandular regions termed the peripheral and central zones (17). These zones contain a complex and histologically distinct ductal system. Ducts of the peripheral zone exit directly laterally from the poster-lateral recesses of the urethral wall. The system consists of small, simple round to oval acinar structures emptying into long narrow ducts surrounded by a stroma of loosely arranged and randomly interwoven muscle bundles. Ducts and acini are lined with simple columnar epithelium. This area is the principle site of prostatitis and CaP although not of BPH. The peripheral zone includes the proximal urethral segment of the prostate. This comprises the region of the prostate between the base of the urinary bladder and the verumontanum (the area where the ejaculatory ducts feed into the urethra). The principle feature of this region, which comprises around 5% of the total prostate mass, is the preprostatic sphincter. The sphincter is a cylindrical sleeve of smooth muscle which stretches from the base of the bladder to the verumontanum.

A number of different animal species, including rats, dogs, and various non-human primates have been used as *in vivo* models of the prostate. Like all model systems, these have their inherent advantages and disadvantages. The principle disadvantage of animal prostates as models for the human organ is the profound structural differences between the single lobed human organ and the multilobed organ found in most other species.

BPH is the most common symptomatic tumor-like condition in humans (18). It is rarely histologically identifiable in men under forty years of age. There is also no evidence to suggest that BPH is a precursor of Cap. The dog is the only animal other than man and the chimpanzee to suffer naturally from benign prostate hyperplasia (BPH). Canine BPH, like

its human counterpart, arises with increasing frequency with age and requires a functional testis. The diseases differ in that human BPH occurs in distinct nodules within the gland, whereas the canine disease is an apparently diffuse process occurring throughout the gland (19). In the dog, BPH causes a general expansion of the gland, which is less anatomically fixed than in man, resulting in compression of the rectum, producing constipation as a symptom as opposed to the urine retention found in humans. The non-human primates might reasonably be expected to have a prostate that is more closely related to the human. Indeed descriptions of the prostates of a range of these animals show that they are anatomically far closer to the human organ than any other animal. Problems of working with these animals fall into two categories. The first is the practical one of obtaining and keeping primates. The second is that primates, with the exception of chimpanzees and humans, do not suffer from BPH (20). McNeal (21) suggested that this is because the region homologous to the human transition zone is absent in other primates.

Prostate diseases

BPH is a leading disorder of the elderly male population. BPH is characterized by a progressive enlargement of prostatic tissue, resulting in obstruction of the proximal urethra and causing urinary flow disturbances. The incidence of BPH increases greatly with age; it is histopathologically evident in 50% of the male population by age 50 years, and in 80% by age 80 years (22). There are 2 mechanisms by which the prostate can cause bladder obstruction. One is by the narrowing of the urethral lumen due to the BPH associated tissue bulk. This is described as the static component of bladder obstruction, because it remains constant except for variation related to size. The dynamic component consists of prostatic

smooth muscle tone, the related pathophysiology is characterized by increased adrenergic tone leading to smooth muscle contraction (23). The progression of BPH can be defined as a deterioration of clinical variables such as lower urinary tract symptoms (LUTS), health-related quality of life and peak flow rate, increased prostate size, or unfavorable outcomes such as acute urinary retention (AUR) and BPH-related surgery (24).

Watchful waiting, pharmacological therapy, and surgery are the three courses of action available to patients with BPH. The simplest and least invasive option is watchful waiting, which could more accurately be termed active surveillance, as it involves regular monitoring for signs of disease progression that may be indicators for treatment (25). The two commonly used classes of drugs are antiadrenergic and antiandrogenic approaches. Alpha1-Adrenoceptor antagonists and 5 alpha-reductase (5AR) inhibitors are well-established representatives of the two categories, respectively. Other antiandrogenic approaches involve gonadotropin-releasing hormone agonists and antagonists (26). Alpha-adrenergic receptor blockers provide the most rapid relief by decreasing the smooth muscle tone in the stroma and prostate capsule, thereby, addressing the dynamic component of prostatic obstruction. 5□-reductase (5AR) inhibitors decrease prostatic bulk, thereby addressing the static component. The two often are combined in patients with moderate to severe symptoms (25). The Medical Therapy of Prostatic Symptoms (MTOPS) trial investigated the use of the alpha-blocker doxazosin and the 5 alpha-reductase inhibitor finersteride, either alone or combined, as inhibitors of symptomatic progression in BPH patients. The trial determined that while either drug was better than no treatment, combined therapy provided better relief from symptomatic progression as determined by increased

American Urological Association (AUA) symptom score than either drug given alone (27). Despite the availability of medical treatments for BPH there is still little true understanding of the role of the biological mechanisms that result in initiation and progression.

Prostate cancer is the now the most common non-cutaneous cancer in men in the Western world (reviewed in (28). It occurs mainly in men who are older than 50 years; prostate cancer incidence is highest in men aged 75 years and older (29). Prostate cancer incidence varies widely between countries and ethnic populations. According to the standardized incidence ratio (SIR) data, the incidence in the USA and Canada is almost five and four times higher compared with the world standardized incidence ratio, respectively. These SIRs are followed by Western and Northern European regions that show a 2–3-fold higher rate. In Europe, prostate cancer incidence decreases when heading from the North towards the Mediterranean regions, a north to south trend that also holds true in the United States, giving rise to a hypothesis that bioavailability of vitamin D may play a suppressive role in prostate cancer pathogenesis (30). Black men are at an increased risk for prostate cancer compared with white men. The lowest incidence of prostate cancer is found in China and India. The risk of dying from prostate cancer is higher in Europe compared with Northern America despite a perceived lower incidence, although these differences are likely reflective of screening and reporting practices. Worldwide, the chance of dying from prostate cancer is small (164), however in Westernized societies this disease is still a major cause of male mortality.

Important prognostic indicators for prostate cancer include the presenting PSA level, clinical stage and Gleason grade. Prior to the widespread use of PSA screening in asymptomatic men, prostate cancer was detected by a simple digital rectal examination or by men presenting with bone fractures symptomatic of metastatic disease. Although the gold standard for prostate cancer still remains a prostatic biopsy, current research in the area of detection and diagnosis of prostate carcinoma are focusing on identification of better sampling protocols, biologic markers and imaging strategies in order to detect disease at an earlier stage (31).

An elevation in serum PSA levels may be caused by prostatic adenocarcinoma and this is widely used in North America as a screening tool. However, prostatitis, BPH and other conditions can also increase serum PSA. Therefore, PSA, although largely prostate-specific, is not a cancer-specific marker. PSA value must be merged with other risk factors of an individual man including ethnicity, family history, as well as the individual's risk aversion to complications from prostate cancer. The future of prostate cancer screening will be built upon incorporation of new biomarkers to the prediction of risk of disease. As these markers move forward in testing, it will no longer be acceptable to move these into clinical usage without formal validation studies and, because of the high frequency of prostate cancer in the general male population, these validation studies will almost certainly have to include measures of prognosis (32).

The Gleason grading system, named after D.F. Gleason who first described it, is now the predominant grading system. In 1993 it was recommended by a World Health Organization (WHO) consensus conference. The Gleason grading system is based on

glandular architecture (33). It assigns a grade to each of the two largest areas of cancer in the tissue samples. Grades range from 1 to 5 (**Figure 1-1**), with 1 being the least aggressive and 5 the most aggressive. The Gleason score is the sum of the primary (most predominant) Gleason grade and the secondary (second most predominant) Gleason grade. Where no secondary Gleason grade exists, the primary Gleason grade is doubled to arrive at a Gleason score. The primary and secondary grades are reported in parenthesis after the Gleason score, i.e. Gleason score 7 (3+4). A score of 2 to 4 is considered low grade; 5 through 7, intermediate grade; and 8 through 10, high grade. Gleason grade has been linked to a number of clinical end points, including clinical stage, progression to metastatic disease, and survival. Gleason grade is often incorporated into nomograms used to predict response to a specific therapy, such as radiotherapy or surgery. Needle biopsy Gleason grade is routinely used to plan patient management and is also often one of the criteria for eligibility for clinical trials testing new therapies (34).

Four major treatment options for prostate cancer are available: surgery, radiotherapy, hormone therapy, and observation. Not only tumor grade and stage, but also patients' life style and wishes should be considered in determining treatment since every treatment has its benefits and risks (35). The advantages of radiotherapy and radical prostatectomy are obvious; the intention of treatment is usually curative. However, side effects of both curative treatments are serious. Aside from sexual dysfunction, radical prostatectomy and radiotherapy on the prostate affect urinary function and can also disrupt bowel function.

Ever since Huggins and Hodges described the relationship between testosterone and prostate cancer (36) androgen deprivation has continued to be an important treatment. This treatment is palliative if the disease has spread, with a median duration of response of about 18 months. Hormone refractory prostate cancer remains a challenge. (37, 38). For example, metastatic prostate cancer, while initially responsive to androgen ablation, eventually becomes hormone-refractory and resistant to many treatments. Unfortunately, there are very few agents in the preclinical stage with a seemingly promising future for hormone-refractory prostate cancer (HRPC) that are being taken through the complete drug development process, including US Food and Drug Administration approval. Many novel strategies are under investigation for treating HRPC target metastatic prostate cancer cells that are neither androgen-dependent nor in the proliferative state (39).

Tissue recombination and subrenal xenografting

Tissue recombinations are made by mixing stromal tissues or cells with epithelial cells to investigate how epithelial and mesenchymal cell populations respond to each other. Tissue recombination models have been extensively used to examine mesenchymal-epithelial interactions in a number of organ systems. Of particular relevance, they have provided important insights into the role of paracrine signaling in the male rodent reproductive tract. The epithelial and stromal cells can be extracted from the same species (homospecific) or from different species (heterospecific). They also can be derived from the same tissue (homotypic) or from different tissues (heterotypic) (40-42). As a model system, tissue recombination has distinct advantages. First, cells can grow in an experimental environment. Secondly, the epithelial and stromal cells can

interact with each other. The third advantage is the flexibility of this system. Historically the model was used to investigate developing tissues from wild type animals. Recombinants of transgenic or knock out tissues extended this model to investigate specific genes or pathways in development and carcinogenesis (43-45).

This model has been used to demonstrate the plasticity of epithelial cells. The induction of prostatic structures from adult bladder epithelial cells by recombination with UGM resembles the process of normal prostate development (46). This experiment also indicates that adult epithelial cells retained the potential to respond to inductive mesenchyma. Tissue recombination has been used to examine the hormonal effects on urogenital tract development. By using Tfm mouse tissues, which have no functional androgen receptor, Cunha and co-workers demonstrated tissue-specific functions of androgen receptors. Specifically control of glandular morphogenesis and epithelial proliferation depends on receptors in mesenchyma, while epithelial receptors play an important role in differentiated function (47-49). Similar controls have since been described as acting in estrogenic and progestogenic control of female genital tract development and function (50, 51).

Tissue rescue is a technique that can be used to generate adult tissues of interest from transgenic or knockout mice that are embryonic lethal. Such tissue can then be used in tissue recombination models to expand cell populations or to interrogate gene function. The first example to use this approach was the rescue of retinoblastoma (Rb) knockout tissue. The Rb knockout mouse dies at 14 days of gestation before the formation of

prostate. The pelvic organs were removed from embryos at 12 days of gestation and grafted beneath the kidney capsule of male host mice to give rise to prostate, bladder, seminal vesicle and other tissues. Then the tissue of interest was microdissected and used for tissue recombination with rat UGM, thus providing a resource of cells to study the biological consequence of Rb loss (43). This study suggests that tissue recombination can use stroma and epithelium from the huge variety of gene knockout and transgenic sources now available. Another example has been the rescue of FoxA1 knockout prostate tissue (52).

A number of graft sites have been used for *in vivo* studies, including chick chorio-allantoic membrane, intra-ocular, renal capsule, subcutaneous and orthotopic grafting into rodents (53). The subcutaneous and subrenal capsule sites are very popular because they are well tolerated by hosts. The efficiency of subcutaneous graft is poor if not improved by Matrigel to increase blood supply. In contrast, the subrenal capsule site is very well vascularized and does not require extra stimulation. Surgery to the renal capsule is slightly more difficult but the graft take is much more efficient. To better observe the metastatic behavior of prostate cancer, Wang's laboratory (among others) have developed and pursued orthotopic xenografting (54). They grafted cells and explants into a pocket created under anterior lobe of the prostatic capsule. Although this method is more technically difficult, it is useful to investigate the mechanisms underlying prostate cancer metastasis and to develop models to test new therapies.

Prostatic mesenchymal-epithelial interaction

There are two major in *vivo* approaches to study epithelial-mesenchymal interactions. Direct grafting of epithelial cells and structures to hosts allows interactions between the host stroma and epithelial cells of interest to be investigated. Alternatively, tissue recombination experiments demonstrated *in vivo* interactions between epithelium and defined mesenchymal populations. These tissue recombinants are normally grafted beneath the renal capsule of rodent host. After a period of growth and development, the host animals are sacrificed and the grafts are removed for analysis. Tissue recombination has demonstrated the two classes of epithelial-mesenchymal interaction: permissive and instructive. Permissive inductions are those in which mesenchyme permits the epithelium to adopt its normal developmental program, while instructive inductions are those in which mesenchyme induces and specifies the epithelium to adopt a new developmental fate. An example of permissive induction is the interaction between UGM and seminal vesical epithelium. Recombinants derived from UGM plus seminal vesicle epithelium differentiate into seminal vesicle, since seminal vesicle epithelium is derived from the mesodermally derived Wolffian duct, whose developmental repertoire has been stably committed to the formation of epididymis, ductus deferens and seminal vesicle, but not prostate (2). In contrast, in instructive inductions, the epithelium responds to a heterologous inductor. This is the mechanism for UGM to elicit prostatic development in the epithelium of UGS derivatives, such as urethra, bladder, and vagina. Recombinants composed of transitional epithelium derived from the adult bladder epithelial cells can be induced by urogenital mesenchyma to give rise to prostatic tissue (22, 46). The induced prostatic acini are filled with secretions that, when adult human bladder epithelium was

used to generate the recombinants expressed human prostate-specific secretory proteins including PSA (22).

Reciprocal interactions between stroma and epithelium are critical in both prostate development and prostatic carcinogenesis (2, 55-57). In the developing prostate, low levels of androgens act on androgen receptors located in the mesenchyme to induce prostatic ductal morphogenesis and epithelial proliferation. In turn, the developing epithelium induces mesenchymal differentiation into prostatic smooth muscle. In the adult prostate, high levels of androgens act on androgen receptors located in both epithelium and mesenchyme. In the adult, androgens act- through the prostatic smooth muscle to maintain differentiation of the epithelium; meanwhile, androgen acts directly on luminal epithelium to induce differentiation and defined secretory activity (5, 57, 58) (**Figure 1-2**). The interactions between epithelium and mesenchyme are believed to be mediated by paracrine signals and Extracellular Matrix (ECM) components secreted from developing mesenchyme that affect adjacent epithelial cells (59).

Prostatic stroma and prostate cancer

Prostate cancer, like the majority of malignant human tumors is a carcinoma (specifically an adenocarcinoma), identified as a tumor that arises from the epithelium. For that reason, much attention has been paid to the epithelial cell type. However, there is growing evidence that the surrounding microenvironmental stroma and the interactions between cancer cells and adjacent stroma are critical for cancer initiation and

progression. The first contribution to this new concept comes from observations of stromal alterations during epithelial carcinogenesis.

Rowley's group demonstrated that the prostatic stroma rapidly responds to carcinoma progression in a number of ways, including elevated stromal cell proliferation, altered expression of matrix components, elevated expression of TGF-β1, neovascularization, and expression of several common stromal markers. In addition, proliferative stromal cells at these sites generally express myodifferentiation markers. These observations indicate that stroma serves not just as a supportive compartment, instead, the tumor stroma is reactive, providing a tumor promoting environment (60, 61). Specific stromal markers, such as reduced desmin and smooth muscle alpha-actin, are hallmarks of cancer-associated reactive stroma. Quantitative analysis of desmin and smooth muscle alpha-actin expression are both significant and independent predictors of recurrence-free survival (62). Several other groups have reported stromal alterations during tumorigenesis in breast, colon, lung and prostate cancer (63). During cancer development and progression, the stroma presents a series of "reactive" changes that occur parallel to tumorigenesis, and are called stromagenesis (64): ECM is modified, more inflammatory cells are recruited, angiogenesis is stimulated, and fibroblasts show a phenotype known variously as "reactive fibroblasts", carcinoma associated fibroblasts (CAF) or myofibroblasts - since they express both fibroblastic markers (vimentin) and a smooth muscle marker (α-smooth muscle actin) (65, 66). These active alterations in stroma that occur during tumorigenesis have changed the traditional concept that stroma is just a

passive structure (67). The importance of stromal-epithelial interactions in cancer gave rise to the concept that the stroma is an essential component of cancer.

To address whether these stromal alterations have a functional role in carcinogenesis, researchers have recombined fibroblasts and epithelial cells and grafted them into host animals. Chung's group demonstrated both the positive and negative effects of fibroblasts on tumor production. Their studies showed that tumorigenic epithelial cells in grafts can respond to signals from the fibroblasts and that the response depends on the molecular changes present in the epithelial cell (68, 69). In an alternative approach using human tissues, Cunha's group tested the effects of CAF on the initiated but non-tumorigenic prostate epithelial cell line BPH1. To determine whether CAF send signals to initiate abnormal epithelial growth or enhance progression of a non-tumorigenic to a tumorigenic cell, they recombined CAFs with BPH1 cells and demonstrated the interaction between these two cell populations. They found that recombinants of CAF and BPH1 cells resulted in tumors from that could exceed five grams in wet weight with a disrupted architecture marked by streaming epithelia with enlarged nuclei (70). This effect was not obtained when normal prostatic fibroblasts were grown with the initiated epithelial cells under the same experimental conditions. In contrast, carcinoma-associated fibroblasts did not affect growth, but did result in squamous differentiation of normal human prostatic epithelial cells under identical conditions (**Figure 1-3**). This experiment demonstrated that carcinoma-associated fibroblasts could direct tumor progression of an initiated but nontumorigenic prostate epithelial cell to form tumors, providing very strong evidence

that prostatic stroma is not just a supportive structure but also is critical for prostate cancer initiation and progression.

TGF-β pathway

The transforming growth factor (TGF)-βs belong to a superfamily of ligands that includes activins, and bone morphogenetic proteins (BMPs). TGF-β ligands act through the transmembrane type I and type II receptors (TβRI and TβRII) to activate a number of downstream signal transduction pathways (71). Following ligand binding to TβRII, TβRI is recruited to the complex. The TβRI kinase is activated by TβRII, and, in turn, phosphorylates Smad2 and Smad3. Smad2/3 then associate with Smad4, and translocate to the nucleus, where they regulate gene transcription (72). The Smad pathway was the first signaling pathway identified to mediate TGF-β effects and remains the best characterized. More recently, multiple non-Smad pathways have been implicated in mediating TGF-β effects downstream of the receptors (Shown in **Figure 1-4**). The involvement of these non-Smad pathways in the changing responses of cells to TGF-β are just beginning to be probed (73, 74). Studies on mammary epithelial cells have demonstrated that TGF-β promotes motility through mechanisms independent of Smad signaling, possibly involving activation of the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and/or mitogen-activated protein kinase (MAPK) pathways (75-77). Additional data suggested that TGF-β rapidly activates RhoA-dependent signaling pathways to induce stress fiber formation and mesenchymal characteristics (78).

Originally named for their transforming activities *in vivo*, TGF-βs play complicated roles including regulating cell proliferation, functional differentiation, ECM production, cell motility, and apoptosis. In tumorigenesis, TGF-β apparently plays dual roles. In the current paradigm, TGF-β acts as a growth suppressor in normal tissue; but in tumors, changes in TGF-β expression and cellular responses tip the balance in favor of pro-oncogenic activity (74). This has been shown in the prostate (79, 80) and many other cancer models (81-83). In benign prostatic epithelia, by eliciting differentiation, inhibiting proliferation and inducing apoptosis, TGF-β provides a mechanism to maintain homeostasis in the prostate (84-86). The ability of TGF-β to enhance tumorigenicity *in vivo* is illustrated by its role in many key processes including; stimulating angiogenesis, inhibiting immune surveillance or promoting the degradation of extracellular matrix (86, 87). TGF-β can also promote local invasion and metastasis through epithelial to mesenchymal transition (EMT) (78, 88, 89) (**Figure 1-5**). EMT is a poorly documented feature of human prostate cancer, arguably because of a lack of good antibodies against the EMT-related transcription factors.

Specific responses to TGF-β relate to a complex of factors including dose of bioavailable ligand, target cell type and cellular context. This reflects the complex network of cross-talking signals that constitute the transduction mechanism by which the cell responds to stimulation by the TGF-β ligand (72). Integration of the TGF-β pathway with other signaling cascades that control the same cellular processes may modulate TGF-β responses. Recently, the discovery of a physical interaction between Akt and Smad3 has presented a mechanism by which cancer cells could avoid TGF-β apoptotic or

growth inhibitory responses (90, 91). Akt, also known as protein kinase B, is a serine-threonine kinase. Following stimulation by growth factors such as insulin-like growth factor (IGF), phosphoinositide 3-kinase (PI-3K) is recruited to the receptor resulting in the production of phosphatidylinositol-3, 4,5-triphosphate (PIP3). This attracts Akt to the membrane where it is phosphorylated at T308 and S473 by phosphoinositide-dependent kinase-1 (PDK-1). The activated Akt is then released from the membrane and translocates to other subcellular compartments (92, 93). Akt has been reported to be constitutively activated in a variety of cancers including prostate cancer (80, 93).

SDF-1/CXCR4 signaling

The human chemokine system includes more than 40 chemokines and 18 chemokine receptors. Chemokines are small secreted proteins that can be separated into two main subfamilies based on whether the two conserved cysteine residues are separated by an intervening amino acid, accounting for CXC or CC chemokines (94). These small pro-inflammatory cytokines bind to specific-G-protein coupled receptors which were initially found on leukocytes, to play an important role in the homing of such cells to sites of inflammation (95). CXC-motif chemokines ligand 12 (CXCL12), originally called Stromal-derived factor-1 (SDF-1) is a chemokine which is constitutively expressed by stromal cells in many tissues, (including lung, liver, lymph nodes, bone marrow, and adrenal glands), and is generally considered to be homeostatic (96). CXCL12 appears to be the sole ligand for the G-protein coupled receptor, CXC-motif chemokines receptor 4 (CXCR4). Mice with targeted deletion of CXCL12 and CXCR4 displayed identical phenotypes: they are lethal with deficient B lymphopoiesis and myelopoiesis, as well as

abnormal neuronal and cardiovascular development (97, 98). Two different isoforms of this chemokine have been identified in humans, CXCL12α and β. These isoform are identical except for an additional four amino acids on the carboxyl terminus of CXCL12β. CXCL12α and β do not appear to differ in their functions as ligands for CXCR4, based on structure–function studies showing that the amino terminal region of CXCL12 is critical for activating the receptor (99). As the only specific G-protein coupled receptor, CXCR4 is detected in a range of adult tissues. SDF-1/CXCR4 has a critical role in migration and patterning of a number of embryonic cell lineages. Signaling via this pathway is also involved in proliferation, survival, homing and retention of primitive hematopoietic CD34+ progenitor cells (HPC) in bone marrow (100, 101). Since CXCR4 is expressed on at least 23 different types of tumor cells, these CXCR4 positive tumor cells may metastasize to organs that secrete/express SDF-1 (e.g., bones, lymph nodes, lung and liver), so that SDF-1 exerts effects regulating processes essential to tumour metastasis (reviewed in (102, 103). The rationale connecting CXCL12/CXCR4 with cancer has been supported by both clinical and laboratory observations. In breast cancer, the expression of functional CXCR4 is elevated when compared to normal breast tissue. Treatment with anti-CXCR4 monoclonal antibody impairs metastasis (104). Similar observations were obtained in the prostate. SDF-1/CXCR4 expression is not seen in normal benign human prostate tissue but has been detected in human prostate cancer cells and tissues (105). Cher's group found that exogenous CXCL12 induced MMP-9 expression and Akt phosphorylation; bone stromal cells and bone tissue conditioned medium induced the migration of prostate cancer cells in a CXCR4-dependent manner.

Neutralizing this pathway has also been demonstrated to block the migration of prostate cancer cells to bone (106, 107).

Beside their involvement in invasion and metastasis, subsequent research has showed that CXCL12/CXCR4 signaling is functional in many other processes of carcinogenesis. The elevated level of ligand CXCR12 secreted from carcinoma-associated fibroblasts could directly stimulate the cancer cells' growth through a paracrine manner. CXCL12 and CXCR4 can activate the PI3K/Akt activity pathway, which functions to inhibit apoptosis and prolong cell survival in many cancer cells (108-110). MAPKinase is an alternative pathway for CXCR4 to regulate cancer cell growth, since brain tumor growth was reduced in mice treated with CXCR4 inhibitor AMD3100, an effect that correlated with loss of Erk activation *in vivo* (109). Secondly, CXCR4 can promote tumor angiogenesis and thus tumor growth by attracting endothelial cells to the tumor microenvironment. When mice carrying the MCF-ras tumor are treated with CXCL12 neutralizing antibody, they exhibited reduced tumor volume and decreased tumor weight due to a reduction in microvascular density (94, 111). All the above studies provided evidence showing the importance of CXCL12/CXCR4 signaling in multiple processes of cancer initiation and progression. We were interested in investigating this function in prostatic epithelium-stroma interactions and determining how this contributes to prostate cancer progression.

Hypothesis

We hypothesize that the prostatic tumor stroma promotes tumorigenesis via secreted molecules which interact with the adjacent epithelial cells. The elevated TGF-β and SDF-1 secreted from CAF in parallel are hypothesized to be necessary communicating factors between the stroma and epithelia. By integrating these two signaling pathways, CAF can modify the epithelial cell response to TGF-β, which enables CAF to induce tumor formation and promote invasion from the initiated but nontumorigenic BPH1 cells.

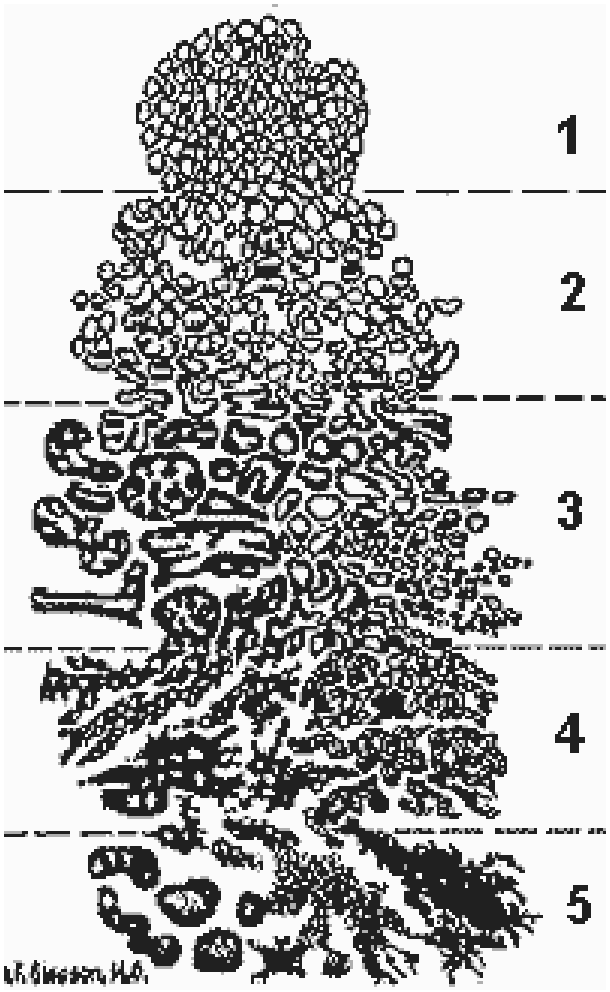
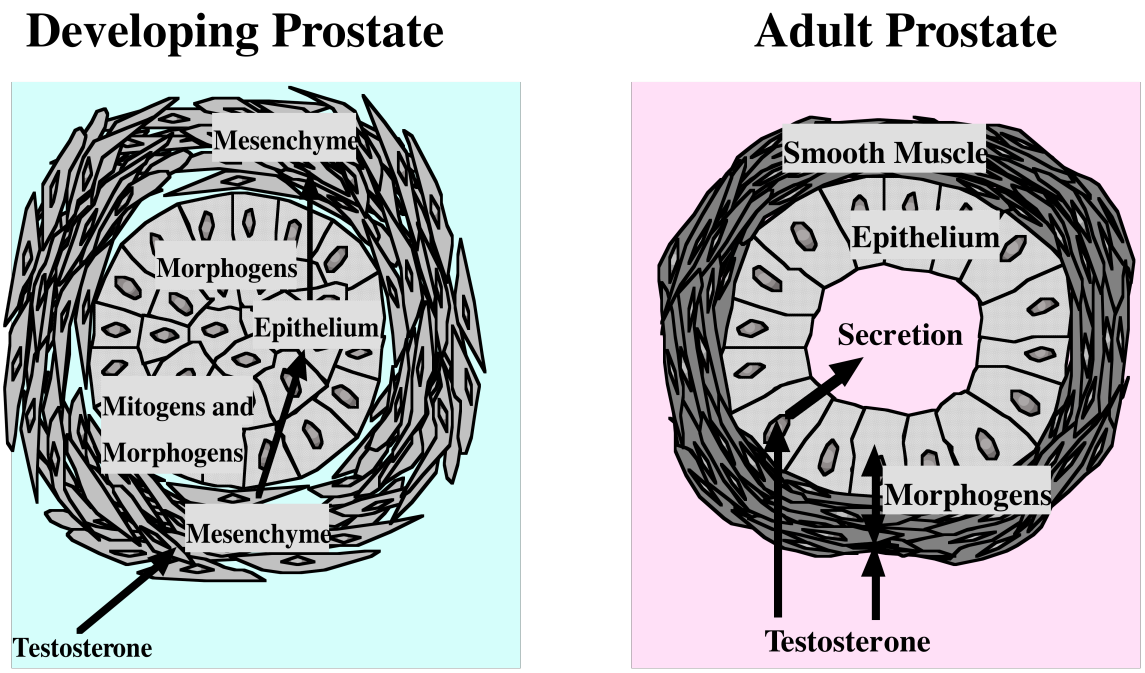


Figure 1-1. The Gleason grading system

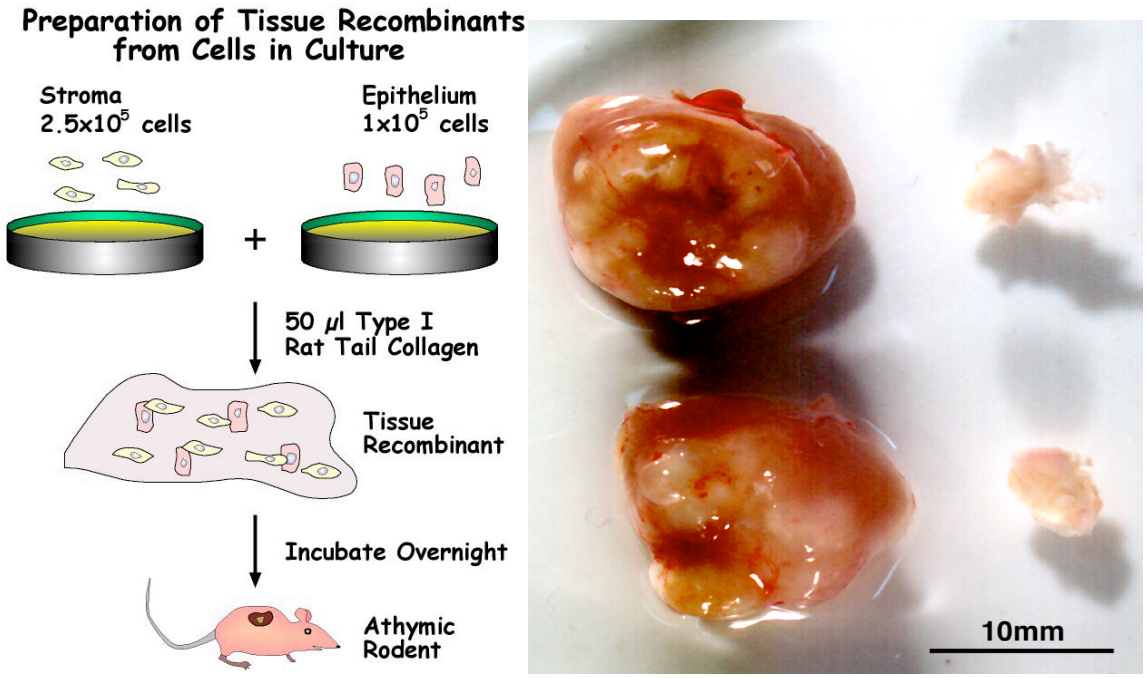
This is a picture, adapted from Gleason's 1977 article, demonstrating the changes in gland pattern as one goes from grade 1 to grade 5. The pathologist looks for a major pattern and a minor pattern to give a Gleason sum between 2 and 10. The glands in grade 1 cancer are small and round. Grade 5 cancer is hardly forming glands at all.



Simon W.Hayward, and Gerald R. Cunha, 2000.

Figure 1-2. Reciprocal epithelial-stromal interactions

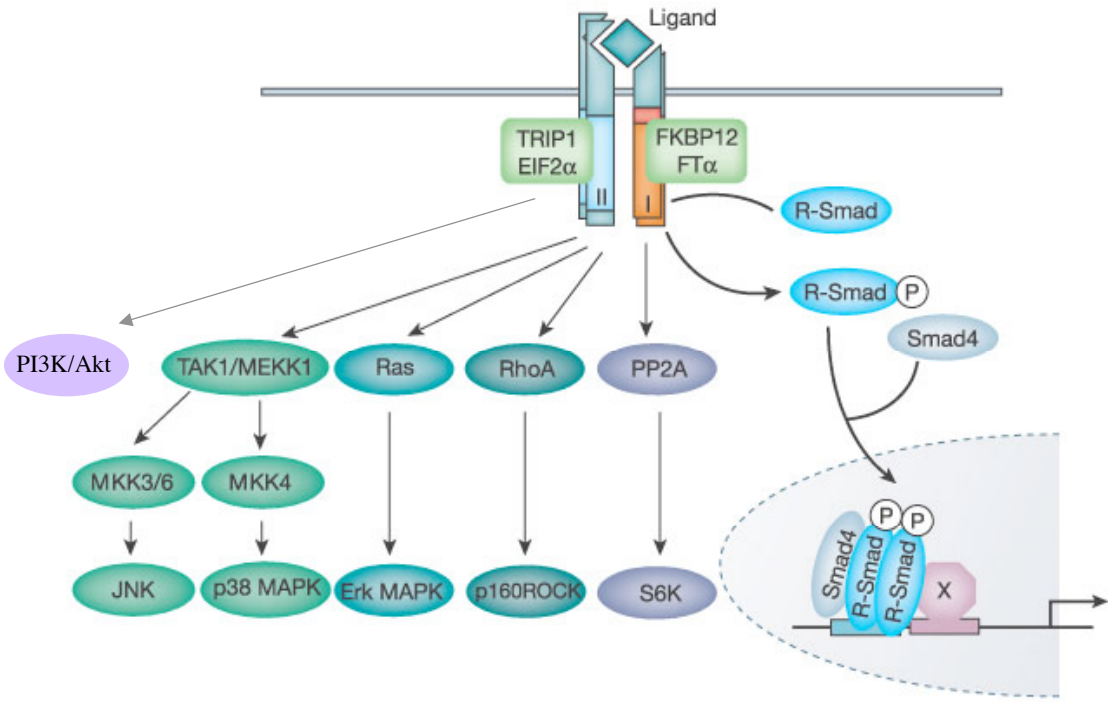
In the developing prostate, low levels of androgens act on the androgen receptor mainly locate in the mesenchyme to induce prostatic ductal morphogenesis, epithelial proliferation and glandular architecture, in turn, the developing epithelium induces mesenchymal differentiation into prostatic smooth muscle. In the adult prostate, high level of androgen act on androgen receptors located in both the epithelium and the mesenchyme. On one side, androgens act through the prostatic smooth muscle to maintain a fully differentiated, growth quiescent epithelium; meanwhile, they also act directly on the luminal epithelium to induce secretory activity.



Olumi et al., Cancer Research 1999

Figure 1-3. Kidney xenografting and CAF induced tumor

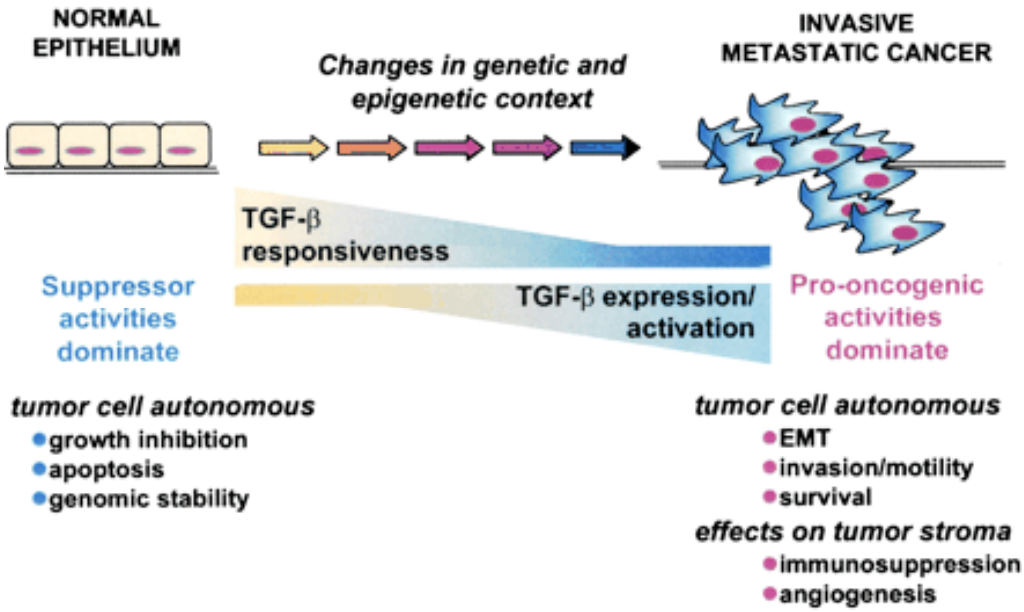
100,000 epithelial cells and 250,000 Normal Prostate Fibroblast (NPF) or Carcinoma Associated Fibroblasts (CAF) cells were resuspended in 50μl of rat tail collagen, the resultant gels were incubated overnight in a 5% CO_2 humidified incubator at 37°C in complete RPMI 1640 and subsequently placed beneath the renal capsule of male athymic mice. The dramatic interaction between CAF and BPH1 resulted in the huge tumor mass (left whole mount image), which is 200-times larger than the NPF + BPH1 control (right whole mount image).



Modified from RIK□DERYNCK AND YING□E.□ZHANG *Nature* **425**, 577 - 584 (09 October 2003)

Figure 1-4. TGF-beta signaling pathway

Following ligand binding to T□RII, T□RI is recruited to the complex. T□RI kinase is activated by T□RII, and, in turn, phosphorylates Smad2 and Smad3 which are known as R-Smads. Smad2/3 then associate with Smad4, and translocate to the nucleus, where they regulate gene transcription. The Smad pathway was the first signaling pathway identified to mediate TGF-□ effects and remains the best characterized. More recently, as indicated, multiple non-Smad pathways have been implicated in mediating TGF-□ effects downstream of the receptors.



From **Anita B. Roberts and Lalage M. Wakefield** PNAS | **July 22, 2003** | vol. 100 | no. 15 | **8621-8623**

Figure 1-5. Two faces of TGF-β in carcinogenesis

TGF-β switches from tumor suppressor in the premalignant stages of tumorigenesis to pro-oncogene at later stages of disease leading to metastasis. Progression to metastatic disease is generally accompanied by decreased or altered TGF-β responsiveness and increased expression or activation of the TGF-β ligand. These perturbations, along with other changes in genetic or epigenetic context of the tumor cell and its stromal environment, combine to alter the spectrum of biological responses to TGF-β

CHAPTER II

MATERIALS AND METHODS

Growth inhibition

Each cell line was seeded at 35,000 cells per well in 24-well plates in serum-free RPMI medium (Invitrogen, Carlsbad, CA.), after growth and attachment overnight, the cells were treated with or without 5ng/ml TGF-β1 (R&D Inc., Minneapolis, MN) in serum-free RPMI medium (Invitrogen). For conditioned medium Co-culture, the culture medium was replaced and the cells were treated with reagents diluted in conditioned medium collected from CAF or NPF cells or in serum-free RPMI medium (Invitrogen). 10μg/ml TGF-β (1,2,3) blocking Ab (Clone 1D11, R&D, Minneapolis, MN), 5ng/ml TGF-β ligand (R&D Inc.), 100ng/ml SDF-1 (Peprotech Inc., Rocky Hill, NJ) were added as indicated in figure legends. Cell growth was terminated by adding Cell Titer 96 Aqueous One solution (Promega, Madison, WI) at indicated time points. After 2 hours incubation at 37°C, absorbance at 490nm wavelength was read using a microplate reader.

Genetic modification of cell lines

DA (constitutively active TGF-β type I receptor) (112), Akt1 (myristylated Akt1) (113), full length human SMAD3 (114) and DN (dominant negative type II receptor) (78, 115) were inserted into a LZRS-EGFP backbone (Nolan Laboratory, Stanford, CA) as previously described (45). Briefly, the CMV promoter was excised from pIRES-EGFP (Clontech, Palo Alto, CA) as a BglII/BamHI fragment. The fragment was ligated into the

BamHI site of the LZRS-EGFP backbone to give C7 and the resultant constructs were used to transfect amphotropic PHNXA packaging cells which were obtained from ATCC [under an MTA from the Nolan laboratory Stanford (www.stanford.edu/group/nolan)] using Lipofectmine 2000 (Invitrogen), the viral supernatant from the transfected cells was centrifuged at 3,000 rpm and passed through a 0.22 m filter. Successive rounds of infection over 5 days were employed. The infected cells were selected either by FACS (Fluorescence Activated Cell Sorting), based upon expression of a bicistronic fluorescent tag or, as appropriate, by antibiotic resistance. The empty vector was used to generate negative controls. The cells were maintained in RPMI-1640 medium per the source lines. 3TP-Luc promotor activity was used to confirm that the TGF- signaling is constitutively active in –DA or –Smad3 cells but inactive in –DN cells (116, 117). Western blotting assay was used to confirm that phosphorylated Akt level is elevated in -Akt cells. The shRNA-CXCR4 or shRNA-GFP control plasmids (kindly provided by Dr. Robert Weinberg) were engineered into BPH1 cells by lentivirus infection as described in (118). Briefly, the plasmids were transiently transfected into 293FT cell line (Invitrogen Co.) using Lipofectmine 2000 (Invitrogen Co.), the virus were harvested at 48 and 72 hours posttransfection and infections were carried out in the same way as retroviral infection process described above. Following transduction, the cells were selected with 5 g/ml Puromycin (Sigma) for 4 days and used for experiments.

Cell and Tissue Immunofluorescence Staining

100k cells suspended in RPMI containing 5% FBS were plated on glass slides, allowed to attach and grow overnight. To test the response to TGF-, BPH1 cells and

BPH1-^{CAFTD}1,3,5 cells were treated with 5ng/ml TGF-β1 for 4 hours after serum starvation for 24 hours. After fixation in methanol for 5 minutes at -20°C, samples were washed twice in PBS, and then blocked for 30 minutes with 5% goat serum (Vector Laboratories, Burlingame, CA). Incubated at room temperature for 1 hour with primary antibodies against Vimentin (1:100, Sigma, St. Louis, MO), wide spectrum keratin (1:100, DAKO, Carpinteria, CA), Smad3 and p21 (1:100,Santa Cruz, CA) and CXCR4 (1:50, R&D) followed with washing for 30 minutes in PBS. The slides were then incubated for 30 minutes with fluorescence-conjugated secondary antibodies, washed and mounted using Vectorshield with DAPI or PI (Propidium Iodide, Vector laboratories).

For histological analysis on tissue, 5μm tissue sections were cut and placed on glass slides. After de-waxing with histo-clear (National diagnostics, Atlanta, GA), the antigen was unmasked by heating samples in antigen unmasking solution (Vector Laboratories). Slides were blocked in 5% goat serum and 2% BSA in PBS for 30 minutes at room temperature before incubating with primary antibodies (1:100) mouse anti-vimentin (V-6630, IgG1, Sigma, which recognizes human, but not mouse, vimentin), mouse anti-SV40 Tag (IgG2a, Santa Cruz Biotechnology, Santa Cruz, CA) and CXCR4 (1:50, R&D) for one hour. After one hour washing in PBS buffer slides were incubated with secondary antibodies (1:200) (Alexa Flor488 anti-mouse IgG1 and Alexa Flor546 anti-mouse IgG2a, Molecular Probes, Eugene, Oregon) for 30 minutes at room temperature. Tissue sections were washed for 30 minutes in PBS and mounted. They were then visualized by conventional fluorescent microscopy.

Cross-linking assay

Cells were cultured in 6-well plates until 100% confluent, washed three times over 30 min with 500 μl of ice-cold 0.1% bovine serum albumin dissolved in Dulbecco's phosphate-buffered saline (D-PBS) containing Ca^{2+} and Mg^{2+}. The cells were then affinity labeled with 100 pM ^{125}I-TGF-β1 with or without a 100-fold molar excess of unlabeled TGF-β1. Using previously described methods (77), the radiolabeled cells were then incubated on a rocking shaker (30 cycles/min) at 4°C for 3 h, washed twice with 2 ml of cold wash D-PBS, and the ligand-receptor complexes were cross-linked with 400μl of 1mM bis (sulfosuccinimidyl) suberate (BS3; Pierce biotech, Rockford, IL) for 10 min on ice. The cross-linking reaction was stopped with the addition of 100μl of 500 mM glycine. Cells were washed twice with 500 μl of D-PBS and solubilized with 125 μl of 20 mM Tris buffer, pH 7.4, containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml pepstatin, and 2μg/ml soybean trypsin inhibitor. Solubilized material was centrifuged for 10 min at 4°C to pellet cell debris. The supernatants were transferred to one-fifth volume of 5 x electrophoresis sample buffer, boiled, and vortex mixed. All samples were analyzed using a gradient (3-12%) SDS-PAGE and visualized by autoradiography.

Cell fractionation

Cells were seeded in 75cm^2 flasks with RPMI1640 medium containing 5% FBS (Atlanta Biologicals). After overnight starvation in serum-free RPMI medium, the cells were treated with 5ng/ml TGF-β1 (R&D) for four hours. The nuclear and cytoplasmic subcellular fractions were prepared using Pierce Extraction Reagent (Pierce biotech,).

Briefly, the cells were trypsinized and washed with cold PBS. Cell pellets were resuspended with appropriate volumes of CERI, CERII and NER successively according to the manufacturer's instruction. The resulting cytoplasmic and nuclear extracts were quantified using the BCA protein assay (Bio-Rad, Hercules, CA) and kept at -80^{0}C until use.

Cell motility Assay

To test cellular motility, wound healing and transwell migration assays were used. For wound healing, a section of a confluent cell monolayer was wounded with a pipette tip, and the ability of cells to migrate into the cleared section was observed at different time points as specified in the results section (119). A Boyden chamber system was used for transwell migration assay. Polycarbonate inserts with 8.0μm pore size (Becton Dickinson Labware, Franklin Lakes, NJ) were coated with 500 l 250 g/ml rat tail collagen I, air dried at room temperature and kept sterile at 4° C before use. One hour before an experiment, the inserts were blocked using 1%BSA in PBS at 37° C. 100 l suspension containing 100,000 cells was loaded into each insert. Various concentrations of FBS (0, 0.5% and 2.5%) in 500μl RPMI medium were applied to the lower chamber. After incubation for 8 hours, cells remaining in the top of the inserts were removed using a cotton swab. The cells which had migrated through the collagen and the filter were fixed with 11% Glutaraldehyde (Sigma) for 20 minutes followed with 0.1% crystal violet staining and counted in five random fields. The mean of the number was used to quantitate the migration. The experiments were performed in triplicate wells.

Western blot Assay

Cells were detached by trypsinization, after washing with cold phosphate-buffered saline, lysed with TNN buffer (50mM tris HCl, 150mM NaCl, 0.5%NP-40, pH 7.4) containing proteinase inhibitor (Roche, Nutley, NJ) and phosphatase inhibitor (Sigma). Lysates were clarified at 13,000 rpm for 20 min at 4 °C, and supernatants were quantified by the BCA protein assay using protein assay dye reagent (Bio-Rad). Proteins were loaded and electrophoresed through 10% NuPAGE BisTris gel (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 1 h in PBS-T containing 5% nonfat dry milk and incubated with primary antibody overnight (vimentin 1:1000, v-6630, Sigma; E-cadherin,1:3000 transduction laboratory, BD Biosciences Pharmingen,; keratin 1:2000, DAKO; Smad3, 1:500, sc-8332, and p21, 1:300, sc-6246, are from Santa Cruz Biotechnology; p-Akt, 1:500, p-Smad2, 1:1000 and p-GSK3□/□ 1:1000 are from Cell Signaling; □-actin, 1:5000, Sigma; Histone H1, 1:5000, ab11079, Abcam Inc., Cambridge, MA), washed 30 minutes in PBS-T, followed with the horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1,000; DAKO) incubation for one hour. ECL detection reagent (Amersham Biosciences UK Ltd, Little Chalfont, Buckinghamshire, UK) was used to visualize protein bands.

Cell Cycle Analysis

BPH1 cells or BPH1 caftd1 cells were starved with serum-free RPMI1640 medium overnight, treated with or without 5ng/ml TGF-□1 for 4 hours until trypsinization. The cells were washed and resuspended in PBS containing 2% FBS at the concentration of 10^6 cells/ml before fixing with 100% ethanol at 4°C one hour. Washing twice with PBS,

the cells were incubated with 1ml PI (propidium Iodide)/RNAse mixture (working dilution: PI: 50□g/ml; RNAse 0.5□g/ml) at 4°C for three hours until analysis with cell flow cytometry.

Tissue recombination and kidney xenografting

Rat UGM (rUGM) was prepared from 18-day embryonic fetuses of pregnant Sprague-Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN, USA). Dissection and separation of urogenital sinus epithelium (UGE) and UGM were performed as previously described (10, 120). Briefly, urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by a 90-min digestion at 4°C with 10 mg/ml trypsin 1:250 (Sigma) followed by mechanical separation. UGM was then additionally reduced to single cells by a 90-min digestion at 37°C with 187U/ml collagenase (Gibco). After digestion, the cells were washed extensively with RPMI 1640. Viable cells were then counted using a hemacytometer.

To prepare tissue recombinants, rUGM or CAF cells were mixed with BPH-1 cells at a ratio of 250,000 stromal cells to 100,000 BPH-1 cells in a sterile microcentrifuge tube inRPMI 1640. The cell mixture or 100K sorted infected BPH1^{CAFTD1}-EV or BPH1^{CAFTD1}-DA epithelial cell lines alone were pelleted and resuspended in 50 μl of rat-tail collagen (pretitrated to pH 7.4) and inoculated in the center of a well of a six-well- plate. After collagen polymerization the "button" was overlaid with normal growth medium (RPMI 1640 plus 5% fetal bovine serum). The grafts were then incubated at 37^{0}C overnight, and the tissue recombinant was grafted under the renal capsule of a SCID mouse using

published protocols from Dr. Cunha's laboratory (46, 47, 121). Eight to twelve weeks after grafting, the hosts were sacrificed. Harvested grafts were fixed in paraffin and embedded for histological and immunohistochemical Analysis.

Flow cytometry for CXCR4

Cells were harvested and resuspended in L-15 medium (Sigma) at a concentration of $2x10^7$ml; 50μl cell suspension was incubated with 50μl monoclonal mouse anti-human CXCR4 (R&D, Inc., 10ug/ml diluted in L-15) on ice for one hour. After three times washing, cells were then incubated with Allophycocyanin (APC) labeled anti-mouse secondary antibody (BD Pharmingen, San Diego, CA) 30 minutes on ice, washed three times before sent for flow cytometry analysis.

Co-culture

Fibroblasts (CAF or NPF) were seeded with 5%FBS RPMI medium (Invitrogen), at the density of 500K per 75cm^2 flask (Corning Incorporation, Corning, NY, USA), allowed to grow and attach overnight, the culture medium was aspired and a suspension of 200K BPH1 cells was seeded on top of fibroblasts in flasks. The mixture of cells was incubated at 37^0C for another 24 hour. The medium was collected, centrifuged and passed through 0.22μm filter (Millipore, Carrigtwohill, Co. Cork, Ireland), stored at $–80^0$C for use.

Treatment on the host mice with TGF-β neutralizing Antibody (2G7)

The host SCID mice were intraperitoneally injected with 2G7 (Eli Lily, Indianapolis, 300 g/mouse (122), starting one day prior to xenografting surgery and subsequently injected twice weekly at the same dose (300 g/mouse) for a period of 8 weeks until the mice were sacrificed.

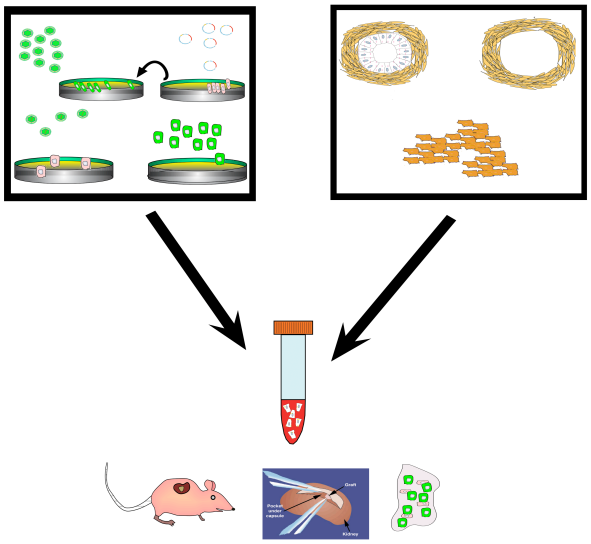


Figure 2-1. Tissue recombination and xenografting

Epithelial cells were genetically modified using retroviral transduction. Cells stably expressing the introduced genes were selected and mixed with rat UGM cells. The cell mixture was resuspended in rat tail collagen gel and allowed to polymerize. After collagen polymerization, the tissue recombinants were grafted beneath kidney capsule of immunocompromised mice.

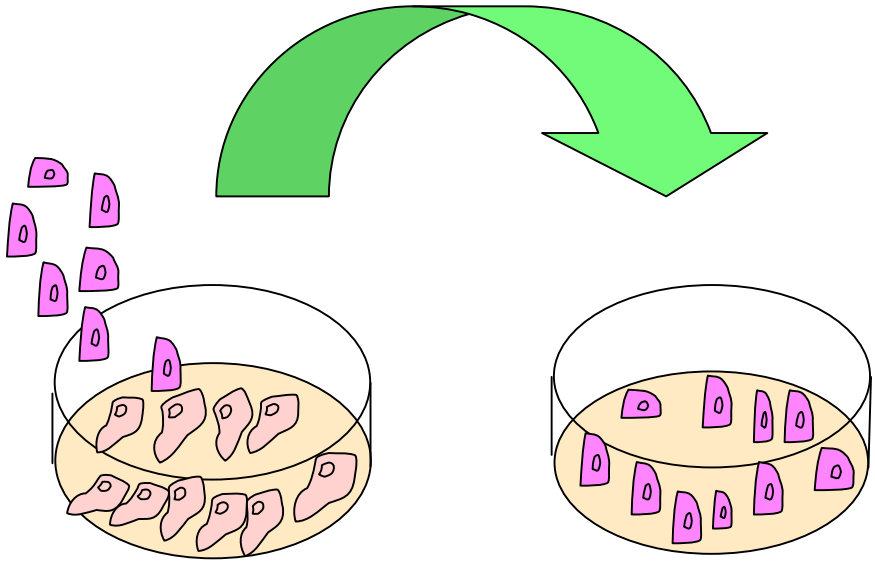


Figure 2-2. Conditioned medium collected from co-culture

Fibroblasts were grown in culture dish until 70-80% confluence. Epithelial cells (10,000 cells/cm^2) were then seeded on top of fibroblastic layer. Conditioned medium was collected 24 hours after the initiation of co-culture. Conditioned medium was used to feed a reporter population of BPH1 cells.

CHAPTER III

TGF-BETA IS AN ESSENTIAL SIGNALING COMPONENT IN CAF-INDUCED TUMORIGENESIS

Introduction

The cancer cell genotype and phenotype have been considered the only determinants supporting cancer initiation and progression, while the microenvironment has long been ignored as a passive supporting meshwork. However a series of observations by both clinical pathologists and experimental scientists have changed this traditional concept about cancer. A host of morphological and biochemical transitions develop in the cancer-associated stromal environment in response to the epithelial cells' carcinogenesis. Further investigations have demonstrated that such reactive tumor stroma promotes the growth and survival of cancer cells and determines the success of cancer colonization at secondary sites (123-125). All of these observations suggest that stroma not only provides fertile soil for cancer growth, but also exerts dominant inductive influences that trigger permanent genetic and phenotypic changes in cancer cells. These significant features become potential biomarkers for cancer prognosis and treatment (62, 126). The concept of the functional tumor stroma has been supported by the studies on breast, colon, and lung cancer, as well as in prostate cancer (60, 61, 64). Olumi et al used tissue recombination and the subrenal capsule xenografting model to extend the understanding about prostatic tumor stroma (61). They recombined a series of human fibroblasts and epithelial cells to observe epithelial-stromal interactions and the resultant tumor formation. Their data showed that CAF and normal fibroblasts exerted different effects

on target BPH1 cells *in vivo*. CAF cells were able to induce a tumor from the initiated but non-tumorigenic BPH1 cells. The same fibroblasts were not capable of inducing this phenotype in genetically normal human prostate epithelial cells (70) (**Figure 3-1**). These data improved the understanding of stroma-epithelial interaction in prostate cancer in the following ways: both genetic and epigenetic changes may be important in human prostate cancer; CAF cells could promote cancer progression of an initiated epithelial cell but normal fibroblasts could not; CAF cells could not initiate genetically normal epithelial cells (reviewed in 55).

Concepts about the role of stroma and the tumor–stroma interaction have been changed such that now many studies have focussed on identifying the signaling between cancer cells and the adjacent tumor stroma. Among the candidate modulators of this communication, there is a large number of soluble factors (chemokines and cytokines). For example, elevated vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF or FGF2) have been shown in tumor-surrounding stromal cells, where they can contribute to the angiogenesis required for maintenance and stimulation of tumor growth (127). The insulin like growth factor-1 (IGF-1) pathway has been shown to be involved with malignant transformation. Prostate cancer cells are sensitive to surrounding IGF-1 levels. Increased IGF-1 levels in stroma may not only be associated with an increased prostate cancer risk but may also be a useful tool for early detection (128). Hepatocyte growth factor/scatter factor secreted from stroma can work through and its receptor c-met in epithelial cells to regulate cancer cell growth and metastasis (129). During the process of tumorigenesis prostate cancer epithelium has been shown to

initiate expression of HGF allowing this normally paracrine-acting growth factor to elicit autocrine functions (130).

In the present study we measured the TGF-β1 secreted by fibroblastic cells of normal or malignant human prostate (NPF and CAF), and demonstrated their effects on target human prostatic BPH1 epithelial cells *in vitro* using a co-culture system, and *in vivo* by tissue recombination. The results replicated the interaction between the tumor stromal cells and initiated epithelial BPH1 cells, and confirmed that CAF have an inductive effect on the initiated BPH1 cells resulting in tumorigenesis. Furthermore, this study demonstrated that the CAF cells secrete elevated levels of TGF-β1, which is essential for CAF cells to stimulate BPH1 cells' proliferation and motility and is required for tumor formation *in vivo*.

Results

CAF conditioned medium promotes proliferation and enhances motility in BPH1 cell culture

To examine the communicating factors active in stromal-epithelial interactions we chose a co-culture system as a first approach to model this interaction. There are two commonly used co-culture methods: one is the use of conditioned medium from one cell type to support the growth of another type of cell; the second method is to use inserts separating the two types of cells, which allows for continuous communication (165). A potential disadvantage of these methods is that the cells have no direct contact. This means that factors which are elevated overall as a function of intercommunication

between the cells would not be detected. We therefore modified the approaches as described in the methodology section, culturing epithelial cells in direct contact with stromal layers to provide continuous and direct contact. We then collected the conditioned medium which contained secreted factors to feed separate epithelial cells. We found that conditioned medium from CAF can stimulate the growth of BPH1 epithelial cells, compared to the conditioned medium collected from the counterpart NPF. The difference in growth rate can be somewhat reduced by using a neutralizing antibody to TGF-β ligands (**Figure 3-2**). In addition to uncontrolled proliferation, tumor cells usually have dysregulated motile ability which is required for cell invasion and metastasis (131). To test the effect of stromal conditioned medium on the motility of BPH1 cells we conducted a wound healing assay. As shown in **Figure 3-3**, the BPH1 cells closed the wound significantly faster when fed with CAF conditioned medium than the cells in NPF conditioned medium; However, when BPH1 cells lose responsiveness to TGF-β by forced expression of the dominant negative truncated type two receptor (TβRII) conditioned media from CAF or NPF had apparently identical effects on epithelial cell motility. These two experiments suggested that TGF-β plays a role in CAF stimulation of BPH1 cell grow and a more important role in epithelial cell motility in cell culture.

TGF-β1 secretion is elevated in CAF as compared to NPF

Since CAF conditioned medium showed effects on BPH1 cells which were distinct from those of NPF conditioned medium, and some of these differences could be accounted for by TGF-β, we examined the secreted TGF-β in conditioned medium from these two types of fibroblasts using ELISA. As shown in **Figure 3-4**, CAF cells have

higher secretion of TGF-β1 than NPF (937ng/ml versa 327ng/ml, $p < 0.01$.) Note that this elevation of TGF-β is consistent in multi-groups of CAF and NPF, which confirmed that TGF-β is a possible functional factor by which CAF might induce tumorigenic responses from BPH1 cells.

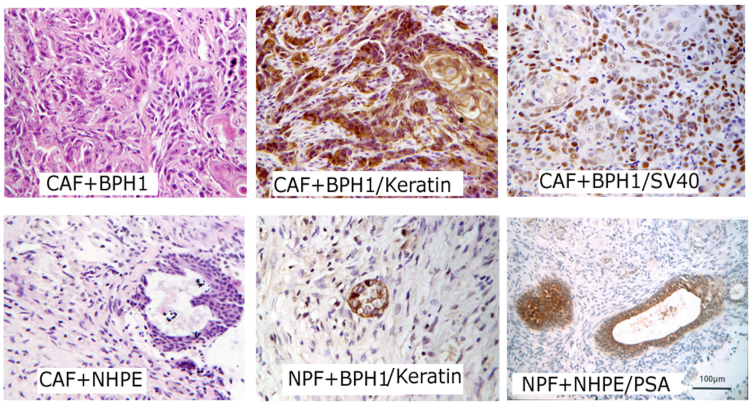
TGF-β is required for CAF to induce tumorigenesis in vivo

As demonstrated by Cunha's group (70), CAF induce tumor formation from the initiated but non-tumorigenic BPH1 cells. To test whether the effects of TGF-β on proliferation and motility contribute to this process, we repeated the experiments using tissue recombination and kidney capsule xenografting. To demonstrate that TGF-β is essential in this process, we took two approaches: 1) BPH1-DN cells (BPH cells engineered with dominant negative type II receptor) were used in tissue recombinants to demonstrate the effects of loss of epithelial response to the TGF-β ligands; 2) host mice were systematically treated with the TGF-β ligand neutralizing antibody 2G7 which limits availability of TGF-β to both epithelial and stromal cells. As shown in **Figures 3-5** & **3-6**, when TGF-β signaling is blocked either by losing responsiveness to ligand in BPH1 epithelial cells or by neutralizing the ligand secreted from CAF, tumor formation was severely impaired: the graft size was significantly reduced and the malignant structure disappeared. These data indicate that TGF-β is necessary for CAF to induce tumor from BPH1 cells. Of interest there were clear phenotypic differences between the two conditions suggesting that TGF-β signaling is playing a role in malignant stromal as well as epithelial phenotype.

Discussion

There is growing evidence demonstrating that stroma and its interactions with cancer cells are important for tumorigenesis. Pathologists have observed stromal changes in tumors with altered morphology and gene expression. Enhanced collagen production and stimulation of hyaluronate synthesis were observed in human basal cell carcinoma stroma (132). Disorganized growth patterns, uncontrolled growth and altered proliferation potential of these fibroblasts in lung carcinoma, pancreatic carcinoma, and melanoma were also reported (133). Once activated to differentiate, myofibroblasts produce a myriad of potential paracrine-acting mediators, including TGF-β, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1). All of these have previously been defined as contributing to the proliferation and expansion of the tumor cell clone by paracrine routes (reviewed in (64). In prostate, it has been shown that carcinoma associated fibroblasts can stimulate tumor progression of initiated nontumorigenic epithelial cells both *in vivo* and *in vitro* (70), which builds on the concept that the stroma is an active rather than passive tissue during carcinogenesis. The present study is a follow up investigation which aims to identify the communicating factors acting between CAF and adjacent epithelial cells *in vitro* and *in vivo*. Experiments were performed to measure the quantity and functions of TGF-β1 secreted from CAF and NPF, to test the effects of TGF-β on BPH1 cell growth, to investigate the cells' motile ability upon TGF-β treatment *in vitro*. Furthermore, tissue recombination and subrenal capsule xenografting were used to elucidate the importance of TGF-β in the tumor formation from the CAF

and BPH1 cell recombinant. The data show that, compared to their normal counterparts, CAF secrete elevated quantities of TGF-β which contributes to the epithelial cells proliferation, enhances the cell motility *in vitro* and contributes to tumor formation *in vivo*. All of the effects of CAF on BPH1 cells which were examined could be impaired when TGF-β signaling is blocked either by neutralizing antibody or by engineering the dominant negative receptor into the epithelial cells. This difference between CAF and NPF supports the concept that tumor stroma develops biochemical alterations during cancer progression and that the altered stroma can promote the tumorigenic phenotype of adjacent epithelial cells. TGF-β signaling is a necessary component in mediating the interaction between prostatic stroma and epithelium. This is an important first step in assessing signal transduction pathways critical in tumor progression that involve stromal-epithelial interactions.



Olumi, A. F. et al. Cancer Res 1999; 59:5002-5011

Figure3-1. Histological appearance of tissue recombinants

Histology of tissue recombinants demonstrates the functional role of CAFs in the stimulation of tumorigenesis and illustrates the observations that were the basis for this project. Normal and carcinoma-associated prostatic fibroblasts were combined with both normal and initiated (non-tumorigenic, Simian virus-40 large T-antigen-expressing, immortalized) human prostatic epithelial cells in a recombinant graft placed under the renal capsule of athymic nude mice. Normal fibroblasts promoted normal or hypoplastic epithelial structures in normal and initiated epithelia, respectively, but CAFs stimulated the formation of abnormal proliferative structures. When combined with normal epithelia, CAFs promote a piling up of epithelia, indicative of a hyperplastic response associated with prostatic intraepithelial neoplasia. When CAFs are combined with initiated epithelia, large tumor masses form with a disrupted architecture marked by streaming epithelia with enlarged nuclei. Recombinants composed of normal epithelium and normal fibroblasts undergo prostatic differentiation with expression of functional markers (PSA).

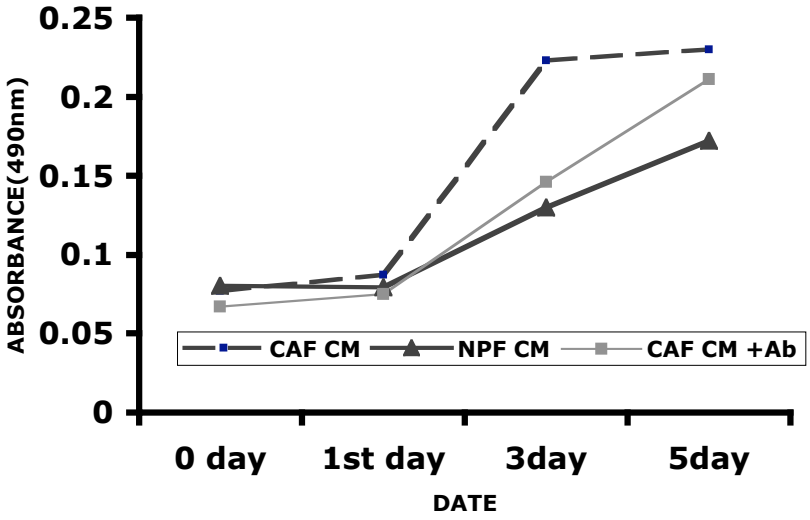


Figure 3-2. TGF-β is necessary for CAF to stimulate BPH1 proliferation

BPH1 cells were seeded in 24-well plates with serum-free RPMI 1640 until attached, conditioned media from CAF or NPF were used to replace the culture medium and the cells were then incubated at 37^0C. CAF conditioned medium stimulated epithelial cell proliferation, an effect which could be blocked by addition of 10μg/ml TGF-β (1,2,3) blocking Ab.

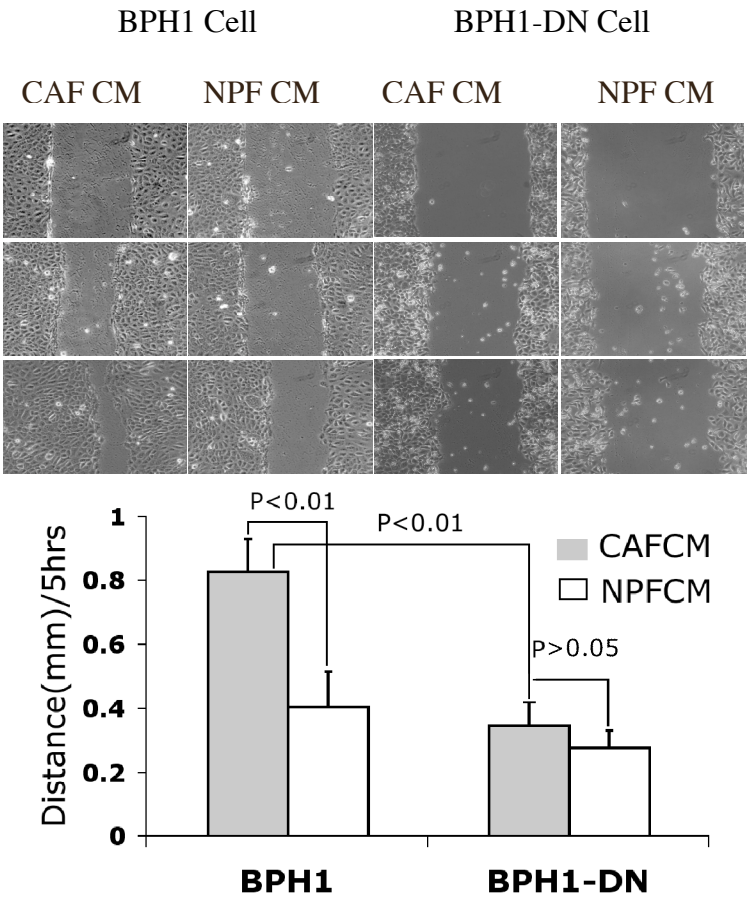


Figure 3-3.TGF-β is necessary for CAF to enhance BPH1 cell motility

Wound Closure assay shows that BPH1 cells exposed to conditioned medium from CAF cells (CAFCM) closed the wound faster than cells exposed to the conditioned medium from Normal Prostate Fibroblasts (NPFCM)($p<0.01$). In contrast cells expressing a dominant negative TGF-β receptor (BPH1-DN) closed wounds more slowly overall and did not show much difference in their response to CAFCM and NPFCM. All experiments were conducted in triplicate; the means were used for graph shown in the lower panel. The error bars show the standard deviation.

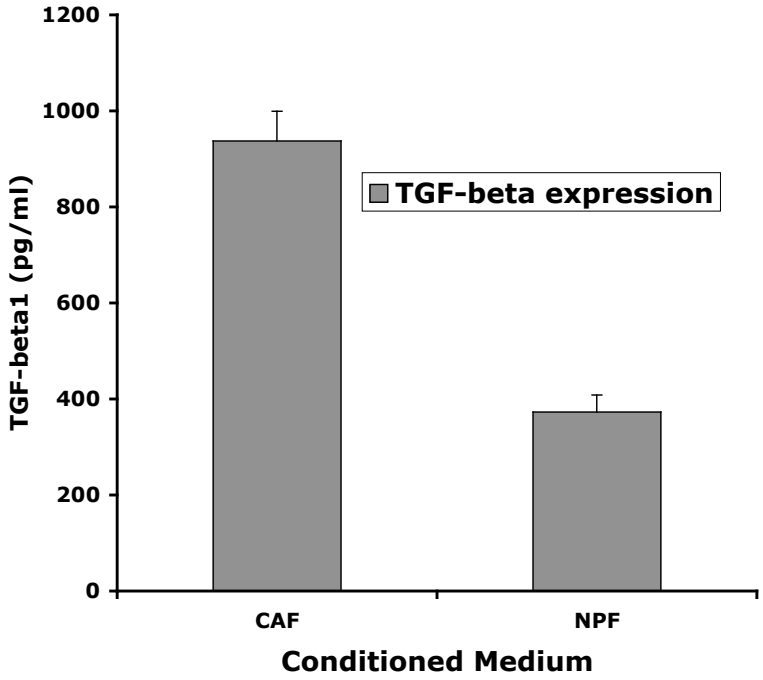


Figure 3-4. CAF has higher TGF-β1 secretion than NPF

ELISA shows that conditioned medium from CAF cells contains elevated levels of active TGF-β1 compared to conditioned medium from NPF. Similar results were gained from three different pairs of conditioned medium, the difference between matched CAF and NPF populations is consistently about three to four-fold. This graph shows representative data. All of the experiments were performed in triplicate; the error bars show the standard deviation.

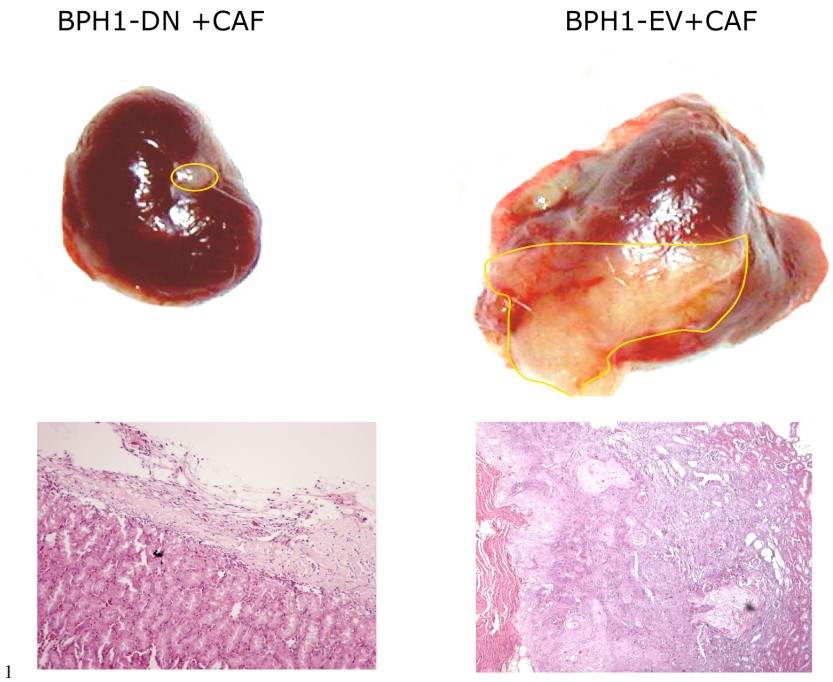


Figure 3-5. Loss of epithelial responsiveness to TGF-β blocks tumor formation induced by CAF

Retrovirally-infected BPH-1 cells which express dominant negative TGF-β type II receptor (BPH1-DN) or empty vector control infected cells (BPH1-EV) were recombined with CAF and grafted to SCID mice for eight weeks. When the epithelial cell responsiveness to TGF-β was suppressed by expression of the DN receptor, CAF did not induce tumor formation. Empty vector carried BPH1 cells in contrast formed large tumors. H&E staining shows that the tumors were composed of poorly differentiated, irregular epithelia cords. In some areas, epithelium formed small glandular nests while in other areas, epithelium appeared as single cells that were intermingled within fibrous stroma. The majority of epithelial cells contained large, pleomorphic nuclei with large nucleoli.

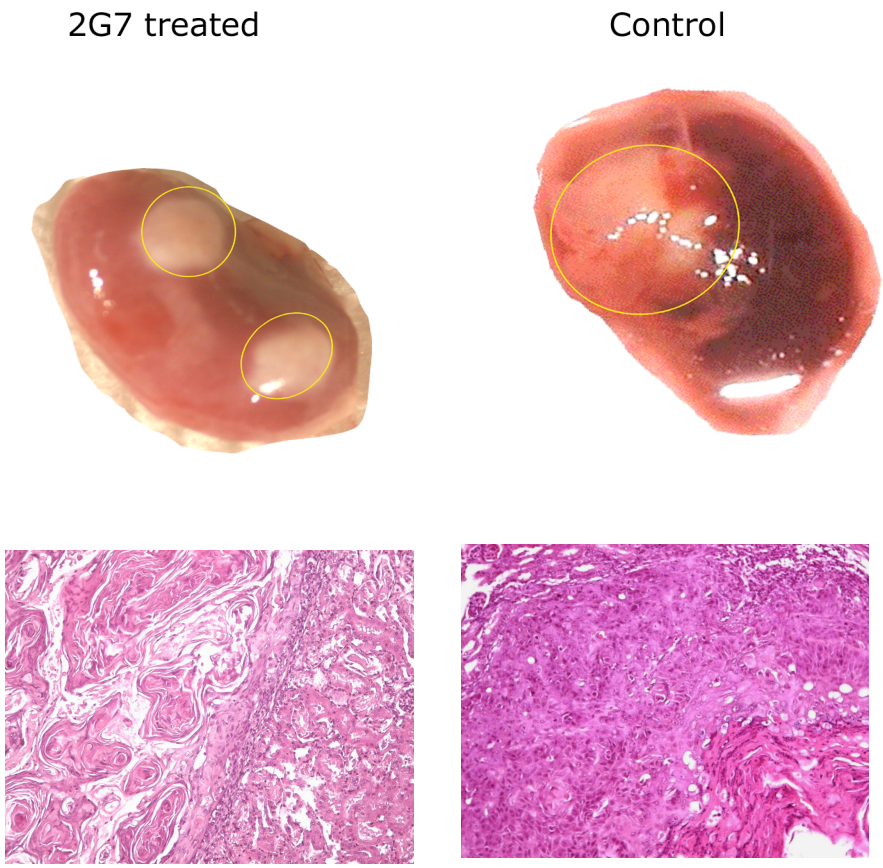


Figure 3-6. Systematic treatment of TGF-β ligand antibody 2G7 abrogates CAF-induced tumorigenesis

BPH1 cells were recombined with CAFs and xenografted beneath kidney capsule of SCID mice. The host mice were treated with or without TGF-β ligand inhibitor 2G7 300μg twice a week for 8 weeks. The control animals displayed a huge and bloody tumor with malignant histology, the streaming epithelia were intermingled into stroma, some areas show small keratin pearls. The inhibitor-treated animal provided significantly smaller and pale grafts filled with many keratin pearls.

CHAPTER IV

PROSTATIC TUMOR STROMA CAN PROMOTE CARCINOGENESIS BY MODIFYING ADJACENT EPITHELIAL CELLS' RESPONSE TO TGF-BETA VIA SDF-1/CXCR4

Introduction

Originally named for their transforming activities *in vivo*, TGF-βs play complicated roles including regulating cell proliferation, functional differentiation, extracellular matrix (ECM) production, cell motility, and apoptosis. In tumorigenesis, TGF-β apparently plays dual roles. In the current paradigm, TGF-β acts as a growth suppressor in normal tissue; but in tumors, changes in TGF-β expression and cellular responses tip the balance in favor of pro-oncogenic activity (74). This has been shown in prostate (79, 80) and many other cancer models (81-83). In benign prostatic epithelia, by eliciting differentiation, inhibiting proliferation and inducing apoptosis, TGF-β provides a mechanism to maintain homeostasis in the prostate (84-86). The ability of TGF-β to enhance tumorigenicity *in vivo* is illustrated by its role in many key processes including; stimulating angiogenesis, inhibiting immune surveillance or promoting the degradation of extracellular matrix (86, 87). TGF-β can also promote local invasion and metastasis through EMT (78, 88, 89).

Specific responses to TGF-β relate to a complex of factors including dose of bioavailable ligand, target cell type, cellular context and microenvironment. This reflects the complex network of cross-talking signals that constitute the transduction mechanism by which the cell responds to stimulation by the TGF-β ligand (72). Integration of the

TGF-β pathway with other signaling cascades that control the same cellular processes may modulate TGF-β responses. As we previously observed, TGF-β inhibits the growth of the initiated but nontumorigenic BPH1 cells (134), reconfirmed in Chapter III. Activating TGF-β signaling does not show effects in motility or protein expression profile which suggest that TGF-β is a tumor suppressor for BPH1 cells (Chapter V). However our current data as shown in chapter III indicate that TGF-β in CAF conditioned medium is playing a tumorigenic role. This conflict suggests that, since TGF-β is only one of the factors secreted from CAF, the aberrant phenomenology is very likely a result of other factor(s) secreted from the stroma (135, 136). We hypothesize that tumor stroma secretes other factors in parallel with TGF-β. TGF-β in this scenario, could integrate the signaling from different pathways; stroma could modulate the epithelial cell's response to the soluble factor milieu, and give rise to the final oncogenic activity.

Results

Stroma can modify BPH1 cells' response to TGF-β

In **Figure 4-1,** the results of a Western blotting assay demonstrated that when exposed to CAF conditioned medium, BPH1 cells showed a relative decrease in phosphorylated Smad2 in response to TGF-β. This observation is consistent with the immunohistochemistry shown in **Figure 4-2,** the tumorigenic grafts containing BPH1 cells plus CAFs have distinctly lower p-Smad2 staining, compared to the levels in the benign-appearing BPH1 plus rUGM recombinants.

SDF-1 expression is elevated in CAF compared to NPF

Previous studies using DNA microarray analysis to compare gene expression in CAF and NPF cells revealed that a number of soluble, potentially growth promoting, factors were overexpressed in CAF cells (137). In an attempt to determine which factor(s) secreted from CAFs might co-operate with TGF to promote growth we screened potential candidates by ELISA analysis of conditioned medium. Stromal cell-derived factor (SDF-1), an alpha-chemokine that binds to the G-protein-coupled CXCR4, plays an important and unique role in the regulation of stem/progenitor cell trafficking and is involved in tumor metastasis and vasculation making it a good candidate to pursue (103). As **Figure 4-3** shows, CAF have elevated SDF-1 secretion compared to NPF.

TGF- secreted from CAF can stimulate CXCR4 expression in BPH1 cells

It is accepted that integration between TGF- and other signaling molecules is one of the mechanisms which modulates the effects of TGF- (72, 138). Based on the data that CAF has both elevated TGF- and SDF-1, further experiments were conducted to identify the platforms for these two signals' interaction. **Figure 4-4** shows that, in the tumors induced by CAF, CXCR4—the specific receptor of SDF-1 - is robustly expressed at levels much higher than that in benign rUGM plus BPH1 recombinants. This mirrors the situation in human prostate cancer (139) where expression of CXCR4 is not seen in benign prostate but is elevated in prostate cancer. To demonstrate that this difference is caused by the TGF- secreted from CAF, we conducted flow cytometry to analyze CXCR4 expression in BPH1 cells upon TGF- treatment. As shown in **Figure 4-5**, after

5ng/ml TGF-β treatment for 72 hours, the expression of CXCR4 increased significantly as displayed by an obvious shift to the right of the expression curve. The induction of CXCR4 by TGF-β was confirmed *in vivo*. Immunofluorescence staining was utilized to test the CXCR4 expression in tissue recombinants containing BPH1 and CAF cells, which were harvested from 2G7 (the TGF-β ligand inhibiting antibody) -treated animals. As **Figure 4-6** shows, when secreted TGF-βs were neutralized, CXCR4 staining was not detected in the epithelial cells within BPH1 plus CAF grafts.

SDF1 is critical for CAF to induce tumorigenesis from BPH1 cells

If our hypothesis that SDF-1 plays a role in converting TGF-β from a tumor suppressor to a tumor promoter is correct, we would expect that without SDF1/CXCR4 signaling, CAF would lose the capacity to induce tumor formation, even if TGF-β secretion is unaffected. To demonstrate the importance of SDF1 signaling in tumor formation induced by CAF, we first used a CXCR4 neutralizing antibody to block the SDF1-CXCR4 axis, and then tested the CAF tumorigenic effects on BPH1 cells *in vitro*. **Figure 4-7** shows that, once signaling in SDF1-CXCR4 is blocked, active Akt induction by CAF was impaired. These experiments suggest that SDF1/CXCR4 signaling is necessary for TGF-β's tumorigenic role. To confirm this conclusion *in vivo*, we knocked down CXCR4 in BPH1 cells by infecting the cells with a lentivirus vector expressing an shRNA, which blocked CXCR4 expression. This effect was demonstrated upon stimulation by CAF or TGF-β to confirm the effect (data not shown). The shRNA-expressing BPH1 cells were then combined with CAFs. After kidney capsule xenografting we observed the tumorigenesis and analyzed the histology, as shown in

Figure 4-8. The shCXCR4 BPH1 cells formed smaller grafts than control (shGFP) infected cells (we grafted 11 mice in total, $p<0.01$), H&E staining results also show different histologic results, the CXCR4 knocked down cells did not form any malignancy. These observations suggested that tumor stroma is inducing epithelial tumorigenesis via inter-communication between SDF-1 and TGF-β, which synergize to give rise to uncontrolled proliferation and dysregulated motility of the adjacent epithelial cells.

Discussion

As shown previously, TGF-β is crucial for tumor stroma to transform the adjacent non-tumorigenic epithelial cells *in vivo* and *in vitro*. However *in vitro* studies also showed that in serum-free conditions, TGF-β inhibits growth of BPH1 target epithelial cells (134). There is an extensive body of evidence to show that TGF-β family signaling pathways operate as part of a signaling network that collects and integrates diverse environmental cues in the cell (reviewed in (138). For example, the interaction between Ras and TGF-β signals has been reported as both opposite and cooperative. Ras signaling stimulates the activation of Cdks; whereas TGF-β signaling induces expression of various Cdk inhibitors that cancel the effects of Cdk activation by Ras (140). Oncogenic Ras in mammary epithelial cells not only attenuates Smad-mediated antiproliferative responses but also endows these cells with the ability to respond to TGF-β with transdifferentiation into a highly invasive and metastatic phenotype (135, 141). Breast cancer cells with a hyperactive Ras pathway (due to EGF receptor gene amplification) respond to TGF-β with an increased ability to metastasize to bone (142). Thus, oncogenic Ras does not merely block Smad signaling, but it "reprograms" the TGF-β response of epithelial cells.

As a different example a growing body of work provides evidence that TGF-β and BMP can activate various MAPK signaling pathways, most prominently the MKK4-JNK and MMK3-p38 pathways (143).

Since TGF-β is growth inhibitory *in vitro* and yet required for tumorigenesis *in vivo* we hypothesized that tumor stroma (CAF) could modify the effects of TGF-β on adjacent epithelial cells through secretion of other factor(s). To test this hypothesis, we checked the secretion of a candidate regulator SDF1/CXCL12 in the conditioned medium of CAF cells. The rationale for us to test this chemokine includes its known involvement in cancer and our preliminary data showing elevated levels of expression in CAF. SDF1 and its specific receptor CXCR4 have been demonstrated to be involved in cell proliferation and cell trafficking. Originally, most of the research in this area was done in lymphocytes (144, 145). More recently since most cancer cells were found to be CXCR4 positive and to respond to its ligand CXC12/SDF1. SDF1/CXCR4 are therefore implicated in cancer cell migration and invasion. For example, treatment with CXCR4 neutralizing antibody can block prostate cancer cells from invading or migrating toward bone marrow which is rich in SDF-1 (106, 119, 146). The second reason for us to measure SDF-1 is as our results showed, CAF cells secret higher levels of SDF1. SDF1/CXCR4 contributes significantly to the increased Akt phosphorylation and enhanced cell motility seen in cells grown in CAF conditioned medium, since the neutralizing antibody against CXCR4 could impair these functions. The importance of SDF1/CXCR4 has been confirmed by knocking down the level of CXCR4 by RNA interference. BPH1 cells infected with the CXCR4 shRNA, did not form tumors upon

recombination with CAFs. These observations are consistent with previous reports that SDF1/CXCR4 is involved in multiple processes in cancer. The more intriguing part of our results is the link between the two parallel pathways connecting stroma and epithelium. CAF cells secrete elevated TGF-β1 and SDF1 in parallel. By stimulating CXCR4 expression in adjacent BPH1 cells, TGF-β triggers SDF1/CXCR4 signaling, in turn, the integration of TGF-β signaling and SDF1/CXCR4 signaling modulates BPH1 cells' response to TGF-β: by converting its growth inhibitory function to a stimulating one, increasing active Akt and enhancing cell motility, it gives rise to the tumor promoting role of TGF-β. This is the first observation demonstrating cross talk between the cytokine TGF-β and chemokine receptor CXCR4, also it is the first report demonstrating mechanisms for tumor stroma to transform adjacent epithelial cells through these signal transductions.

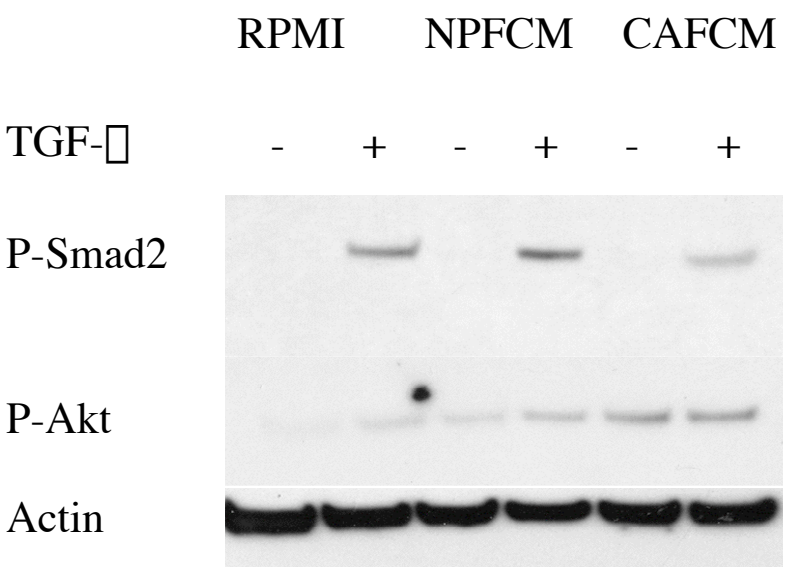


Figure 4-1. Stroma modifies epithelial cell response to TGF-β in co-culture

Normal prostatic fibroblast and CAF conditioned medium (NPFCM and CAFCM respectively) were collected as described in methodology. Conditioned medium was added to serum starved BPH1 cells and incubated for another 24 hours. Cells grown in RPMI showed a robust phosphorylation of Smad2 in response to TGF-β challenge with a mild phosphorylation of Akt. Cells grown in NPF CM had low basal levels of P-Akt which increased in moderately response to TGF-β; they also showed a robust Smad2 phosphorylation. Cells grown in CAF CM exhibited an obviously reduced Smad phosphorylation in response to TGFβ, and also exhibited increased basal level of Akt phosphorylation. These effects were consistent in multiple repeats of the experiment.

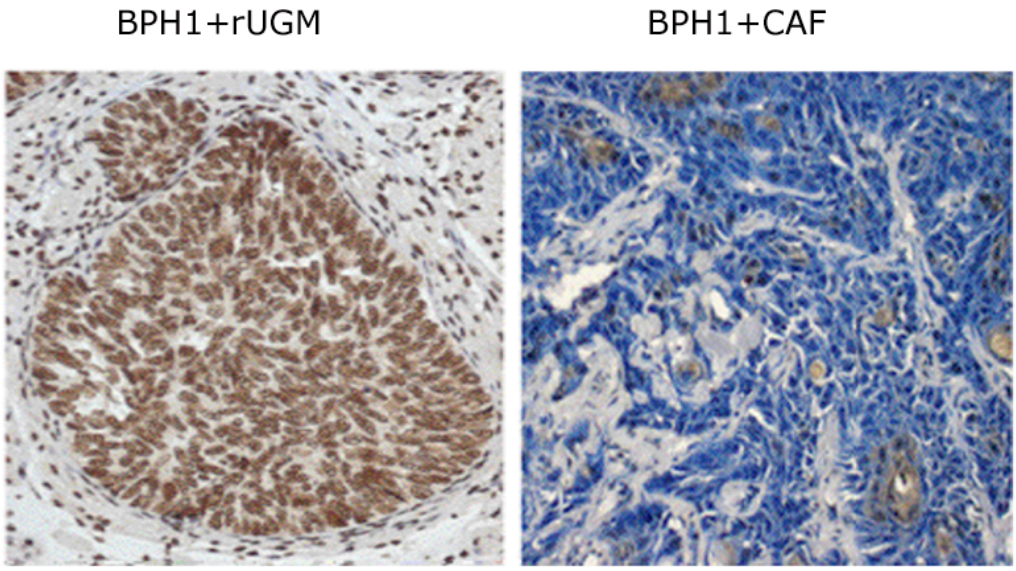


Figure 4-2. Tumor stroma decreases Smad2 phosphorylation *in vivo*

After being recombined with rUGM, BPH1 cells form organized solid cords in which BPH1 cells (and the rat stromal cells) have a strong positive signal for p-Smad2. In contrast when recombined with CAF, BPH1 cells form poorly differentiated, irregular epithelial tumors. The BPH1 cells intermingled within a fibrous stroma exhibits suppressed p-Smad2 expression.

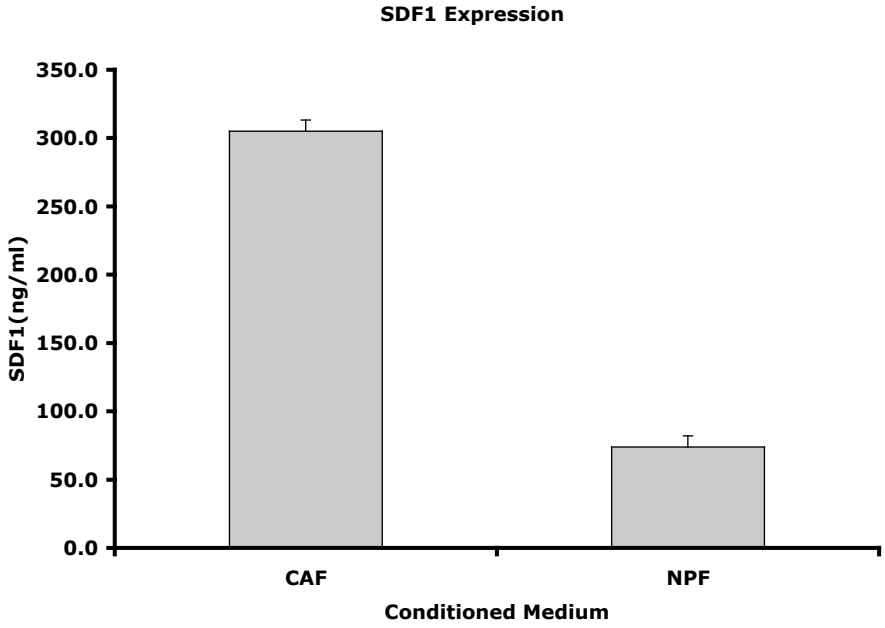


Figure 4-3. CAF conditioned medium contains elevated SDF1 than NPF

ELISA was used to quantitate SDF1 secreted from CAF and NPF into the culture medium. CAF cells secrets higher titers of SDF1 than do NPF (305pg/ml verse 73pg/ml). The experiments were performed in triplicate. The mean of the volume was used for the graph. The error bars show the standard deviation.

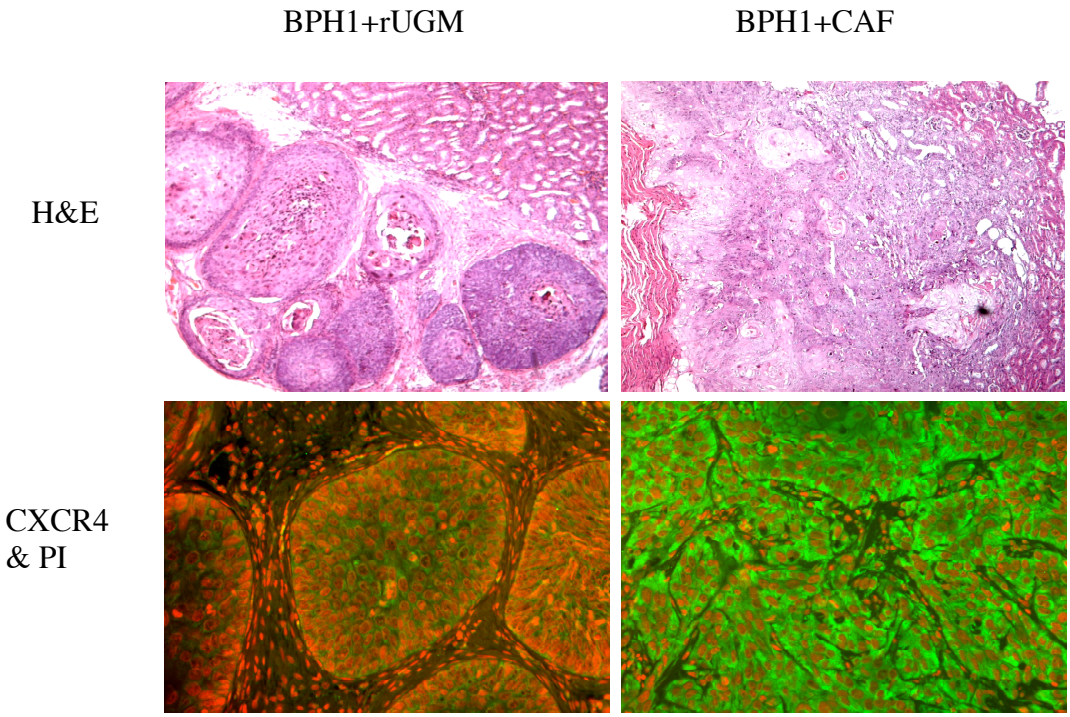


Figure 4-4. CAF stimulate CXCR4 expression in BPH1 cells *in vivo*

Upper panels show the histology of recombinants from BPH1 plus rUGM or BPH1 plus CAF. As described previously, in the presence of UGM the epithelial cells form well organized, benign-appearing solid cords, while in the presence of CAF rapidly growing tumors are formed. The lower panels show immunofluorescence staining of CXCR4. When BPH1 cells were combined with CAF, CXCR4 expression (Green) was robustly stimulated, compared to the tissue from BPH1 cells combined with rUGM. This pattern mirrors the progression of CXCR4 expression seen in benign and malignant human prostate tissues. Nuclear Propidium Iodide (PI) was stained in red.

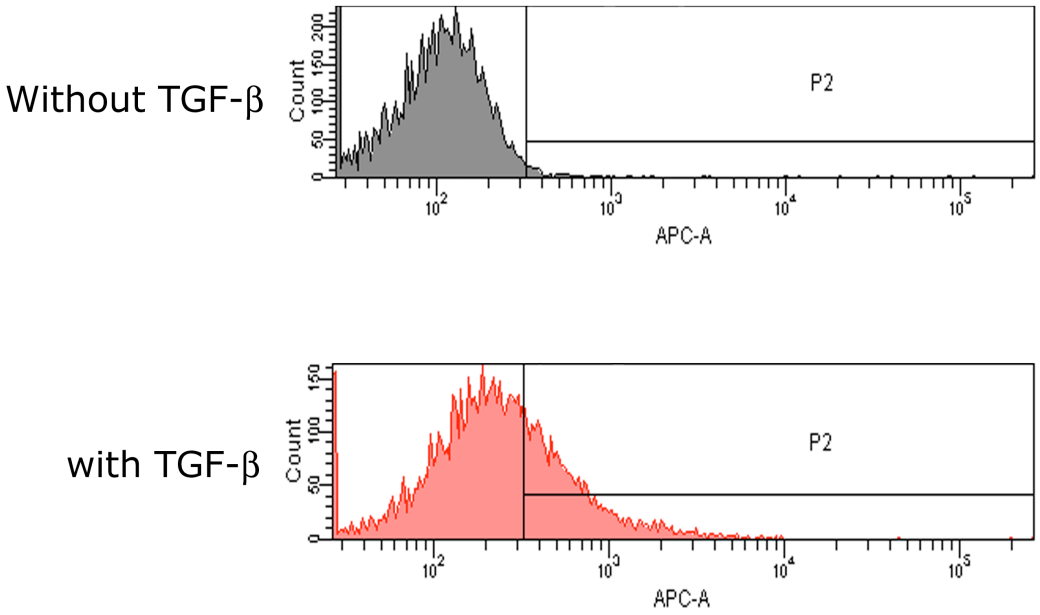


Figure 4-5. TGF-beta induces CXCR4 expression in BPH1 cells

BPH1 cells were seeded in serum-free RPMI with or without 5ng/ml TGF-β for 72 hours, then harvested and stained using CXCR4 primary antibody and APC conjugated secondary antibody. Flow cytometry showed that TGF-β treatment induced CXCR4 expression (cell population in high CXCR4-expressing population (P2 area) is increased from 1.2% in the untreated cells to 25.1% in the TGF-β treated population).

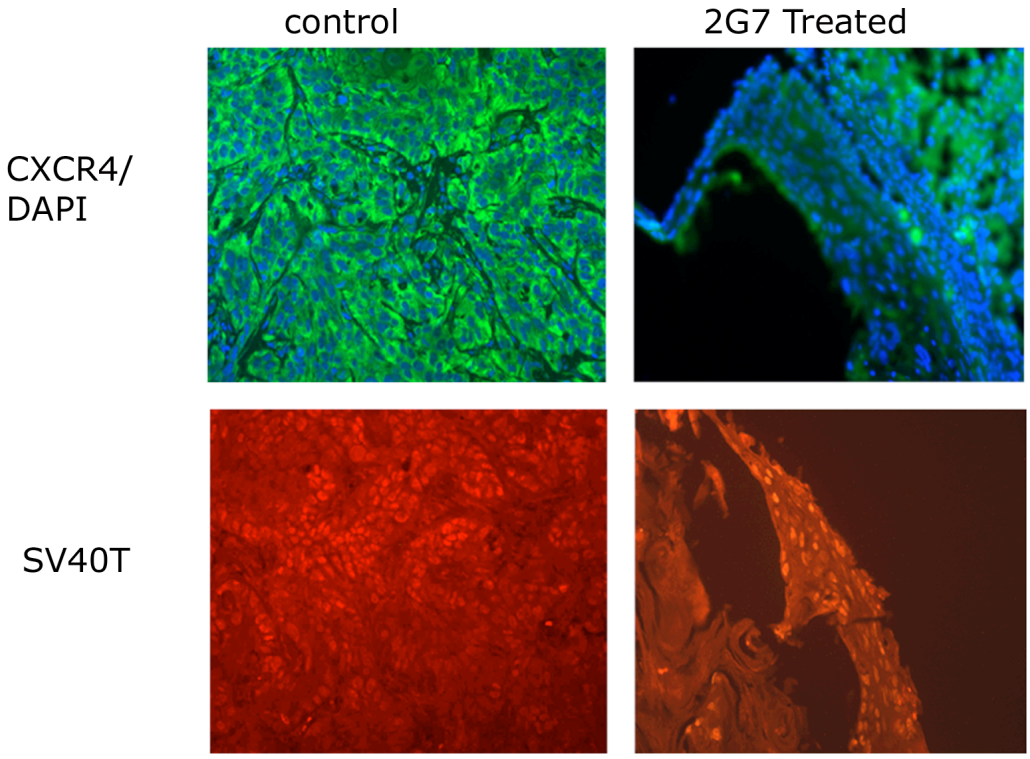


Figure 4-6. Neutralizing of TGF-beta ligand blocks CXCR4 stimulation *in vivo*

Tissue recombinants of BPH1 and CAF cells were subrenal capsule xenografted into host mice which either were (right panels) or were not (left panels) treated using the TGF-β blocking antibody 2G7. The grafts were harvested 8 weeks afterwards. Immunofluorescence was conducted to check CXCR4 expression (upper panels), and SV40 large T antigen (lower panels) was used to trace the BPH1 cells. CXCR4 expression is widespread in grafts to the untreated mice but in treated mice is only seen in the host kidney (top right corner of upper right panel).

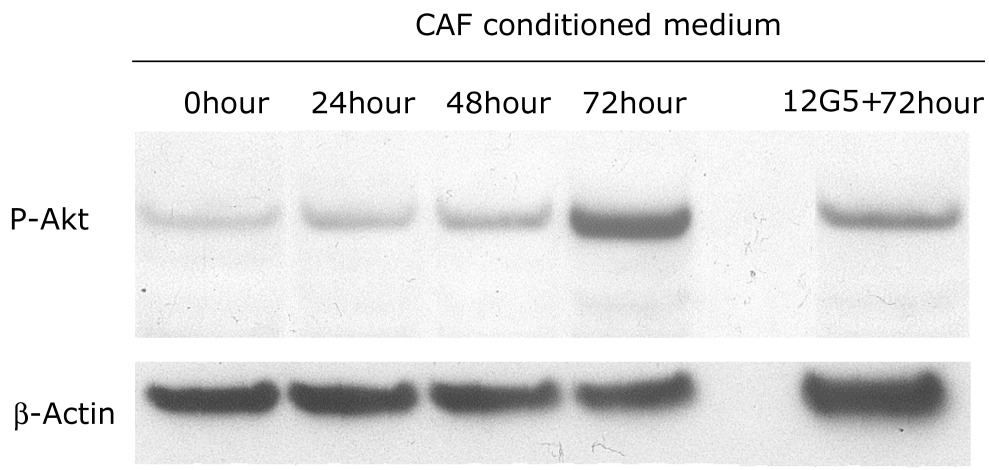


Figure 4-7. Neutralizing CXCR4 inhibits Akt activation by CAF

BPH1 cells were cultured in conditioned medium collected from CAF cells with or without the CXCR4-blocking antibody 12G5. The whole cell lysate was probed using phosphorylated Akt antibody; β-actin was used as loading control. This experiment shows that CAF conditioned medium can induce Akt phosphorylation in BPH1 cells, and that this induction is impaired when CXCL12/CXCR4 signaling is blocked by the CXCR4 neutralizing antibody.

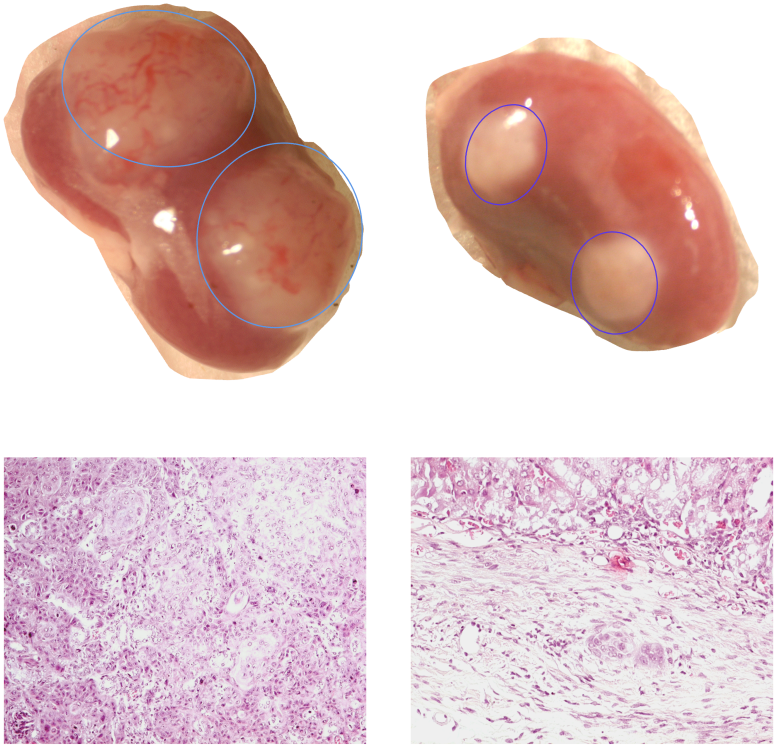


Figure 4-8. Inhibiting CXCR4 RNA expression will impair tumor formation in CAF and BPH1 tissue recombinants

Tissue recombinants containing CAF with either control BPH1 GFPshRNA cells (left panels) or with BPH1 CXCR4shRNA cells, in which CXCR4 expression is suppressed, (right panels) were grafted to host animals. The gross images in the upper panels show that by the time of harvest the control cells formed a much bigger tumor than the CXCR4 knocked down BPH1 cells. The H&E staining in the lower panel shows the histological difference: the control cells displayed typical adenocarcinoma appearance while a few benign-appearing cords were seen in the CXCR4-suppressed cells.

CHAPTER V

TGF-BETA MEDIATED EPITHELIAL TO MESENCHYMAL TRANSFORMATIONS CAN ENHANCE HUMAN PROSTATIC EPITHELIAL CELL INVASION

Introduction

The ability of TGF-β to enhance tumorigenicity *in vivo* is illustrated by its role in many key processes including; stimulating angiogenesis, inhibiting immune surveillance and promoting the degradation of extracellular matrix (86, 87). TGF-β can also promote local invasion and EMT (78, 88, 89). EMTs are associated with tumor metastasis due to the need for cells to be motile and invasive. The use of MEK inhibitors and DN TβRII helped to demonstrate that TGF-β is an inducer of EMT (78, 88, 147, 148). Cells expressing DN RhoA and $p160^{ROCK}$ also inhibited EMT (44) implicating the ROCK/Rho pathways in TGF-β-mediated EMT. A survey of relevant literature shows that either MAPK/ERK, PI3K, or both are important in TGF-β mediated EMT and/or motility in transformed and metastatic mammary epithelial cells (149, 150). EMT is a poorly documented feature of human prostate cancer, arguably because of a lack of good antibodies against the EMT-related transcription factors. The present study investigates the potential of TGF-β signaling to play a role in mediating the promotion of human prostate cancer. To approach this question the effects of TGF-β signaling on the BPH1-derivative tumorigenic $BPH1^{CAFTD}$ lines were investigated (134). These tumorigenic strains were permanently transformed by exposure to human prostatic carcinoma associated fibroblasts (151), and have a series of characteristic genetic changes resulting

from their exposure to a cancer stroma environment (152). This communication records the profound differences in the responses of the parental and derivative lines to stimulation by TGF-β and illustrates mechanisms by which a single ligand can play different roles depending upon the cell type and context in which it is acting.

Results

TGF-β1 Effects on BPH1^{CAFTD} cells

As shown in chapter 3, TGF-β1 at a concentration of 5ng/ml inhibited the growth of the initiated but non-tumorigenic BPH1 cells over a 5-day period, consistent with previously published observations (134). In contrast none of the tumorigenic BPH1^{CAFTD} cell lines were growth inhibited by TGF-β, with treated and untreated cells growing at statistically identical rates (**Figure 5-1**). Parental BPH1 cells were phenotypically unchanged by TGF-β treatment, exhibiting a classical epithelial cobblestone morphology in culture with continued expression of keratin and E-cadherin. In contrast the tumorigenic BPH1^{CAFTD} lines responded to TGF-β by subtle morphologic changes suggesting cellular elongation (**Figure 5-2**). Vimentin expression in these cells was robustly induced by TGF-β while keratin expression was repressed (**Figure 5-3)**. Western blotting assays confirmed the initial immunofluorescence observations demonstrating induction of vimentin, suppression of E-Cadherin and Keratin (**Figure 5-4**).

Cellular Response to TGF-β

First we wished to determine whether the difference between the parental and tumorigenic cell response to TGF-β resulted from a loss of TGF-β signaling components,

or the alteration of downstream signal transduction pathways resulting from changes in expression of other proteins which modulate signaling (73, 153). We used a cross linking assay to check the binding of iodinated TGF-β to receptors and also examined the phosphorylation of Smad2 in response to TGF-β stimulation. The cross-linking assay demonstrated that TβR1, TβRII and the class three TβRs were all expressed in both the BPH1 cells and all of the BPH1^{CAFTD} cell lines (**Figure 5-5**). This result was confirmed with a Western blotting assay using a specific procedure described in methodology (data not shown). Western blotting assays showed that downstream of the receptor, Smad2 phosphorylation in response to TGF-β stimulation was identical in parental and tumorigenic cells. In marked contrast the levels of phosphorylated Akt are very different between the two cell types with the tumorigenic BPH1^{CAFTD} lines having much higher basal levels of expression than the non tumorigenic BPH1 cells (**Figure 5-6).**

Elevated P-Akt Blocks Smad3 Translocation and Reduces Nuclear p21 to Release the Cell Cycle Arrest

To test the potential of elevated P-Akt to modulate the response to TGF-β, we checked R-Smad (Smad 2 and 3) localization upon TGF-β treatment in parental BPH1 cells and BPH1^{CAFTD}1 cells. Both Western blotting and immunofluorescence staining show that before TGF-β treatment, Smad3 is predominantly seen in the cytoplasm. Within 4 hours of TGF-β treatment Smad3 was translocated into the nucleus in BPH1 cells. However in BPH1^{CAFTD}1 cells this process was apparently only partial with a substantial amount of Smad3 maintained in the cytoplasm, Smad3 localization was quantitated by applying NIH Image J software to captured images of immunofluorescence staining (**Figure 5-7**). To confirm that this phenomenon is caused

by the endogenous levels of P-Akt, we performed two complimentary experiments. To examine a gain of function a constitutively active myristylated Akt1 (myrAkt) was expressed in the BPH1 cells. To demonstrate loss of function the BPH1^{CAFTD}1 cells were pretreated with the PI3K/Akt inhibitors Wortmannin (Sigma) or LY294002 (Calbiochem, San Diego, CA) (75) before TGF-β administration. These experiments showed that myrAkt overexpression in BPH1 cells inhibits nuclear translocation of Smad 3 in response to TGF-β with a portion of the Smad3 remaining in the cytoplasm of the non-tumorigenic BPH1 cells. In contrast blocking PI3K/Akt enhanced Smad3 translocation into the nucleus of the BPH1^{CAFTD} cells (**Figure 5-8**). Unlike Smad3, Smad2 activation and localization showed little difference between BPH1 and BPH1^{CAFTD} cells consistent with recently published observations suggesting distinct control mechanisms in relation to the two proteins (154).

Activated Smads regulate the transcription of target genes, including cell cycle inhibitors such as p21, which then mediate the anti-proliferative response and partially explain the tumor suppressive action of the TGF-β pathway (155, 156). To further assess the influence of TGF-β on genes downstream of the Smad-pathway we examined the subcellular localization of the growth inhibitor p21^{CIP}. **Figure 5-10 A** shows that in tumorigenic BPH1^{CAFTD}1 cells, TGF-β treatment results in almost all p21 becoming localized to the cytoplasm. This is in marked contrast to the non-tumorigenic BPH1 cells where p21 is evenly distributed between the cytoplasm, and the nucleus. This differential distribution pattern involves P-Akt since in BPH1 cells overexpressing myristylated Akt1 all p21 was seen to be localized in the cytoplasm. Pretreatment of BPH1^{CAFTD} cells with

Wortmannin resulted in cytoplasmic and nuclear distribution of p21. Since the inhibitory function of $p21^{CIP}$ occurs in the nucleus, its exclusion from the nucleus allows the cells to go through G1 phase, which could allow $BPH1^{CAFTD}1$ cells skip the cell cycle arrest upon TGF-β treatment. Cell cycle analysis (**Figure 5-10 B &C**) is consistent with this concept.

PI3K/Akt signaling is involved in Vimentin induction by TGF-β

To further study the cellular response to TGF-β, we used cell lines expressing a dominant active TGF-β receptor I (DA), myristylated Akt1 (Akt) or full length Smad3 (Smad3). The empty vector (EV) was transduced as a negative control. In line with observations on the effect of ligand (**Figure 5-1**) in non-tumorigenic BPH1 cells, the overexpression of DA receptor, Akt1 or Smad3 did not result in obvious changes either in cell morphology or protein expression profile (**Figure 5-11**). In contrast in tumorigenic $BPH1^{CAFTD}$ cells expressing either the DA receptor or myrAkt, both Western blotting and immunofluorescence staining demonstrated that the mesenchymal marker vimentin was robustly induced, while the epithelial markers cytokeratin and E-Cadherin were concurrently suppressed. At the same time the morphology changed from a cobblestone to a more elongated phenotype **(Figure 5-12**). There were no phenotypic changes induced in these cells by overexpression of Smad3. These results showed that in the tumorigenic cell line $BPH1^{CAFTD}1$, the activation of TβRI or overexpression of Akt1 led to an expression profile suggestive of EMT, which is characterized by morphological changes, induction of vimentin and suppression of cytokeratin and E-Cadherin.

The results described above showed that overexpression of Akt1 elicited similar effects on BPH1^{CAFTD}1 cells to treatment with TGF-β or to overexpression of DATβRI. To further investigate whether PI3K/Akt is necessary for TGF-β to elicit these effects, we infected BPH1^{CAFTD}1 cells with a dominant negative Akt (DN-Akt, K179M) adenovirus (157). As an additional separate test we pretreated cells with the PI3Kinse inhibitor Wortmannin, before exposure to TGF-β. The Western blotting results (**Figure 5-13**) showed that the induction of vimentin by TGF-β was impaired in BPH1^{CAFTD}1 cells when the PI3K/Akt pathway was blocked by Wortmannin or DN-Akt, indicating that the PI3K/Akt pathway is involved in the EMT response to TGF-β. As a downstream target gene of PI3K/Akt, GSK3β can be inhibited by Akt mediated phosphorylation (158). GSK3β was used to confirm that DNAkt cells lost the kinase activity in the experiment.

Activation of TGF-β receptor I or Akt signaling elicits enhancement of motility

In addition to vimentin induction, the BPH1^{CAFTD}1 cells show enhanced motile ability in wound closure and transwell migration assays. In a wound healing assay, the motility was checked by quantifying the rate of wound closure **(Figure 5-14)**. The results showed that, the BPH1^{CAFTD}1 cells overexpressing DA or Akt closed wounds significantly faster (Student's T test, $p<0.01$) than empty vector control (EV) cells. Smad3 overexpressing cells showed no significant difference from EV cells. In a Boyden Chamber assay as shown in **Figure 5-15**, the ability of cells to invade collagen and migrate to the underside of the inserts is determined by a 12 hour response to conditioned medium containing different concentrations of FBS in the lower chamber. The data showed that more -DA cells and -Akt cells migrated to the underside of the filter, compared to the empty vector

control cells (Student's T test, $p<0.01$). Overexpression of full length Smad3 showed a trend towards slower migration which did not reach significant difference from EV controls. These two assays demonstrated that tumorigenic $BPH1^{CAFTD}1$ cells acquired enhanced motility when either type I receptor or Akt but not Smad3 is forcibly activated. The same experiments were performed on parental BPH1 cells, no significant changes in wound closure or motility were found.

EMT cells lead invasive front *in vivo*

To confirm that the results observed *in vitro* corresponded to *in vivo* phenomena and to test whether EMT is associated with invasion, we suspended $BPH1^{CAFTD}1$ cells expressing either empty vector (control) or DA TGF-β receptor in collagen gel and xenografted beneath the kidney capsule of male athymic hosts. Two months later, the grafts were harvested for analysis. From gross images, the cells overexpressing DATβRI generated larger tumors (data not shown). At a histologic level the EV control cells grow within a very limited area which has a sharply delineated border with the host kidney. This is consistent with previous descriptions of the growth of $BPH1^{CAFTD}1$ tumors which exhibit very little invasive activity (151). In contrast, the DA overexpressing cells show obvious invasion into the adjacent kidney. This is clearly illustrated when SV40 T antigen is used to identify the grafted epithelial cells, the transgenic DA cells are clearly seen to have a more aggressive behavior compared with the empty vector control cells with cells invading the kidney parenchyma and surrounding kidney tubules. Double staining with antibodies against SV40T and vimentin shows that in the grafts of control cells containing the empty vector there was no vimentin expression to the tumor margin

(**Figure 5-16**). In stark contrast the invasive DAT□R1 cells expressed vimentin along the invasive front, but interestingly not in the body of the tumor. Cells co-expressing SV40T antigen and vimentin are clearly visible along this front confirming their EMT nature. This finding demonstrates not only that EMTs can occur in tumorigenic human prostatic epithelium but also that the expression of this phenotype, even in the face of constitutive TGF□ signaling, is context dependent.

Discussion

The TGF-□ system is a potential tumor suppressing pathway in the prostate based upon its ability to inhibit cell proliferation and induce apoptosis in most prostatic epithelial cells (84, 159-161). The role of TGF-□ in maintaining prostatic homeostasis is further demonstrated by the decreased expression of TGF-□ type I and type II receptors (T□RI and T□RII) in malignant prostatic epithelium, which is correlated with increased Gleason Score and poor prognosis (162, 163). There are several reports that loss of responsiveness to TGF-□ could induce malignancy (164). The LNCaP human prostatic cancer cell line lacks T□RII, while forcibly expressing wild type T□RII can restore the cells responsiveness to TGF-□ and reduce colony formation (85, 165). However, while the above lines of evidence suggest that TGF-□ is a tumor suppressor there are also paradoxical responses to this ligand. Many studies have demonstrated that TGF-□ plays a dual role in carcinogenesis depending on the disease stage being inhibitory for normal epithelial cell growth but not for the growth of cancer cells (166). Cancer cells can acquire resistance to growth inhibition by TGF-□ through mutation or deletion of components of the receptors or signaling cascade, or, more often, through the regulation

of the downstream signaling molecules (73, 74, 89, 167). The local concentration of extracellular TGF-β1 is significantly elevated in prostate cancer compared to normal or benign prostate tissues (79). Since such an elevated level of a normally growth suppressive factor would act to inhibit progression it is clear that a loss in the ability of tumor cells to perceive TGF-β as a growth inhibitor would provide a significant growth advantage and would thus be expected to be an early change seen in tumor evolution. This elevated level TGF-β1 is suggested to be indirectly involved in the development of prostate cancer, for example, stimulating angiogenesis and inhibiting immune responses directed against tumour cells (168). Elevated TGF-β levels can also affect the cancer cells directly, since loss of sensitivity to inhibition of growth by TGF-β by most tumor cells is not synonymous with complete loss of TGF-β signaling (153, 169).

In the present study the response to TGF-β signaling was examined in a related group of non-tumorigenic parental and tumorigenic derivative cell lines. During the course of malignant transformation by their stromal environment these cells have lost the growth suppressive response to TGF-β and have acquired a constitutively activated Akt pathway. Elevation of Akt is commonly a response to the loss of the PTEN tumor suppressor (170-172). Deletions of PTEN are common in human prostate cancer (173, 174), so this molecular characteristic of these cells is typical of the differences between normal and malignant cells in many prostate cancer patients. It was noteworthy that the receptor signaling response to TGF-β is apparently intact in the tumorigenic BPH1^{CAFTD}1 cells as measured by their ability to phosphorylate Smad3 in response to TGF-β stimulation. However we observed that in the tumorigenic cells (in contrast to the non-tumorigenic

cells) the nuclear translocation of the Smad complex in response to TGF-β signaling was impaired. We were able to demonstrate that the elevated Akt levels in the tumorigenic cells was sufficient for this response, since nuclear translocation was restored by blocking the PI3K/Akt pathway. To confirm this we expressed a myristylated Akt in the benign cells and saw the same inhibition of Smad nuclear translocation as in the tumorigenic cells.

EMT is another way for TGF-β to promote carcinogenesis (78, 88). The development of the mesodermal germ layer is dependent upon epithelial to mesenchymal transformations that allow its derivation from the ectodermal epithelium. Without this phenomenon animals with three germ layers would not exist (175). In both normal tissue differentiation and carcinogenesis, select populations of epithelial cells may undergo EMT, lose epithelial polarity, form actin stress fibers, and differentiate into a mesenchymal, fibroblast phenotype (165). Additionally, delocalization of cell adhesion markers, including E-cadherin, ZO-1, and integrin β1 allow cells to digest and to migrate through the ECM (176). EMTs have been associated with tumor metastasis due to the need for cells to be motile and invasive.

The role of EMTs in human cancers occurring *in vivo* is still controversial and their presence or absence in specific tumor types is still under investigation. A major problem is the ability of pathologists to positively identify such cells given that their appearance and location would be consistent with mesenchymal cells. A firm resolution of the situation in tumors in patients will await the development and application of good

antibodies to transcription factors such as snail and twist which are involved in the EMT phenomenon. In the meantime model systems with inbuilt tracers provide a useful means of investigating the phenomenology and underlying mechanisms.

The use of MEK inhibitors and DN TβRII helped demonstrate that TGF-β is an inducer of EMT (88, 148, 165). Cells expressing DN RhoA and $p160^{ROCK}$ also inhibited EMT (44). Either MAPK/ERK, PI3K, or both are important in TGF-β-mediated EMT and/or motility in transformed and metastatic mammary epithelial cells (149). The mechanisms that led to TGF-β-mediated motility and EMT involve PI3K/Akt and Ras/ERK signaling. Most reports have used mouse breast cancer as a model, to our knowledge no data have been reported concerning the role of TGF-β on the induction of human prostatic EMT, the effect of EMT on prostatic cancer progression and the significance of these TGF-β-mediated pathways remain unknown.

In this study, we saw that TGF-β-induced EMT, characterized as mesenchymal marker induction, epithelial marker suppression and associated enhanced cell motility, in the tumorigenic but not in the non-tumorigenic cells. Induction of an EMT phenotype in these cells was impaired by blocking the PI3K/Akt pathway suggesting that this non-Smad pathway is involved in and required for mediating this response in these tumorigenic cells. These data suggest that while the elevated levels of Akt seen in the tumorigenic cells are sufficient to inhibit nuclear translocation of the Smad complex they are insufficient, of themselves, to elicit an EMT. However stimulation by TGF-β on this background or, alternatively massive overexpression of Akt is sufficient to elicit an EMT

response. This suggests two possibilities, the first that an EMT can be a direct result of sufficiently high levels of Akt activation, this could be the product of a dose effect composed of the high background level compounded by additional Akt activation resulting from TGF-β stimulation. Or alternatively that TGF-β stimulation of these cells can trigger other pathways such as MAPK which can elicit an EMT due to additive effects on the high background levels of activated Akt.

In vivo expression of DATβRII in BPH1^{CAFTD}1 cells results in the formation of tumors which are much more invasive than the empty vector controls. EMT cells lead the aggressive invasive front but are apparently not present in the non-invading body of the tumor. These observations reflect the importance of cellular context upon the responses to specific pathways and support the concept of cells as signaling integrators. The observations also provide important *in vivo* confirmation and refinement of the *in vitro* observations.

This study supports a view that TGF-β has different roles in this model of human prostatic carcinogenesis. In the benign cells, TGF-β acts as a tumor suppressor by inhibiting cell growth and likely supporting differentiation. However its growth inhibitory function can be lost early in tumorigenesis as a result of apparently commonly occurring changes, such as loss of PTEN activity resulting in enhanced Akt activity. The ability of TGF-β, which is present at elevated levels in prostatic tumors, to stimulate invasion via the induction of an EMT may then become an important contributor to the carcinogenic process.

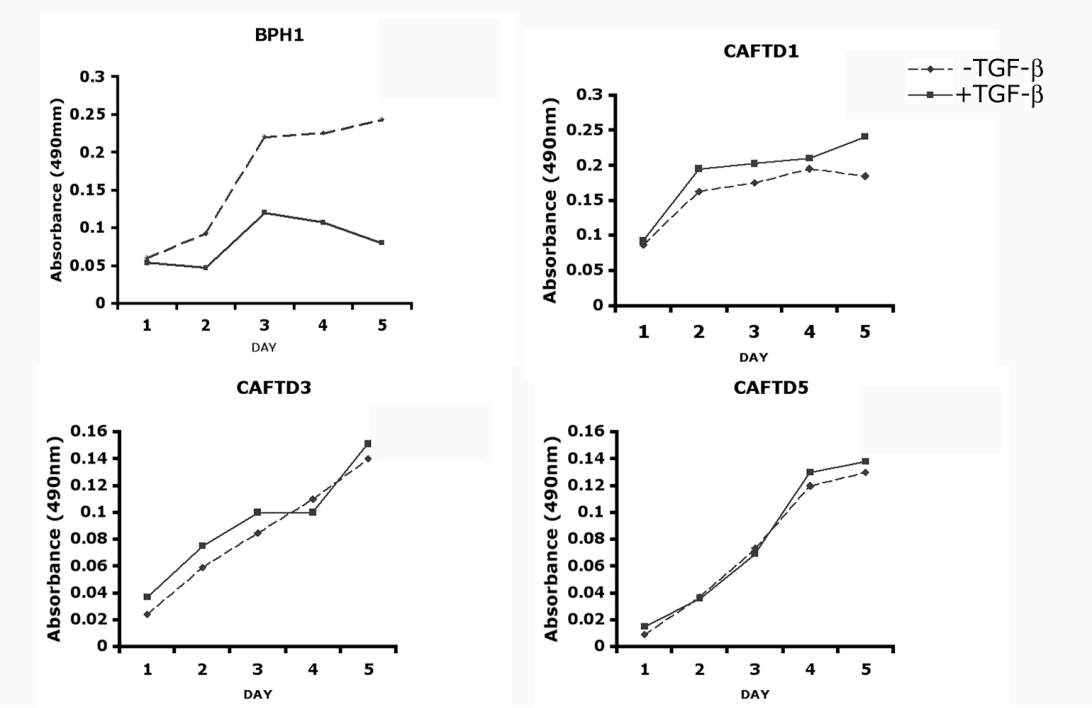


Figure 5-1. Growth response of BPH1 and BPH1^{CAFTD}1, 3, and 5 cells (CAFTD1, 3, 5) to TGF-β1

The non-tumorigenic parental BPH1 cells and tumorigenic CAFTD1, 3, 5 cells were grown in serum-free RPMI-1640 with or without 5ng/ml TGF-β1. BPHI cells showed growth inhibition upon TGF-β treatment. In contrast the tumorigenic sublines showed no significant change in their proliferation rate in response to TGF-β.

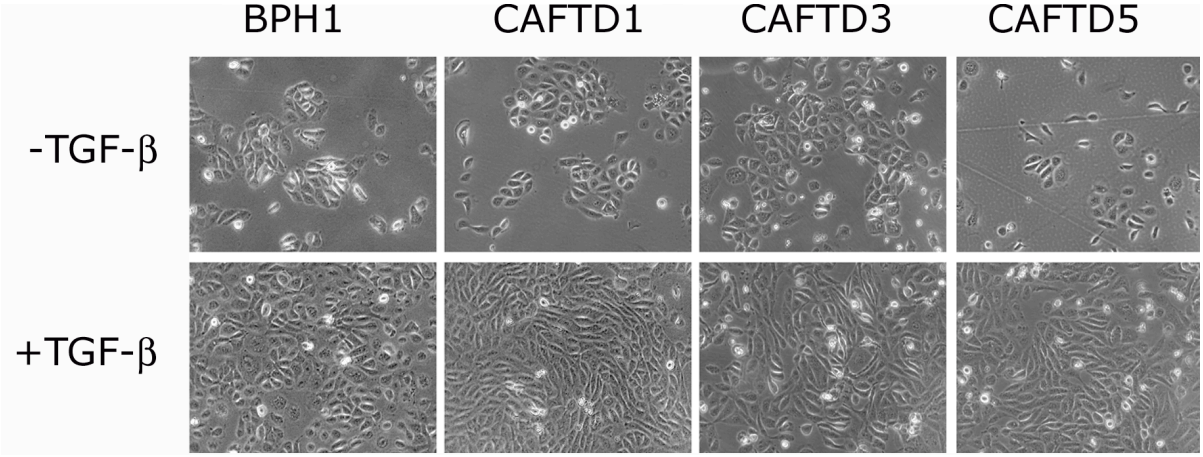


Figure 5-2. TGF-β treatment changed the BPH1-^{CAFTD} cells morphology

BPH1 cells and BPH1-^{CAFTD} 1,3,5 cells were seeded in culture flasks. Before treated with TGF-β, all the cells displayed cobblestone shape; after treatment with 5ng/ml TGF-β1 for 72 hours, the CAFTD cells became elongated while the parental cells retained a cobblestone morphology.

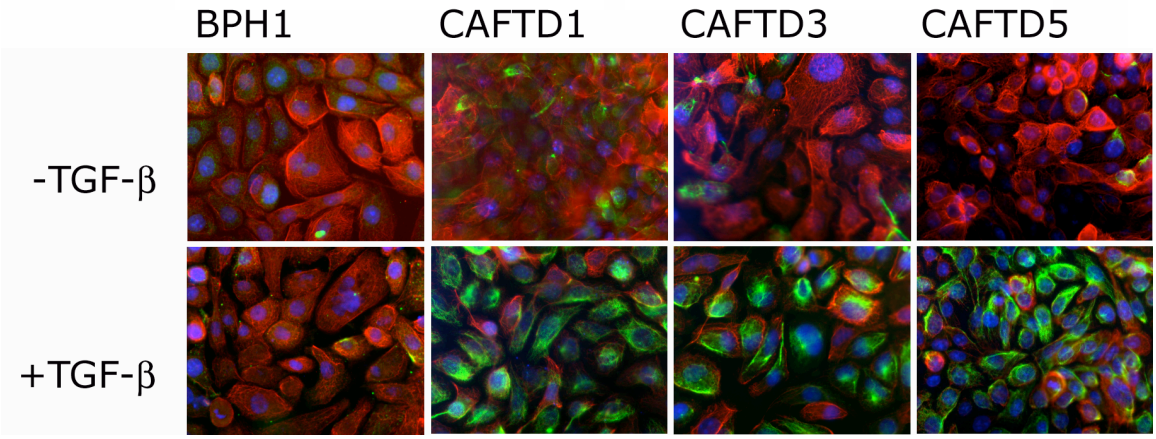


Figure 5-3. TGF-β elicits alterations in the profile of intermediate filament proteins expressed in CAFTD cells but not in BPH1 cells

Phenotypic response of BPH1 and BPH1^{CAFTD}1, 3, and 5 cells (CAFTD1, 3, 5) to 72 hours of treatment with 5ng/ml TGF-β1. Immunofluorescence staining reveals changes in protein expression. Cytokeratin expression was visualized in red, and vimentin in green, nuclear DNA was stained in blue using DAPI. In serum-free medium all four cell lines express cytokeratin with little detectible vimentin. In response to treatment with TGF-β1 the tumorigenic cells, but not the non-tumorigenic parental cells initiate expression of vimentin and lose keratin expression.

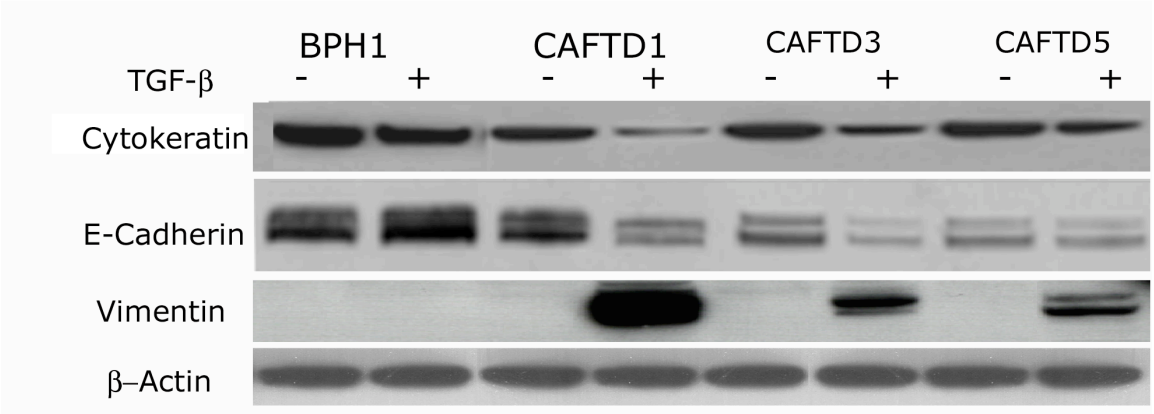


Figure 5-4. TGF-β changed protein profile in CAFTD cells but not in BPH1 cells

BPH1 and BPH1^{CAFTD} cells were cultured in RPMI-1640 medium with or without 5ng/ml TGF-β. 72 hours post treatment the cells were harvested and the whole cell lysate were used for Western blotting to check the protein expression. Without treatment, all of the cells expressed cytokeratin and E-cadherin while vimentin could not be detected. Upon TGF-β treatment, CAFTD cells robustly induced expression of vimentin while cytokeratin and E-Cadherin expression was suppressed.

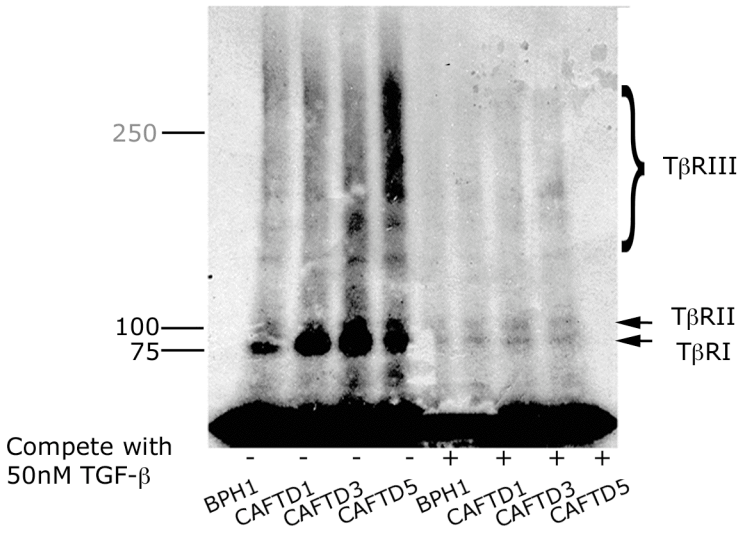


Figure 5-5. TGF-β receptors are intact in the tumorigenic cell lines

TGF-β receptors were assayed by cross-linking following binding of ^{125}I-TGF-β1 to cells at 4 °C in the presence or absence of 50nM unlabeled TGF-β1. Cell lysates, normalized to cell number, were subjected to SDS-PAGE and autoradiography. The data show that all the three types of receptors; TβRI, II and III are present in both non-tumorigenic BPH1 cells and tumorigenic BPH1 ^{CAFTD} cells.

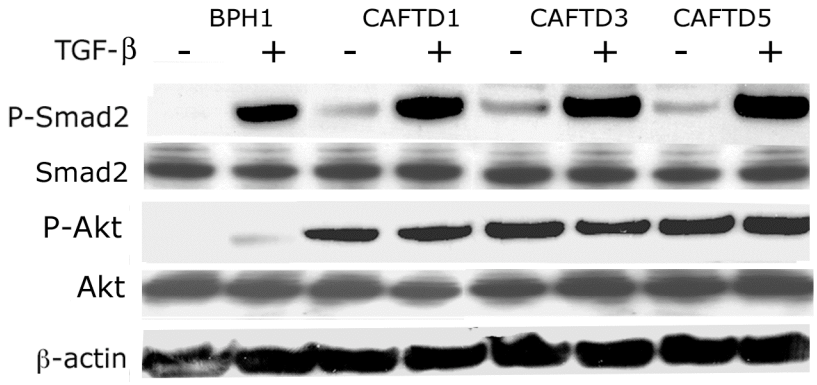


Figure 5-6. TGF-β signaling mechanisms are intact in the tumorigenic cell lines

To confirm the presence of downstream signaling components, cells were incubated with or without TGF-β1 for 4 hours. Western blot analysis using anti-P-Smad2 and P-Akt, showed no differences in Smad2 phosphorylation. In contrast the non-tumorigenic BPH1 cells show low basal levels of P-Akt which is mildly stimulated by TGF-β1 while the tumorigenic BPH1 ^{CAFTD} cells exhibit an elevated P-Akt level, both in the basal and TGF-β stimulated state. Total amount of Smad2 or Akt were not different in BPH1 cells and the BPH1 ^{CAFTD} cells.

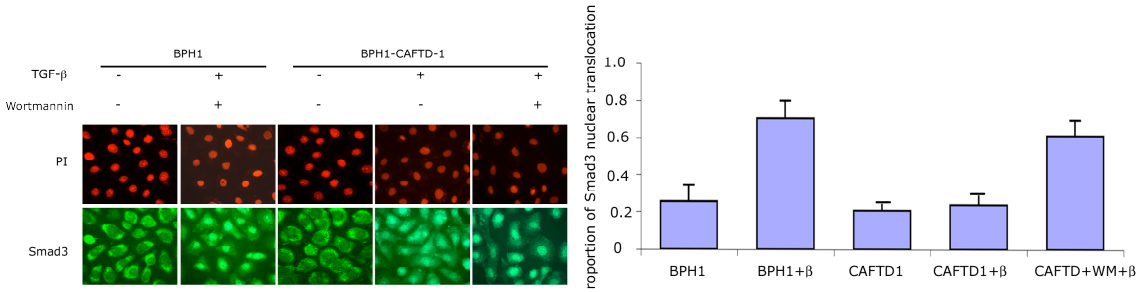


Figure 5-7. P-Akt blocks Smad3 translocation to the nucleus in human prostatic epithelial cells

Immunofluorescence staining illustrates Smad3 localization in cells with or without TGF-1 treatment. Smad3 is visualized in green while nuclei are stained using propidium iodide and are shown in red. Without treatment, Smad3 was localized in the cytoplasm both in BPH1 cells and BPH1^{CAFTD}1 cells. After TGF- treatment, almost all of the Smad3 was translocated into the nucleus in BPH1 cells, however in BPH1^{CAFTD}1 cells both nuclear and cytoplasmic localization were seen. Pretreatment with Wortmannin enhanced nuclear localization of Smad3 in BPH1^{CAFTD}1 cells. Smad3 translocation was quantitated by NIH Image J, the average photons locating in the nucleus or in the whole cell body were measured in five random views, and the percentage of the nuclear distribution was used for the graph. The error bars show the standard deviation.

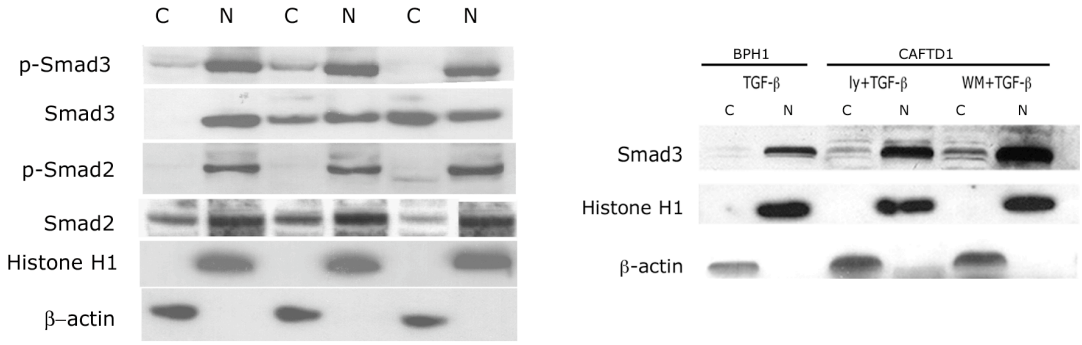
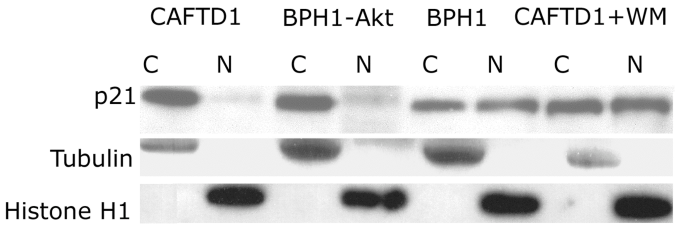


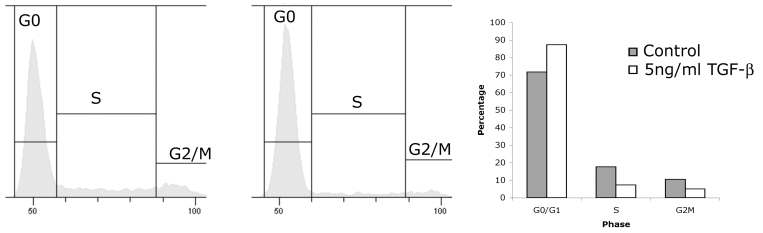
Figure 5-8. P-Akt blocks Smad3 translocation to the nucleus in human prostatic epithelial cells

Cells were incubated with 5ng/ml TGF-1 for four hours then extracted and fractionated to give cytoplasmic (C) and nuclear (N) extracts. Specificity was confirmed using histone H1 and -actin as nuclear and cytoplasmic markers and loading controls. Analysis of Smad3 localization showed that in non-tumorigenic BPH1 cells Smad3 was predominantly in the nucleus after TGF-1 treatment. In contrast, in BPH1^{CAFTD}1 (CAFTD1) cells Smad3 was seen at similar levels in both the nuclear and cytoplasmic fractions. Overexpression of myristylated Akt1 in BPH1 cells (BPH1-Akt) inhibited Smad3 translocation to the nucleus, while treatment using the PI3K inhibitors Ly294002 (10M) or Wortmannin (WM-100nM) enabled Smad3 to translocate into the nucleus of BPH1^{CAFTD}1. In contrast, total and phosphorylated Smad2 localization were essentially unchanged in response to the state of Akt activation.

A.



B.



C.

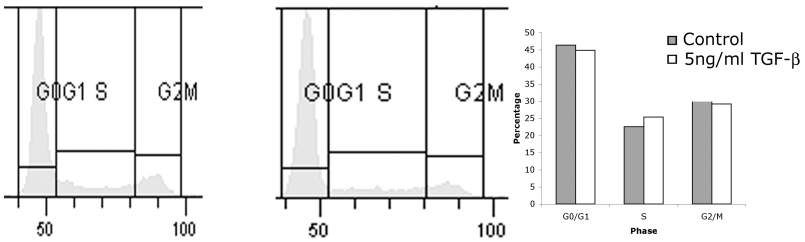


Figure 5-9. p-Akt can reduce nuclear p21 and cause the release of G1-S arrest upon TGF-β

A. BPH1 cells and BPH1^{CAFTD}1 (CAFTD1) cells were treated with TGF-β1 for 4 hours and extracted as nuclear and cytosolic fractions. Western blotting results show that in BPH1 cells, p21 locates both in the cytosolic (C) and nuclear (N) fractions. In BPH1^{CAFTD}1 cells only cytoplasmic p21 is seen. Overexpression of myristylated Akt1 in BPH1 cells profoundly reduces p21 levels in the nuclear fraction while treatment with Wortmannin (WM) maintains nuclear p21 levels in BPH1^{CAFTD}1 cells. The p21 distribution pattern is confirmed by immunofluorescence staining. **B and C.** Flow cytometric analysis demonstrates that after 4 hour of TGF-β1treatment, BPH1 cells are arrested at G1 to S phase (B) while BPH1^{CAFTD}1 cells (C) skipped the cell cycle check.

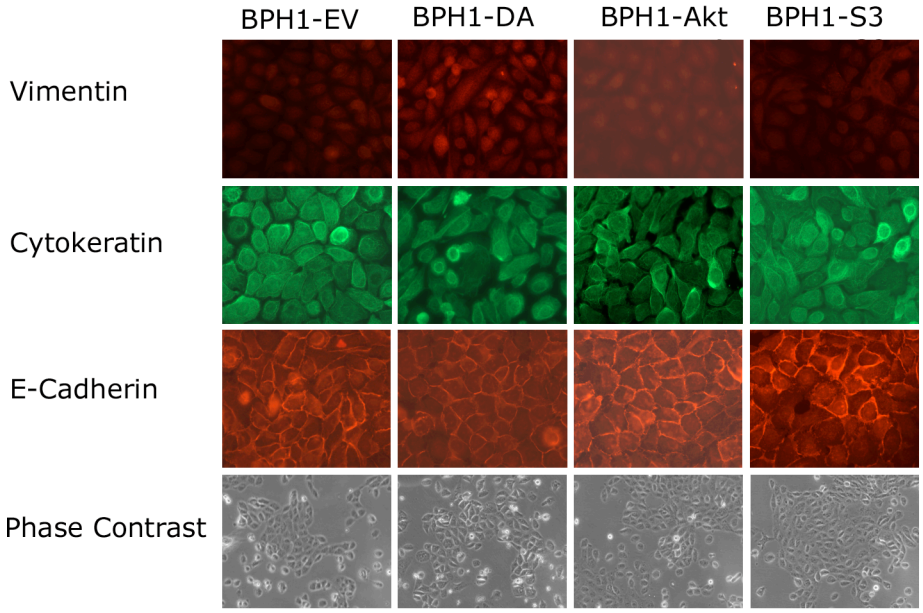


Figure 5-10. TGF-β Signaling did not change protein profiles in BPH1 cells

Immunofluorescence staining of BPH1 cells expressing empty vector (EV-control), dominant active TGF-β receptor 1 (DA), myristylated Akt (Akt) and Smad3 (S3) retroviral constructs. Cells are stained to visualize expression of vimentin, keratin and E-cadherin. In the non-tumorigenic BPH1 cells a cobblestone epithelial phenotype was maintained under all conditions with expression of E-cadherin and cytokeratin being maintained and little to no immunoreactivity to a vimentin antibody.

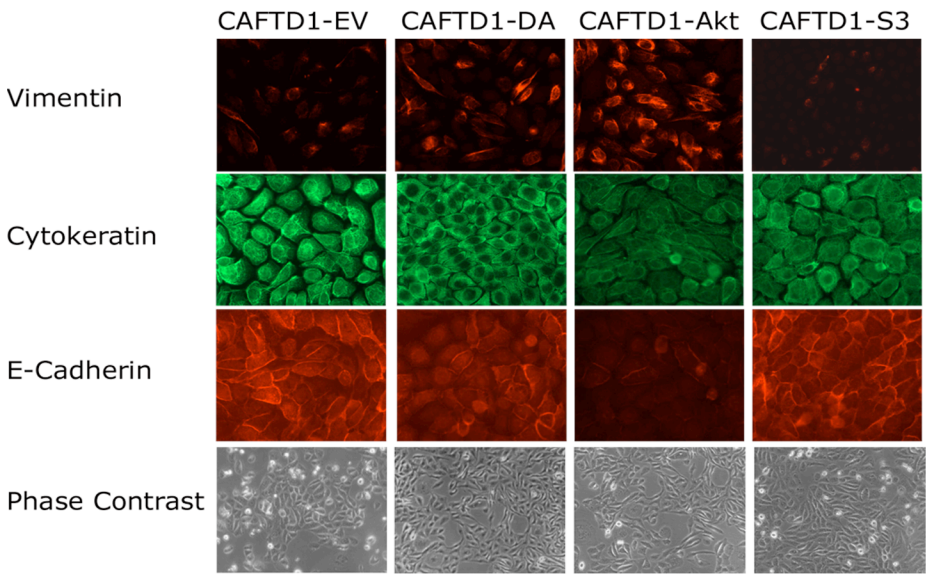


Figure 5-11. TGF-β Signaling changed protein expression in BPH1^{CAFTD}1 cells

Immunofluorescence staining BPH1^{CAFTD}1 (CAFTD-1) cells expressing empty vector (EV-control), dominant active TGF-β receptor 1 (DA), myristylated Akt (Akt) and Smad3 (S3) retroviral constructs. Cells are stained to visualize expression of vimentin, keratin and E-cadherin. In the tumorigenic BPH1^{CAFTD}1 cells show vimentin induction and suppression of E-cadherin when either the DA receptor or myrAkt1 are expressed. Of note stimulation of the SMAD pathway in these tumorigenic cells does not elicit phenotypic changes consistent with EMT.

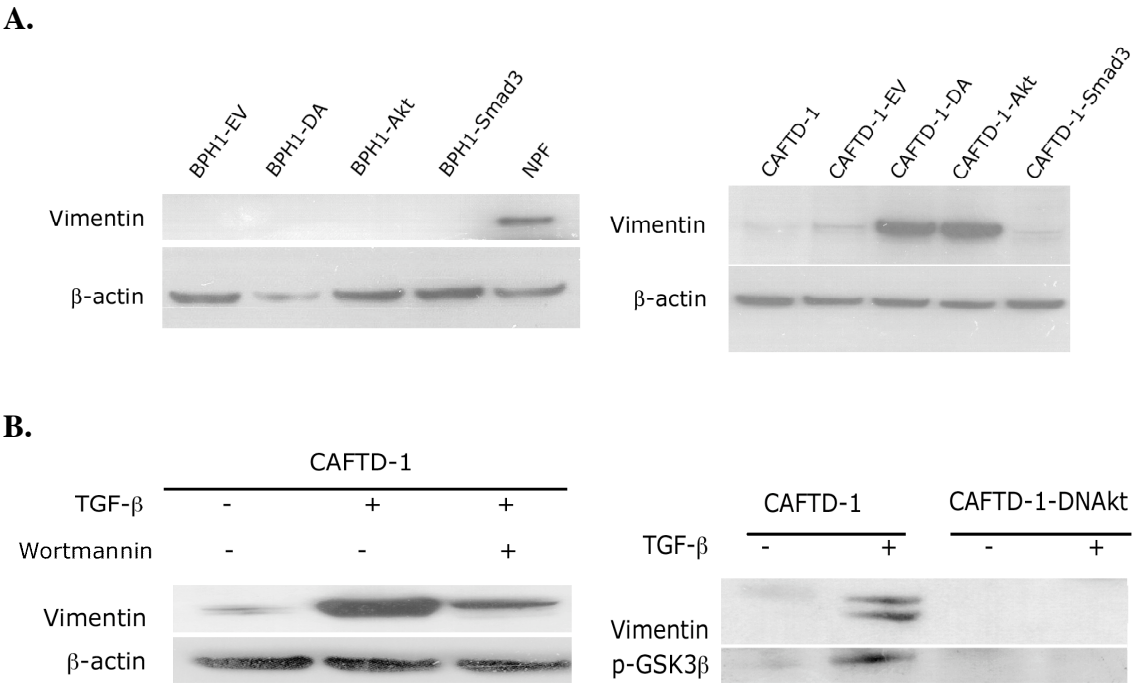


Figure 5-12. PI3/Akt is Involved in Vimentin Induction by TGF-β Signaling

A. Western blot analysis confirms the immunocytochemical observations with no vimentin expression seen in BPH1 cells under any circumstance while BPH1^{CAFTD}1 cells show marked increases in vimentin expression when either the DA receptor or myrAkt1 were expressed. Normal prostate fibroblasts (NPF) were used for a positive control.

B. BPH1^{CAFTD}1 cells (CAFTD-1) were serum starved overnight and treated with TGF-β1 (5ng/ml) for 60 hours, in the presence or absence of PI3Kinase inhibitor Wortmannin (100nM). The cell lysates were probed with vimentin antibody. After pretreatment with Wortmannin, vimentin induction by TGF-β was impaired. In a parallel study BPH1^{CAFTD}1 cell were infected by DNAkt adenovirus 24 hours before treatment with TGF-β1. The result indicates that after DNAkt infection, TGF-β does not induce vimentin expression in these cells.

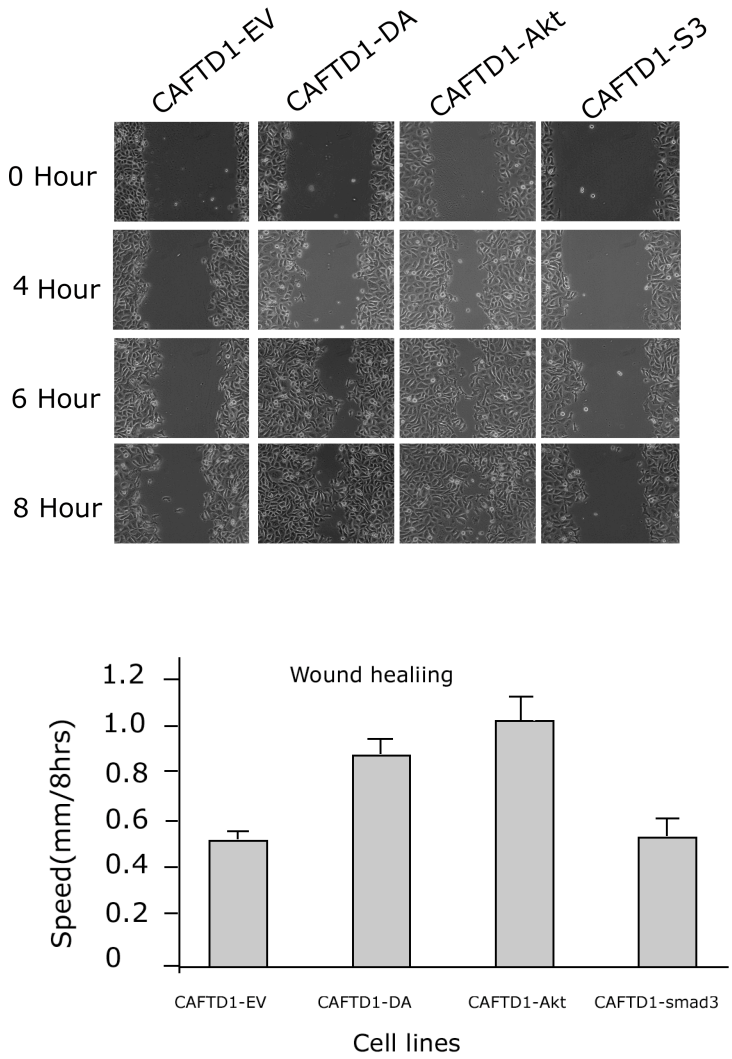


Figure 5-13. Activation of TGF-β or Akt Signaling Enhances Cell Motility in Wound Healing Assay

In a wound healing assay a measure of the speed of BPH1^{CAFTD}1 cells closing the wound demonstrated that once both DATβRI and myrAkt1 enhanced cell mobility compared to empty vector while Smad3 expression had no effect. All the experiments were performed in triplicates, mean and standard deviation shown (Student's t-test, $p<0.01$).

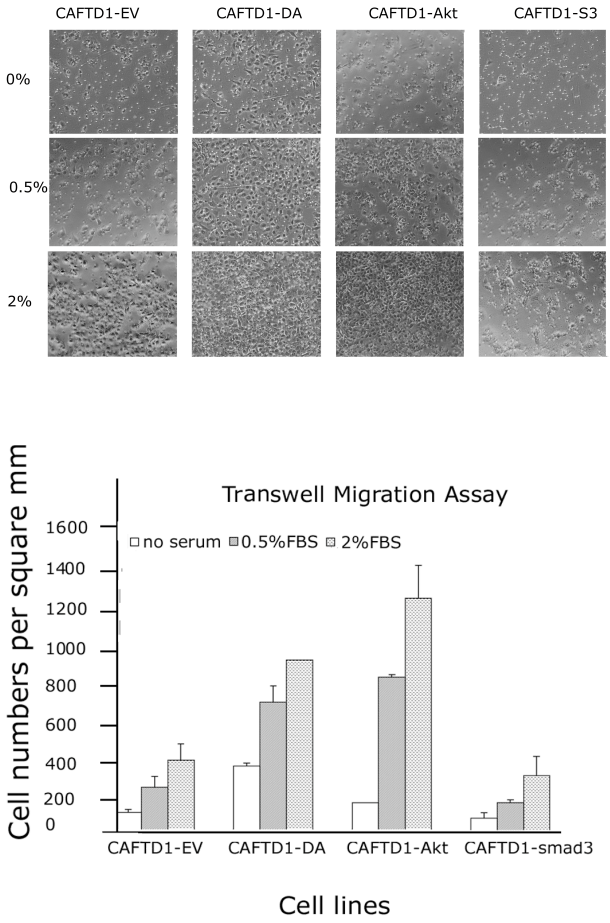


Figure 5-14. Activation of TGF-β or Akt Signaling Enhances Cell Motility in Transwell Migration Assay

A transwell migration assay showed that both DATβRI and myrAkt1 significantly enhancing invasion in the presence of serum while Smad3 overexpression had a non-significant inhibitory effect. The top figure shows the cells migrated to the undersurface of the inserts in response to different serum concentration as indicated. The migrated cells were fixed and counted and the numbers were used to plot the lower figure. All the experiment was performed in triplicates, mean and standard deviation shown (Student's t-test, $p<0.01$).

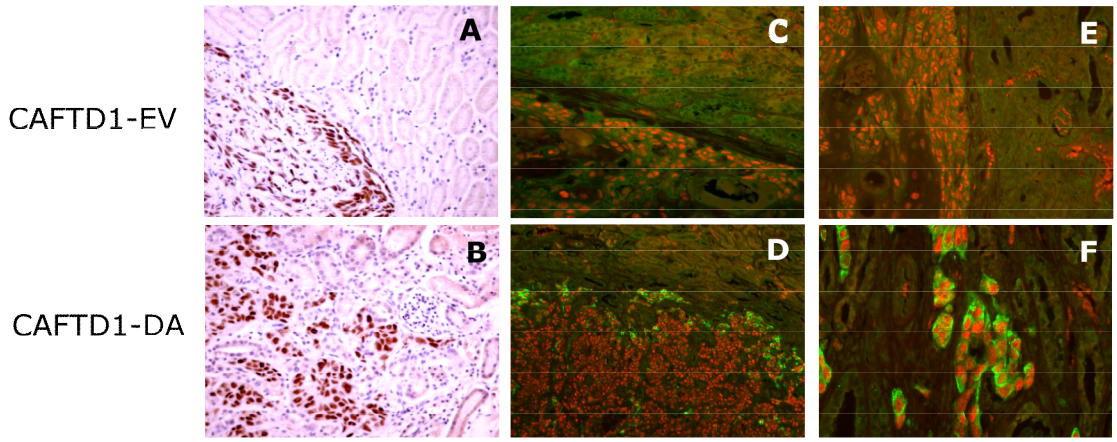


Figure 5-15. TGF-β Stimulates Cell Invasion *in vivo*

Grafts of BPH1^{CAFTD}1 cells carrying empty vector control (CAFTD-1-EV; panels A, C, E) or dominant active TGF-β receptor 1 (CAFTD-1-DA; panels B, D, F). Cells were grafted to the renal capsule of SCID mice and harvested after two months. Sections were stained for expression of SV40T antigen (A and B) or double stained for SV40T antigen (red) and vimentin (green) using immunofluorescence (C-F). BPH1^{CAFTD}1 cells were chosen for this assay, as they are minimally invasive, an observation confirmed in the EV controls (A, C, E). These tumors grow on the surface of the kidney with limited invasion and no expression of vimentin. In contrast cells expressing DATβRI invaded the host kidney infiltrating between, and surrounding, kidney tubules (B, D, F). Vimentin expression by the epithelial cells was located specifically at the invading front and not in the tumor body (D, F). Co-expression of vimentin and SV40T (shown in detail in panel F) confirms that the cells which expressed vimentin were derived from the BPH1^{CAFTD}1.

CHAPTER VI

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This project used human prostatic cell lines to study the effects of factors mediating stromal-epithelial interactions during human prostatic tumorigenesis. The parallel application of *in vitro* co-culture systems, *in vivo* tissue recombination and xenografting models confirmed transformation of the initiated but non-tumorigenic epithelial cells (BPH-1) upon exposure to human prostatic tumor stromal cells (CAF). This study identified TGF-β as an essential factor communicating between the stromal and adjacent epithelial cells. An elevated level of secreted TGF-β by the tumor stroma was found to play a critical role in inducing tumor formation, with roles in regulating epithelial proliferation and increasing cell motility. These findings provide another piece of evidence that the interaction between cancer cells and the surrounding stroma is essential for prostatic carcinogenesis. During cancer development, the stromal cells acquired a range of properties which allowed them to maintain and promote the tumorigenic condition. One of these mechanisms is to secrete factors, for example, TGF-β, which exert a paracrine influence on adjacent epithelial cells.

As an immortalized but non-tumorigenic cell line, BPH1 cells form very organized solid cords when recombined with rat UGM. However, when BPH1 cells are recombined with CAFs, they form malignant structures with streaming epithelia intermingled into

fibrous stroma (70). Additionally, when host animals carrying BPH1 + rUGM recombinants were treated with testosterone and estradiol-17beta, epithelial cells metastasized from the renal capsule graft site to liver, lung and renal lymph nodes (177). For these reasons, BPH1 cells and their derivatives represent a progression model including different stages of prostate cancer with the ability to move cells from one stage to the next. These cells also provide a unique model to interrogate the functions of TGF-β during disease progression. In early stage cancer, TGF-β acts as a tumor suppressor by inhibiting cell proliferation; in late stage, TGF-β acts a prooncogenic role by inducing EMT which leads to increased invasive ability. Our data demonstrated that parental BPH1 cells display growth inhibition in response to TGF-β, while the tumorigenic $BPH1^{CAFTD}$ derivatives skip growth inhibition of TGF-β, instead responding to TGF-β by undergoing EMT with loss of epithelial markers such as keratin and E-Cadherin and acquisition of the mesenchymal marker vimentin. Additionally, these tumorigenic cells displayed morphological changes and increased migration in culture, characteristics which have been demonstrated to correspond with the invasion *in vivo*. The EMT cells lead the invasive front in tissue recombinants and xenografts.

This study on TGF-β signaling enhanced our understanding of at least in two aspects of the pathway. The first important concept is that the final biological function of TGF-β is the net effect of the integration of multiple signaling events. It is well known that TGF-β inhibits cell growth in normal epithelial cells. One key event that leads to this function is the induction of expression of the CDK inhibitors $p15^{INK4B}$ (140) and/or $p21^{CIP1}$ (178), depending on the cell type. The inhibitor $p21^{CIP1}$ interacts with

complexes of CDK2 and cyclin A or cyclin E and thereby inhibits CDK2 activity, preventing progression of the cell cycle. By contrast, $p15^{INK4B}$ interacts with and inactivates CDK4 and CDK6, or associates with cyclin D complexes of CDK4 or CDK6. The latter interaction not only inactivates the catalytic activity of these CDKs but also displaces $p21^{CIP1}$ or the related $p27^{KIP1}$ from these complexes, allowing them to bind to and inactivate the CDK2 complexes with cyclin A and E (reviewed in (167). Induction of $p15^{INK4B}$ or $p21^{CIP1}$ expression in response to TGF-β is brought about by Smad-mediated transcriptional activation (179). However, alterations in the TGF-β signaling pathway contribute to increased tumor progression, invasion and metastasis by different routes. First, early genetic loss of signaling components, such as TRII, leads to increased synthesis of DNA, more rapid tumor growth and clonal expansion, which results in an increased probability of accumulating further mutations and cytogenetic changes (165). Second, and more frequently, the TGF-β signaling pathway remains intact but is perturbed by epigenetic mechanisms, such as activation of other signaling factors, which leads to a diminished growth inhibitory response but accentuation of activities that enhance tumor progression. Synergism between an elevated TGF-β signaling pathway and other signaling pathways leads to a direct increase in tumor cell plasticity, invasion and metastasis. The present study demonstrated that integration between TGF-β signaling and SDF-1/CXCR4 signaling is one of the mechanisms which could result in a pro-oncogenic role of TGF-β.

With regard to these experiments, the function of TGF-β on BPH-1 cells is modified by the stromal input through integration with SDF-1/CXCR4 signaling. CAF secreted elevated levels of SDF-1 in parallel to TGF-β. Since TGF-β stimulates CXCR4 expression in BPH1 cells, upon exposure to tumor stroma, the signaling from TGF-β and that from SDF-1 cooperate and result in Akt activation. To our knowledge this is the first report of such an interaction. This is an important result providing a mechanism by which cells can ignore the growth inhibitory effects of TGF-β. Activation of Akt stimulated proliferation and enhanced motility, characteristics which favor tumorigenicity. The second important feature of TGF-β is that the recipient cell context can determine the response to TGF-β. As we demonstrated, BPH1 cells and the tumorigenic derivatives BPH1-^{CAFTD} lines showed different responses to TGF-β treatment due to the constitutively higher levels of phosphorylated Akt in the BPH1^{CAFTD} cells. These higher levels of active Akt block translocation of Smad3 and p21 into the nucleus upon TGF-β stimulation thus ameliorating the growth inhibitory effects of TGF-β. At the same time TGF-β induces EMT in these tumorigenic cells thus contributing to increased invasive ability *in vivo*.

The present study identified a signal transduction mechanism between tumor stroma and epithelial cells. It identified an underlying mechanism by which tumor stroma can induce a tumorigenic response from adjacent epithelium and can subsequently contribute to tumor progression. Thus, these data have contributed to the further understanding of prostatic stromal function in tumorigenesis. These results shed some light on the interaction between epithelium and stroma. Clearly this is not the end of the story. Future directions include the further study of the mechanisms by which TGF-β induces CXCR4

in BPH1 cells; an examination of which other paracrine signals are changed upon exposure to tumor stroma exposure; a determination of the role of autocrine effects on epithelial cells; the role of juxtacrine signaling between the tumor and surrounding normal stroma; and, a determination of the connections between the signaling condition, clinical diagnosis, therapy and prognosis. Thus, this work represents an early step in an ongoing process to more fully understand the details of the signaling environment in carcinogenesis.

REFERENCES

1. Aumuller, G. [Functional morphology of the prostate]. Urologe A, *28:* 306-310, 1989.

2. Cunha, G. R., Donjacour, A. A., Cooke, P. S., Mee, S., Bigsby, R. M., Higgins, S. J., and Sugimura, Y. The endocrinology and developmental biology of the prostate. Endocr Rev, *8:* 338-362, 1987.

3. Berry, S. J., Coffey, D. S., Walsh, P. C., and Ewing, L. L. The development of human benign prostatic hyperplasia with age. J Urol, *132:* 474-479, 1984.

4. Cunha, G. R., Hayward, S. W., Dahiya, R., and Foster, B. A. Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. Acta Anat (Basel), *155:* 63-72, 1996.

5. Hayward, S. W. and Cunha, G. R. The prostate: development and physiology. Radiol Clin North Am, *38:* 1-14, 2000.

6. Hayward, S. W., Baskin, L. S., Haughney, P. C., Foster, B. A., Cunha, A. R., Dahiya, R., Prins, G. S., and Cunha, G. R. Stromal development in the ventral prostate, anterior prostate and seminal vesicle of the rat. Acta Anat (Basel), *155:* 94-103, 1996.

7. Hayward, S. W., Baskin, L. S., Haughney, P. C., Cunha, A. R., Foster, B. A., Dahiya, R., Prins, G. S., and Cunha, G. R. Epithelial development in the rat ventral prostate, anterior prostate and seminal vesicle. Acta Anat (Basel), *155:* 81-93, 1996.

8. Wang, Y., Hayward, S., Cao, M., Thayer, K., and Cunha, G. Cell differentiation lineage in the prostate. Differentiation, *68:* 270-279, 2001.

9. Cunha, G. R., Battle, E., Young, P., Brody, J., Donjacour, A., Hayashi, N., and Kinbara, H. Role of epithelial-mesenchymal interactions in the differentiation and spatial organization of visceral smooth muscle. Epithelial Cell Biol, *1:* 76-83, 1992.

10. Cunha, G. R. and Donjacour, A. Stromal-epithelial interactions in normal and abnormal prostatic development. Prog Clin Biol Res, *239:* 251-272, 1987.

11. Lilja, H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J Clin Invest, *76:* 1899-1903, 1985.

12. Lilja, H. and Lundwall, A. Molecular cloning of epididymal and seminal vesicular transcripts encoding a semenogelin-related protein. Proc Natl Acad Sci U S A, *89:* 4559-4563, 1992.

13. Peehl, D. M. Cellular biology of prostatic growth factors. Prostate Suppl, *6:* 74-78, 1996.

14. Seitz, J. and Aumuller, G. Biochemical properties of secretory proteins from rat seminal vesicles. Andrologia, *22 Suppl 1:* 25-32, 1990.

15. Lowsley, O. S. The development of the human prostate gland with reference to the development of other structures at the neck of the urinary bladder. Am. J. Anat., *13:* 299-349, 1912.

16. Hutch, J. A. and Rambo, O. S., Jr. A study of the anatomy of the prostate, prostatic urethra and the urinary sphincter system. J Urol, *104:* 443-452, 1970.

17. McNeal, J. E. and Bostwick, D. G. Anatomy of the prostatic urethra. Jama, *251:* 890-891, 1984.

18. Kissane, J. M. Anderson's Pathology, 9th edition, p. 72. St. Louis: Mosby, 1989.

19. DeKlerk, D. P., Coffey, D. S., Ewing, L. L., McDermott, I. R., Reiner, W. G., Robinson, C. H., Scott, W. W., Strandberg, J. D., Talalay, P., Walsh, P. C., Wheaton, L. G., and Zirkin, B. R. Comparison of spontaneous and experimentally induced canine prostatic hyperplasia. J Clin Invest, *64:* 842-849, 1979.

20. Steiner, M. S., Couch, R. C., Raghow, S., and Stauffer, D. The chimpanzee as a model of human benign prostatic hyperplasia. J Urol, *162:* 1454-1461, 1999.

21. McNeal, J. E. Relationship of the origin of benign prostatic hypertrophy to prostatic structure of man and other mammals. *In:* F. J. Hinman (ed.), Benign Prostatic Hypertrophy, pp. 152-166. New York: Springer-Verlag, 1983.

22. Aboseif, S., El-Sakka, A., Young, P., and Cunha, G. Mesenchymal reprogramming of adult human epithelial differentiation. Differentiation, *65:* 113-118, 1999.

23. Burnett, A. L. and Wein, A. J. Benign prostatic hyperplasia in primary care: what you need to know. J Urol, *175:* S19-24, 2006.

24. Fitzpatrick, J. M. The natural history of benign prostatic hyperplasia. BJU Int, *97 Suppl 2:* 3-6; discussion 21-22, 2006.

25. McConnell, J. D., Barry, M. J., and Bruskewitz, R. C. Benign prostatic hyperplasia: diagnosis and treatment. Agency for Health Care Policy and Research. Clin Pract Guidel Quick Ref Guide Clin 1-17, 1994.

26. Tiwari, A., Krishna, N. S., Nanda, K., and Chugh, A. Benign prostatic hyperplasia: an insight into current investigational medical therapies. Expert Opin Investig Drugs, *14:* 1359-1372, 2005.

27. McConnell, J. D., Roehrborn, C. G., Bautista, O. M., Andriole, G. L., Jr., Dixon, C. M., Kusek, J. W., Lepor, H., McVary, K. T., Nyberg, L. M., Jr., Clarke, H. S., Crawford, E. D., Diokno, A., Foley, J. P., Foster, H. E., Jacobs, S. C., Kaplan, S. A., Kreder, K. J., Lieber, M. M., Lucia, M. S., Miller, G. J., Menon, M., Milam, D. F., Ramsdell, J. W., Schenkman, N. S., Slawin, K. M., and Smith, J. A. The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia. N Engl J Med, *349:* 2387-2398, 2003.

28. Postma, R. and Schroder, F. H. Screening for prostate cancer. Eur J Cancer, *41:* 825-833, 2005.

29. Siesling, S., van Dijck, J. A., Visser, O., and Coebergh, J. W. Trends in incidence of and mortality from cancer in The Netherlands in the period 1989-1998. Eur J Cancer, *39:* 2521-2530, 2003.

30. Schwartz, G. G. Vitamin D and the epidemiology of prostate cancer. Semin Dial, *18:* 276-289, 2005.

31. Scattoni, V., Sangalli, M., Roscigno, M., Raber, M., Gallina, A., Fabbri, F., Sacca, A., Salonia, A., Montorsi, F., and Rigatti, P. Detection and diagnosis of prostate cancer: what's new. Arch Ital Urol Androl, *77:* 173-179, 2005.

32. Thompson, I. M., Bermejo, C., Hernandez, J., Basler, J. A., and Canby-Hagino, E. Screening for prostate cancer: opportunities and challenges. Surg Oncol Clin N Am, *14:* 747-760, 2005.

33. Montironi, R., Mazzuccheli, R., Scarpelli, M., Lopez-Beltran, A., Fellegara, G., and Algaba, F. Gleason grading of prostate cancer in needle biopsies or radical prostatectomy specimens: contemporary approach, current clinical significance and sources of pathology discrepancies. BJU Int, *95:* 1146-1152, 2005.

34. Coard, K. C. and Freeman, V. L. Gleason grading of prostate cancer: level of concordance between pathologists at the University Hospital of the West Indies. Am J Clin Pathol, *122:* 373-376, 2004.

35. Hara, I. [Advancement of treatment for prostate cancer]. Gan To Kagaku Ryoho, *33:* 178-182, 2006.

36. Huggins, C. and Hodges, C. V. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin, *22:* 232-240, 1972.

37. Wilson, S. S. and Crawford, E. D. Controversies of androgen ablation therapy for metastatic prostate cancer. Curr Pharm Des, *12:* 799-805, 2006.

38. Gillessen, S. and Strasser, F. [Treatment possibilities for patients with advanced prostate cancer]. Ther Umsch, *63:* 157-162, 2006.

39. Isaacs, J. T. New strategies for the medical treatment of prostate cancer. BJU Int, *96 Suppl 2:* 35-40, 2005.

40. Cunha, G. R. and Donjacour, A. A. Mesenchymal-epithelial interactions: Technical considerations. *In:* D. S. Coffey, N. Bruchovsky, W. A. Gardner, M. I. Resnick, and J. P. Karr (eds.), Assessment of Current Concepts and Approaches to the Study of Prostate Cancer, pp. 273-282. New York: A.R. Liss, 1987.

41. Hayward, S. W., Haughney, P. C., Rosen, M. A., Greulich, K. M., Weier, H. U., Dahiya, R., and Cunha, G. R. Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. Differentiation, *63:* 131-140, 1998.

42. Haughney, P. C., Hayward, S. W., Dahiya, R., and Cunha, G. R. Species-specific detection of growth factor gene expression in developing prostatic tissue. Biol. of Reproduction, *59:* 93-99, 1998.

43. Wang, Y., Hayward, S. W., Donjacour, A. A., Young, P., Jacks, T., Sage, J., Dahiya, R., Cardiff, R. D., Day, M. L., and Cunha, G. R. Sex hormone-induced carcinogenesis in Rb-deficient prostate tissue. Cancer Res, *60:* 6008-6017, 2000.

44. Bhowmick, N. A., Chytil, A., Plieth, D., Gorska, A. E., Dumont, N., Shappell, S., Washington, M. K., Neilson, E. G., and Moses, H. L. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science, *303:* 848-851, 2004.

45. Williams, K., Fernandez, S., Stien, X., Ishii, K., Love, H. D., Lau, Y. F., Roberts, R. L., and Hayward, S. W. Unopposed c-MYC expression in benign prostatic epithelium causes a cancer phenotype. Prostate, *63:* 369-384, 2005.

46. Cunha, G. R., Fujii, H., Neubauer, B. L., Shannon, J. M., Sawyer, L., and Reese, B. A. Epithelial-mesenchymal interactions in prostatic development. I. morphological observations of prostatic induction by urogenital sinus mesenchyme in epithelium of the adult rodent urinary bladder. J Cell Biol, *96:* 1662-1670, 1983.

47. Cunha, G. R. and Chung, L. W. Stromal-epithelial interactions--I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. J Steroid Biochem, *14:* 1317-1324, 1981.

48. Cooke, P. S., Young, P., and Cunha, G. R. Androgen receptor expression in developing male reproductive organs. Endocrinology, *128:* 2867-2873, 1991.

49. Donjacour, A. A. and Cunha, G. R. Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. Endocrinology, *131:* 2342-2350, 1993.

50. Kurita, T., Lee, K. J., Cooke, P. S., Taylor, J. A., Lubahn, D. B., and Cunha, G. R. Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract. Biol Reprod, *62:* 821-830, 2000.

51. Kurita, T., Lee, K. J., Cooke, P. S., Lydon, J. P., and Cunha, G. R. Paracrine regulation of epithelial progesterone receptor and lactoferrin by progesterone in the mouse uterus. Biol Reprod, *62:* 831-838, 2000.

52. Gao, N., Ishii, K., Mirosevich, J., Kuwajima, S., Oppenheimer, S. R., Roberts, R. L., Jiang, M., Yu, X., Shappell, S. B., Caprioli, R. M., Stoffel, M., Hayward, S. W., and Matusik, R. J. Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. Development, *132:* 3431-3443, 2005.

53. Cunha, G. R., Hayward, S. W., and Wang, Y. Z. Role of stroma in carcinogenesis of the prostate. Differentiation, *70:* 473-485, 2002.

54. Wang, Y., Xue, H., Cutz, J. C., Bayani, J., Mawji, N. R., Chen, W. G., Goetz, L. J., Hayward, S. W., Sadar, M. D., Gilks, C. B., Gout, P. W., Squire, J. A., Cunha, G. R., and Wang, Y. Z. An orthotopic metastatic prostate cancer model in SCID mice via grafting of a transplantable human prostate tumor line. Lab Invest, *85:* 1392-1404, 2005.

55. Grossfeld, G., Hayward, S., Tlsty, T., and Cunha, G. The role of stroma in prostatic carcinogenesis. Endocrine-related cancer, *5:* 253-270, 1998.

56. Hayward, S. W., Rosen, M. A., and Cunha, G. R. Stromal-epithelial interactions in the normal and neoplastic prostate. Br J Urol, *79 Suppl 2:* 18-26, 1997.

57. Hayward, S. W., Cunha, G. R., and Dahiya, R. Normal development and carcinogenesis of the prostate: A unifying hypothesis. Ann N Y Acad Sci., *784:* 50-62, 1996.

58. Cunha, G. R., Cooke, P. S., and Kurita, T. Role of stromal-epithelial interactions in hormonal responses. Arch Histol Cytol, *67:* 417-434, 2004.

59. Hill, R., Song, Y., Cardiff, R. D., and Van Dyke, T. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell, *123:* 1001-1011, 2005.

60. Rowley, D. R. What might a stromal response mean to prostate cancer progression? Cancer Metastasis Rev, *17:* 411-419, 1998.

61. Tuxhorn, J. A., Ayala, G. E., Smith, M. J., Smith, V. C., Dang, T. D., and Rowley, D. R. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clin Cancer Res, *8:* 2912-2923, 2002.

62. Ayala, G., Tuxhorn, J. A., Wheeler, T. M., Frolov, A., Scardino, P. T., Ohori, M., Wheeler, M., Spitler, J., and Rowley, D. R. Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer. Clin Cancer Res, *9:* 4792-4801, 2003.

63. Micke, P. and Ostman, A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer, *45 Suppl 2:* S163-175, 2004.

64. Beacham, D. A. and Cukierman, E. Stromagenesis: The changing face of fibroblastic microenvironments during tumor progression. Semin Cancer Biol, 2005.

65. Bosman, F. T., de Bruine, A., Flohil, C., van der Wurff, A., ten Kate, J., and Dinjens, W. W. Epithelial-stromal interactions in colon cancer. Int J Dev Biol, *37:* 203-211, 1993.

66. De Wever, O. and Mareel, M. Role of tissue stroma in cancer cell invasion. J Pathol, *200:* 429-447, 2003.

67. Cheng, J. D. and Weiner, L. M. Tumors and their microenvironments: tilling the soil. Commentary re: A. M. Scott et al., A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. Clin. Cancer Res., 9: 1639-1647, 2003. Clin Cancer Res, *9:* 1590-1595, 2003.

68. Camps, J. L., Chang, S. M., Hsu, T. C., Freeman, M. R., Hong, S. J., Zhau, H. E., von Eschenbach, A. C., and Chung, L. W. Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. Proc Natl Acad Sci U S A, *87:* 75-79, 1990.

69. Chung, L. W., Gleave, M. E., Hsieh, J. T., Hong, S. J., and Zhau, H. E. Reciprocal mesenchymal-epithelial interaction affecting prostate tumour growth and hormonal responsiveness. Cancer Surv, *11:* 91-121, 1991.

70. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res, *59:* 5002-5011, 1999.

71. Massague, J. TGF-beta signal transduction. Annu Rev Biochem, *67:* 753-791, 1998.

72. Massague, J. How cells read TGF-beta signals. Nat Rev Mol Cell Biol, *1:* 169-178, 2000.

73. Akhurst, R. J. and Derynck, R. TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol, *11:* S44-51, 2001.

74. Wakefield, L. M. and Roberts, A. B. TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev, *12:* 22-29, 2002.

75. Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L., and Arteaga, C. L. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem, *275:* 36803-36810, 2000.

76. Bakin, A. V., Rinehart, C., Tomlinson, A. K., and Arteaga, C. L. p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci, *115:* 3193-3206, 2002.

77. Dumont, N. and Arteaga, C. L. Targeting the TGF beta signaling network in human neoplasia. Cancer Cell, *3:* 531-536, 2003.

78. Bhowmick, N. A., Ghiassi, M., Bakin, A., Aakre, M., Lundquist, C. A., Engel, M. E., Arteaga, C. L., and Moses, H. L. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell, *12:* 27-36, 2001.

79. Bello-DeOcampo, D. and Tindall, D. J. TGF-betal/Smad signaling in prostate cancer. Curr Drug Targets, *4:* 197-207, 2003.

80. Danielpour, D. Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate. Eur J Cancer, *41:* 846-857, 2005.

81. Tang, B., Vu, M., Booker, T., Santner, S. J., Miller, F. R., Anver, M. R., and Wakefield, L. M. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest, *112:* 1116-1124, 2003.

82. Dumont, N. and Arteaga, C. L. The tumor microenvironment: a potential arbitrator of the tumor suppressive and promoting actions of TGFbeta. Differentiation, *70:* 574-582, 2002.

83. Roberts, A. B. and Wakefield, L. M. The two faces of transforming growth factor beta in carcinogenesis. Proc Natl Acad Sci U S A, *100:* 8621-8623, 2003.

84. Kyprianou, N. Activation of TGF-beta signalling in human prostate cancer cells suppresses tumorigenicity via deregulation of cell cycle progression and induction of caspase-1 mediated apoptosis: significance in prostate tumorigenesis. Prostate Cancer Prostatic Dis, *2:* S18, 1999.

85. Guo, Y. and Kyprianou, N. Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. Cancer Res, *59:* 1366-1371, 1999.

86. Lee, C., Sintich, S. M., Mathews, E. P., Shah, A. H., Kundu, S. D., Perry, K. T., Cho, J. S., Ilio, K. Y., Cronauer, M. V., Janulis, L., and Sensibar, J. A. Transforming growth factor-beta in benign and malignant prostate. Prostate, *39:* 285-290, 1999.

87. Barrack, E. R. TGF beta in prostate cancer: a growth inhibitor that can enhance tumorigenicity. Prostate, *31:* 61-70, 1997.

88. Ellenrieder, V., Hendler, S. F., Boeck, W., Seufferlein, T., Menke, A., Ruhland, C., Adler, G., and Gress, T. M. Transforming growth factor beta1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. Cancer Res, *61:* 4222-4228, 2001.

89. Muraoka, R. S., Dumont, N., Ritter, C. A., Dugger, T. C., Brantley, D. M., Chen, J., Easterly, E., Roebuck, L. R., Ryan, S., Gotwals, P. J., Koteliansky, V., and Arteaga, C. L. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest, *109:* 1551-1559, 2002.

90. Remy, I., Montmarquette, A., and Michnick, S. W. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. Nat Cell Biol, *6:* 358-365, 2004.

91. Conery, A. R., Cao, Y., Thompson, E. A., Townsend, C. M., Jr., Ko, T. C., and Luo, K. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. Nat Cell Biol, *6:* 366-372, 2004.

92. Vanhaesebroeck, B. and Alessi, D. R. The PI3K-PDK1 connection: more than just a road to PKB. Biochem J, *346 Pt 3:* 561-576, 2000.

93. Nicholson, K. M. and Anderson, N. G. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal, *14:* 381-395, 2002.

94. Burger, J. A. and Kipps, T. J. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood, *107:* 1761-1767, 2006.

95. Loetscher, P., Moser, B., and Baggiolini, M. Chemokines and their receptors in lymphocyte traffic and HIV infection. Adv Immunol, *74:* 127-180, 2000.

96. Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y., and Nagasawa, T. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). Proc Natl Acad Sci U S A, *100:* 5319-5323, 2003.

97. Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature, *393:* 591-594, 1998.

98. Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T., and Springer, T. A. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci U S A, *95:* 9448-9453, 1998.

99. Crump, M. P., Gong, J. H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J. L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. Embo J, *16:* 6996-7007, 1997.

100. Aiuti, A., Webb, I. J., Bleul, C., Springer, T., and Gutierrez-Ramos, J. C. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med, *185:* 111-120, 1997.

101. Broxmeyer, H. E., Cooper, S., Kohli, L., Hangoc, G., Lee, Y., Mantel, C., Clapp, D. W., and Kim, C. H. Transgenic expression of stromal cell-derived factor-1/CXC chemokine ligand 12 enhances myeloid progenitor cell survival/antiapoptosis in vitro in response to growth factor withdrawal and enhances myelopoiesis in vivo. J Immunol, *170:* 421-429, 2003.

102. Balkwill, F. The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol, *14:* 171-179, 2004.

103. Kucia, M., Jankowski, K., Reca, R., Wysoczynski, M., Bandura, L., Allendorf, D. J., Zhang, J., Ratajczak, J., and Ratajczak, M. Z. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. J Mol Histol, *35:* 233-245, 2004.

104. Kang, H., Watkins, G., Douglas-Jones, A., Mansel, R. E., and Jiang, W. G. The elevated level of CXCR4 is correlated with nodal metastasis of human breast cancer. Breast, *14:* 360-367, 2005.

105. Sun, Y. X., Wang, J., Shelburne, C. E., Lopatin, D. E., Chinnaiyan, A. M., Rubin, M. A., Pienta, K. J., and Taichman, R. S. Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) in vivo. J Cell Biochem, *89:* 462-473, 2003.

106. Taichman, R. S., Cooper, C., Keller, E. T., Pienta, K. J., Taichman, N. S., and McCauley, L. K. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res, *62:* 1832-1837, 2002.

107. Cooper, C. R., Chay, C. H., Gendernalik, J. D., Lee, H. L., Bhatia, J., Taichman, R. S., McCauley, L. K., Keller, E. T., and Pienta, K. J. Stromal factors involved in prostate carcinoma metastasis to bone. Cancer, *97:* 739-747, 2003.

108. Kayali, A. G., Van Gunst, K., Campbell, I. L., Stotland, A., Kritzik, M., Liu, G., Flodstrom-Tullberg, M., Zhang, Y. Q., and Sarvetnick, N. The stromal cell-derived factor-1alpha/CXCR4 ligand-receptor axis is critical for progenitor survival and migration in the pancreas. J Cell Biol, *163:* 859-869, 2003.

109. Rubin, J. B., Kung, A. L., Klein, R. S., Chan, J. A., Sun, Y., Schmidt, K., Kieran, M. W., Luster, A. D., and Segal, R. A. A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. Proc Natl Acad Sci U S A, *100:* 13513-13518, 2003.

110. Luo, J., Manning, B. D., and Cantley, L. C. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell, *4:* 257-262, 2003.

111. Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V. J., Richardson, A. L., and Weinberg, R. A. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell, *121:* 335-348, 2005.

112. Wieser, R., Wrana, J. L., and Massague, J. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. Embo J, *14:* 2199-2208, 1995.

113. Barthel, A., Kohn, A. D., Luo, Y., and Roth, R. A. A constitutively active version of the Ser/Thr kinase Akt induces production of the ob gene product, leptin, in 3T3-L1 adipocytes. Endocrinology, *138:* 3559-3562, 1997.

114. Bernard, D. J. Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells. Mol Endocrinol, *18:* 606-623, 2004.

115. Dumont, N. and Arteaga, C. L. A kinase-inactive type II TGFbeta receptor impairs BMP signaling in human breast cancer cells. Biochem Biophys Res Commun, *301:* 108-112, 2003.

116. Liu, X., Yue, J., Frey, R. S., Zhu, Q., and Mulder, K. M. Transforming growth factor beta signaling through Smad1 in human breast cancer cells. Cancer Res, *58:* 4752-4757, 1998.

117. Nagarajan, R. P., Chen, F., Li, W., Vig, E., Harrington, M. A., Nakshatri, H., and Chen, Y. Repression of transforming-growth-factor-beta-mediated transcription by nuclear factor kappaB. Biochem J, *348 Pt 3:* 591-596, 2000.

118. Stewart, S. A., Dykxhoorn, D. M., Palliser, D., Mizuno, H., Yu, E. Y., An, D. S., Sabatini, D. M., Chen, I. S., Hahn, W. C., Sharp, P. A., Weinberg, R. A., and Novina, C. D. Lentivirus-delivered stable gene silencing by RNAi in primary cells. Rna, *9:* 493-501, 2003.

119. Mochizuki, H., Matsubara, A., Teishima, J., Mutaguchi, K., Yasumoto, H., Dahiya, R., Usui, T., and Kamiya, K. Interaction of ligand-receptor system between stromal-cell-derived factor-1 and CXC chemokine receptor 4 in human prostate cancer: a possible predictor of metastasis. Biochem Biophys Res Commun, *320:* 656-663, 2004.

120. Ishii, K., Shappell, S. B., Matusik, R. J., and Hayward, S. W. Use of tissue recombination to predict phenotypes of transgenic mouse models of prostate carcinoma. Lab Invest, 2005.

121. Cunha, G. R. Androgenic effects upon prostatic epithelium are mediated via trophic influences from stroma. Prog Clin Biol Res, *145:* 81-102, 1984.

122. Kobie, J. J., Wu, R. S., Kurt, R. A., Lou, S., Adelman, M. K., Whitesell, L. J., Ramanathapuram, L. V., Arteaga, C. L., and Akporiaye, E. T. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Res, *63:* 1860-1864, 2003.

123. Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., Guise, T. A., and Massague, J. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell, *3:* 537-549, 2003.

124. Chambers, A. F., Groom, A. C., and MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer, *2:* 563-572, 2002.

125. Kauffman, E. C., Robinson, V. L., Stadler, W. M., Sokoloff, M. H., and Rinker-Schaeffer, C. W. Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site. J Urol, *169:* 1122-1133, 2003.

126. Chung, L. W., Baseman, A., Assikis, V., and Zhau, H. E. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. J Urol, *173:* 10-20, 2005.

127. Berger, A. P., Kofler, K., Bektic, J., Rogatsch, H., Steiner, H., Bartsch, G., and Klocker, H. Increased growth factor production in a human prostatic stromal cell culture model caused by hypoxia. Prostate, *57:* 57-65, 2003.

128. Djavan, B., Bursa, B., Seitz, C., Soeregi, G., Remzi, M., Basharkhah, A., Wolfram, R., and Marberger, M. Insulin-like growth factor 1 (IGF-1), IGF-1 density, and IGF-1/PSA ratio for prostate cancer detection. Urology, *54:* 603-606, 1999.

129. Lail-Trecker, M., Gulati, R., and Peluso, J. J. A role for hepatocyte growth factor/scatter factor in regulating normal and neoplastic cells of reproductive tissues. J Soc Gynecol Investig, *5:* 114-121, 1998.

130. Hurle, R. A., Davies, G., Parr, C., Mason, M. D., Jenkins, S. A., Kynaston, H. G., and Jiang, W. G. Hepatocyte growth factor/scatter factor and prostate cancer: a review. Histol Histopathol, *20:* 1339-1349, 2005.

131. Kassis, J., Lauffenburger, D. A., Turner, T., and Wells, A. Tumor invasion as dysregulated cell motility. Semin Cancer Biol, *11:* 105-117, 2001.

132. Bauer, E. A., Uitto, J., Walters, R. C., and Eisen, A. Z. Enhanced collagenase production by fibroblasts derived from human basal cell carcinomas. Cancer Res, *39:* 4594-4599, 1979.

133. Knudson, W., Biswas, C., and Toole, B. P. Interactions between human tumor cells and fibroblasts stimulate hyaluronate synthesis. Proc Natl Acad Sci U S A, *81:* 6767-6771, 1984.

134. Hayward, S. W., Dahiya, R., Cunha, G. R., Bartek, J., Deshpande, N., and Narayan, P. Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. In Vitro Cell Dev Biol Anim, *31:* 14-24, 1995.

135. Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev, *10:* 2462-2477, 1996.

136. Wong, Y. C. and Wang, Y. Z. Growth factors and epithelial-stromal interactions in prostate cancer development. Int Rev Cytol, *199:* 65-116, 2000.

137. Joesting, M. S., Perrin, S., Elenbaas, B., Fawell, S. E., Rubin, J. S., Franco, O. E., Hayward, S. W., Cunha, G. R., and Marker, P. C. Identification of SFRP1 as a candidate mediator of stromal-to-epithelial signaling in prostate cancer. Cancer Res, *65:* 10423-10430, 2005.

138. Massague, J. and Chen, Y. G. Controlling TGF-beta signaling. Genes Dev, *14:* 627-644, 2000.

139. Sun, Y. X., Schneider, A., Jung, Y., Wang, J., Dai, J., Cook, K., Osman, N. I., Koh-Paige, A. J., Shim, H., Pienta, K. J., Keller, E. T., McCauley, L. K., and Taichman, R. S. Skeletal localization and neutralization of the SDF-1(CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites in vivo. J Bone Miner Res, *20:* 318-329, 2005.

140. Hannon, G. J. and Beach, D. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature, *371:* 257-261, 1994.

141. Oft, M., Heider, K. H., and Beug, H. TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol, *8:* 1243-1252, 1998.

142. Yin, J. J., Selander, K., Chirgwin, J. M., Dallas, M., Grubbs, B. G., Wieser, R., Massague, J., Mundy, G. R., and Guise, T. A. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest, *103:* 197-206, 1999.

143. Adachi-Yamada, T., Nakamura, M., Irie, K., Tomoyasu, Y., Sano, Y., Mori, E., Goto, S., Ueno, N., Nishida, Y., and Matsumoto, K. p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in Drosophila wing morphogenesis. Mol Cell Biol, *19:* 2322-2329, 1999.

144. Nagasawa, T. A chemokine, SDF-1/PBSF, and its receptor, CXC chemokine receptor 4, as mediators of hematopoiesis. Int J Hematol, *72:* 408-411, 2000.

145. Moser, B. and Loetscher, P. Lymphocyte traffic control by chemokines. Nat Immunol, *2:* 123-128, 2001.

146. Singh, S., Singh, U. P., Grizzle, W. E., and Lillard, J. W., Jr. CXCL12-CXCR4 interactions modulate prostate cancer cell migration, metalloproteinase expression and invasion. Lab Invest, *84:* 1666-1676, 2004.

147. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer, *72:* 1-22, 1997.

148. Miettinen, P. J., Ebner, R., Lopez, A. R., and Derynck, R. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol, *127:* 2021-2036, 1994.

149. Janda, E., Lehmann, K., Killisch, I., Jechlinger, M., Herzig, M., Downward, J., Beug, H., and Grunert, S. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol, *156:* 299-313, 2002.

150. Dumont, N., Bakin, A. V., and Arteaga, C. L. Autocrine transforming growth factor-beta signaling mediates Smad-independent motility in human cancer cells. J Biol Chem, *278:* 3275-3285, 2003.

151. Hayward, S. W., Wang, Y., Cao, M., Hom, Y. K., Zhang, B., Grossfeld, G. D., Sudilovsky, D., and Cunha, G. R. Malignant transformation in a nontumorigenic human prostatic epithelial cell line. Cancer Res, *61:* 8135-8142, 2001.

152. Phillips, J. L., Hayward, S. W., Wang, Y., Vasselli, J., Pavlovich, C., Padilla-Nash, H., Pezullo, J. R., Ghadimi, B. M., Grossfeld, G. D., Rivera, A., Linehan, W. M., Cunha, G. R., and Ried, T. The consequences of chromosomal aneuploidy on gene expression profiles in a cell line model for prostate carcinogenesis. Cancer Res, *61:* 8143-8149, 2001.

153. Piek, E. and Roberts, A. B. Suppressor and oncogenic roles of transforming growth factor-beta and its signaling pathways in tumorigenesis. Adv Cancer Res, *83:* 1-54, 2001.

154. Song, K., Wang, H., Krebs, T. L., and Danielpour, D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. Embo J, *25:* 58-69, 2006.

155. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev, *9:* 1831-1845, 1995.

156. Moustakas, A., Pardali, K., Gaal, A., and Heldin, C. H. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett, *82:* 85-91, 2002.

157. Shin, I., Bakin, A. V., Rodeck, U., Brunet, A., and Arteaga, C. L. Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. Mol Biol Cell, *12:* 3328-3339, 2001.

158. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature, *378:* 785-789, 1995.

159. Martikainen, P., Kyprianou, N., and Isaacs, J. T. Effect of transforming growth factor-beta 1 on proliferation and death of rat prostatic cells. Endocrinology, *127:* 2963-2968, 1990.

160. Hsing, A. Y., Kadomatsu, K., Bonham, M. J., and Danielpour, D. Regulation of apoptosis induced by transforming growth factor-beta1 in nontumorigenic rat prostatic epithelial cell lines. Cancer Res, *56:* 5146-5149, 1996.

161. Larisch-Bloch, S., Danielpour, D., Roche, N. S., Lotan, R., Hsing, A. Y., Kerner, H., Hajouj, T., Lechleider, R. J., and Roberts, A. B. Selective loss of the transforming growth factor-beta apoptotic signaling pathway in mutant NRP-154 rat prostatic epithelial cells. Cell Growth Differ, *11:* 1-10, 2000.

162. Kim, I. Y., Ahn, H. J., Zelner, D. J., Shaw, J. W., Lang, S., Kato, M., Oefelein, M. G., Miyazono, K., Nemeth, J. A., Kozlowski, J. M., and Lee, C. Loss of expression of transforming growth factor beta type I and type II receptors correlates with tumor grade in human prostate cancer tissues. Clin Cancer Res, *2:* 1255-1261, 1996.

163. Kundu, S. D., Kim, I. Y., Zelner, D., Janulis, L., Goodwin, S., Engel, J. D., and Lee, C. Absence of expression of transforming growth factor-beta type II receptor is associated with an aggressive growth pattern in a murine renal carcinoma cell line, Renca. J Urol, *160:* 1883-1888, 1998.

164. Tang, B., de Castro, K., Barnes, H. E., Parks, W. T., Stewart, L., Bottinger, E. P., Danielpour, D., and Wakefield, L. M. Loss of responsiveness to transforming growth factor beta induces malignant transformation of nontumorigenic rat prostate epithelial cells. Cancer Res, *59:* 4834-4842, 1999.

165. Guo, Y. and Kyprianou, N. Overexpression of transforming growth factor (TGF) beta1 type II receptor restores TGF-beta1 sensitivity and signaling in human prostate cancer cells. Cell Growth Differ, *9:* 185-193, 1998.

166. Wakefield, L. M., Piek, E., and Bottinger, E. P. TGF-beta signaling in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia, *6:* 67-82, 2001.

167. Derynck, R., Akhurst, R. J., and Balmain, A. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet, *29:* 117-129, 2001.

168. Wikstrom, P., Damber, J., and Bergh, A. Role of transforming growth factor-beta1 in prostate cancer. Microsc Res Tech, *52:* 411-419, 2001.

169. Derynck, R. and Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature, *425:* 577-584, 2003.

170. Cantley, L. C. and Neel, B. G. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A, *96:* 4240-4245, 1999.

171. Brader, S. and Eccles, S. A. Phosphoinositide 3-kinase signalling pathways in tumor progression, invasion and angiogenesis. Tumori, *90:* 2-8, 2004.

172. Besson, A., Robbins, S. M., and Yong, V. W. PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis. Eur J Biochem, *263:* 605-611, 1999.

173. Davies, M. A., Koul, D., Dhesi, H., Berman, R., McDonnell, T. J., McConkey, D., Yung, W. K., and Steck, P. A. Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. Cancer Res, *59:* 2551-2556, 1999.

174. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, *275:* 1943-1947, 1997.

175. Hay, E. D. An overview of epithelio-mesenchymal transformation. Acta Anat (Basel), *154:* 8-20, 1995.

176. Yue, J. and Mulder, K. M. Activation of the mitogen-activated protein kinase pathway by transforming growth factor-beta. Methods Mol Biol, *142:* 125-131, 2000.

177. Ricke, W. A., Ishii, K., Ricke, E. A., Simko, J., Wang, Y., Hayward, S. W., and Cunha, G. R. Steroid hormones stimulate human prostate cancer progression and metastasis. Int J Cancer, *118:* 2123-2131, 2006.

178. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A, *92:* 5545-5549, 1995.

179. Feng, X. H., Lin, X., and Derynck, R. Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. Embo J, *19:* 5178-5193, 2000.