Human prostate cell culture models for studies on prostate cancer progression and chemoprevention

By

Salmaan Taher Abdul Quader

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Master of Science

Department of Zoology

1999

ABSTRACT

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Prostate cancer is the leading cancer in men in the United States. Progress in research on prostate cancer has been hindered due to a lack of well-characterized human cell lines. This study presents characterization of five new human prostatic epithelial cell lines that exhibit varying degrees of malignant characteristics. These cell lines are unique because they are derived from the same parent cell line RWPE-1. These cells were derived by stepwise immortalization of normal prostatic epithelial cells followed by malignant transformation. Characterization is based on morphology, cytoskeletal protein expression, growth in monolayer, anchorage independent growth, response to growth factors, invasive ability, and tumorigenicity. These cell lines display different levels of malignancy with WPE-1-NA22 being the least malignant and WPE1-NB26 the most malignant. The other cell lines represent intermediate stages in the process of malignant progression. This is the first prostate cell culture model system where cell lines derived from the same parent cell line display varying degrees of malignancy. The following sequence was determined from the least to the most malignant: WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-NB26. This family of cell lines can be used to

examine the molecular basis of the multi-step process of carcinogenesis and tumor progression and has applications in the prevention and treatment of prostate cancer.

To my parents and my mentor

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Mukta M. Webber for her continuous support, guidance patience and for helping me think like a scientist. I thank my committee members Dr. Stephen C. Bromley, Dr. William Kamanda, Dr. Daniel E. Williams for their advice and support. I thank Dr. Diana Bello-DeOcampo for her invaluable instruction, assistance, support and friendship. I thank Nestor D. Deocampo for his advice and showing me the ropes in performing cell culture research. I would like to express my appreciation towards Dr. Hynda K. Kleinman for her assistance with tumorigenicity studies. I thank felllow lab members William W. Chu, Rebekah M. Sharp Nick Trakul and Brandi Purcell for their support and friendship. I would also like to acknowledge Beth Roestel and Natasha Nesbitt for their invaluable assistance.

I extend my deepest gratitude to my parents, Ather and Parveen Quader, for their unconditional love and suppport and my sister Sabeeha for her patience for all those days I couldn't come home and "play." I thank my sister Sameena and her husband Ahsan Basha for being there when I needed an ear to bend. I thank my fiancee Asra Fatima Hamzavi, who, contrary to her opinion, was a source of strength and inspiration rather than a distraction while I was writing this thesis. Finally, I would like to thank Allah (SWT) for giving me the ability, knowledge, and faith to complete this project.

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5α-DHT	5α-dihydrotestosterone
AR	androgen receptor
BEC	basal epithelial cells
bFGF	basic fibroblast growth factor
BPE	bovine pituitary extract
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CPA	Cyproterone acetate
CAMs	cell adhesion molecules
CFE	colony forming efficiency
CK	Cytokeratin
DAB	3,3'-diamino-benazadine
ECM	Extracellular matrix
EGF	epidermal growth factor
FBS	fetal bovine serum
H & E	Haematoxylin and eosin
HPV	human papilloma virus
IGF	insulin-like growth factor
IL	Interleukin
INT	p-iodonitrotetrazolium violet
KSFM	Ketatinocyte serum free medium
LW	Lobund-Wistar
MNU	*N*-methyl-*N*-nitrosourea
MRPs	mouse reconstituted prostate
MTT	3-[4,5-dimethylthiazol-2-yl]2,5-di-phenyltetrazolium bromide
NEC	Neuroendocrine cells
PAHs	polycyclic aromatic hydrocarbons
PAP	prostatic acid phosphatase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PIN	prostatic intraepithelial neoplasia
PKC	protein kinase C
PSA	prostate specific antigen
ROS	reactive oxygen species
SS	stem cells
TGF-β	Transforming growth factor-β
TNF	tumor necrosis factor
TP	Testosterone propionate
TPA	Tetradecanoyl phorbol-13-acetate
TURP	Transurethral resection of the prostate
UGE	urogenital sinus epithelium
UGM	urogenital sinus mesenchyme

Introduction

Prostate cancer is the most common non-skin malignancy and the second leading cause of cancer death among men in the United States (1). It is estimated that 185,500 new cases were reported and 39,200 men died of the disease in 1998 (1). Prostate cancer incidence increases with age and three-fourths of all prostate carcinomas are diagnosed in men over the age of 65 (1). Improved methods for detection, coupled with the large aging male population, have contributed to increased prostate cancer incidence and heightened public awareness of the disease in the United States (2). Approximately 11 million men between ages 45 and 50 are believed to have latent, nonmetastatic carcinoma of the prostate. It is predicted that this latent carcinoma will develop into metastatic prostate cancer in one in ten men (3,4). The invasive potential of latent carcinoma remains a mystery, as researchers and physicians are currently unable to determine what causes latent carcinoma to develop into metastatic disease and which cells are most likely to become malignant. This problem makes prognosis of early stage prostate cancer difficult leaving physicians unsure of how aggressively patients afflicted with the disease should be treated. It is, thus, imperative to uncover information leading to the understanding of the molecular events associated with prostate cancer progression. Such an understanding at the molecular level would only be possible through *in vitro*

study. However, researchers currently lack the tools that would allow them to explore this area of investigation.

In vitro research on prostate cancer progression has been made difficult in the past due to a lack of suitable human cell models. The majority of human prostate cancer cell models, currently in use, represent a highly metastatic state. This is hardly surprising because most of them were derived from biopsy specimens of metastasized prostate cancer (5). Therefore, these models provide little information regarding the early events associated with prostate carcinogenesis. Thus, it is imperative to establish cell models that more accurately represent the early stages of prostate carcinogenesis and tumor progression in order to gain a meaningful understanding of these processes.

Five such cell models have been developed in Dr. Mukta M. Webber's laboratory. The non-neoplastic human prostatic epithelial cell line, RWPE-1, was treated with the chemical carcinogen, *N*-methyl-*N*-nitrosourea, giving rise to the WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-NB26 cell lines. The primary objective of this study is the characterization of these cells to establish their human prostatic epithelial origin.

These cell models may provide important clues for gaining a stronger understanding of carcinogenesis at the molecular level. The understanding of the molecular events associated with carcinogenesis will provide the knowledge necessary for the discovery of chemical agents that will prevent cancer from manifesting at an early stage. The application of chemical agents to this end is termed chemoprevention. Chemoprevention, as defined by Sporn, "is the use of

pharmacological or natural agents that inhibit the development of invasive cancer either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of premalignant cells in which such damage has already occurred " (6,7). The concept of chemoprevention, therefore, includes the use of drugs to prevent development of preneoplastic lesions and their progression to invasive cancer.

References

1. American Cancer Society. Cancer Facts and Figures-1998.

2. Potosky AR; Miller BA; Albertsen PC; Kramer BS. The role of increasing detection in the rising incidence of prostate cancer. JAMA, 273:548-552, 1995.

3. Carter HB; Coffey DS. The prostate: an increasing medical problem. Prostate, 16:39-48, 1990.

4. Foster CS. Predictive factors in prostatic hyperplasia and neoplasia. Hum Pathol, 21:575-577, 1990.

5. Webber MM; Bello D; Quader S. Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications part 2. Tumorgenic cell lines. Prostate, 30:58-64, 1997.

6. Greenwald P. Chemoprevention of cancer. Sci Am, 274:96-00, 1996.

7. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science, 278:1073-1077, 1997.

Hypotheses

The main focus of this study is to develop and characterize tumorigenic, human prostatic epithelial cell lines by transformation of the immortalized non-tumorigenic cell line RWPE-1, with the chemical carcinogen, *N*-methyl-*N*-nitrosourea (MNU). These cell lines are of varying degrees of malignancy and mimic the early stages of cancer progression *in vivo*. The proposed hypotheses are that:

1. The MNU-transformed cell lines are of prostatic epithelial origin.

2. The MNU-transformed cell lines exhibit characteristics of malignant epithelial cells.

3. Although malignant, the MNU-transformed cell lines retain and express differentiated functions of prostatic epithelial cells unlike currently used malignant prostatic epithelial cell lines.

4. The MNU-transformed cell lines represent different stages of cancer progression from early, non-invasive, latent carcinoma to late, metastatic prostate cancer.

Part I

Literature Review

Chapter 1

The Normal Prostate

Abstract

The purpose of this chapter is to provide background knowledge of structure, function and histology of the normal prostate. This will provide a frame of reference when describing the changes that occur during carcinogenesis and tumor progression and ultimately aid in the comparison of normal prostatic epithelial cells with prostate cancer cells. In addition to providing information on the development, structure and function of the prostate, this review will describe the basal, secretory, and neuroendocrine epithelial cell types and the stem cell population from which they are derived. The interactions that occur between the epithelial and stromal compartments of the prostate will also be discussed. The major growth factors associated with the human prostate, namely EGF, FGF, and TGF-β will be described in the context of prostate growth regulation. The cellular interactions and the balance between stimulatory and inhibitory growth factor expression are of great importance in understanding the etiology of prostate cancer.

Introduction

The etymology of the gland, known as the prostate, is from the Greek word literally meaning "to stand in front of." This designation was given over two thousand years ago and is in reference to the location of the prostate in relation to the bladder (1). Although the existence of the prostate has been known for centuries, our understanding of the gland in regards to internal structure, physiology and pathology has only recently been established.

Interest in the gland has been enhanced because of the high incidence of prostate cancer during the last fifty years. This can be attributed to the increasing population of aging males who are susceptible to diseases of the prostate. In addition, introduction of new diagnostic and treatment options for diseases of the prostate have also led to the recent interest in prostatic diseases.

Before embarking on a discussion regarding prostate cancer, it is imperative to have a solid understanding of the development, structure and function of the normal gland. This includes the zonal anatomy, the normal histology, including the different cell types and their function, as well as growth factors involved in the regulation of growth in human prostate epithelium.

Development, Anatomy and Function of the Human Prostate

Development

The development of the prostate gland can be divided into four phases from embryogenesis to adulthood: 1) during the early embryonic phase, endodermally-derived epithelial cells from the urogenital sinus infiltrate and proliferate within the surrounding mesenchymal tissue. Preprostatic epithelial cells are divided into four subanatomic regions, each giving rise to a separate and subanatomically defined component of the mature gland (2,3); 2) the late embryonic/post natal phase is a period of morphogenesis and epithelial and mesenchymal cell regression (2,3); 3) the infantile phase is often referred to as the "resting phase" lasting from the second post natal month to 10-12 years of age. Although considered to be a resting period of relative inactivity, it is hypothesized that molecular events that determine future phenotypes of individual cell groups within the epithelium and stroma occur during this phase (2,3) and 4) the period of pubertal maturation begins at about 12 years of age and lasts until the age of 18. This phase is characterized by enhanced cellular proliferation, differentiation and tissue morphogenesis (2,3). Following this phase, the prostatic components are involved in a continuous range of activities, including cell proliferation, apoptosis, variable response to hormonal influences and transdifferentiation, which persist through old age. The term transdifferentiation denotes the process by which a cell type takes on

characteristics of cells of a different lineage. The next section explores the structure of the adult human prostate.

Anatomy

The prostate is a walnut-sized gland that forms part of the male reproductive system. It is located in front of the rectum and just below the bladder and surrounds the urethra (Figure 1-1). The normal adult human prostate weighs approximately 20 grams (5). The prostate is a compound tubuloalveolar exocrine gland consisting of a large series of branching ducts that enter the prostatic urethra (6). For many years the anatomy of the prostate was described in terms of lobes in reference to its supposed lobular structure (5). In recent years it has been demonstrated that this description arose from confusion between the anatomy of the normal prostate and the prostate that has undergone hyperplastic changes (5). Urologists often refer to midline and laterally projecting nodules of benign prostatic hyperplasia (BPH) as middle and lateral lobes, respectively (5). These "lobes," however, exist only in BPH and are not reference points of the normal prostate (5).

The most widely accepted concept, currently used to describe the structure of the prostate, is expressed in terms of concentric zones as established by McNeal (7). McNeal, along with his collaborators, was the first to systematically explore the entire adult prostate using serial sections through the entire gland in multiple planes rather than transverse sections as was formerly

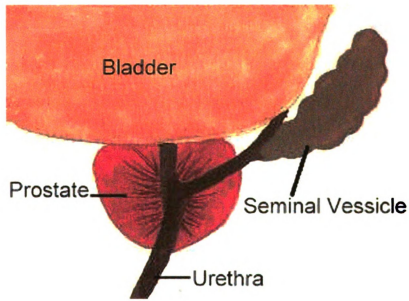

Figure 1-1: Diagram of the prostate gland in relation to the surrounding organs. (From http://www.uro.com/prostate.htm, Copyright 1996,97,98,99 MedAtlantic Corporation/Intelligent Communications Corporation - All Rights Reserved)

the common practice. Thus, in lieu of dividing the prostate into arbitrary lobes, McNeal identified zones that have morphologic, functional, and pathologic significance (5). The prostatic urethra serves as an anatomic landmark defining the zones since it makes contact with a specific portion of each zone (5). These zones include the peripheral, central and transition zones (Figure 1-2) along with the anterior fibromuscular stroma.

The peripheral zone in the normal prostate comprises the majority (approximately 75%) of the glandular tissue. It extends around the postero-lateral peripheral aspects of the gland from its apex to its base (1). It is a flat disk of secretory tissue whose ducts branch out laterally on either side of the distal urethra (5). Its histological appearance is characterized by small, simple, acinar spaces lined by tall columnar secretory epithelial cells. The majority of prostatic carcinomas arise in this zone (7).

The central zone comprises 25% of the glandular volume and is the second largest component of the normal prostate. The ducts of the central zone join the urethra at the bladder neck or verumontanum (5). The prostatic ducts branch towards the base of the prostate and join their acinar lobules. The central zone surrounds the ejaculatory ducts and makes up the majority of the prostatic base. Histologically the central zone is identified by the presence of relatively large acini with irregular contours that are lined by cuboidal to low columnar epithelium. Histological differences between the central and peripheral zones suggest significant biological differences (7).

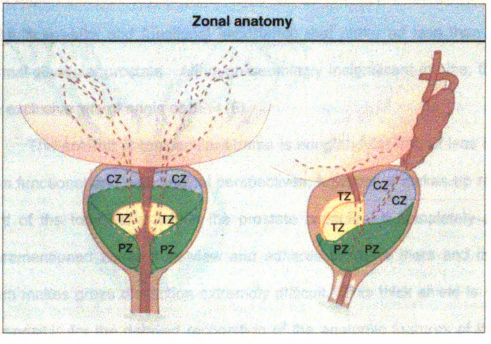

Figure 1-2: Schematic diagram of the zonal anatomy of the prostate. This diagram displays the central, peripheral and transition zones of the prostate. The preprostatic zone as described by McNeal would include the area designated by the transition zone. The anterior fibromuscular stroma encompasses the entire anterior surface of the gland (Kirby et al., 1996, Ref. #1).

The preprostatic region is the smallest zone of the prostate comprising only about 5-10% of the total volume. The term "preprostatic" is in reference to the sphincteric function of this zone at the time of ejaculation to prevent the reflux of seminal fluid into the bladder (5). The main component of this zone is a cylindrical smooth muscle sphincter surrounding the entire preprostatic urethra. This region includes the transition zone composed of a small group of ducts of insignificant size and functional importance that make up less than 5% of the normal glandular prostate. Although seemingly insignificant in size, this zone is the exclusive site of origin of BPH (5).

The anterior fibromuscular stroma is nonglandular and of less importance from functional and pathological perspectives, however, it makes up roughly one third of the total tissue within the prostate capsule. It completely shields the aforementioned zones from view and adheres tightly to them and its fusion to them makes gross dissection extremely difficult. This thick shield is considered responsible for the delayed recognition of the anatomic features of the prostate gland (7). The preceding sections have provided background on the development and structure of the human prostate that is required before examining the function of the gland.

Function

The main function of the prostate is to contribute to the fluid that helps carry sperm produced by the testes. The fluid supplies nourishment to the sperm and aids in making the vaginal canal less acidic, thus, allowing sperm to survive

in the environment. The physiological role of the prostate involves slow accumulation and occasional rapid discharge of relatively small volumes of fluid. A lesser-known function of the prostate is the transport of a variety of substances initially from the blood transmurally (across epithelial basal cell membrane, through the cell and out of the apical end) into the glandular lumen (6). This secretion and transport occurs constantly and not only during ejaculation (6). Studies by W.W. Scott proving the continuous output of basal prostatic fluid without active ejaculation involved the collection of 24-hour urine samples of young and old human males and females and the measurement of urinary acid phosphatase (6). It was discovered that males past puberty displayed a threefold increase in urinary acid phosphatase output compared to both same-aged females and prepubertal males. This high urinary level was diminished to female levels in males having undergone total prostactomies (6). Studies by Aumuller et al. (2) proved that foreign compounds could also enter the prostatic fluid. This study was done by injecting humans, intravenously, with the antibiotic rosamicin and subsequently measuring the amount of the antibiotic in the prostatic tissue, fluid, and blood serum (2). It was discovered that the ratio of the antibiotic in prostatic tissue to blood serum was greater than one, thus indicating that the prostate has the ability to concentrate rosamicin from the blood. Furthermore, the ratio of the antibiotic in the prostatic fluid to blood was even more elevated indicating that rosamicin was transported into the glandular fluid (6). Comparative studies of basal prostatic fluid between populations of low and high prostatic cancer incidence followed the studies described above with the hope of

identifying markers for the disease. These comparative studies gave rise to the prostate specific antigen (PSA) screening method currently employed for the early detection of prostatic disease. PSA is expressed by secretory epithelial cells of the prostate, one of the cells types discussed in the following section.

Cell Types and Their Functions

The normal prostate is a highly complex tissue. Populations of epithelial and stromal cells interact with one another under a constant state of proliferation, differentiation, elimination and selective secondary replenishment so that functional integrity and homeostasis in the tissue is maintained (3). Retention of cells is necessary for the normal prostatic tissue to maintain its structure and function. There are two primary mechanisms by which replacement of cells occurs when lost: 1) mitosis within a population of undifferentiated or partially differentiated cells, and 2) replacement by selective differentiation following mitotic proliferation of a precursor stem cell population (3). Prostatic stem cells give rise to three epithelial cell types that make up the prostatic epithelium: secretory epithelial cells, basal cells, and neuroendocrine cells. Mesenchymal stromal cells of the prostate interact with the epithelial cells aiding in epithelial differentiation.

Typically, in glands with cell-regenerating populations, a steady-state flow of cells exists from reserve, sedentary, stem cells to a more rapidly proliferating transient population that eventually proceeds to the formation of mature, nondividing, terminally differentiated secretory cells. These cells are then

programmed to undergo senescence and die. This generalized mechanism is still poorly understood in the normal prostate (5).

Luminal Cells

Tall columnar secretory epithelial cells are the most common cell type in the prostate gland. These cells are easily distinguished by their morphologic structure, enzymes and secretory granules that stain for prostate-specific antigen (PSA), prostate acid phosphatase (PAP), and other enzymes. These secretory cells are arranged next to one another in rows, are connected by cell adhesion molecules and are attached at their base to a basement membrane via integrin receptors. Characteristics of these cells include a basal location of nuclei just below a clear zone of abundant Golgi apparatus and an upper region that is rich in secretory granules and enzymes. Microvilli are located at the apical plasma membrane facing the lumen and secretions move out into the lumen of the acini that drain into the ducts connecting to the urethra (5).

Basal Cells

Basal cells are far less abundant in number and much smaller than the secretory cells. These cells are more round, have little cytoplasm, and possess large nuclei. They form a layer separating the secretory cells from the basement membrane and stroma. Basal cells are hardly noticeable in routine preperations, however, immunohistochemical staining with basal cell-specific keratin shows this layer to be continuous. Basal cells are more abundant in the central zone

than in the other regions of the prostate. They have been found to be the proliferative compartment of the prostate epithelium, normally dividing and maturing into secretory cells.

Neuroendocrine Cells

Neuroendocrine cells exist in all zones of the prostate and are randomly scattered throughout. Three types of prostate neuroendocrine cells have been identified to date. The major type produces both serotonin and thyroid-stimulating hormone. The two minor types produce calcitonin and somatostatin (8). The neuroendocrine cells rest on the basal cell layer between secretory cells and often have dendritic processes. The specific role of these cells in prostate physiology is unclear but they are believed to have paracrine function in response to neural stimulation (9).

Stem Cells

Stem cells are located in the basal cell layer around the periphery of prostatic terminal ducts, ductules and acini (3). These elongated cells contain little cytoplasm and separate the underlying basement membrane from overlying secretory epithelial cells (3). Upon androgenic stimulation of immature prostatic epithelium during puberty, the stem cells give rise to basal cells. A schematic diagram of the inter-relationship of the various prostatic epithelial cell-types can be seen in Figure 1-3 (3). The prostatic stem cells (SS) within the basal cell layer give rise to basal epithelial cells (BEC), neuroendocrine cells (NEC) and luminal

epithelial cells (LEC) or secretory epithelial cells (SEC). It is believed that some BEC's retain the ability to proliferate and to give rise to additional BEC's or to undergo further differentiation into LEC's and NEC's (3). The combined effects of circulating steroid hormones along with a local nonsteroidal paracrine effect regulate the differentiation processes within the prostate cell system in a balanced manner. Figure 1-3 also makes reference to the two functional compartments of the prostatic epithelium. The androgen-independent proliferative compartment localized within the basal cell layer and the androgen-dependent secretory compartment composed of secretory epithelial cells of limited proliferative potential (3). The following section examines the interatction of the prostatic epithelial cells with the surrounding stroma.

Stromal Epithelial Interactions

The role of epithelial cells of the prostate, involving synthesis and secretion of marker proteins including PAP, PSA, as well as polyamines and citrate, is well characterized while that of the stromal compartment is more nebulous. The stroma is composed of mesenchymal cells and a supporting scaffold known as the extracellular matrix (ECM). Along with providing a supporting scaffolding for cells, the ECM has been shown to play an important role in the development and control of cellular function (5). The stromal compartment contains the majority of 5α–reductase activity, thus, allowing it to metabolize the androgen 5α–dihydrotestosterone (5α-DHT) (10). The normal differentiation of the urogenital sinus in the male is dependent on the presence of

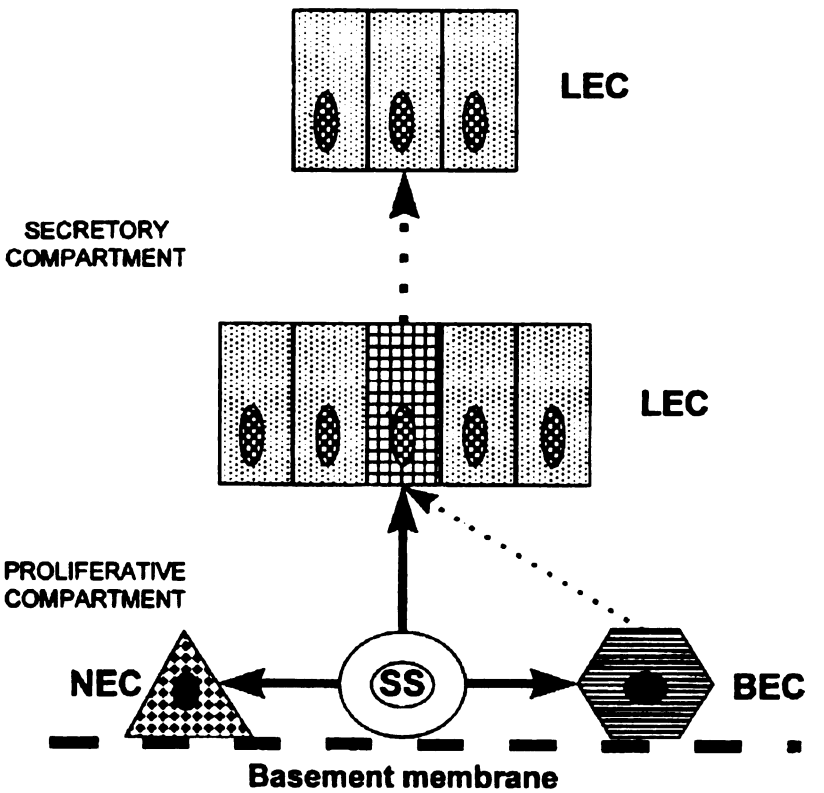

Figure 1-3: Interrelationships of the Various Prostatic Epithelial Cell Types. Schematic diagram indicating the inter-relationship of the various prostatic epithelial cell-types in the fully differentiated normal adult gland. The prostatic stem cells (SS) are shown to give rise to the neuroendocrine cells (NECs), basal epithelial cells (BECs) and luminal epithelial cells (LEC) (secretory cells). In addition, the BECs are shown to be possible precursors of the LECs (Foster et al., 1997, Ref. #3).

androgens and, thus, on the stroma, as demonstrated in the prostate reconstitution studies conducted by Cunha et al. (11). Androgen receptors are detectable by autoradiography in the prostatic mesenchyme but not in the epithelial cells of the urogenital sinus during development (10). Cell-cell and cell-matrix interactions via cell adhesion molecules (CAMS) and integrins, respectively, mediate stromal epithelial interactions (Figure 1-4). These interactions form direct structural linkages and allow communication between the stroma and epithelial cell DNA (5). Studies conducted by Isaacs et al. (6) provided evidence that the prostatic stroma regulates epithelial cell growth. Experiments, using canine prostatic epithelial cells, were conducted to compare proliferation on plastic and a biomatrix of the stroma and tissue matrix. The cells grew rapidly as primary outgrowth on plastic, but when plated on biomatrix, growth was reduced and the cells maintained their morphology and secretory ability closely resembling *in vivo* behavior. This observation indicates that the stromal component of the prostate acts as a brake by keeping cell proliferation in check, thus, allowing the maintenance of functional differentiation and secretion. Removal of the cells from this normal state, in effect, removes the brake, thus, allowing the epithelial cells to proliferate. The removal of the stromal brake in BPH and cancer is currently an area of intense investigation. The stromal-epithelial interaction is based largely on growth factors.

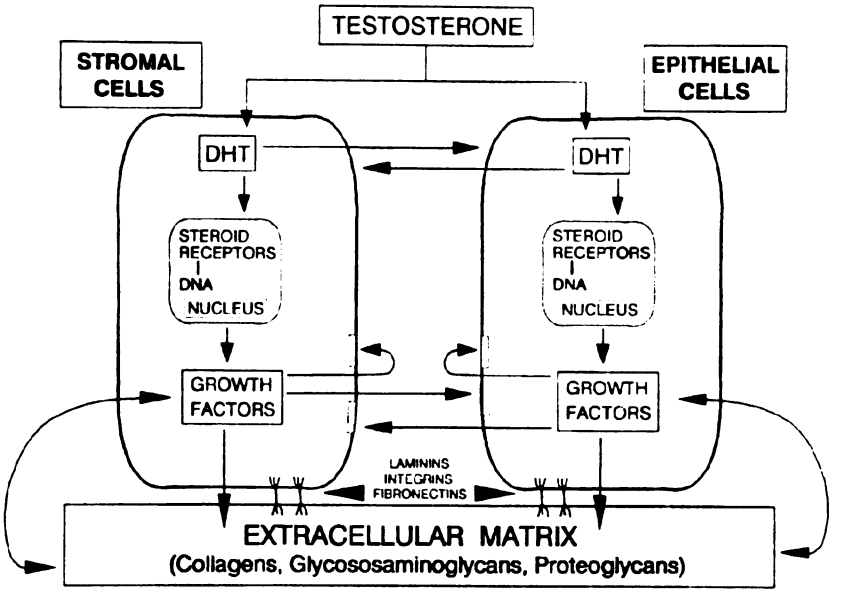

Figure 1-4: Stromal-Epithelial Interactions in Information transfer and Regulation within the Prostate. Testosterone and growth factors interact on and between stromal and epithelial cells. The formation of DHT, the production of growth factors, and ECM components regulate stromal-epithelial interactions (Coffey et al., 1992. Ref. #5).

Growth Factors Involved in Growth Regulation

One would not expect the adult prostate to contain many active growth factors because of its slow rate of growth. Interestingly, however, many slow growing adult tissues, including the prostate, have high levels of growth factors. It is believed that components of the ECM may sequester many of the prostate growth factors (5). This sequestering of growth factors can be regarded as the suppressor element in the balance that exists in all types of growth between growth stimulating elements and growth suppressing elements. This balance is further demonstrated when considering the fact that androgens and growth factors can stimulate the synthesis and degradation of the ECM components, thus, altering the response of cells to steroids and growth factors. Although the mechanism of the effect of growth factors on any cell tends to be complicated, growth regulation for simplicity's sake may be generically described as having four phases: synthesis, secretion, target cell interactions and effects (5) (Figure 1-5).

Environmental conditions typically trigger a signal that causes a cells to produce a growth factor. Some growth factors, such as TGF-β, are usually in an inactive, pro-form are and activated by proteolysis. The growth factor is secreted extracellularly and either serves as a growth signal on the cell from which it was produced (autocrine) or on a nearby cell (paracrine). Upon secretion the growth factor may be sequestered by binding to the ECM or it may bind to specific receptors that reside on the plasma membrane of target cells. The binding of a growth-stimulating growth factor to its receptor triggers a cascade of events that

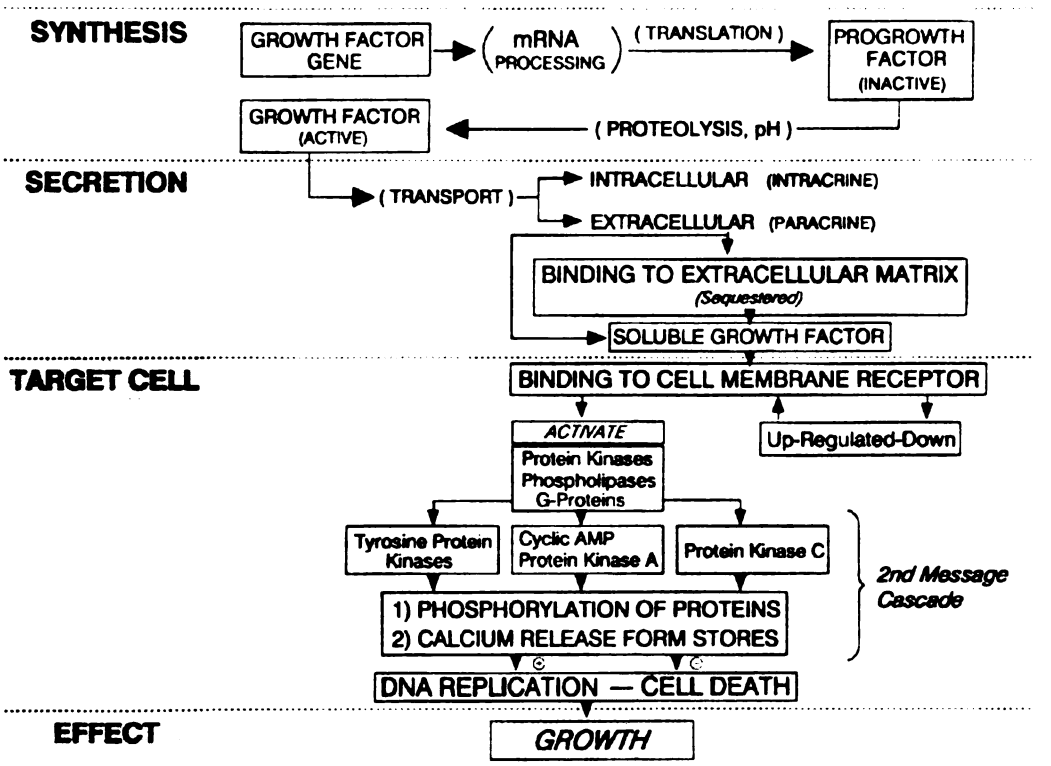

Figure 1-5: Mechanisms involved in growth factor action The synthesis, secretion, and interaction with target cells of growth factors can vary, but generally follow the diagram (Coffey et al., 1992, Ref. #5).

ultimately induce the cellular machinery to initiate DNA synthesis and cell replication. As mentioned above, there is a balance between the rate of cell replication and cell death and growth factors affect this balance by either stimulating or suppressing growth (5). The growth factors known to stimulate prostatic growth include fibroblast growth factor (FGF) and epidermal growth factor (EGF), while transforming growth factor-β (TGF-β) is inhibitory to normal prostatic epithelial cells. Other growth factors involved in the prostate include insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF), however, EGF, FGF, and TGF-β have received more attention in scientific literature and will, thus, be the focus of the following sections.

Fibroblast Growth Factors (FGFs)

The fibroblast growth factors are a family of growth factors that are produced predominantly by cells of embryonic mesoderm or neuroectoderm origin and can be found in a variety of tissues. They are also found in the extracts of a variety of tumors and are produced by many types of cells in culture. All FGFs bind to heparin and are sometimes referred to as heparin-binding growth factor (13). The binding of FGFs to heparin protects them from degradation by proteases (13). The FGF family consists of at least 18 members of related peptides which include acidic FGF (aFGF) and basic FGF (bFGF). bFGF is thought to be the more important growth factor in the prostate. Studies have shown that bFGF is the main growth factor produced by human fibroblasts (14,15). It has been shown to be a mitogenic factor present in human prostatic

extracts and it is capable of stimulating the growth of both epithelial and mesenchymal cells. The inactive, pro-form of the protein is composed of 155 amino acid units while the active form contains 146 amino acids. bFGF is regarded as a tumor angiogenesis factor due to its ability to stimulate capillary growth (5). Among other growth factors having a growth stimulatory effect on prostatic cells is EGF.

Epidermal Growth Factor (EGF)

Urogastrone was the term used to refer to the 53 amino acid peptide isolated from human urine that inhibited gastric acid secretions (16). It is now known to be similar to mouse epidermal growth factor. Both normal and malignant cells secrete EGF. The EGF receptor is an integral, cell membrane receptor. It consists of an extracellular binding domain, a trans-membrane domain and a cytoplasmic domain that encodes a tyrosine kinase. Binding of EGF to its receptor leads to phosphorylation of intracellular proteins and activation of second messenger systems (17).

EGF has a mitogenic role in the prostate gland. Human prostatic secretions were found to contain the highest EGF levels of all biological fluids (18). It is a potent stimulator of proliferation in cultured epithelial and stromal cells (19). EGF expression in the prostate is androgen regulated. Androgen withdrawal by castration in mice leads to reduced prostatic EGF levels (20). Characterization of the communication between the androgen receptor (AR) and growth factor transduction cascades is an area of intense study in prostate

cancer research. This communication is very important considering the observation that in androgen-independent tumors AR is nevertheless expressed. This suggests an androgen signaling cascade in advanced androgen-deprived tumors (21,22).

EGF shares its receptor with a related peptide, transforming growth factor-α (TGF-α). TGF-α represents one of two families of TGFs described in the following section.

Transforming Growth Factors (TGFs)

The two growth factor families, TGF-α and TGF-β, which although share very similar names, are otherwise unrelated. They were named because of their ability to promote cell colony formation in agar cultures (5). TGF-α contains 160 amino acid residues in its pro-form and 50 amino acids in the active form. It is structurally similar to EGF and it exerts its effect by acting through the EGF receptor. TGF-α and EGF have several biological effects in common. TGF-α has not been detected in normal human prostatic tissue. Advanced prostatic tumors display an increased expression of TGF-α leading to the hypothesis that increased TGF-α expression is indicative of a more malignant phenotype (23,24).

TGF-β is expressed by two genes that give rise to two forms sharing 70% homology (5). The pro-form of the protein is composed of 391 amino acids while the cleaved active form contains 112 amino acids. There are a total of three forms of TGF-β that can result when the two gene products combine with each other forming a heterodimer or with themselves to form a homodimer. Both

TGF-β_1 and TGF-β_2 are expressed in prostate tissue, but TGF-β_2 exhibits greater expression in benign prostatic hyperplasia (BPH) while TGF–β_1 levels remain the same in both normal prostate and BPH (25). TGF–β has different effects on different types of cells. For example, while it negatively regulates epithelial cells growth, it also has the ability to stimulate stromal cell proliferation (5). This response may be altered in cancerous tissues in which epithelial cell growth may be stimulated by TGF–β. This hypothesis is supported by studies of Dunning tumor cell lines that express increased levels of the TGF-β_1 isoform compared to normal levels (26). Further support is provided by a study conducted by Merz et al. (27) in which epithelial and mesenchymal tissues from the mouse urogenital sinus were transfected with *ras* and *myc* oncogenes. Recombination of both components and grafting to the renal capsule resulted in poorly differentiated adenocarcinoma with elevated TGF-β_1 and TGF-β_3 isoforms. Together, these results suggest a stimulatory role of TGFβs in advanced tumors.

The study of growth factors increases in complexity when considering the fact that many growth factors affect the synthesis of other growth factors. For example, TGF–β_1 has been shown to stimulate production of IGF in bone and PDGF has exhibited the ability to make cells more competent to respond to other growth factors (5). When taking these elements into consideration one can gain a better appreciation of the delicate balance maintained by growth factors.

Intermediate Filaments

Intermediate filaments are major components of the cytoskeleton. They are organized as a network surrounding the nucleus and spreading out into the cytoplasm and terminating at the plasma membrane (28,29). Among the functions of intermediate filaments is the maintenance of cell shape, intracellular support and cell locomotion, receptor-mediated endocytosis and positioning of cellular organelles (28,29). Intermediate filaments are divided into five classes: cytokeratins, desmin, vimentin, neurofilaments, and glial filaments (28-29). Other cytoskeletal components include micortubules and microfilaments. Microtubules and microfilaments are present in all cell types while intermediate filaments are expressed in a cell-type specific manner. The focus of this section will be on cytokeratins and vimentin.

Cytokeratins (CKs)

Cytokeratins are expressed by epithelial cells. Screening for cytokeratin expression is often performed in order to confirm the epithelial origin of cells. They are commonly expressed as pairs composed of a basic and an acidic cytokeratin protein (28). The acidic and basic combinations are expressed as sets characteristic of a specific cell type. The combination expressed in the human prostatic luminal, secretory cells, are cytokeratin 8 (basic) and cytokeratin 18 (acidic) (29-33).

A reduction in cytokeratin 8 and 18 expression in prostate carcinomas, compared to levels in normal and hyperplastic prostate, have been reported (33).

This decrease in cytokeratin expression is postulated as being a consequence of the dedifferentiation that occurs during transformation from a normal to a malignant phenotype (34). Differentiation-inducing agents may reverse this dedifferentiation process. Retinoids are a family of vitamin A derived agents known to induce differentiation and have been reported to increase cytokeratin expression in prostatic and breast epithelial cells (35,36). The decrease in cytokeratin expression observed in cancer of the prostate is sometimes associated with increased expression of the intermediate filament, vimentin.

Vimentin

Vimentin is a cytoskeletal component normally not expressed in epithelial cells but is expressed by cells of mesenchymal origin (29). However, epithelial cells that develop into carcinomas may co-express both cytokeratins and vimentin. This co-expression is occasionally observed when epithelial cells are placed in cell culture. High levels of vimentin expression have been reported in hormone-independent, highly metastatic tumors, while levels in normal and benign prostatic epithelium are significant lower (37).

Conclusion

Prostate cancer is the leading cancer in men in the United States. A fundamental understanding of the normal prostate structure and function is imperative in order to gain a better understanding of the etiology of the disease. This chapter provides information about the development and zonal anatomy of

the prostate along with the active function of the gland. The cell types and growth factors involved in growth regulation were also described. Taken together, this information facilitates a better understanding of the delicate balance that exists between the normal and neoplastic state. For example, the discussion of the progenitor stem cells, giving rise to the epithelial cells of the prostate, helps to explain how genetic events that occur within embryonic stem cells may predispose an individual to prostate cancer (3). In addition, this chapter promotes a better understanding of how perturbation of the delicate balance, existing between the stromal and epithelial compartments or expression of growth factors or receptors, may lead to a malignant state. The stronger our understanding of the normal prostate structure and function the better equipped we will be to understand the events leading to prostate cancer.

References

1. Kirby RS, Christmas TJ, Brawer M. Prostate Cancer. Turin, Italy, Mosby, 170 p., 1996.

2. Aumuller G. Morphologic and endocrine aspects of prostatic function. Prostate, 4:195-214, 1983.

3. Foster CS, Ke Y. Stem cells in prostatic epithelia. Int J Exp Pathol, 78:311-329, 1997.

4. Aumuller G, Groos S, Renneberg H, Konrad L, Aumuller M. Embryology and postnatal development of the prostate. *In* Foster, C.S.; Bostwick, D.G. (Eds.): Pathology of the prostate. Philadelphia, W.B. Saunders Company, pp. 1-18, 443 p., 1998.

5. Coffey DS. The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. *In* Walsh, P.C.; Retik, A.B.; Stamey, T.A.; Vaughan, E.D. Jr. (Eds.) Campbell's urology. Philadelphia, W.B. Saunders Company, pp. 221-266, 1992.

6. Isaacs JT. Prostatic structure and function in relation to the etiology of prostatic cancer. Prostate, 4:351-366, 1983.

7. McNeal JE. The zonal anatomy of the prostate. Prostate, 2:35-49, 1981.

8. Abrahamsson PA, Lilja H. Partial characterization of a thyroid-stimulating hormone-like peptide in neuroendocrine cells of the human prostate gland. Prostate, 14:71-81, 1989.

9. McNeal JE. Anatomy and normal histology of the human prostate. *In* Foster, C.S.; Bostwick, D.G. (Eds.): Pathology of the prostate. Philadelphia, W.B. Saunders Company, pp. 19-34, 1998.

10. Tenniswood MP, Montpetit ML, Leger JG, Wong P, Pineault JM, Rouleau M. Epithelial-stromal interactions and cell death in theprostate. *In* Farnsworth, W.E.; Ablin RJ (Eds.) The prostate as an endocrine gland. Boca Raton, CRC Press Inc., 213 p., 1990.

11. Cunha GR, Fujii H, Neubauer BL, Shannon JM, Sawyer L, Reese BA. Epithelial-mesenchymal interactions in prostatic development: I. Morphological observations of prostatic induction by urogenital sinus mesenchyma in epithelium of the adult rodent urinary bladder. J Cell Biol, 96:1662-1670, 1983.

12. Wilding G, Valverius E, Knabbe C, Gelmann EP. Role of transforming growth factor-alpha in human prostate cancer cell growth. Prostate, 15:1-12, 1989.

13. Saksela O, Moscatelli D, Sommer A, Rifkin DB. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J Cell Biol, 107:743-751, 1988.

14. Story MT, Livinstron B, Baeten L, Swartz SJ, Jacobs SC, Begun FP, Lawson RK. Cultured human prostate-derived fribroblasts produce a factor that stimulates their growth with properties indistinguishable from basic fibroblast growth factor. Prostate, 15:355-365, 1989.

15. Nishi N, Matuo Y, Kunitomi K, Takenaka I, Usami M, Kotake T, Wada F. Comparative analysis of growth factors in normal and pathologic human prostates. Prostate, 13:39-48, 1988.

16. Gospodarowicz D. Growth factors and their effects on the proliferation of normal and transformed cells. *In*: Receptors In Tumor Biology. C.M. Chadwick (ed.), Cambridge, Cambridge University Press, 257 p, 1986.

17. Hill DJ. Growth factors and their cellular action. J Reprod Fertil, 85:723-734, 1989.

18. Gregory H, Willshire IR, Kavanagh JP, Blacklock NJ, Chowdury S, Richards RC. Urogastrone-epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy. Clin Sci, 50:359-363, 1986.

19. McKeehan WL, Adams PS, Rosser MP. Direct mitogenic effect of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. Cancer Res, 4:1998-2010, 1984.

20. Hiramatsu M, Kashimata M, Minami N, Sato A, Murayama M. Androgenic regulation of epidermal growth factor in the mouse ventral prostate. Biochem Int, 17:311-317, 1988.

21. Sadi MV, Walsh PC, Barrack ER. Immunohistochemical study of androgen receptors in metastatic prostate cancer-comparison of receptor content and response to endocrine therapy. Cancer, 67:3057-3064, 1991.

22. Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res, 55:3068-3072, 1995.

23. Hofer DR, Sherwood ER, Bromberg WD, Mendelsohn J, Lee C, Kozlowski JM. Autonomous gorwth of androgen-independent human prostatic carcinoma cells: Role of transforming growth factor α. Cancer Res, 51:2780-2785, 1991.

24. Connolly JM, Rose DP. Production of epidermal growth factor and transforming growth factor-α by the androgen-responsive LNCaP human prostate cancer cell line. Prostate, 16:209-218, 1990.

25. Mori H, Maki M, Oishi K, Jaye M, Igarashi K, Yoshida O, Hatanaka M. Increased expression of genes for basic fibroblast growth factor and transforming growth factor type beta 2 in human benign prostatic hyperplasia. Prostate, 16:71-80, 1990.

26. Steiner MS, Barrack ER. Expression of transforming growth factor β1 in prostate cancer. Endocrinology, 135:2240-2247, 1994.

27. Merz VW, Miller GJ, Krebs T, Timme TL, Kadmon D, Park SH, Egawa S, Scardino PT, Thompson TC. Elevated TGF-β1 and β3 mRNA levels are associated with *ras* + *myc*-induced carcinomas in reconstituted mouse prostate: Evidence for a paracrine role during progression. Mol Endocrinol, 5:503-513, 1991.

28. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalogue of human cytokeratins: patterns of expression in normal epithelial, tumor and cultured cells. Cell, 31:11-24, 1082.

29. Traub P. Intermediate filaments: a review. New York Springer-Verlag, pp. 2-144, 1985.

30. Molinolo AA, Meiss RP, Leo P, Sens AI. Demonstration of cytokeratins by immunoperoxidase staining in prostatic tissue. J Urol, 134:1037-1040, 1985.

31. Sherwood ER, Berg LA, McEwan RN, Pasciak RM, Kozlowski JM, Lee CJ. Two-dimensional protein profile of cultured stromal and epithelial cells from hyperplastic human prostate. J Cell Biochem, 40:201-214, 1989.

32. Hendrick L, Epstein JI. Use of keratin 903 as an adjunct diagnosis of prostate carcinoma. Am J Surg Pathol, 13:389-396, 1989.

33. Peehl DM, Leung GK, Wong ST. Keratin expression: a measure of phenotypic modulation of human prostatic epithelial cells by growth inhibitory factors. Cell Tissue Res, 277:11-18, 1994.

34. Ramaekers FC, Verhagen AP, Isaacs JT, Feitz WF, Moesker O, Schaart G, Schalken JA, Vooijs GP. Intermediate filament expression and the progression of prostate cancer as studied in the Dunning R-3327 rat prostate carcinoma systems. Prostate, 14:323-329, 1989.

35. Peehl DM, Wong ST, Stamey TA. Vitamin A regulates proliferation and differentiation of human prostatic epithelial cells. Prostate, 23:69-78, 1993.

36. Jing YK, Zhang J, Waxman S, Mira-y-Lopez R. Retinoids upregulate cytokeratins 8 and 18 in T47D human breast cancer cells. Proc Am Assoc Cancer Res, 36:513, 1995.

37. Bussemakers MJ, Verhaegh GW, van-Bokhoven A, Debruyne FM, Schalken JA. Differential expression of vimentin in rat prostatic tumors. Biochem Biophys Res Commun, 182:1254-1259, 1992.

Chapter 2

Prostate Cancer In Man: Incidence and Mortality

Abstract

Prostate cancer is the most common non-skin malignancy and the second leading cause of cancer-related death among men in the United States. It is estimated that there were 184,500 new cases reported and 39,200 deaths due to prostate (1). Several factors have been implicated in giving rise to and exacerbation of the disease. The most salient of these factors are old age, race, and diet. Other potential factors exhibiting a connection with the disease include plasma testosterone levels, genetic predisposition, occupational hazards, socioeconomic status, and marital status. Although the above factors are believed to influence an individual's risk for developing of prostate cancer, the one reason for the apparent increase in incidence of the disease is the increasingly sensitive methods of screening and detection of prostate cancer.

Introduction

There has been a general increase in prostate cancer indicdence and mortality during the past two decades. The purpose of this chapter is to explore the factors that are suspected to lead to the increased incidence and mortality of the disease. Among the main risk factors are old age, race, and diet. A sudden increase in prostate cancer incidence occurred between the late 1980s and early 1990s. This increase in incidence was not accompanied by a proportional increase in mortality. This disparity may be explained by improved, increasingly sensitive methods for early detection of prostate cancer.

Risk Factors

Age

Several studies have confirmed that incidence and mortality of prostate cancer increase with age (2-3). Each year over three-fourths of the new cases of the disease occur in men above the age of sixty-five. Cancer of the prostate is extremely rare in younger men with less than one case per 100,000 for men under the age of forty (1) (Figure 2-1). The slow-growing nature of prostate cancer causes it to be an increasing concern for the male population with an increasing life expectancy. Longer life spans allow the cancer more time to manifest and metastasize leading to more prostate cancer-related deaths. Old age also affects the ability to treat prostate cancer. Older patients are unable to endure aggressive treatment and are, thus, more likely to succumb to cancer of the prostate.

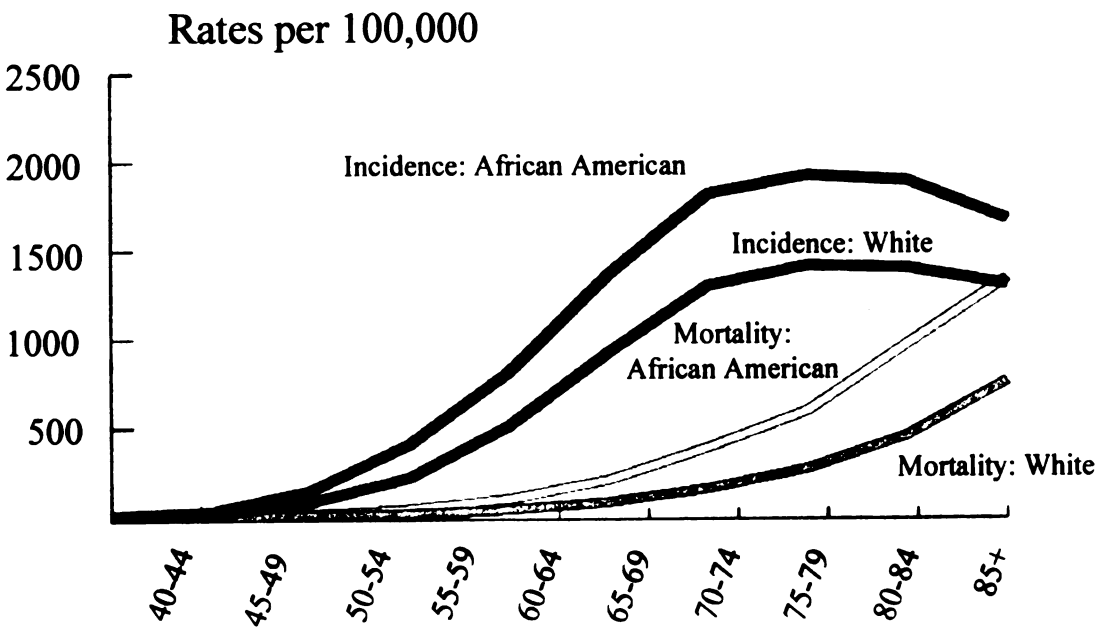

Figure 2-1: Prostate Cancer, Age-Specific Incidence and Mortality Rates by Race, US, 1990-1994. Per 100,000, age-adjusted to the 1970 US standard population (American Cancer Society, Surveillance Research, 1998 Ref. #1).

Race

Epidemiological studies indicate that African-American men in the United States have a greater predisposition for developing prostate cancer than any other ethnic group. At all ages, the incidence of disease in African-American men is nearly double that of white men (234/100,000 vs. 135/100,000 respectively) (1). In addition, mortality rates in African-American men are at least twice that of other racial and ethnic groups in the United States. The reason for this disparity is currently an area of intense research. Recent studies, testing prostate specific antigen (PSA) levels in black and white men without prostate cancer, found that black men tend to have higher PSA levels and greater PSA densities than similarly aged white men (4,5). There is a strong correlation between elevated PSA levels and the presence of prostate cancer. Thus, the increased PSA in African-American males is indicative of the possibility that this ethnic group may have a predisposition for the development of prostate cancer.

Conversely, prostate cancer incidence among Asian-American male populations tends to be far less than that in white males in the United States. Although there is a great deal of variation in incidence between the specific Asian-American ethnicities (24.2, 88.0, 6.6, and 19.9 per 100,000 for Korean, Japanese, Chinese and Hawaiian men, respectively), the incidence in each of these groups is significantly less than that in white American males (1) (Figure 2-2). It is believed that differences in diet may contribute to this disparity in incidence between different ethnic groups.

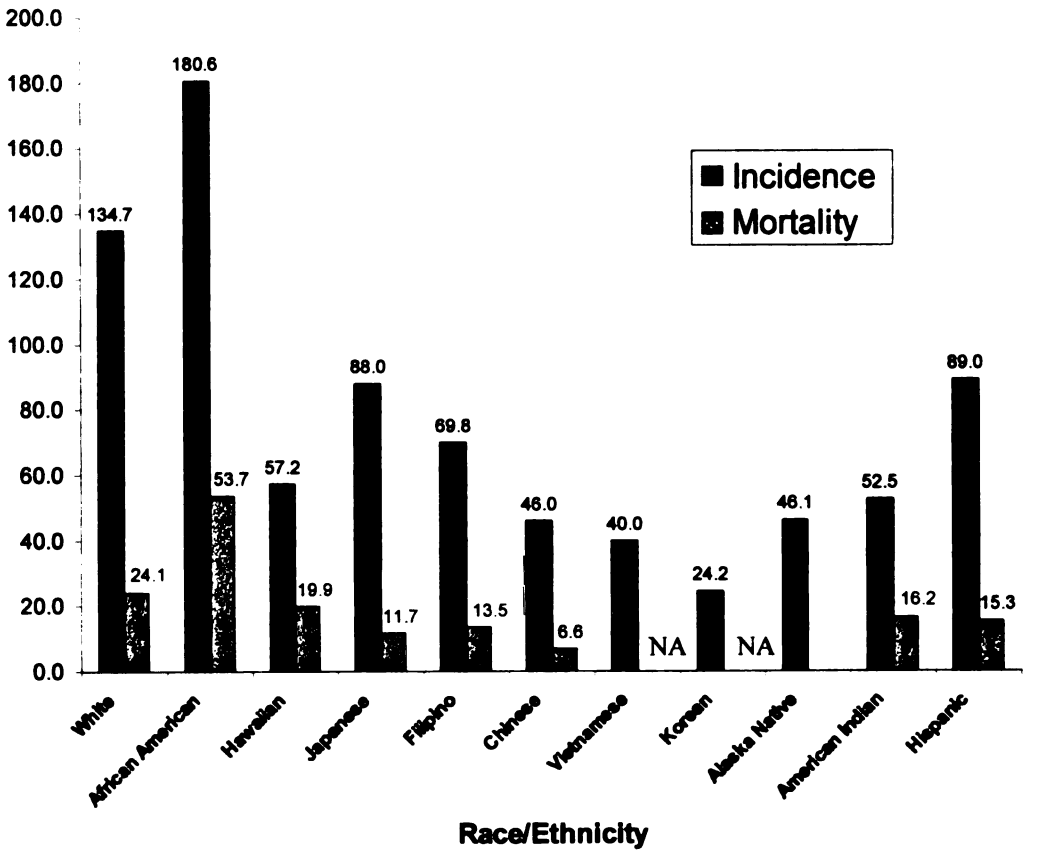

Figure 2-2: Prostate Cancer—Incidence and Mortality Rates by Race and Ethnicity, US, 1988-1992. Per 100,000, age-adjusted to the 1970 US standard population (American Cancer Society, Surveillance Research, 1998. Ref.# 1).

Diet

Dietary habits may influence the possibility of development of prostate cancer. Studies suggest that a diet high in fat may increase the risk of developing prostate cancer (1). The differences in incidence of prostate cancer between different racial groups strongly suggest a link between diet and disease. Asian men traditionally consume a diet high in soy products. Such a diet has been reported to retard the development of prostatic adenocarcinoma in animal models (6). This potentially high consumption of soy products by Asian-American men, compared to white and African-American males, may lead to the observed reduced incidence rates.

Dietary supplementation of vitamin E and vitamin A are believed to influence the development of prostate cancer (7). A study involving a form of vitamin E, α-tocopherol, along with β-carotene, was carried out to examine the effects of each agent alone and in combination on prostate cancer in male smokers (7). Men on diets supplemented with α-tocopherol displayed a 32% decrease in incidence and 41% decrease in mortality from prostate cancer. Surprisingly, subjects receiving β-carotene had a 23% higher incidence and 15% higher mortality rate (7).

It is believed that vitamin E exerts its effect as an antioxidant and prevents the propagation of free radical damage to critical cellular structures including DNA and proteins. Other potential mechanisms of the protective effect of vitamin E may be through the enhancement of the immune functions and the

reduction of activity of the cellular signal transducer protein kinase C which regulates cell proliferation (7).

Beta-carotene is the major precursor of vitamin A and is believed to exert its effect through modulation of gene expression leading to induction of differentiation, inhibition of cell proliferation and apoptosis (8,9). Results of studies involving treatment of prostate cancer with β-carotene have been inconsistent in that it either has no effect or shows an increase in incidence of the disease (7). The adverse effects of β–carotene may arise from its toxicity. Synthetic vitamin A derivatives with decreased toxicity levels have been developed and have shown great promise in *in vitro* and *in vivo* studies (10). The application of these synthetic retinoids, as described by Heinonen et al. (7), may prove that dietary supplement of these agents will effectively reduce prostate cancer incidence and mortality.

Other Factors

Several factors, aside from those described above, are associated with development of prostate cancer. The growth of the normal prostate is dependent on androgens and, thus, men with high plasma testosterone levels are believed to be at greater risk of developing prostate cancer (1). Studies have shown a two to three-fold increase in the risk for developing prostate cancer in men with a family history of prostate cancer indicating that there may be a genetic predisposition (1). In addition, higher failure rates in the treatment of familial

prostate cancer as well as greater relapse rates indicate that familial prostate cancer may be biologically more aggressive (11).

Other factors that appear to have a weaker link with prostate cancer are occupation, marital and socioeconomic status. Occupation-associated factors involved in increased prostate cancer incidence and mortality are observed through epidemiological studies. The conclusions drawn from such studies are predicated primarily on the exposure of the worker to occupational hazards. For example, farmers and agricultural workers in general are considered to be at higher risk for the development of prostate cancer. This increased risk is more likely linked to herbicides and pesticides to which the worker may be exposed rather than other day to day activities associated with the farming lifestyle (12). Similarly, chemical workers may be at risk for development of prostate cancer through exposure to chemicals in the workplace or environment that may interfere with the endocrine system (13). These chemicals include acetic acid and acetic anhydride, both of which are used in manufacturing plants for the synthesis of cellulose triacetate (13).

In studies on marital status and prostate cancer mortality, it has been reported that married men with prostate cancer had a longer median survival time than divorced, single, separated, or widowed men (14). The investigators attributed this finding to the beneficial psycho-neuroimmunological effects of marriage on social support and/or mood (14). There are conflicting reports on the association between marital status and prostate cancer incidence (15-16). Previous studies assert that greater lifetime frequency of intercourse associated

with married men decreases the risk of prostate cancer (16). It was discovered in more recent studies that there is a 20% increase in incidence for ever-married men, however, the same study reported that marriage has a favorable effect on survival of men with prostate cancer (15).

Finally, studies on the effect of socioeconomic status in survival of prostate cancer indicate that individuals of higher socioeconomic groups tend to display better survival from prostate cancer. It is likely that this finding is closely associated with differences in the access and quality of treatment between men of different socioeconomic status (15). This fact may also partially explain the geographic patterns of prostate cancer where advanced screening protocols and quality of treatment affect the incidence and mortality rates due to prostate cancer throughout the world.

Effects of Improved Detection on Incidence and Mortality

One must proceed with caution when considering statistics of prostate cancer incidence over time and between geographic locations. According to Figure 2-3 the incidence of the disease in the United States slowly increased between the years 1974-1989 then suddenly doubled between the years 1989-1992 and finally slightly decreased between 1992-1994. This graph suggests that a prostate cancer epidemic occurred between 1989-1992, however, the mortality rates remained steady during this period and have only slightly increased during the twenty-year period. This observation is reflective of the increased sensitivity of methods for the detection of prostate cancer (Figure 2-3).

Transurethral resection of the Prostate (TURP)

TURP is a procedure using a small wire loop to remove the cancerous section of the prostate gland by placing the loop in the prostate through the urethra (1). This procedure is routinely employed for the treatment of benign prostatic hyperplasia and early detection of prostate cancer. This procedure may have contributed to the apparent increase in prostate cancer incidence rates between 1973 and 1986 (17). The use of this procedure has declined in recent years in favor of prostate needle biopsy as the primary surgical procedure employed for detecting prostate cancer (17). But a serum prostate specific antigen (PSA) test, that aids in the detection of asymptomatic disease, often precedes the needle biopsy.

Prostate Specific Antigen (PSA)

Prostate specific antigen (PSA) is a protein secreted specifically by prostate epithelial cells and its level can be measured in the blood. PSA blood test levels of less than 4 ng/ml are considered normal while a result of over 10 ng/ml is considered high and a result between these two values is borderline. The higher the PSA level the greater the likelihood of the presence of prostate cancer. Although there is a strong correlation between high PSA and prostate cancer, PSA screening does not represent a definitive method of detection of the

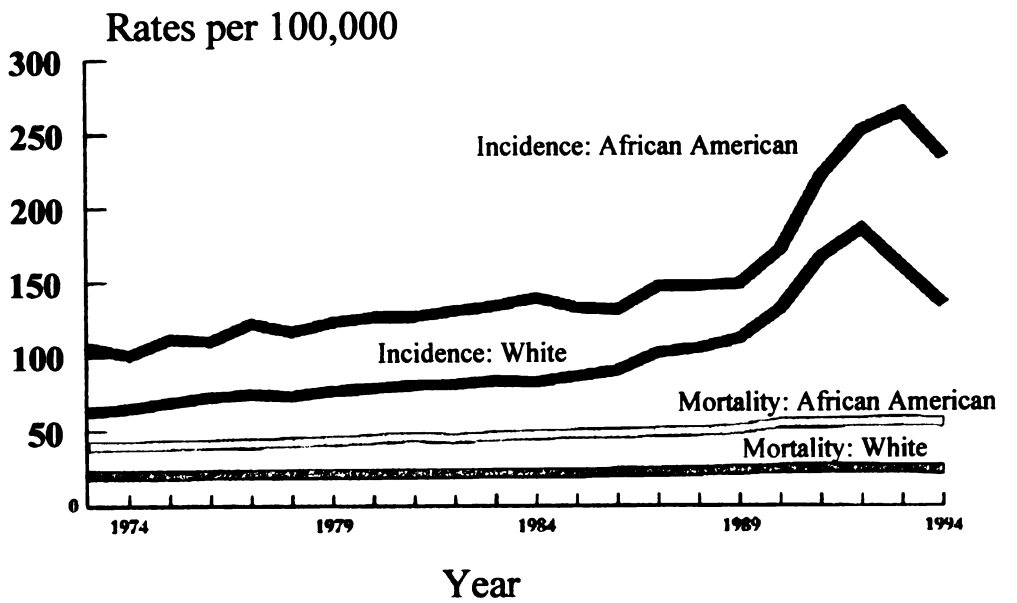

Figure 2-3: Prostate Cancer—Incidence and Mortality Rates by Race, US, 1973-1994. Per 100,000, age-adjusted to the 1970 US standard population (American Cancer Society, Surveillance Research, 1998. Ref. # 1).

disease. However, men with elevated PSA are strongly urged to undergo biopsy while men with borderline PSA levels should have a percent-free PSA test performed. PSA exists in one of two isoforms: one form is complexed to α1-antichymotrypsin while the other is uncomplexed, free PSA (18). A higher level of the complexed PSA is found in patients with prostate cancer while patients with BPH have less of the complexed antigen and, thus, a greater percentage of free PSA (19). This innovation in PSA screening allows physicians to fine-tune the procedure and make more confident conclusions based on the results.

Conclusion

The use of PSA screening for detection of asymptomatic prostate cancer may be the one reason that explains the sudden increase in incidence of the disease after 1988. Heightened awareness of the potential presence of prostate cancer has contributed to the widespread screening for PSA levels, thus, leading to the apparent ever-increasing incidence rates over the next 4-5 years. This sudden surge in incidence was followed by an inevitable decline. This marks the point at which the majority of men, who were formerly scarcely aware of the presence of the asymptomatic cancer in their prostate, were diagnosed with the disease.

It is likely that the long-term effects of early detection of prostate cancer through PSA screening will be reflected in decreased rates of mortality. Although early detection of the disease increases the likelihood of catching the disease at

an early stage, when it is still localized to the prostate gland, the effects of early detection may not always be beneficial. More sensitive tests reveal a broader spectrum of cases whose natural history and response to treatment are poorly understood (17). The inability at present to alter the natural progression of prostate cancer diminishes the significance of earlier detection of a tumor. A stronger comprehension of the molecular events associated with carcinogenesis and tumor progression is, thus, invaluable to the patient and society. *In vitro* cell models are the means to obtain an understanding such molecular events. The following chapter examines the commonly used cell models for the study of prostate cancer and why they may not be adequate for the study of prostate carcinogenesis. It is proposed that better cell models , such as those described in the present study, will enhance our understanding of prostate carcinogenesis and its prevention.

References

1. American Cancer Society. Cancer Facts and Figures-1998.

2. Krongrad A, Lai H, Lamm SH, Lai S. Mortality in prostate cancer. J Urol, 156:1084-1091, 1996.

3. Krongrad A, Lai H, Shenghan L. Competing risks of mortality in prostate cancer. J Urol, 158:865-868, 1997.

4. Eastham JA, May RA, Whatley T, Crow A, Venable DD, Sartor O. Clinical characteristics and biopsy specimen features in African-American and white men without prostate cancer. J Natl Cancer Inst, 90:756-760, 1998.

5. Abdalla I, Ray P, Ray V, Vaida F, Vijayakumar S. Comparison of serum prostate-specific antigen levels and PSA density in African-American, white, and Hispanic men without prostate cancer. Urology, 51:300-305, 1998.

6. Dalu A, Haskell JF, Coward L, Lamartiniere CA. Genistein, a component of soy, inhibits the expression of the EGF and ErbB2/Neu receptors in the rat dorsolateral prostate. Prostate, 37:36-43, 1998.

7. Heinonen OP, Albanes D, Virtamo J, Taylor PR, Huttunen JK, Hartman AM, Haapakoski J, Malila N, Rautalahti M, Ripatti S, Maenpaa H, Teerenhovi L, Koss L, Virolainen M, Edwards BK. Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial. J Natl Cancer Inst, 90:440-446, 1998.

8. Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahi M, Hartman AM, Palmgren J, Freedman LS, Haapakoski J, Barrett MJ, Pietinen P, Malila N, Tala E, Liippo K, Salomaa E-R, Tangrea JA, Teppo L, Askin FB, Taskinen E, Erozan Y, Greenwald P, Huttunen JK. α-tocopherol and β-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. J Natl Cancer Inst, 88:1560-1570, 1996.

9. Betram JS, Hossain MZ, Pung A, Rundhaug JE. Development of *in vitro* systems for chemoprevention research. Prev Med, 18:562-575, 1989.

10. Lotan R. Retinoids and apaptosis: implications for cancer chemoprevention and therapy. J Natl Cancer Inst, 87:1655-1657, 1995.

11. Kupelian PA, Klein EA, Witte JS, Kupelian VA, Suh JH. Familial prostate cancer: a different disease? J Urol, 158:2197-2201, 1997.

12. Keller-Byrne JE, Khuder SA, Schaub EA. Meta-analyses of prostate cancer and farming. Am J Ind Med, 31:580-586, 1997.

13. Whorton MD, Amsel J, Mandel J. Cohort mortality study of prostate cancer among chemical workers. Am J Ind Med, 33:293-296, 1998.

14. Krongrad A, Lai H, Burke MA, Goodkin K, Lai S. Marriage and mortality in prostate cancer. J Urol, 156:1696-1700, 1996.

15. Harvei S, Kravdal O. The importance of marital and socioeconomic status in incidence and survival of prostate cancer. Prev Med, 26:623-632, 1997.

16. Mandel JS, Schuman LM. Sexual factors and prostate cancer: results from a case-control study. J Gerontol, 42:259-264, 1987.

17. Potosky AR, Miller BA, Albertsen PC, Kramer BS. The role of increasing detection in the rising incidence of prostate cancer. JAMA, 273:548-552, 1995.

18. Lin DW, Noteboom JL, Blumenstein BA, Ellis WJ, Lange PH, Vessella RL. Serum percent free prostate-specific antigen in metastatic prostate cancer. Urology, 52:366-371, 1988.

19. Wojno KJ, Vashi AR, Schellhammer PF, Wright GL Jr, Montie JE. Percent free prostate-specific antigen values in men with recurrent prostate cancer after radical prostatectomy. Urology, 52:474-478, 1998.

Chapter 3

Commonly Used Human Prostate Cancer Cell Culture Models

Abstract

In vitro research of prostate cancer is made difficult by the lack of available human prostate cancer cell models. The three most widely used human prostate cancer cell models-DU-145, PC-3 and LNCaP-have contributed significantly to our understanding of prostate cancer. However, upon careful examination it is apparent that these cell lines represent advanced cancer and yield little information about the early stages of the disease. The purpose of this chapter is to provide a brief characterization of the commonly used human prostate cancer cell culture models and why they are not well suited for the study of prostate carcinogenesis.

Introduction

In vitro cell models provide invaluable information about the molecular characteristics of an organism. *In vitro* systems allow investigators to study cellular characteristics in a controlled environment. For example, one may wish to study the effects of a particular growth factor on a particular cell type and this would be possible using a well-characterized cell line. In essence, specific characteristics may be isolated and studied *in vitro* whereas such studies would be obscured by uncontrollable physiological variables *in vivo*.

The lack of well-characterized prostate cell models is a major obstacle for *in vitro* prostate cancer research. Current prostate cancer cell models, including DU-145, PC-3 and LNCaP, are often used to test different modes of treatment for the disease. The establishment of a relationship between the behavior these cell models and the *in vivo* prostatic tumor behavior can aid in predicting the response of a tumor to a particular treatment (1). This chapter describes the most widely used prostate cancer cell models (Table 3-1) and why it is imperative that new models be established, especially for studying the process of carcinogenesis in the prostate.

The DU-145 Human Prostate Cancer Cell Line

The DU-145 cell line was derived from a brain metastasis of a 69-year old patient and introduced by Stone, Mickey et al. (2,3). The metastatic lesion, from which DU-145 cells were derived, was composed of moderately differentiated carcinoma with foci of poorly differentiated cells.

DU-145 cells produce high levels of EGF and TGF-α as well as high levels of EGF receptor, suggesting that these cells are subject to autocrine growth stimulation. It was mentioned earlier that the expression of TGF-α is strongly indicative of a more malignant phenotype. Therefore, TGF-α expression by DU-145 cells provides evidence for the highly progressed nature of this cell line. DU-145 cells express FGF receptor and respond to exogenous bFGF treatment and also produce large amounts of bFGF (4). These cells exhibit little response when exposed to androgens. This lack of androgen responsiveness may be attributed to the increased production of endogenous growth factors by these cells, thus, masking the effect of exogenous androgen treatment. Exposure of DU-145 cells to TGF-β, in culture, results in monolayer growth inhibition (5). Studies conducted by MacDonald et al. (6) indicate that DU-145 cells are not very responsive to exogenous EGF stimulation. This suggests that DU-145 cells endogenously produce EGF for maximal autocrine stimulation.

DU-145 cells express cytokeratins 8 and 18, which confirms their epithelial origin (7). However, the expression of the specific prostatic markers, PSA and AR, has not yet been definitively confirmed. This lack of expression of prostatic markers diminishes the potential utility of this cell line for the study of prostate cancer.

The DU-145 cells proliferate rapidly with a doubling time of ~34 hours (2). These cells also show anchorage-independent growth and form tumors when injected into nude mice (7). DU-145 cells are highly invasive and are used as a 100% positive control for invasion studies in our laboratory.

The PC-3 Human Prostate Cancer Cell Line

The PC-3 cell line was developed by Kaighn et al. (8) from a metastatic tumor from a lumbar vertebra of a 62-year old patient. This cell line is tumorigenic and is considered to represent a poorly differentiated prostatic carcinoma (7).

PC-3 cells express high levels of EGF receptor and TGF-α which contribute to autocrine growth (9,10). Studies conducted by Jarrard et al. (11) show that treatment of PC-3 cells with exogenous EGF stimulates the invasion potential of the cell line by stimulating urokinase plasminogen activator expression. This cell line has also been shown to express large amounts of bFGF (4). This high endogenous bFGF expression explains why PC-3 cells respond weakly to exogenous bFGF treatment. PC-3 cells are inhibited by TGF-β and express TGF-β receptors (5).

PC-3 cells were initially shown not to express PSA (8), but more recent studies have shown weak PSA staining (12). It has been proposed that certain sub-populations of the PC-3 cell line are positive for AR and will express PSA (7).

PC-3 cells are very rapidly proliferating cells with a doubling time of 19.8 hours (13). This cell line has also been shown to grow in an anchorage-independent manner and is tumorigenic when injected into nude mice (7).

The LNCaP Human Prostate Cancer Cell Line

The LNCaP cell line was derived from a lymph node metastasis of a 50-year old patient. The cell line was introduced by Horoszewicz et al. (14) as a moderately differentiated prostate cancer.

LNCaP cells express EGF and TGF-α but not at the levels produced by DU-145 cells (15,16). Exogenous supplementation of androgen or estrogen leads to an increase in EGF receptor expression indicating that growth stimulation by hormones may be mediated by growth factors (7). Growth of LNCaP cells is not inhibited by TGF-β (17). There are conflicting reports as to whether or not LNCaP cells express bFGF (4,18), however, it is generally accepted that LNCaP cells do not produce endogenous bFGF but respond to exogenous bFGF (4,18).

LNCaP cells, unlike the other widely used human prostate cancer cell lines, unquestionably express both PSA and AR (19,20). However, the AR of LNCaP cells is mutated, allowing it to bind estradiol and progesterone in addition to androgens (17). The promiscuity of the LNCaP AR, however, severely diminishes the usefulness of this cell line because this is the only one of the three widely used human prostate cancer cell lines that is sensitive to androgen exposure. A corresponding mutation of the AR in prostatic tumor cells *in vivo* has not yet been reported in scientific literature.

The LNCaP cell line is the slowest growing of the three prostate cancer cell models with a doubling time of 60 hours (21). LNCaP cells, like the DU-145

and PC-3 cell lines, growth anchorage independently and form tumors when injected into nude mice (21).

TABLE 3-1: Summary of Characteristics of DU-145, PC-3 and LNCaP Cells

	DU-145	PC-3	LNCaP
Origin	Brain metastasis	Metastatic tumor from lumbar vertebra	Lymph node metastasis
Prostatic Markers	No PSA and conflicting reports of AR expression	Conflicting reports of both AR and PSA expression	Express both AR and PSA but AR is mutated and binds estrogens, progesterone and androgens
Growth Factor Expression and Response	Produce and respond to EGF, TGF-α, and bFGF and inhibited by TGF-β	Produce high levels of EGF, TGF-α, and bFGF but have little response to exogenous treatment with these growth factors. Inhibited by TGF-β treatment	Produce lower levels of EGF and TGF-α than DU-145. Do not express bFGF but respond to bFGF stimulation. Are not inhibited by TGF-β
Doubling Time	34 hours	19.8 hours	60 hours
Anchorage-Independent Growth	yes	yes	yes
Form Tumors in Nude Mice	yes	yes	yes

Conclusion

The DU-145, PC-3 and LNCaP cell lines have served as important tools for the *in vitro* study of prostate cancer research for nearly two decades. However, the significance of the discoveries these cell models have yielded is of a limited scope. The characterization of the commonly used cell models are summarized in Table 3-1. Each cell model has significant flaws which diminish their applicability to the *in vivo* disease. For example, DU-145 cells do not express PSA and their androgen receptor status and androgen responsiveness is, at best, debatable. The autocrine growth and highly invasive nature of the DU-145 cell line indicates that it represents a highly progressed, dedifferentiated cancer. Similar conclusions can be drawn about the PC-3 cell line in that it too represents a highly progressed cancerous state. Both DU-145 and PC-3 cell lines express high levels of growth-inducing growth factors such that exogenous growth factor exposure fails to significantly augment cell proliferation. Although the LNCaP cells are more differentiated, their practical application is diminished by the mutated AR. The applicability of these cell models resides in the treatment of highly progressed prostate cancer. Unfortunately, metastasized prostate cancer is currently extremely difficult to treat leaving patients with little chance for survival of more than 5 years. The increasing sensitivity of screening tests for the presence of prostate cancer has made it possible to detect the disease at an earlier, localized stage. The cell models discussed in this chapter are poor *in vitro* representatives of localized prostate cancer. Therefore, there is a definite need of cell models representing earlier stages of cancer. Such

models would allow investigators to elucidate the molecular events that occur during carcinogenesis and cancer progression. The understanding of these molecular events will further allow for the blocking and eventual prevention of prostate cancer rather than the mere treatment of the disease, which is the limitation of the current models. It is important to gain a fundamental understanding of the multi-step process of carcinogenesis before embarking on a quest to establish a cell model representing early-stage cancer. The following chapter describes the multi-step process of carcinogenesis and the carcinogen *N*-methyl-*N*-nitrosourea that is often used to induce *in vitro* malignant transformation.

References

1. Webber MM. In vitro models for prostatic cancer: summary. *In* Models for Prostate Cancer. *In* Murphy GP (ed): "Models for Prostate Cancer." Prog in Clin Biol Res, 37:133-147, 1980.

2. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line (DU-145). Int J Cancer, 21:274-281, 1978.

3. Mickey DD, Stone KR, Wunderli H, Mickey g, Paulson DF. Characterization of a human prostate adenocarcinoma cell line (DU-145) as a monolayer culture and a solid tumor in athymic mice. *In* Murphy GP (ed): "Models for Prostate Cancer." Prog in Clin Biol Res, 37:67-84, 1980.

4. Nakamoto T, Chang C, Li A, Chodak GW. Basic fibroblast growth factor in human prostate cancer cells. Cancer Res, 52:571-577, 1992.

5. Wilding G, Zugmeir G, Knabbe C, Flanders K, Gelmann E. Differential effects of transforming growth factor β on human prostate cancer cells *in vitro*. Mol Cell Endocrinol, 62:79-87, 1989.

6. MacDonald A, Habib FK. Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. Br J Cancer, 65:177-182, 1992.

7. Webber MM, Bello D, Quader S. Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications. Part 2. Tumorigenic cell lines. Prostate, 30:58-64, 1997.

8. Kaighn ME, Narayan S, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol, 17:16-23, 1979.

9. Carruba G, Leake RE, Rinaldi F, Chalmers D, Comito L, Sorci C, Pavone-Macaluso, M, Castagnetta LAM. Steroid-growth factor interaction in human prostate cancer. 1. Short-term effects of transforming growth factors on growth of human prostate cancer cells. Steroids, 59:412-420, 1994.

10. Ching KZ, Ramsey E, Pettigrew N, D'Cunha R, Jason M, Dodd JG. Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cell lines. Mol Cell Biochem, 126:151-158, 1993.

11. Jarrard DF, Blitz BF, Smith RC, Patai BL, Rukstalis DB. Effect of epidermal growth factor on prostate cancer cell line PC3 growth and invasion. Prostate, 24:46-53, 1994.

12. Garde SV, Sheth AR, Proter AT, Pienta KJ. A comparative study on expression of prostatic inhibin peptide, prostate acid phosphatase and prostate specific antigen in androgen independent human and rat prostate carcinoma cell lines. Cancer Lett, 70:159-166, 1993.

13. Rowley DR, Dang TD, Larsen M, Gerdes MJ, McBride L, Lu B. urification of a novel protein (ps20) from urogenital sinus mesenchymal cells with growth inhibitory properties *in vitro*. J Biol Chem, 270: 22058-22065, 1995.

14. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA. The LNCaP cell line—A new model for studies on human prostatic carcinoma. *In* Murphy GP (ed): "Models for Prostate Cancer." Progr in Clinical Biol Res, 37:115-132, 1980.

15. Connolly JM, Rose DP. Secretion of epidermal growth factor and related polypeptides by the DU145 human prostate cell line. Prostate, 15:177-186, 1989.

16. Connolly JM, Rose DP. Production of epidermal growth factor and transforming growth factor-α by the androgen-responsive LNCaP human prostate cancer cell line. Prostate, 16:209-218, 1990.

17. Schuurmans AL, Bolt J, Veldscholte J, Mulder E. Regulation of growth of LNCaP human prostate tumor cells by growth factors and steroid hormones. J Steroid Biochem Mol Biol, 40:193-197, 1991.

18. Gleave ME, Hsieh JT, von-Eschenbach AC, Chung LW. Prostate and bone fibroblasts induce human prostate cancer growth *in vivo*: implications for bidirectional tumor-stromal cell interaction in prostate carcinoma growth and metastasis. J Urol, 147:1151-1159, 1992.

19. Berns EMJJ, de Doer W, Mulder E. Androgen-dependent growth regulation of and release of specific protein(s) by the androgen receptor containing human prostate tumor cell line LNCaP. Prostate, 9:247-259, 1986.

20. Langeler EG, van Uffelen CJC, Blankenstein MA, van Steenbrugge GJ, Mulder E. Effect of culture conditions on androgen sensitivity of the human prostatic cancer cell line LNCaP, Prostate, 23:213-223, 1993.

21. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP. LNCaP model of human prostatic carcinoma. Cancer Res, 43:1809-1818, 1983.

Chapter 4

The Multi-Step Process of Carcinogenesis and *N*-methyl-*N*-nitrosourea

Abstract

Carcinogenesis is the study of the development of cells from a normal to a malignant state. Current studies in carcinogenesis focus on attempting to understand the molecular events involved during this transformation and how they may be blocked or reversed. While some means of prevention are quite obvious, such as the cessation of smoking, others remain shrouded in mystery. One of the most intense areas of study in cancer research is prevention through chemical agents. Screening for potential chemicals or chemopreventive agents, which may prevent cancer, are performed through *in vivo* and *in vitro* models. This chapter describes the steps involved in carcinogenesis with the use of *N*-methyl-*N*-nitrosourea (MNU) as a chemical carcinogen in studies on carcinogenesis.

Introduction

Malignant transformation results by the action of an agent, a carcinogen, that alters the cellular machinery giving rise to a malignant phenotype. A carcinogen most often interacts with the cellular DNA leading to mutations. The process of carcinogenesis may be divided into two general steps, initiation and promotion. These steps themselves may be further divided, however, for the pupose of this review attention will be given to the general process. Certain types of carcinogens will also be discussed with particular attention focused upon MNU.

The Multi-Step Process of Carcinogenesis

Carcinogenesis is the multi-step process which describes the events that occur during the transformation of normal cells to a cancerous state. It is generally recognized that there are two main stages, initiation and promotion, in the multi-step process. Each of these stages itself consists of multiple steps. The first stage, initiation, involves the exposure of the normal cells to a carcinogen. The carcinogen causes irreversible and permanent changes in a cell's genome (1). Although the DNA of the cell is permanently altered, this altered genome may not necessarily survive. The natural defenses of the cell may prevent it from further dividing and cause it to undergo apoptosis or the initiated cell may merely remain latent and not proliferate. This leads to the second stage, promotion, in which the initiated cell requires a promoter to induce cell proliferation and clonal expansion (1). The promoter is essentially neither

carcinogenic nor mutagenic (1). Once a cancer cell appears, further steps, constituting tumor progression, occur during which the initiated cancer cell evolves into a neoplasm (Figure 4-1).

Current research in carcinogenesis places emphasis on the process by which carcinogens act, the steps in the process, the factors that enhance or suppress the effect of the carcinogens and how chemical carcinogenesis relates to biological factors in the target organism. The focus is not so much on the animal or the cell line but on the actual molecular processes involved.

Initiation and promotion were first discovered through experiments done on mouse skin carcinogenesis using polycyclic aromatic hydrocarbons (PAHs) (2-4). The PAH (initiator) was applied at a dose that did not lead to the tumor formation during the lifetime of the animal. The initiation was followed by the periodic application of a solution of croton oil (promoter) which lead to the formation of numerous tumors. Varying the time of application of the promoter made no difference. In fact, application of croton oil following one year of PAH treatment led to the same results as when the promoter was applied just one week after carcinogen treatment (2-4). The active material in croton oil was discovered to be tetradecanoylphorbol-13-acetate (TPA) which is now widely used as a promoter in carcinogenesis studies. Several other naturally occurring promoters have been identified including phenol, dodecane, and anthralin (5).

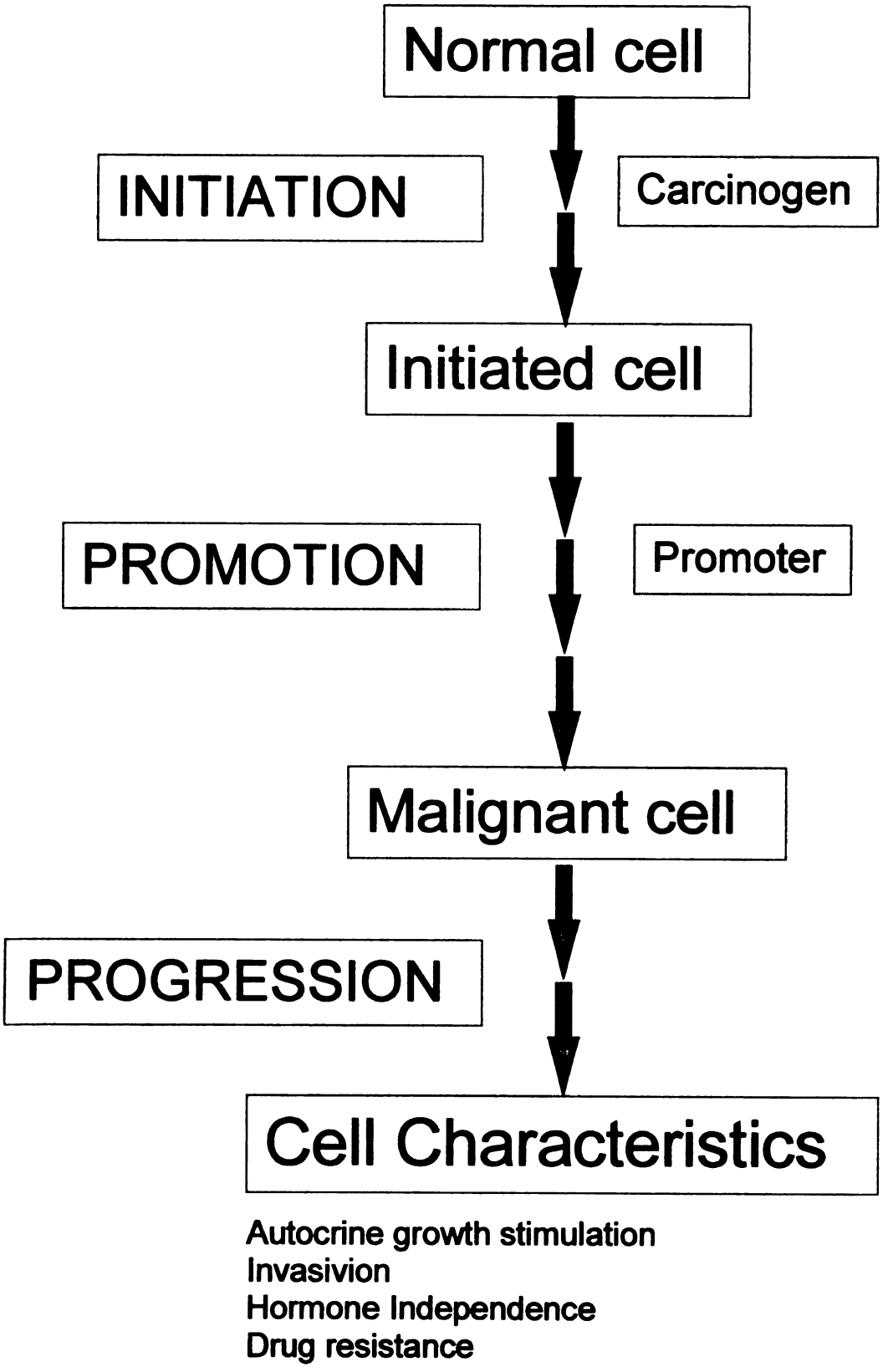

Figure 4-1: Multi-step Process of Carcinogenesis and Progression

The primary mode of action of the initiating carcinogens is through perturbation of gene function by mutation. These agents can be classified into two classes: direct acting and indirect acting carinogens. Direct acting carcinogens, as the name implies, directly interact with the genetic material of the cell while the indirect acting carcinogens require metabolic activation (1). The focus of this study will be on the direct acting carcinogen, *N*-methyl-*N*-nitrosourea (MNU).

N-methyl-*N*-Nitrosourea (MNU)

The *N*-nitrosamide compound, MNU, is a direct-acting carcinogen that does not require enzymatic activation (6). The direct-acting nature of MNU makes it an attractive agent for the study of carcinogenesis both *in vitro* and *in vivo*. Early molecular studies involving MNU demonstrated that this carcinogen was capable of causing significant enhancement of sister-chromatid exchange, a cytological manifestation of DNA damage (7). More recent studies have implicated MNU in the activation of members of the *ras* class of proto-oncogenes (8-10). However, in a recent study conducted by Rhim et al., involving the *in vitro* transformation of an immortalized human prostatic epithelial cell line, no *ras* or p53 mutations were observed. The investigators instead discovered deletions and translocations elsewhere in the genome (11). The effects of MNU described above can be attributed to its ability to act as an alkylating agent (12-15). In essence, MNU exposure results in the aberrant methylation patterns of DNA, causing perturbed gene expression and disruption of normal cell-cycle control,

resulting in altered regulation of cell proliferation (16). Some investigators claim that the methylation patterns of MNU are random while others are convinced that the patterns are nonrandom. Kanduc et al. (14) made the observation that hypermethylation occurs in hepatic DNA of rats following partial hepatectomy in the presence of MNU. This observation is indicative of the fact that proliferating cells are more susceptible to the effects of MNU. In a study conducted by Mironov et al. (13), treatment of rat liver DNA with MNU resulted the presence of O^6-methylguanine in the H-*ras* gene. Miyamoto et al. (7) found that treatment of mouse mammary epithelial cells with MNU caused a specific G→A point mutation resulting in mutation of c-Ki-*ras* proto-oncogenes. A study conducted by Mathison et al. (15) reports the methylation of guanine at the N7 position by MNU. This methylation was blocked by the presence of a neighboring 5-methylcytosine. 5'-CpG-3' dinucleotides are frequent sites for 5-methylcytosine modification and, thus, induction of 5-methylcytosine at such sites may prevent the mutagenic effects of MNU.

Although the mechanism by which MNU exerts its mutagenic effects by acting as an alkylating agent of DNA has been established, there is not enough evidence at present to determine whether the damage induced by MNU is aimed at specific sequences in the genome. However, this fact does not diminish the potential usefulness of this agent for studies of carcinogenesis. MNU may also be used for studies of cancer progression by applying it to malignant cells in effort to enhance transformation.

Enhancement of Transformation by MNU

Several studies, involving the both the *in vitro* and *in vivo* transformation of cells by MNU, have been conducted with both the inclusion and exclusion of promoting agents. Some recent studies have focused on the enhancement of MNU transformation by promoters or other initiators.

A study by Okamoto et al. (17) explores the possibility of the enhancement of MNU-induced transformation by cytokines. This study was based on the idea that chronic inflammation, the subsequent inflammatory response and release of cytokines, increases the possibility of developing bladder cancer. It was discovered that amongst IL-1, IL-6, IL-8 and TNF, IL-6 was the most effective cytokine in enhancing the transformation of rat urothelial cell line, MYP3 by MNU. Treatment with IL-6 resulted in a 4-fold increase in colony formation in agar compared to MNU treatment alone. Cells were treated with IL-6 in several different ways. In one experiment the cells were treated with MNU for one hour and after twenty-four hours they were plated in agar in the presence of the cytokine. In another set of experiments MYP3 cells were treated with MNU as described above but were then cultured for one week with or without IL-6 before being grown in soft agar in the presence or absence of IL-6. Interestingly, the colony formation, in the case of cells exposed to MNU followed by a week in culture in the absence of IL-6 and plating in soft agar with IL-6 treatment, was nearly twice that of when the cells were plated in agar with the cytokine one day after carcinogen treatment. The experiment, in which cells were exposed to IL-6 for one week following MNU treatment and were plated in agar with IL-6, resulted

in only a slightly higher number of colonies than in the experiment where IL-6 was absent for the week following MNU treatment but was present in soft agar (17). One would expect a greater enhancement in transformation through immediate application of the cytokine in liquid medium in which it is allowed to flow over and interact more freely with initiated cells. It is possible, however, that the cells did not have enough time to undergo initiation immediately following carcinogen treatment, thus, promotion would not be possible. However, initiated cells have more than adequate time during the agar assay to undergo the changes required for promotion to be possible and may then interact with the IL-6 rich, albeit rigid matrix giving rise to promotional events. It can be stated with confidence that IL-6 acts as a promoter in this experiment due to the lack of colony formation upon treatment of any kind with the cytokine in the absence of MNU. It was discovered that IL-6 causes a dose-dependent increase in growth of the MYP3 cells (17). During the transformation experiments this stimulation in growth would aid in increasing the number of surviving initiated cells by successfully moving them past their cell cycle check points. Another study, which examined the effects of chronic inflammation by killed *Escherichia coli* on enhancement of MNU transformation *in vivo* in rat urothelium, yielded similar results (18).

In yet another investigation, involving the causative aspects of chronic inflammation, the effect of hydrogen peroxide on transformation with and without MNU was studied. Reactive oxygen species (ROS), including hydrogen peroxide, are present at sites of chronic inflammation and are known to act as

carcinogens, causing mutations, and inducing the expression of proto-oncogenes (19). Hydrogen peroxide (0.1 mM) alone successfully led to the transformation of MYP3 cells and was, in fact, more effective in inducing transformation than MNU (50 μg/ml) alone. When used in combination, the two carcinogens appear to have an additive effect. Thus, ROS are thought to act at both initiation and promotion levels (19).

The effect of TPA, as a promoter of carcinogenesis, was mentioned earlier in this paper. Studies by Garrett et al. (20) and Parsa et al. (21) employed TPA in combination with MNU and achieved significant enhancement of transformation. In the study conducted by Garrett et al. (20), human keratinocytes were treated with MNU alone but failed to form tumors upon injection into mice, however, when both MNU and TPA were applied, all mice injected with the combination displayed tumor formation (14). Parsa et al. (21) applied a single treatment of MNU to pancreatic cells followed by TPA and achieved results upon injection into mice similar to those when multiple treatments of MNU alone were applied (21). TPA may exert its promotional effect through several mechanisms. It is a known protein kinase C (PKC) activator. PKC is a key enzyme in the intracellular signal transduction and growth control. TPA also stimulates production of reactive oxygen species (ROS) and possibly other free radicals by polymorphonuclear leukocytes. The promotional effect of TPA, by stimulation of ROS, is further supported by the observation that antioxidants are effective inhibitors of chemical carcinogenesis

and skin tumor promotion (1). Inhibition of carcinogenesis will be examined further in the following section.

The final study involving enhancement of transformation, is quite interesting in that MNU was employed to improve on its own transformation. The study conducted by Azuma et al. (9) involves the conversion of low-grade rat urinary bladder carcinoma cells initially transformed by MNU to malignant, high-grade carcinoma cells. Further exposure to MNU led to *ras* mutations, altered cell morphology, increased invasive carcinomas upon injection into nude mice, and elevated plasminogen activator activity (9).

Conclusion

Carcinogenesis is the multi-step process involving initiation by a carcinogen and promotion by a promoting agent that leads to expression of the genetic alterations caused during initiation. The important role of promoters is to induce cell proliferation, which provides the environment for initiated cells to undergo clonal expansion. MNU is a potent, direct-acting carcinogen that causes significant damage to DNA. Often, in *in vitro* and *in vivo* studies, MNU is administered at such high concentrations that it alone induces its own promotion and gives rise to transformation. Although this is exciting in that this is a new area in the study of cancer, it needs to be significantly improved if we are to learn more about the cellular and molecular mechanisms involved in it. Systems involving an initatior and a promoter, in which carcinogenesis is more subtle, are required, thus, mirroring the actual mechanism by which this process acts in

humans can be studied more closely. Carcinogenesis initiated by MNU, may be enhanced at both the initiation and promotion stages. The *in vitro* and *in vivo* models of transformation by MNU, described in the following chapter serve only as the initial steps in understanding the complex multi-step process of carcinogenesis. Future studies, to expand our knowledge of the actual mechanisms involved in carcinogenesis along with establishing better models and standardized methods for transformation, will aid in achieving a better understanding of the process. The possibility that this process might be blocked, reversed or inhibited before cells and tissues reach the cancer stage, has been the driving force behind research on chemoprevention.

References

1. Waalkes MP, Ward JM-eds. "Carcinogenesis." Raven Press, New York, 1994.

2. Berenblum I, Shubik P. The role of croton oil applications, associated with a single painting of a carcinogen, in tumour induction of the mouse's skin. Br J Cancer, 1:379-382, 1947.

3. Berenblum I, Haran-Ghera N. The induction of the initiatiing phase of skin carcinogenesis in the mouse by oral administration of 9:10-dimethyl-1:2-benzanthracene, 20-methylcholanthrene, 3:4-benzpyrene, and 1:2:5:6-dibenzanthracene. Br J Cancer, 11:85-87, 1957b.

4. Berenblum I, Haran-Ghera N. A quantitative study of the systemic initiating action of urethane (ethyl carbamate) in mouse skin carcinogenesis. Br J Cancer, 11:77-84, 1957a.

5. Fujiki H, Sugimura T. New classes of tumor promoters: teleocidin, aplysiatoxin and palytoxin. Adv Cancer Res, 49:223-264, 1987.

6. Delp CR, Treves JS, Banerjee MR. Neoplastic transformation and DNA damage of mouse mammary epithelial cells by *N*-methyl-*N*-nitrosourea in organ culture. Cancer Lett, 55:31-37, 1990.

7. Manoharan K, Banerjee MR. Measurements of chemical carcinogen-induced sister-chromatid exchanges in a whole organ in vitro. Mutat Res, 147:165-169, 1985.

8. Miyamoto S, Sukumar S, Guzman RC, Osborn RC, Nandi S. Transforming c-Ki-*ras* mutation is a preneoplastic event in mouse mammary carcinogenesis induced in vitro by *N*-methyl-*N*-nitrosourea. Mol Cell Biol, 10:1593-1599, 1990.

9. Mangold KA, Hubchak S, Mangino MM, Laconi S, Scarpelli DG. Carcinogenesis, 15:1979-1984, 1994.

10. Azuma M, Momose H, Oyasu R. *In vitro* malignant conversion of low grade rat urinary bladder carcinoma cells by exposure to N-methyl-Ninitrosourea. Cancer Res, 50:7062-7067, 1990.

11. Rhim JS, Uin S, Jung M, Thraves PJ, Kuettel MR, Webber MM, Hukku B. Malignant transformation of human prostate epithelial cells by N-nitroso-N-methylurea. Cancer Res, 57:576-580, 1997.

12. Wurdeman RL, Douskey MC, Gold B. DNA methylation by N-methyl-N-nitrosourea: methylation pattern changes in single- and double-stranded DNA, and in DNA with mismatched or bulged guanines. Nucleic Acids Res, 21:4975-4980, 1993.

13. Mironov NM, Bleicher F, Martel-Planche G, Montesano R. Nonrandom distribution of O^6-methylguanine in H-ras gene sequence from DNA modified with *N*-mtheyl-*N*-nitrosourea. Mutat Res, 288:197-205, 1993.

14. Kanduc D, Aresta A, Farber E. Hypermethylation of replicating hepatic DNA following *N*-methyl-*N*-nitrosourea administration. Int J Cancer, 58:436-439, 1994.

15. Methison BH, Said B, Shank RC. Effect of 5-methylcytosine as a neighboring base on methylation of DNA guanine by *N*-methyl-*N*-nitrosourea. Carcinogenesis, 14:323-327, 1993.

16. Jones PA. DNA methylation and cancer. Cancer Res, 46:461-466, 1986.

17. Okamoto M, Kawamata H, Kawai K, Oyasu, R. Enhancement of transformation *in vitro* of a nontumorigenic rat urothelial cell line by interleukin 6. Cancer Res, 55:4581-4585, 1995.

18. Kawai K, Kawamata H, Kemeyama S, Rademaker R, Oyasu R. Persistence of carcinogen-altered cell population in rat urothelium which can be promoted to tumors by chronic inflammatory stimulus. Cancer Res, 54:2630-2632, 1994.

19. Okamoto M, Kawai K, Reznikoff CA, Oyasu R. Transformation in vitro of a nontumorigenic rat urothelial cell line by hydrogen peroxide. Cancer Res, 56: 4649-53, 1996.

20. Garrett, L.R, Perez-Reys N, Smith PP, McDougall JK. Interaction of HPV-18 and nitrosomethylurea in the induction of squamous cell carcinoma. Carcinogenesis, 14:329-332, 1993.

21. Parsa I, Marsh WH, Sutton AL. An *in vitro* model of pancreas carcinogenesis. Am J Pathol, 98:649-655, 1980.

Chapter 5

In Vivo and *In Vitro* Transformation of Prostatic Epithelial Cells with *N*-methyl-*N*-nitrosourea (MNU)

Abstract

Human prostate carcinogenesis is a poorly understood phenomenon. Model systems simulating the events that occur during carcinogenesis are necessary for gaining insight into the etiology of prostate cancer. The direct acting chemical carcinogen *N*-methyl-*N*-nitrosourea may be applied to cell culture model systems to induce carcinogenesis. The standardization of such a model would not only improve our understanding of the disease, but may also provide the tools necessary for screening potential chemoprevention agents which block carcinogenesis.

The purpose of this chapter is to examine the existing *in vivo* and *in vitro* models of prostate carcinogenesis and to evaluate their respective advantages and disadvantages. The models will also be assessed for potential use to study prostate cancer prevention in humans.

Introduction

The multiple steps in prostate carcinogenesis are poorly understood. Epidemiological study of the disease is made difficult due to the old age of prostate cancer patients, and the long latent period of the cancer (1). Several causative factors are believed to influence the transition from normal prostatic epithelium to invasive adenocarcinomas. Therefore, there is a need for model systems that may provide a better insight into understanding the causative factors as well as the potential preventive measures that may be taken. The ultimate goal of any such model system is to mimic the true disease as closely as possible. The majority of research in prostate carcinogenesis has involved *in vivo* studies in which tumors are induced in experimental animal by exposing them to chemical carcinogens. *In vitro* studies have been initiated only recently in an effort to better understand prostate carcinogenesis at the molecular level. While *in vivo* and *in vitro* studies have their respective advantages and disadvantages, they share a common link in the carcinogen, *N*-methyl-*N*-nitrosourea (MNU) that is often used to induce carcinogenesis.

The purpose of this chapter is to describe individual studies along with the advantages and disadvantages of *in vivo* and *in vitro* transformation of prostatic cells. The *in vivo* experiments examined will primarily involve rat and canine models while the *in vitro* models will involve human prostatic epithelial cell lines. Both types of models will be assessed for applicability in studying human prostate carcinogenesis.

In Vivo and *In Vitro* Models

The drawbacks one encounters when working with the *in vivo* models for prostatic carcinogenesis may appear to be so daunting on the surface that one may question whether such studies are required at all. For example, the studies tend to be extremely time consuming with tumors requiring up to and often more than a year to grow to a satisfactory size from which histological sections may be taken. In addition, such studies leave the investigator at the mercy of the normal physiology of the test animal with little control over the animal's metabolic regulatory mechanisms. This often results in excessive inter-experimental variability. *In vitro* studies of transformation, on the other hand, often require little more than a month between treatment of cells with the carcinogen and detection of transformed cell. Furthermore, *in vitro* assays allow for greater control over the variables giving the investigator more flexibility when conducting experiments. *In vitro* assays also allow the investigator to examine the molecular events associated with transformation upon exposure of the cells to the carcinogen. Such events include mutations, morphological alterations, increased invasive ability, increased growth rate, and changes in protein expression. It would, thus, appear that an investigator should not trouble him/herself with the arduous task of conducting *in vivo* transformation experiments. However, the indispensability of animal models may be illustrated by a simple fact: no *in vitro* assay is fully predictive of a cell's behavior *in vivo* (2). Although the phenotypes of the transformed cells *in vitro* may be consistent with cancer, they are not necessarily equivalent. Any correlation between *in vitro* transformed cells and

cancer must be verified *in vivo*. For example, injection of *in vitro* MNU transformed prostatic epithelial cells, that display cancerous behavior, into animals is required to confirm the cancerous nature of the cells through tumor formation.

A reproducible model of *in vitro* transformation of human prostatic epithelial cells, whose malignancy is confirmed through injection and tumor formation in animals, would prove invaluable in chemopreventive studies. Such a model would allow investigators to rapidly assess the chemopreventive ability of potential agents in a relatively short time frame, whereas, *in vivo* chemopreventive studies may take several months before any results are obtained. However, the value of *in vivo* data should not be underestimated.

A model for transformation would serve as a useful tool for chemopreventive studies only if it is possible for the transformation to be blocked. If the model overwhelmingly favors transformation, any potential effect of a chemopreventive agent may be masked. An appropriate analogy would be to try and stop a jet-propelled vehicle with hand brakes from a bicycle. *In vivo* carcinogenesis studies may prove useful in this area by the ability of the investigator to "fine tune" the level of transformation by adjusting the carcinogen dose to a level where tumors may be present in some but not all subjects, for example, a tumor incidence of 60-75% as opposed to 100%.

The following section begins the description of *in vivo* prostate carcinogenesis models by MNU. The characteristics of what makes a good model will be discussed followed by individual studies.

In Vivo Models

It is important for *in vivo* models of prostate carcinogenesis to share as many characteristics as possible with the disease in man. We must, therefore, examine the requirements for appropriate animal models of prostate carcinogenesis before describing specific examples.

Bosland, one of the leading investigators in the field of *in vivo* prostate carcinogenesis, outlined (Table 5-1) the definitive list of requirements mentioned above (1). It is highly unlikely that an animal model will satisfy all the requirements listed in Table 5-1, however, the more requirements that are satisfied, the more appropriate would be the model. All the requirements listed above are obvious analogs of prostate cancer in man with the exception of the description of embryological requirements. Bosland specifically mentions rodents because these animals are the most widely utilized in studies of prostate carcinogenesis. The human prostate consists of one fused unit while rodent prostate is comprised of four paired lobes; the dorsal, lateral, anterior, and ventral which have not merged into one anatomic structure. Of these four lobes only the dorsal, lateral, and anterior lobes are homologous to zones in the human prostate while the ventral rodent prostate lobes have has no apparent human homologue. This lack of homology in the ventral prostate is extremely important in light of the fact that several animal models display tumor formation selectively in the ventral lobes which has little meaning in the context of human prostate carcinogenesis.

Table 5-1. Requirements for Appropriate Animal Models of Prostatic Carcinogenesis

Histology:	Adenocarcinoma
Biologic behavior	Invasive and metastasizing, preferably to bone; Androgen-sensitive, responding to hormonal therapy, ultimately relapsing to hormone-insensitive state; Slow growing, but not so slowly as to preclude feasible experiments
Embryology	In rodents, not developing from the ventral prostate, but from the dorsal, lateral, and/or anterior prostate lobes
Natural History	Mimic pathogenesis of human prostate cancer, with identifiable precursor lesions and a "latent" carcinoma stage
Genetic Alterations	Similar molecular/genetic alterations to those found in human prostatic carcinomas
Feasibility	Simple yet relevant induction procedure; Predictable and adjustable incidence

(Modified from Bosland, 1992, Ref. #1)

A striking characteristic of human prostate cancer is the slow growth of tumors which do not occur with appreciable incidence until after the fifth decade of life. Thus, although the length of time involved in animal studies of prostate carcinogenesis was earlier labeled as a disadvantage in the context of convenience, it may also be regarded as an advantage in the context of human mimicry. A relatively long latent period and slow growth rate are desirable traits of an animal model of prostate carcinogenesis. However, according to Bosland, carcinoma latency exceeding 12 months is not practical (1). Based on the guidelines provided in Table 5-1, we may proceed to examine examples of transformation of animal prostatic epithelial cells *in vivo* by MNU.

Wistar Rat Model: Prostate carcinogenesis using the Wistar rat model has been studied since 1983 (3). This research focuses largely upon the theory that prostatic cancer in man develops in atrophic areas of the gland. It is believed that temporary atrophy, induced by chemical castration followed by exogenous renewal of testosterone action on the prostate epithelium, is a prerequisite for the development of prostatic carcinogenesis in rats upon treatment with MNU (3). In order to enhance transformation with MNU, Bosland et al. (4) increased cell proliferation in the prostate during treatment with the carcinogen by treatment with androgen, thus, ensuring that DNA lesions leading to mutations are fixed in the cell (4). Wistar rats, which have a known low incidence of spontaneous cancer, served as the models in these studies. Bosland et al. performed several

similarly designed experiments which follow a general scheme in which the rats are pretreated with cyproterone acetate (CPA) daily for three days to induce chemical castration, which leads to prostatic atrophy. CPA (50mg/kg) treatment was followed by prostatic epithelial cell growth stimulation achieved through daily administration of testosterone propionate (TP) (100mg/kg) for three consecutive days. Finally, MNU (50 mg/ml in earlier studies and 10 mg/ml in more recent investigations) was administered to the rats intravenously with no additional treatments of androgen following the exposure of the animals to the carcinogen.

Initial experiments yielded exciting data where 25% of the Wistar rats developed prostatic adenocarcinoma in the dorsolateral region of the prostate after MNU treatment (3). Dorsolateral refers to the apparent fusion of the dorsal and lateral lobes of the prostate. Further experiments involved essentially the same design as described above, but were more refined with a greater number of control groups (e.g. no TP, recovery time between CPA treatment and MNU injection). Once again, adenocarcinomas located in the dorsolateral region, were found in several groups while the ventral lobes were always free of malignant tumors (4). This rodent tumorigenesis model exhibits a strong relationship to prostate carcinogenesis in man because the tumors are adenocarcinomas, they are invasive, capable of metastasis, and they do not originate from the ventral prostate. Curiously, control groups, with administration of MNU alone to assess the unaided transformation ability of the carcinogen, were not included in the study. Also, there were no groups that received only CPA and TP. Thus, it is impossible to isolate the specific effects of the three agents (CPA, TP, and MNU)

individually, which may provide a greater understanding of the types of changes that occur when they are administered in combination.

The Wistar rat model has been a valuable tool for studying prostate carcinogenesis (3,4). Although these rats are currently commercially available, at one time the general unavailability of these animals led investigators to perform carcinogenesis experiments on more readily available rat models such as the Sprague-Dawley, and Fisher F334 rats. Unfortunately, transformation by CPA, TP and MNU resulted in proliferative lesions of the ventral prostate and seminal vesicle rather than the dorsolateral region, thus, diminishing the adequacy of these models (5). A more promising model is the Lobund-Wistar rat that is the focus of the carcinogenesis studies described below (6-8).

Lobund-Wistar Rat Model: The Lobund-Wistar (LW) rat is unusual in its susceptibility to metastatic prostate adenocarcinoma (6). The incidence of spontaneous development of prostate adenocarcinomas in LW rats is 26% over an average of 26 months. This unusual behavior exhibited by these rats makes them suitable candidates for induction of carcinogenesis by MNU.

The carcinogenesis studies conducted using Lobund-Wistar rats differ from those of the Wistar rats described in the previous section in that investigators have applied the former model for examining the multi-step process of carcinogenesis in their experiments and in focusing on the initiation and promotion events. The rats are not pretreated with castrating agents or

hormones prior to exposure to MNU, the initiator, and are subsequently treated with a promoting agent, testosterone propionate (TP) (6).

The first study involved four experimental groups of animals: 1) untreated control, 2) MNU (30 mg/kg) alone, 3) TP (50 mg) alone, and 4) TP (50 mg) and MNU (30 mg/kg) in combination. No prostate adenocarcinomas were observed in the untreated group within 20 months. While MNU and TP individually did not display a very high incidence of prostatic tumor formation (20% and 18%, respectively), the combination of the two agents yielded an incidence of 65-80% in 11 months (6). Although this experiment was nicely designed with proper controls, there was no mention of which region of the prostate exhibited tumor formation. Subsequent studies, however, explain that the large size of the tumors had obscured the region where they originated. It was confirmed, through further experimentation, that the MNU (30 mg/kg) and TP treated LW rats develop tumors primarily in the dorsolateral and anterior regions of the prostate and to a lesser degree in the seminal vesicles (7). This confirmation satisfies the homology requirement between human prostate carcinogenesis and the rat model.

The final study, using the LW rat model, involves the latency period between initiation and promotion of carcinogenesis. Pollard et al. attempted to establish an experimental procedure for investigating dormancy of adenocarcinomas in accessory sex organs that are initially not apparent upon exposure to MNU but are activated by the promotional effects of testosterone (8). The procedure involves detection of tumors arising in the prostate-seminal

vesicle complex following exposure of LW rats to MNU (30 mg/kg) followed by activation of prostate-seminal vesicle tumor cells by TP. Animals were treated with the androgen at intervals of 3, 6, or 12 months following MNU exposure. The results proved very interesting with tumors detected in 100%, 89.5%, and 90% of LW rats respectively, prior to initiation of TP treatment. The longer the time between MNU and TP, the shorter the subsequent latent period. The average latency of tumor formation following TP treatments was 6.4, 4.7 and 2.1 months for the 3, 6, and 12-month intervals, respectively. The control group that received MNU alone exhibited no tumor formation 12 months following initial carcinogen exposure. These results suggest that the latent period following TP was reduced in rats with longer MNU-TP intervals. The investigators stated that the results suggest that the number of tumor cells had increased with time (8). Although this is apparently true, it must not be forgotten that LW rats are inherently susceptible to the formation of prostatic tumors. Therefore, it is unclear whether the tumor cells that are activated by TP were initiated spontaneously or by MNU. A more striking inconsistency of this study is the lack of tumor formation following the test group exposed to MNU alone. Although this result nicely illustrates the necessity of the promotional event that gives rise to tumor formation, it contradicts the first study by Pollard in which 20% of LW rats treated with MNU alone exhibit prostatic tumors after an average of 12.3 months following exposure to the carcinogen (6). These details, however, are relatively minor when juxtaposed with the possible applications of this model to study prostate carcinogenesis and possible modes of prevention. The LW rat model

satisfies many of the requirements outlined by Bosland et al. in Table 1 (1) and further research may provide more evidence to support the correlation between this model and prostate carcinogenesis in man.

The LW rat model may serve as a suitable model for screening potential chemopreventive agents. The only modification in the protocol used by Pollard et al. (8) would be the inclusion of the chemopreventive agent prior to, as well as after exposure of the animal to MNU. The agent would ideally be included in the diet of the animal rather than through injection thereby increasing its practical application.

Other Potential Models: The studies described above are the only currently reported investigations involving *in vivo* carcinogenesis with MNU. There are, however, other model systems to which carcinogenesis induced by MNU may be applied. The first system to be discussed involves a canine model. A system involving a reconstituted mouse prostate will also be discussed.

The dog is the only nonhuman species in which spontaneous prostate cancer occurs frequently (9). Prostate cancer in this animal frequently metastasizes to the lymph nodes, lung, and bone bearing a striking similarity to the disease in man. In addition, the canine and human prostates share greater homology than that shared between rodents and humans. Recent findings by Waters (9,10,11) report the occurrence of prostate intraepithelial neoplasia (PIN) showing cytological features identical to the human counterpart including cell crowding, loss of polarity, and nuclear and nucleolar enlargement. His study is

the first of its kind to describe PIN in dogs without clinically apparent cancer. This is an extremely important discovery suggesting that canine PIN, like human PIN, represents a precancerous stage in the progression from benign epithelium to carcinoma. There is an interesting correlation between prostate cancer in man and dogs when considering geography. Prostate cancer primarily affects males in the western world and dogs are perhaps the most "westernized" domesticated animal exposed to many of the same environmental carcinogens and promoters as humans. The dog, thus, represents the ultimate model for the study of prostate carcinogenesis. The greatest impediments in the development of canine models for carcinogenesis are likely to be availability, cost and time. Rodent models are more readily available, are relatively inexpensive and can be used within a relatively short time frame because the induction of carcinogenesis can be initiated in rodents eight weeks of age which is unlikely to be possible in canine models. Although canine models may not be as feasible as rodent models, they show the strongest correlation of a nonhuman species to prostate carcinogenesis in man.

One of the greatest drawbacks of *in vivo* carcinogenesis studies is the length of time required for transformation to occur and for subsequent detection of tumors. Application of the prostate reconstitution system described for mice (12) may alleviate this problem. The general procedure involves isolation of urogenital sinus tissue from mouse fetuses and trimming the anterior and posterior regions thereby separating the portion that gives rise to the prostate gland. The urogenital sinus epithelium (UGE) is isolated from the urogenital

sinus mesenchyme (UGM). Once isolated, the UGE may be exposed to MNU for an hour. The cells are then mixed in a predetermined ratio (UGE:UGM=3:7) to reconstitute the fetal urogenital sinus tissue. The mouse reconstituted prostates (MRPs) are suspended in collagen, incubated overnight, and grafted under the renal capsules of adult isogenic male hosts. Potential chemopreventive agents may be incorporated into the diet of the host to block carcinogenesis. After seven weeks of growth in the host, the MRPs are harvested and could be sectioned or cultured and tested for malignancy. The time of MRP harvesting may be varied to observe the progression of the cells from a normal to malignant state. This *in vitro-in vivo* procedure would greatly decrease the time involved in studying induction of prostate carcinogenesis in animals.

The above example underscores the importance of having characteristics of both *in vitro* and *in vivo* protocols for constructing the ideal model of prostate carcinogenesis. The isolation of the UGE gives the investigator control over the transformation while the grafting of the MRP into hosts ensures that the cancer will grow in a physiological environment and finally the harvesting of the MRP allows the study of the cells at a molecular level. The next section focuses on *in vitro* models for prostate carcinogenesis which would allow the investigator to control and closely monitor the transformation at the molecular level.

In Vitro Cell Models

Although there are several proposed *in vitro* models for carcinogenesis induced by MNU, there are very few that involve the prostate. To date, there have only been two studies involving *in vitro* transformation of human prostatic epithelial cells. This section will evaluate the investigations and provide potential applications of the *in vitro* models.

One study of chemical transformation of human prostatic epithelial cells employed MNU as the carcinogen (13). The cell model used in the experiments was the non- tumorigenic RWPE-1 human prostatic epithelial cell line immortalized by human papillomavirus-18. The experimental design involves the exposure of one day old RWPE-1 cell cultures to 50-250 μg/ml MNU for one hour. The cells are then grown to confluency whereupon they are subcultured and exposed to two additional rounds of MNU treatment. The cells are plated in soft agar following the third round of exposure to MNU and colonies are counted following 21 days of anchorage-independent growth. It is not mentioned whether all three rounds of MNU treated cells were plated in agar or whether only the third was grown in agar cultures. The control group was not exposed to MNU and had a low colony forming efficiency (CFE) (0.01%) while cultures exposed to 50 μg/ml MNU exhibited a CFE of 0.40%. Although anchorage-independent growth of cells in agar provides strong evidence of malignancy, the cells must also be injected into animals to confirm that they are tumorigenic. Cells transformed with 50 and 100 μg/ml MNU exhibited high tumor formation upon injection into nude mice while no tumors were observed in the control and in the

250 μg/ml MNU treated groups. It was not specified as to whether the cells used for injection into mice were isolated from colonies in agar or monolayer cultures after undergoing three rounds of treatment. The use of isolated colonies would explain the high tumor incidence in the 50 and 100 μg/ml MNU groups.

The standardization of the experiment described above would serve as an invaluable tool for the study of prostate carcinogenesis and the screening of potential chemopreventive agents that may be applied to the model system. First, it is necessary to prove that MNU transformation of human prostatic epithelial cells can be repeated with consistency to show similar CFEs upon plating the cells in soft agar. Once transformation is standardized, potential chemopreventive agents may be included immediately following exposure of the cells to MNU. Successful chemoprevention would exhibit fewer colonies in soft agar than a control receiving MNU without the chemopreventive agent. The molecular events associated with transformation may be described in unprecedented detail by noting the changes following each round of MNU treatment.

Okamoto et al. transformed the RWPE-1 and PWR-1E cell lines with MNU (14). PWR-1E is also a non-tumorigenic, human prostate epithelial cell line immortalized by infection with adenovirus-12/simian virus-40 hybrid virus (15). The experimental design is similar to that described above, but greater detail is provided. One day-old cultures were exposed to 50 μg/ml MNU for one hour and allowed to grow for 24 hours whereupon they were plated in soft agar for anchorage-independent growth. Okamoto and his collaborators were

interested in examining the effects of epidermal growth factor (EGF) in promoting anchorage-independent growth and, therefore, did not include EGF in the culture medium when plating the cells and during feeding. Some test groups received EGF when plated in agar while others did not. An additional aim of this study was to investigate the effects of interleukin-6 (IL-6) in anchorage-independent growth of the cells and included the cytokine in some groups plated in agar as well. The agar colonies were grown for 28 days and colonies were counted. Addition of EGF and IL-6 significantly increased colony formation in soft agar of both cell lines pretreated with MNU. Very few colonies were observed in the untreated groups receiving no MNU. Groups pretreated with MNU without further cytokine or growth factor treatment formed a modest number of colonies while groups pretreated with a combination of MNU and EGF or MNU and IL-6 displayed a significantly higher CFE. It is likely that the study conducted by Rhim et al. (13) included EGF when plating the cells in agar, thus, giving rise to a high CFE. MNU transformants were isolated from the agar dishes and cultured. Transformants isolated from agar cultures, treated with MNU alone, displayed a higher growth potential than the parent cell lines. Although, anchorage-independent growth and increased growth potential are characteristic of malignant cells, it cannot be stated that these cells are, in fact, malignant until tumor formation is observed upon injection of the cells into animals. However, the purpose of this investigation was to establish the promoting effects of EGF and IL-6 on anchorage-independent and anchorage-dependent growth of the MNU treated cells. Although the transformation experiments conducted by Rhim

et al. (13) need to be repeated, they have yielded some valuable information. In view of these observations, the study described in this thesis was initiated and includes transformation and characterization of human prostatic epithelial cells.

Cells transformed by exposure of RWPE-1 cells to MNU were injected into nude mice. The tumors were removed and cells were isolated and cultured. The cultured cells were again injected into nude mice and again cells from these second generation tumors were cultured and expanded into several cell lines, five of which are currently undergoing characterization. The characteristics of the MNU-transformed cell lines vary considerably. The morphology and anchorage dependent and independent growth rates differ from one cell line to another. A spectrum of morphologies exists from an epithelial shape, resembling the parent RWPE-1 cells, to an elongated fibroblast-like morphology. Interestingly, cells with an epithelial morphology grow at a slower rate both in the anchorage-dependent monolayer cultures, and in the anchorage-independent agar cultures, as compared to the elongated cells. These observations imply that the MNU transformed cell lines may represent varying degrees of malignancy from the early stage carcinoma to the late stage carcinoma. Thus, these cell lines may provide invaluable information regarding the molecular events associated with the different stages of carcinogenesis. The characterization of these cell lines is the focus of my research reported in this thesis.

Conclusion

In vivo and *in vitro* models of prostate carcinogenesis are the key to gaining a better understanding of the disease. The choice of model depends largely on primary interests of the investigator. Simulating prostate carcinogenesis as closely as possible requires the use of *in vivo* models which show strong correlation with the human disease. If scrutinizing the molecular events associated with carcinogenesis is the primary goal, *in vitro* models are better suited for this task.

Currently, the Lobund-Wistar rat is the most promising *in vivo* model of prostate carcinogenesis. Prostate tumors can be induced with relative ease by treatment with MNU and the origin of the tumors appears to be homologous to that in man. Although a very strong correlation between canine and human prostate carcinogenesis is apparent, canine models lack in feasibility and are, thus, not widely used.

The recent development of *in vitro* models in our laboratory, for the study of prostate carcinogenesis by treatment with MNU, has provided a means of gaining a better understanding of the molecular events associated with carcinogenesis. The possibility of establishing cell models, representing different stages in carcinogenesis, is very encouraging. A standardized model for *in vitro* transformation of human prostatic epithelial cells by MNU would be immensely useful in establishing a system for testing of chemopreventive agents. Both *in vitro* and *in vivo* models of transformation of prostatic epithelial cells by MNU provide valuable information for understanding the etiology of prostate

carcinogenesis and may ultimately be applied to discovering agents for chemoprevention. However, in order to establish the utility of any cell model, it is necessary for it to undergo thorough characterization. In the case of malignant cells, certain criteria must be established to determine whether these cells are, in fact, transformed and to what degree. These criteria are the subject of the following chapter.

References

1. Bosland M. Animal models for the study of prostate carcinogenesis. J Cell Biochem, Suppl, 16H:89-98, 1992.

2. Welch DR. Technical considerations for studying cancer metastasis *in vivo*. Clin Exp Metastasis, 15:272-306, 1997.

3. Bosland MC, Prinsen MK, Kroes R. Adenocarcinomas of the prostate induced by *N*-nitroso-*N*-methylurea in rats pretreated with cyproterone acetate and testosterone. Cancer Lett, 18:69-78, 1983.

4. Bosland MC, Prinsen MK. Induction of dorsolateral prostate adenocarcinomas and other accessory sex gland lesions in male Wistar ratsby a single administration of *N*-methyl-*N*-nitrosourea, 7, 12-dimethylbenz(*a*)anthracene, and 3,2'-dimethyl-4-aminobiphenyl after sequential treatment with cyproterone acetate and testosterone propionate. Cancer Res, 50:691-699, 1990.

5. Bosland MC, Prinsen MK, Rivenson A, Silverman J, Fiala E, Williams GM, Kroes R, Weisburger JH. Induction of proliferative lesions of ventral prostate, seminal vesicle, and other accessory sex glands in rats by *N*-methyl-*N*-nitrosourea: effect of castration, pretreatment with cyproterone acetate and testosterone propionate, and rat strain. Prostate, 20:339-353, 1992.

6. Pollard M, Luckert PH, Snyder D.L. The promotional effect of testosterone on induction of prostate-cancer in MNU-sensitized L-W rats. Cancer Lett, 45:209-212, 1989.

7. Pollard M, Luckert PH. Early manifestations of induced prostate tumors in Lobund-Wistar rats. Cancer Lett, 67:113-116, 1992.

8. Pollard M, Luckert PH. Activation of dormant cancer cells in the prostates and seminal vesicles of Lobund-Wistar rats. Cancer Lett, 82:141-144, 1994.

9. Waters DJ, Bostwick DG. Prostatic intraepithelial neoplasia occurs spontaneously in the canine prostate. J Urol, 157:713-716, 1997.

10. Waters DJ, Hayden DW, Bell FW, Klausner JS, Qian J, Bostwick DG. Prostatic intraepithelial neoplasia in dogs with spontaneous prostate cancer. Prostate, 30:92-97, 1997.

11. Waters DJ, Bostwick DG. The canine prostate is a spontaneous model of bintraepithelial neoplasia and prostate cancer progression. Anticancer Res, 17:1467-1470, 1997.

12. Slawin K, Kadmon D, Park SH, Scardino PT, Anzano M, Sporn MB, Thompson TC. Dietary fenretinide, a synthetic retinoid, decreases the tumor incidence and the tumor mass of *ras+myc*-induced carcinomas in the mouse prostate reconstitution model system. Cancer Res, 53:4461-4465, 1993.

13. Rhim JS, Jin S, Jung M, Thraves PJ, Kuettel, MR, Webber MM, Hukku, B. Malignant transformation of human prostate epithelial cells by *N*-nitroso-*N*-methylurea. Cancer Res, 57:576-580, 1997.

14. Okamoto M, Webber MM, Quader S, Oyasu R. Interleukin-6 and epidermal growth factor promote anchorage-independent growth of immortalized human prostatic epithelial cells treated with *N*-methyl-*N*-nitrosourea. Prostate, 35:255-262, 1998.

15. Webber MM, Bello D, Kleinman HK, Wartinger DD, Williams DE, Rhim JS. Prostate specific antigen and androgen receptor induction and characterization of an immortalized adult human prostatic epithelial cell line. Carcinogenesis, 17:1641-1646, 1996.

Chapter 6

Markers of Transformation

Abstract

Carcinogenesis studies require the establishment of criteria for determining whether transformation has occurred. Such criteria may also be applied towards determining the degree to which cells are transformed allowing investigators to distinguish between more malignant cells from those that are less malignant. This chapter describes definitive markers for the assessment of *in vitro* transformation. The markers examined include morphological alterations, increased growth rate, anchorage-independent growth, invasive ability, and tumorigenicity.

Introduction

Once cells have been exposed to single or multiple treatments of MNU, the next step is to investigate whether MNU has induced transformation. In general, the primary characteristics, confirming malignant transformation of cells are anchorage-independent growth and tumorigenicity when injected into nude mice (1-3). Although these characteristics are adequate for confirming as to whether transformation has occurred, additional tests of other characteristics of transformed cells may be performed, thus, providing a more accurate description of the extent of transformation. In addition, the use of several different transformation markers is essential when comparing characteristics of different cell lines. For example, testing five different cell models only for anchorage-independent growth and tumorigenicity may not provide adequate data for determining with confidence, whether one transformed cell type is more malignant than another. Each additional test for transformation and malignancy provides an increasing level of stratification allowing the investigator to delineate the order of progression between cell lines. Among the definitive markers for cell transformation are morphological alterations, enhanced growth, anchorage independent growth, enhanced invasiveness and the ability of the transformed cells to induce tumors in nude mice (4). Many other interesting methods for testing malignant transformation were implemented in the investigations reviewed here and will be examined in detail with focus on transformation by MNU.

Studies on Cell Morphology

There is a great deal of valuable information that may be obtained through observing the morphology of cells. Many investigations examined in this chapter paid close attention to the morphological and histological effects of MNU on the cell model (4-12). Many investigators reported morphological alterations after the cells were treated with MNU. These alterations vary from cell type to cell type with the primary objective of identifying carcinogen-treated cells with morphology different from that of the parent cells. Other characteristics that were studied include abnormal growth, changes in cytokeratin expression and decreased differentiation. Although morphological changes are not definitive markers for transformation, they aid in the monitoring of the effects of MNU on the treated cells. A study conducted by Russo et al. (7) involved a multinucleation assay on the cells treated with MNU for which an increase in the number of nuclei per cell strongly suggested that transformation took place. The premise behind this assay is the ability of normal and neoplastic cells to respond to cytochalasin B differently (13). Normal cells show control of nuclear division upon treatment with cytochalasin B while neoplastic cells are highly multinucleated with continuous nuclear division following cytochalasin B treatment. Cytoplasmic division is prevented by cytochalasin B in both cases (13). A study was conducted by Azuma et al. (8) in which rat bladder epithelial cells, exhibiting an altered morphology following exposure to MNU, were isolated and cloned. These cells of altered morphology proved to be much more tumorigenic than the parent cells (8). The establishment of altered morphology of carcinogen-treated cells may be

followed by comparing the doubling time of the transformed cells with that of the parent cells.

Increased Growth Rate

Malignant transformed cells often exhibit and enhanced rate of growth. Hyperplasia of transformed cells may eventually lead to a neoplasm. This elevated growth rate may be monitored in a number of ways. A hyperplastic state may be observed in histological sections of tumors that result after injection of MNU-transformed cells into mice (10,11,14,15).

A simple and quantifiable method of comparing growth rate of cells before and after treatment with MNU, that was not reported in the investigations reviewed here, may be through a microplate growth assay. The same number of MNU-treated and untreated parent cells are plated in separate wells and allowed to grow for a fixed number of days, during which time they are fed at regular intervals. Upon fixing and staining the plates, the growth curves for each cell type may be established along with their doubling time. This allows the investigator to elucidate the effect of MNU on the growth rate of the treated cells. Although the exact procedure was not reported, the study conducted by Calaf et al. (4) involved the comparison of the doubling times of MNU-treated and untreated human breast epithelial cells. Although increased monolayer growth rate and doubling time may suggest transformation, anchorage-independent growth in soft agar bears a stronger correlation with malignant transformation.

Anchorage-Independent Growth

Normal epithelial cells *in vivo* rest on a basement membrane that is required for their polarization and growth. In cell culture, normal cells (except lymphocytes) requires a substrate for attachment before they undergo DNA synthesis and cell division. This phenomenon is called anchorage independence. If normal cells are placed in suspension, such as in soft agar, they do not proliferate. Upon malignant transformation, cells show varying degrees of loss of anchorage dependence so that if placed in soft agar or methylcellulose, they have the ability to proliferate and form colonies (16,17). This ability of cancer cells to grow in soft agar is associated with autocrine secretion of growth factors and secretion of proteases involved in invasion, during the late stages of carcinogenesis. Soft agar assay provides an *in vitro* method to identify and quantify the number of potentially transformed cells. A strong correlation between increased cloning efficiency in agar and tumor formation has been demonstrated. The assay for loss of anchorage dependence is based on the ability of cells to form colonies in soft agar. These colonies may be stained with a tetrazolium dye (18) as seen in Figure 6-1, thus, facilitating the quantification of colony number. This assay was developed by MacPherson and Motagnier (16). The varying ability to grow in soft agar may be correlated with the degree of acquisition of malignant characteristics in a continuum/multistep process of carcinogenesis. However, exhibition of anchorage-independent growth is a relatively late marker and may be correlated with tumorigenicity (4).

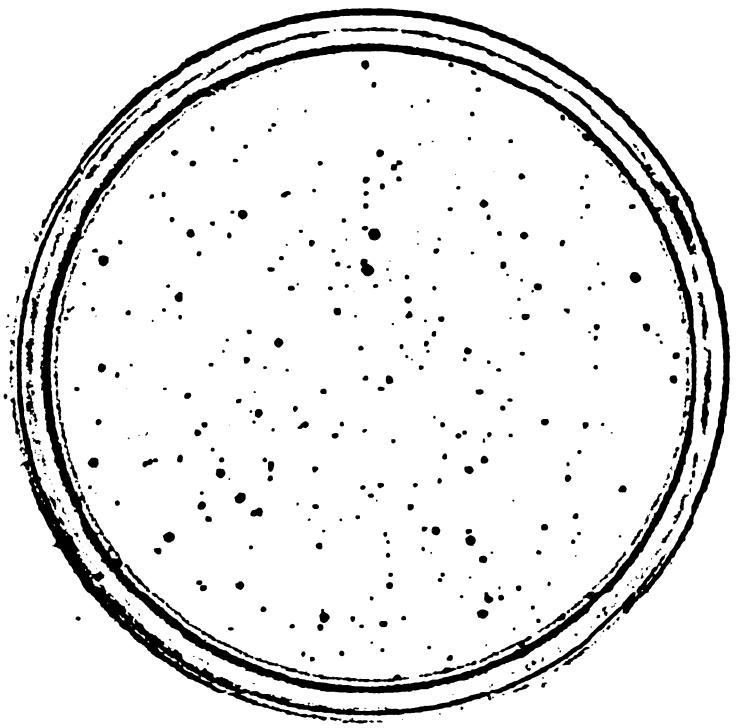

Figure 6-1: Anchorage-independent growth of WPE-1-NB14 cells. Cells were plated at a density of 12,500 in a 30 mm culture dish in an agar-medium suspension and allowed to grow for 21 days and stained with INT.

Several investigators employed the soft agar assay for anchorage independent growth along with injection of MNU treated cells into nude mice. Although it takes three to four weeks for the assay to run its course, it is significantly shorter than the time required for the growth of tumors in mice, and it may serve as a preview to the events that will be observed upon injection *in vivo*. It should be noted, however, that just because cells are able to grow in soft agar does not necessarily indicate that they will form tumors *in vivo* (7).

One very significant feature of the soft agar assay for anchorage-independent growth is that it allows the investigator to purify the transformed cell population. This may be done by removing colonies from the soft agar dish after allowing time for the colony to form and then placing the colony in culture medium. The resulting population will have a higher percentage of transformed cells and will be a more pure population. It is surprising that none of the investigations employed this method to increase transforming efficiency. Although there is a strong correlation between the ability of a cell line to form colonies in agar and its tumorigenicity, colony-forming ability is not synonymous with malignant transformation. Invasive ability has a stronger correlation with malignancy than anchorage-independence.

Invasive Ability

A very good marker for the confirmation of transformation is the effect MNU has on invasive ability of treated cells. Invasion and metastasis are the processes by which malignant tumors spread throughout the body. The steps

include: 1) attachment to the basement membrane, 2) degradation of the basement membrane by induction and release of matrix degrading proteases, 3) migration of tumor cells toward chemoattractants of the target tissue, and 4) tumor cell proliferation forming a metastasis (19). These events may be simulated *in vitro* through the blind well chamber assay. A schematic diagram of the blind well is provided in figure 6-2. A nuclepore filter coated with the reconstituted basement membrane, Matrigel, separates the two chambers of the blind well. Cells are plated in the top chamber while the bottom chamber contains a chemoattractant towards which the cells migrate. The cells must degrade the Matrigel in order to pass to the underside of the filter and to migrate to the lower chamber. The migrated cells are quantified after being allowed appreciable time to invade and the greater the number of migrated cells, the greater the invasive ability of the cell line.

It is surprising that only one investigator tested MNU treated cells for enhanced invasive ability (4). The investigation conducted by Azuma et al. (8) indirectly assayed for invasive ability by monitoring the activity of plasminogen activator. The plasminogen activator plays an important role in the cascade of events leading to the release of enzymes involved in the degradation of the basement membrane. Treatment with MNU resulted in a 4 to 9-fold increase in plasminogen activator activity as compared to the parent cells (8).

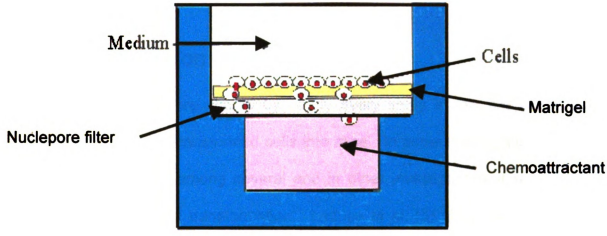

Figure 6-2: Schematic Diagram of a Blind Well Chamber used to assess Invasive Ability. The chamber consists of tow compartments separated by a nucleopore filter coated with the reconstituted basement membrane, Matrigel. Cells are plated in the top chamber and migrate towards the chemoattractant in the bottom chamber. Normal cells are unable to penetrate the Matrigel layer while invasive cells degrade the Matrigel, pass through the filter into the bottom chamber. Cells in the bottom chamber are quantified.

The *in vitro* methods for detection of transformation examined thus far provide valuable information as to whether transformation has occurred and to what degree. However, there is no substitute for the ultimate *in vivo* test to determine whether malignant transformation has taken place. This involves testing for tumorigenicity by injection of cells into nude mice.

Tumor Formation

The one feature shared by nearly every study in this chapter was the injection of suspect transformed cells into mice. In some investigations this was the primary marker among several and in other investigations it was the only marker for malignant transformation. Laduca et al. (5) chose not to use mice stating that there are too many problems associated with *in vivo* experiments. These investigators favor an entirely *in vitro* system in which cells are transformed and tumors are grown "*in vitro*" with the rationale that carcinogenesis may be better understood when tumor formation is visualized in culture without the use of any animal transplantation (5). The general procedure involves subculturing and mixing the MNU-treated cell suspension with collagen in a 12- well plate and adding appropriate amounts of culture medium and changing the medium every 3 days. The MNU treated cells soon began to form "microtumors," consisting either of clusters of cells when small, or spherical bodies when larger (5). This may be true, however, it is unlikely that this *in vitro* method can serve as a replacement for the *in vivo* observation of tumorigenicity. In other words, an *in vitro* system, in which MNU treated cells form "tumors,"

does not necessarily mean that the same result would be achieved *in vivo*. The *in vivo* model involves injection of MNU treated cells into mice and waiting for either a fixed time period or until palpable tumors may be harvested from the animal. At this point the tumors are removed from the mice and placed back into culture. Following injection into nude mice it is important to confirm the origin of the tumor cells a) by screening for markers characteristic of the particular organ or gland from which the cells were originally derived and b) by chromosome analysis to ensure that the cells of the same species and type, which were originally injected, are being taken out (6). This is especially important if a transformed cell line is being established because it is possible that the injected cells may consist of cells from the organ along with cells of the surrounding tissue. Although this *in vivo* method is much more time consuming than the *in vitro* collagen gel matrix model, it is a definitive method by which tumorigenicity is assayed. I applied the aforementioned markers for transformation during the characterization of the MNU-transformed human prostate cancer cell lines described in Chapter 7. The following subsections describe additional transformation markers that may be utilized.

Other Markers

Cancer is a disease that arises from the disruption of gene function. Changes in the expression of genes associated with growth regulation, such as, oncogenes and suppresser genes are characteristic of malignant cells. Comparison of gene expression may be accomplished by directly staining MNU- treated and untreated cells with antibodies for specific proteins encoded by oncogenes. More specific results may be obtained by detection of actual mutations through examination of the gene sequence of MNU-treated and untreated cells. The latter method was employed for the detection of *ras* mutations in a study conducted by Mangold et al. (20). An interesting finding in this study was that K-*ras* mutations could be modified by the nature and dose of MNU as well as the frequency and duration of exposure (20).

Examination of the karyotype, the array of chromosomes the cells possess, is also a very useful tool for the assessment of transformation. Chromosomal analysis of MNU-treated and untreated cells provides a map for comparison of genetic mutations that may have taken place and the genes that may have been altered. Garrett et al. (15) studied tumor suppressor genes and discovered abnormalities in cells treated with MNU that were absent in untreated cells.

With respect to the transformation markers reviewed for this chapter, there are a few markers that are not widely employed but proved interesting. Among these markers are lectin reactivity, levels of O^6-methyl guanine, and response to

modulation by serum and growth factors in the medium with which the MNU cells are nourished.

Lectins are sugar-binding proteins and in malignant cells there is deletion of some terminal sugar residue causing the lectin receptor of subterminal sugar residues to be more unmasked than normal. Tumor cells have the ability to agglutinate when mixed with certain lectins, including concanavalin A and wheat germ agglutinin, whereas normal cells may not agglutinate with the same lectin (21). In a study of human breast epithelial cells treated with carcinogens, the lectins, concanavalin A and soybean agglutinin, had an increased intensity of reaction upon treatment of cells with MNU (7).

Levels of O^6-methyl guanine were monitored in the DNA of hamster dermal fibroblasts in a study conducted by Shiner et al. (22). The increased level of O^6-methyl guanine found after treatment with MNU is significant because this type of modified base has been implicated as a promutagenic and procarcinogenic lesion (22). Treatment with MNU formed high levels of O^6-methyl guanine. O-alkylation has been implicated in the induction of thymic lymphomas in mice suggesting a positive correlation between the extent of alkylation at the O^6 position of guanine in DNA of the target tissue and the ability of MNU to induce thymic lymphomas in mice (22).

In a study conducted by Mangold et al. (20), MNU-treated hamster pancreatic duct cells were maintained in serum-free and EGF-free medium. Initially the culture exhibited extensive cell death, however, after three passages, cell death decreased leaving a population of isolated transformed cells.

MNU-treated cells, grown in the serum and EGF deprived conditions, were highly tumorigenic (tumors induced in 29/30 mice) when compared to MNU treated cells which were grown in media containing serum and EGF (tumors induced in 8/30 mice) (22). Thus, selection of MNU-transformed cells by serum and EGF deprivation is highly reproducible and effective (20). Studies conducted by Guzman et al. (10) and Miyamoto et al. (11) did the exact opposite of this by inducing MNU transformation of mammary epithelial cells by the addition of a variety of growth factors and hormones. Transformation was achieved in both cases.

Conclusion

Although there are many characteristics, associated with malignant transformation, that could be examined through experimentation to confirm whether cells have been transformed, investigators often examine only a few to obtain just enough evidence to confirm that transformation has taken place. The real challenge lies in elucidating how this transformation occurred. If transformation is the primary goal then only a few experiments are necessary for confirmation. However, in order to gain a better understanding of the cellular and molecular events that take place during transformation, it becomes imperative to examine most of the transformation markers described in this section. In this manner, the investigator will not only be able to confirm whether transformation has taken place, but can also report results on a host of other characteristics including, morphology, monolayer growth rate and doubling time, colony forming

efficiency in agar, invasive ability and tumorigenicity. I examined all of these characteristics for the comparative study of five human prostate cancer cell lines derived by transformation with MNU. A study of these characteristics made it possible to discover that each cell line represents a different level of transformation and cancer progression.

References

1. Wahrman MZ, Augenlicht LH, Higgins PJ. Alterations in growth properties and cellular proteins of cloned hamster lung fibroblasts during loong-term culture. Oncology, 41:49-54, 1984.

2. Su ZZ, Luo ZY, Guo LP, Lu SZ, Liu YL. Transformation of cultured human fetal gastric fibroblasts with physio-chemical agents. Scientia Sinica (Series B), 31:850-859, 1988.

3. Horowitz AT, Treves AJ, Voss R, Okon E, Fuks Z, Davidson L, Biran S. A new human ovarian carcinoma cell line: establishment and analysis of tumor associated markers. Oncology, 42:332-337, 1985.

4. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis, 14:483-492, 1993.

5. Laduca JR, Sinha DK. *In vitro* carcinogenesis of mammary epithelial cells by N-nitroso-N-methylurea using a collagen gel matrix culture. In Vitro Cell Dev Biol, 29A:789-794, 1993.

6. Rhim,JS. Neoplastic transformation of human epithelial cells *in vitro*. Anticancer Res, 9:1345-1366, 1989.

7. Russo J, Reina D, Frederick J, Russo IH. Expression of phenotypical changes by human breast epithelial cells treated with carcinogens *in vitro*. Cancer Res, 48:2837-2857, 1988.

8. Azuma M, Momose H, Oyasu R. *In vitro* malignant conversion of low grade rat urinary bladder carcinoma cells by exposure to N-methyl-N-nitrosourea. Cancer Res, 50:7062-7067, 1990.

9. Parsa I, Marsh WH, Sutton AL. An *in vitro* model of pancreas carcinogenesis. Am J Pathol, 98:649-655, 1980.

10. Guzman RC, Osborn RC, Bartley JC, Imagawa W, Asch BB, Nandi, S. *In vitro* transformation of mouse mammary epithelial cells grown serum-free inside collagen gels. Cancer Res, 47:275-280, 1987.

11. Miyamoto S, Guzman RC, Osborn RC, Nandi S. Neoplastic transformation of mouse mammary epithelial cells by *in vitro* exposure to N-methyl-N-nitrosourea. Proc Natl Aacad Sci USA, 85:477-481, 1988.

12. Rhim JS, Jin S, Jung M, Thraves PJ, Kuettel MR, Webber MM, Hukku B. Malignant transformation of human prostate epithelial cells by *N*-nitroso-*N*-methylurea. Cancer Res, 57:576-580, 1997.

13. O'Neill FJ. Selective destruction of cultured tumor cells with uncontrolled nuclear division by cytochalasin B and cytosine arabinoside. Cancer Res, 35:3111-3115, 1975.

14. Delp CR, Treves JS, Banerjee MR. Neoplastic transformation and DNA damage of mouse mammary epithelial cells by N-methyl-N'-nitrosourea in organ culture. Cancer Lett, 55:31-37, 1990.

15. Garrett LR, Perez-Reys N, Smith PP, McDougall JK. Interaction of HPV-18 and nitrosomethylurea in the induction of squamous cell carcinoma. Carcinogenesis, 14:329-332, 1993.

16. MacPherson I, Montagnier L. Agar suspension culture for the selective assay of cells transformed by polynoma virus. Virology, 23:291-294, 1964.

17. Tsao MS, Earp HS, Grisham JW Gradation of carcinogen-induced capacity for anchorage independent growth of cultured rat liver epithelial cells. Cancer Res, 45:4428-4432, 1985.

18. Alley MC, Uhl CB, Lieber MM. Improved detection of drug ctyotoxicity in the soft agar colony formation assay through use of a metabolizable tetrazolium salt. Life Sci, *31:* 3071-3078, 1982.

19. Terranova, V.P., Hujanen, E.S., Martin, G.R. Basement membrane and the invasive activity of metastatic tumor cells. J Natl Cancer Inst, 77:311-316, 1986.

20. Mangold, K.A., Hubchak, S., Mangino, M.M., Laconi, S., Scarpelli, D.G. *In vitro* carcinogenesis of hamster pancreatic duct cells: cellular and molecular alterations. Carcinogenesis, 15:1979-1984, 1994.

21. Webber MM. In vitro models for prostatic cancer: summary. *In* Models for Prostate Cancer. *In* Murphy GP (ed): "Models for Prostate Cancer." Prog Clin Biol Res, 37:133-147, 1980.

22. Shiner, A.C., Newbold, R.F., Cooper, C.S. Morphological transformation of immortalized hamster dermal fibroblasts following treatment with simple alkylating carcinogens. Carcinogenesis, 9:1701-1709, 1988.

Part 2

Characterization of *N*-methyl-*N*-nitrosourea-transformed cell lines

Chapter 7

Characterization of *N*-methyl-*N*-nitrosourea-transformed cell lines

Abstract

Prostate cancer is the leading cancer in men in the United States. Research on prostate cancer has been difficult due to a lack of well-characterized human cell lines. Current cell models share few characteristics with the disease *in vivo,* thus, diminishing their use in the study of the disease. This study presents characterization of five new human prostatic epithelial cell lines (WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-NB26) that exhibit varying degrees of malignant characteristics. These cells were derived by stepwise immortalization of normal prostatic epithelial cells followed by malignant transformation. Characterization is based on morphology, cytoskeletal protein expression, growth in monolayer, anchorage-independent growth, response to growth factors, invasive ability, and tumorigenicity. These cell lines display different levels of malignancy with WPE-1-NA22 being the least malignant, and WPE1-NB26, the most malignant. The other cell lines represent intermediate stages in the process of malignant progression. This is the first prostate cell culture model system where cell lines derived from the same parent cell line display varying degrees of malignancy. The following sequence was determined from the least to the most malignant: WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-NB26. This unique model system can be used to understand the molecular basis of the multi-step process of tumor progression in prostate cancer and has important applications in the prevention and treatment of prostate cancer.

Introduction

Prostate cancer is the most common non-skin malignancy and the second leading cause of cancer death among men in the United States (1). It is estimated that 185,500 new cases were reported and 39,200 men died of the disease in 1998 (1). Prostate cancer incidence increases with age and three-fourths of all prostate carcinomas are diagnosed in men over the age of 65 (1). Improved methods for detection, coupled with the large aging male population, have contributed to increased prostate cancer incidence and heightened public awareness of the disease in the United States (2). Approximately 11 million men between ages 45 and 50 are believed to have latent carcinoma of the prostate. It is predicted that this latent carcinoma will develop into metastatic prostate cancer in one in ten men (3,4). The invasive potential of latent carcinoma remains a mystery, as researchers and physicians are currently unable to determine what causes latent carcinoma to progress to metastatic disease and which cells are most likely to become malignant. This problem makes prognosis of early stage prostate cancer difficult leaving physicians unsure of how aggressively the patients, afflicted with the disease, should be treated. It is, thus, imperative to uncover information leading to the understanding of the molecular events associated with prostate cancer progression. Such an understanding at the molecular level would only be possible through *in vitro* study. However, researchers have lacked the tools that would allow them to explore this area of investigation.

In vitro research on prostate cancer progression has been made difficult in the past due to a lack of suitable human cell models. The most widely used human prostate cancer cell models, described in chapter 3, represent a highly metastatic state. This is hardly surprising considering the fact that most of them were derived from biopsy material of metastasized prostate cancer (5). Therefore, these models provide little information regarding the early events associated with prostate carcinogenesis. Thus, it is imperative to establish cell models that more accurately represent the early stages of prostate carcinogenesis and tumor progression in order to gain a meaningful understanding of these processes.

Five such cell models have been developed in Dr. Mukta M. Webber's laboratory. The non-neoplastic, immortalized, human prostatic epithelial cell line, RWPE-1 (6), was treated with the chemical carcinogen, *N*-methyl-*N*-nitrosourea (MNU), giving rise to the WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-NB26 cell lines. The primary objective of this study is to characterize these cells to establish their human prostatic epithelial origin and to rank them on a scale of malignancy by examining their malignant characteristics.

These cell models may provide important clues for gaining a better understanding of carcinogenesis at the molecular level. The understanding of the molecular events associated with carcinogenesis will provide the knowledge necessary for the discovery of chemical agents that will prevent cancer from manifesting at an early stage. The application of chemical agents to this end is termed chemoprevention. Chemoprevention, as defined by Sporn, "is the use of pharmacological or natural agents that inhibit the development of invasive cancer

either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of premalignant cells in which such damage has already occurred " (7,8). The concept of chemoprevention, therefore, includes the use of drugs to prevent development of preneoplastic lesions and their progression to invasive cancer. The MNU-transformed cell lines provide *in vitro* cell models for testing agents for cancer prevention.

Hypotheses

The main focus of this study is to develop and characterize tumorigenic, human prostatic epithelial cell lines by transformation of the immortalized non-tumorigenic cell line RWPE-1, with the chemical carcinogen, *N*-methyl-*N*-nitrosourea (MNU). These cell lines are of varying degrees of malignancy and mimic the early stages of cancer progression *in vivo*. The proposed hypotheses are that:

1. The MNU-transformed cell lines are of prostatic epithelial origin.

2. The MNU-transformed cell lines exhibit characteristics of malignant epithelial cells.

3. Although malignant, the MNU-transformed cell lines retain and express differentiated functions of prostatic epithelial cells unlike currently used malignant prostatic epithelial cell lines.

4. The MNU-transformed cell lines represent different stages of cancer progression from early, non-invasive, latent carcinoma to late, metastatic prostate cancer.

The Origin of Cell Lines

Transformation of human prostatic epithelial cells with the chemical carcinogen, MNU, was recently reported (9). This investigation did not explore questions beyond the malignant transformation of the cells. Well-characterized cell lines of human prostatic epithelial origin, transformed with MNU, were established in our laboratory by the following method as described, in general terms, in the flow chart shown in Figure 7-1.

Normal human prostatic epithelial cells were isolated from a white male and immortalized using a plasmid pSHPV-18 m containing a single copy of the HPV-18 genome (6). Normal cells senesce within approximately five passages in culture. After 6-7 weeks immortalized cell colonies appeared that were isolated, propagated, and thoroughly characterized in our laboratory to establish their prostatic epithelial origin (6,10). This characterization gave rise to the RWPE-1 cell line. RWPE-1 cells show growth response to androgens and express AR and PSA upon exposure to the synthetic androgen mibolerone (6). RWPE-1 cells are not tumorigenic when injected into nude mice, show a positive growth response when treated with exogenous EGF and bFGF and their growth is inhibited by TGF-β (6). RWPE-1 cells were plated at a density of 100,000 cells/80-cm^2 flask, and after 25 hours, were exposed to 50, 100 and 250 μg/ml MNU for one hour. Following MNU treatment the cultures were grown to confluence with medium being changed every 3 days. At confluence the cells were passaged and treated for two additional rounds of MNU treatment as described above. They were then subcultured and prepared for examination of

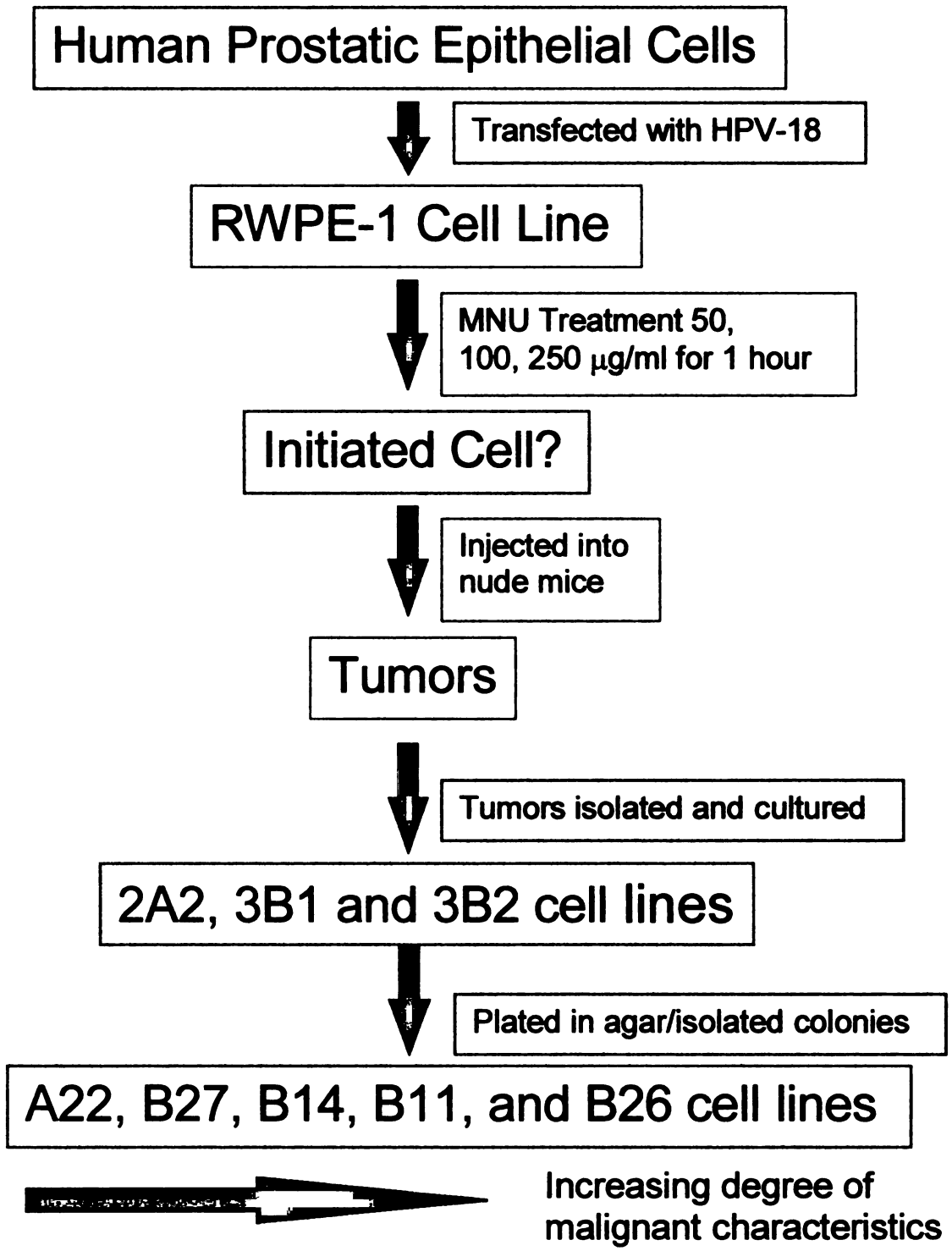

Figure 7-1: Derivation of MNU-Transformed Cell Lines

in vivo tumorigenesis. Cells treated with 50 μg/ml MNU were injected into nude mice with and without Matrigel by Dr. Hynda Kleinman. Ten weeks following injection, several tumors were removed from the mice and passed on to Dr. Mukta M. Webber. No tumors formed in the absence of Matrigel. Among the tumors were three termed 2A, 2B, 3A and 3B that were cultured by Dr. Webber. Cells were injected similarly again into nude mice to obtain second generation tumors, which were designated as 2A1, 2A2, 3B1, and 3B2. Cells from these tumors were placed in culture and approximately three weeks later they were plated in soft agar for isolation of pure colonies (soft agar assay described in detail in the Methods section). Single colonies were excised from the agar culture while viewing the colony through a microscope using a sterile razor blade. The excised colony was isolated using sterile forceps and transferred to a well of a 24-well plate containing 1.0 ml of medium. The cloned cell lines obtained through this colony isolation were termed 2A22, 3B14, 3B11, 3B27, and 3B26. The cell lines were subjected to a second round of isolation from soft agar to attain a more pure cell population. Five MNU-transformed cell lines have been designated WPE1-NA22, WPE1-NB14, WPE1-NB11, WPE1-NB27, and WPE1-NB26.

Materials and Methods

Materials

Keratinocyte-serum free medium (K-SFM) no 17005-042, antibiotic/antimycotic mixture no. 15240-013, Gibco-BRL, Grand Island, NY; RPMI-1640 no. CC261, Celox Laboratories, Hopkins, MN; Dulbecco's phosphate buffered saline Ca^{2+}/Mg^{2+}-free (PBS) no. 28374, Pierce, Rockford, IL; fetal bovine serum (FBS) no. 1020-75, Intergen, Purchase, NY; mibolerone no. W-300, BIOMOL Biomolecular Research Laboratories, Plymouth Meeting, PA; epidermal growth factors (EGF) no. 4001, transforming growth factor β (TGF-β) no. 40039, Collaborative Research, Bedford, MA; recombinant basic fibroblastic growth factor (bFGF) no. 234-FS, R & D Systems, Minneapolis, MN; monoclonal antibody to prostate specific antigen no. M0750, DAKO, Carpinteria, CA; polyclonal antibody to androgen receptor no. PA1-110, Affinity BioReagents, Neshanic Station, NJ; monoclonal antibodies to: cytokeratin 8 no. C-5301, cytokeratin 18 no. C-8541, and bovine serum albumin (BSA) no. A-2153, p-iodonitrotetrazolium violet (INT) no. I-8377, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) no. M-2128; *N*-nitroso-*N*-methylurea (MNU) no. N-1517, Sigma, St. Louis, MO; monoclonal antibody to p53 no. OP09, Oncogene Science, Cambridge, MA; monoclonal antibody to pRb no. 14001A, Pharmingen, San Diego, CA; Vectastain Elite ABCPeroxidase Kit no. PK-6102 and 3,3'-diamino-benzadine (DAB) Substrate Kit no. SK-4100, Vector Laboratories, Burlingame, CA; HEMA-3 Stain Set no. 67-56-1, Curtin-Matheson, Wood Dale, IL; 24 well plates (Falcon) no. 08-7721; 12 mm circle coverslips no.

12-545-80; Cell Strainers 40 μm (Falcon) no. 08-771-1, Fischer Scientific, Ithasca, IL; Nucleopore membrane, 8 μ pore size no. 1550446, 96 well plates 3596, Costar, Cambridge, MA; Bacto agar (0140-01), Difco, Detroit, MI; 35 mm dishes no. 3001, Becton Dickinson, Lincoln Park, NJ; Matrigel was a kind gift from Dr. H. K. Kleinman, NIH.

Cell Culture

All cell lines derived from RWPE-1 by transformation with MNU, were maintained in complete K-SFM medium containing 50 μg/ml bovine pituitary extract (BPE), 5 ng/ml epidermal growth factor (EGF), and 1% antibiotic/antimycotic mixture (Penicillin, 100 U/ml, Streptomycin 100 μg/ml and Fungizone, 25 μg/ml). Cells were passaged upon confluence and seeded at 1-2x10^6 cells/T-25 flask. The human prostate carcinoma cell line, DU-145, was maintained in RPMI-1640 medium containing 5% fetal bovine serum (FBS).

Anchorage-dependent growth *in vitro*

Microplate growth assays were performed to assess the population growth rates of the MNU-transformed cell lines. Cells were plated in 96-well plates at a density of 5,000 cells/well in sextuplicate. Medium was changed every 48 hours. Plates were stained on days 3, 6, and 9 with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). MTT was dissolved in PBS at a stock concentration of 5 mg/ml and filtered with a 0.45 μm filter. The day the cultures were ready to be stained, the MTT stock was diluted 1:5 in pre-warmed (37°C)

culture medium. Staining involved the direct addition of 50 μl 5 mg/ml MTT to each well of the plate to be fixed without removing any medium. The plates were incubated for 4 h at 37°C. The MTT solution was also added to control wells that contained no cells. The media and MTT were removed and the dye was released by the addition of DMSO and absorbance was measured at 540 nm with a Titertek microplate reader. This experiment was performed twice and the average of the two experiments plotted.

Anchorage-independent growth *in vitro*

Soft agar assays were performed to test the anchorage-independent growth ability of the MNU-transformed cell lines by colony formation. A solution of 0.5% agar containing medium (40% KSFM, 40% 1.25% agar, 10% PBS, 10% FBS) was made and kept in a 44°C water bath when not in use during the assay. A volume of 2 ml of the 0.5% agar medium was added to 35 mm dishes evenly and left for 30 minutes to solidify. Cells were subcultured, filtered through a 40 μm cell strainer and counted. A 1.0 ml suspension containing 37,500 cells in KSFM with 10% FBS was then made. Each tube containing the 1.0 ml cell suspension received 2.0 ml of the 0.5% agar medium for a final agar percentage of 0.33% in the medium. The suspension was mixed by triturating twice and 1.0 ml of the suspension was added on top of the 0.5% bottom layer of agar in three 35 mm dishes with each dish receiving 12,500 cells. Controls consisted of dishes containing agar and no cells. The dishes were placed in a 37°C incubator for 21 days. Colonies were stained with the metabolizable INT dye. INT was dissolved in deionized H_2O at a concentration of 1.0 mg/ml and sterilized in an autoclave

for 20 minutes. After 21 days in agar culture the colonies were stained with 1.0 ml of a 1.0 mg/ml solution of INT for 12 h. Plates were fixed with a 10% formalin solution and colonies were counted immediately using a Biotran 2 Automated Colony Counter or stored at 4°C. Colony forming efficiency was calculated by dividing the total number of colonies formed by the total number of cells plated and multiplying by 100 to obtain a percentage. The experiment was repeated and the results of the two experiments were pooled and plotted.

Effects of Growth Factors on Growth of MNU-Transformed Cells

The response of the MNU-transformed cell lines to the effects of the growth stimulatory growth factor, EGF, and growth inhibitory growth factor, TGF-β, was examined. EGF was dissolved in sterile distilled water and TGF-β in 4 mM HCl in D-PBS containing 1 mg/ml BSA. All growth factor stocks were aliquoted and stored at –70°C. Growth factor dilutions were made in the final medium at 1:1000, thus, giving a final vehicle concentration of 0.1%, which did not affect growth. The microplate assay was used with 5,000 cells/well plated for EGF experiments and 10,000 cell/well plated for TGF-β experiments in triplicate in complete KSFM. A greater cell number was used for TGF-β treatment because this growth factor was expected to have an inhibitory effect on the cells that would be more easily detected with a higher initial cell number. Conversely, fewer cells were plated for EGF treatment as this growth factor was expected to have a stimulatory effect on the growth of the MNU-transformed cells. After 48 h, cells were switched to test media consisting of K-SFM with BPE only and

supplemented with the vehicle or growth factor concentrations of 0.1, 1.0 or 10 ng/ml for EGF studies. The medium used for TGF-β treatments consisted of complete K-SFM supplemented with the vehicle or growth factor concentrations of 0.1, 1.0, or 10 ng/ml. The medium was changed every 48 h. Controls consisted of untreated and vehicle-treated cells. Test plates were prepared for absorbance readings five days after treatment as described earlier. Results were plotted as percentages of the vehicle control.

Haematoxylin and eosin staining for cell morphology and immunostaining for the expression of cytokeratins and vimentin

Cells grown on coverslips for each cell line were stained with haematoxylin and eosin (H & E) to examine cell morphology. Protein expression was detected by a modified avidin-biotin immunoperoxidase Vector protocol using monoclonal antibodies. In 24-well plates, 20,000 cells/well were plated on coverslips. At 75% confluency, cells were fixed in 50/50 methanol/acetone at room temperature and processed the same day or stored at -20°C. Antibody dilutions were made in normal horse serum. The following sequential steps were conducted at room temperature and cells were rinsed twice with PBS between steps after application of the primary Ab: cells were blocked with normal horse serum for 1h, incubated with the appropriate antibody for 1h, followed by biotinylated secondary Ab (1:200) for 30 min, treated with 3% H_2O_2 for three min to quench endogenous peroxidase activity, incubated with the avidin-biotin-peroxidase complex for 30 min, developed with DAB-nickel chloride for five min, dehydrated and mounted on alcohol washed slides. Control coverslips lacked primary

antibody. Primary antibody dilutions were: for CK-8 1:200, CK-18 1:800 and vimentin 1:40.

Immunostaining for PSA and AR expression

MNU-transformed cells were tested for PSA and AR expression to establish their prostatic origin. Cells grown on coverslips for immunostaining were pretreated in K-SFM medium containing 5 nM mibolerone, a non-metabolizable androgen, for six days, beginning 48h after plating. Mibolerone was dissolved in absolute ethanol and stored at –70°C. Controls consisted of ethanol treated cultures where ethanol was diluted in the culture media at a ratio of 1:1000 giving a final ethanol concentration of 0.1% in the medium. Cells on coverslips were fixed after reaching 75% confluency with 50/50 methanol/acetone at room temperature and processed the same day or stored at –20°C. The primary Ab dilutions and incubation temperature and times were: PSA 1:20, 4°C, 24h; and AR 1:100, 25°C, 2h.

Invasion Assay

The ability of the cell lines to invade through Matrigel, a reconstituted basement membrane, was examined using a Blind-well chamber *in vitro* invasion assay (11) modified in our laboratory (6). The prostate cancer cell line DU-145, which has a high invasive ability, were used as controls for the invasion experiments. The assay was conducted as follows: on the day prior to running the assay, cells were plated at density of four million cells in T-75 flasks in eight ml of media.

Matrigel was diluted 1:20 with cold (4°C), sterile distilled water on ice for a final concentration of 500 μg/ml. Each Nuclepore filter was coated with 25 μg Matrigel in 50 μl of distilled water and left to dry overnight at room temperature under sterile conditions. Cells were rinsed with D-PBS 18 h after plating and incubated in 3 ml of 0.53 mM EDTA for seven minutes, dislodged by tapping, suspended in three ml K-SFM containing 0.1% BSA (RWPE-1 and MNU-transformed cell lines) or serum-free RPMI containing 0.1% BSA (DU-145 cell line) and recovered by centrifugation. Pellets were resuspended to obtain 1 million cells/ml in K-SFM or RPMI containing 0.1% BSA. The bottom of the Boyden chamber contained 220 μl of NIH/3T3 cell conditioned medium that served as the chemoattractant. For preparation of the conditioned medium, subconfluent NIH-3T3 cultures in 100 mm plates were fed with seven ml of serum-free DMEM containing 50 μg/ml ascorbic acid. Conditioned medium was collected 24h later, centrfuged to remove cell debris and stored at –20°C. A cell suspension containing 200,000 cells in 200 μl medium was added to the top chamber and allowed to remain undisturbed for five min before overlaying with 650μl of K-SFM containing 0.1% BSA for the RWPE-1 derived cell lines or serum-free RPMI medium for DU-145 cells containing 0.1% BSA. Controls consisted of K-SFM and RPMI serum-free medium containing 0.1% BSA. Cells were allowed to invade for 24 h at 37°C at which time the filters were stained and prepared for absorbance reading. The filters were fixed, stained with HEMA-3 and allowed to hydrate in distilled water for five min. Non-migrated cells on the Matrigel-coated side of the filter were removed by wiping with a cotton swab. Stain was extracted by placing filters

individually in wells of a 24-well plate containing 300 μl of 0.1 N HCl for 15 min and 200 μl from each well was then placed in a 96-well plate and the absorbance was measured at 620 nm using a Titertek microplate reader. In addition to evaluating the number of migrated cells on the filter, cells that had migrated to the bottom chamber were also counted. One hundred microliters were taken from each of the triplicate chambers, pooled into cuvettes containing 10 ml Isoton, triturated and counted. The percentages from the chamber counts and filters were added to obtain total invasion. Percent absorbance and cell counts for the cell lines were calculated using DU-145 as 100%. The invasion experiments were conducted at least three times with the MNU-transformed cell lines and the average of three experiments is depicted in the results.

Growth in Nude Mice

Experiments to determine the tumorigenicity of cell lines were conducted by Dr. Hynda Kleinman at the National Institute of Dental Research (NIDR), National Institutes of Health, Bethesda, MD. Cells were tested for tumorigenicity by injecting $5x10^5$ cells with Matrigel and $1x10^6$ cells without Matrigel, subcutaneously, into left and right flanks of nude mice, respectively, that were maintained for about three months. Tumors were removed and placed in culture. A portion of each tumor was fixed for histological examination. The tumor size was measured and the tumors were photographed.

Statistical Analysis

Results are expressed as means with standard error of the mean (sem). The differences between means were considered significant if $P<0.05$. Data were analyzed using one way analysis of variance (ANOVA) for growth factor effects. Response to individual concentrations was compared to the control using the Tukey-Kramer multiple comparison test or a paired t-test, as appropriate. GraphPad InStat version 3 was used for these analyses.

Results

Growth in Monolayer

The growth curves for the MNU-transformed cells are plotted in Figure 7-2. The MNU-transformed cell lines have different proliferative capacities. The WPE-1-NA22 cells are the slowest growing with a doubling time of ~ 96 h for this cell population, followed by the WPE-1-NB27 (doubling time ~ 62 h) and WPE-1-NB14 (doubling time ~ 45 h) cell lines which have similar growth rates. The WPE-1-NB11 (doubling time ~ 40 h) cells proliferate more rapidly as do the WPE-1-NB26 cells (doubling time ~ 40 h) that have the greatest rate of growth. The growth rate of WPE-1-NB26 cells is nearly three times that of the parent RPWE-1 cells (doubling time ~ 120 h). All five cell lines have a greater rate of growth compared to the parent RPWE-1 cells. Not only do the MNU cell lines show a more rapid growth rate than the parent cell line, but they also appear to have different rates of growth compared to one another.

Anchorage-Independent Growth

The WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-NB26 cell lines were plated in agar at a density of 12,500 cells/35 mm dish to assess anchorage independent growth. The results for anchorage independent growth of the MNU-transformed cell lines are plotted in Figure 7-3. RWPE-1 cells were included in this experiment in order to compare the anchorage independent growth of the MNU-transformed cell lines with that of the parent cells. The RWPE-1 cells failed to show anchorage independent growth

while the MNU-transformed cell lines exhibited varying degrees of colony forming ability (Figure 7-3). All MNU-transformed cell lines displayed a statistically significant enhanced colony forming efficiency compared to the RPWE-1 parent cell line; WPE-1-NA22, p=0.0159; WPE-1-B14, p=0.0049; WPE-1-NB11, p=0.023; WPE-1-NB26, p=0.0174; WPE-1-NB27, p=0.001). The following order from lowest to highest colony forming ability was established: WPE-1-NA22 (0.06%), WPE-1-NB14 (1.85%), WPE-1-NB11 (2.04%), WPE-1-NB26 (2.58%), and WPE-1-NB27 (3.24%). The differences in anchorage-independent growth ability between each cell line is statistically significant with the exception of WPE-1-NB14 and WPE-1-NB11, WPE-1-NB14 and WPE-1-NB26, and WPE-1-NB26 and WPE-1-NB27.

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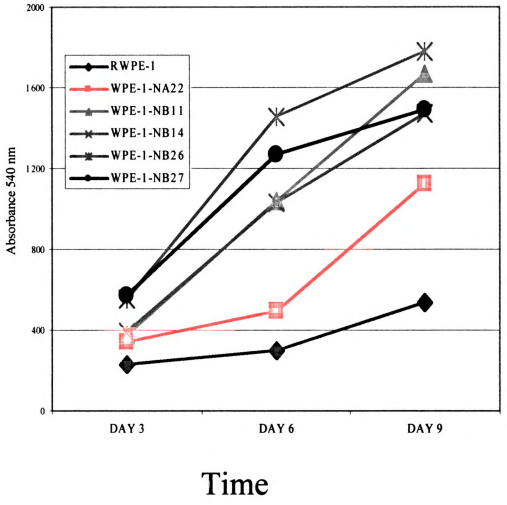

Figure 7-2: Growth Curves for MNU-Transformed Cell Lines. Cells were plated at a density of 5,000 per well in 96-well plates in complete KSFM. Plates were fixed at 3, 6 and 9 days and absorbance was plotted against time. The results plotted are an average of two experiments.

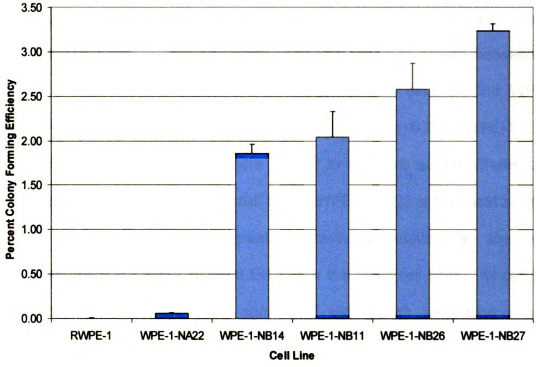

Figure 7-3: Anchorage Independent Growth of MNU- Transformed cell Lines. Cells were plated in agar at a density of 12,500 cells/dish in triplicate. The agar media mixture contained 40% 1.25% agar, 40% complete KSFM, 10% FBS, and 10% PBS. Colonies were stained with INT after 21 days of growth and colonies larger than 0.2 mm were counted. This Graph is an average of two experiments, +/- standard error of the mean.

Effect of Growth Factors

The effects of EGF and TGF-β on the growth of the five cell lines are shown in Figures 7-4 and 7-5, respectively. EGF has a growth stimulating effect on all the MNU-transformed cell lines. The difference in growth between the lowest and highest concentration of EGF is significant for each cell line, WPE-1-NA22, $p<0.01$; WPE-1-NB27, $p<0.001$; WPE-1-NB14, $p<0.001$; WPE-1-NB11, $p<0.01$; WPE-1-NB26, $p<0.01$. There appear to be some subtle differences in the response to EGF amongst the cell lines. WPE-1-NA22 is the most sensitive to EGF treatment exhibiting greater growth stimulation at the lower concentrations of 0.1 and 1.0 ng/ml EGF than the other cell lines. All cell lines appear to respond similarly at 10 ng/ml EGF with growth stimulation of about 130% of control. WPE-1-NB14 cells are slightly more sensitive at this concentration exhibiting 139% stimulation. Conversely, WPE-1-NB26 cells are not as sensitive at 10 ng/ml EGF displaying only 122% growth stimulation.

An inverse relationship was established by measuring the growth rates of the MNU-transformed cell lines treated with increasing concentrations of TGF-β (Figure 7-5). The difference between dose response of the lowest and highest concentration of TGF-β is significant for each cell line, WPE-1-NA22, $p<0.001$, WPE-1-NB27, $p<0.001$; WPE-1-NB14, $p<0.01$; WPE-1-NB11, $p<0.0497$, WPE-1-NB26, $p<0.05$. Similar to EGF response, the cell lines did not exhibit an identical response to TGF-β. WPE-1-NB11 cells are much more sensitive to TGF-β than the other cell lines having a similar inhibitory effect at 0.1 ng/ml as 10 ng/ml TGF-β had on the rest of the cell lines (30-40% growth inhibition).

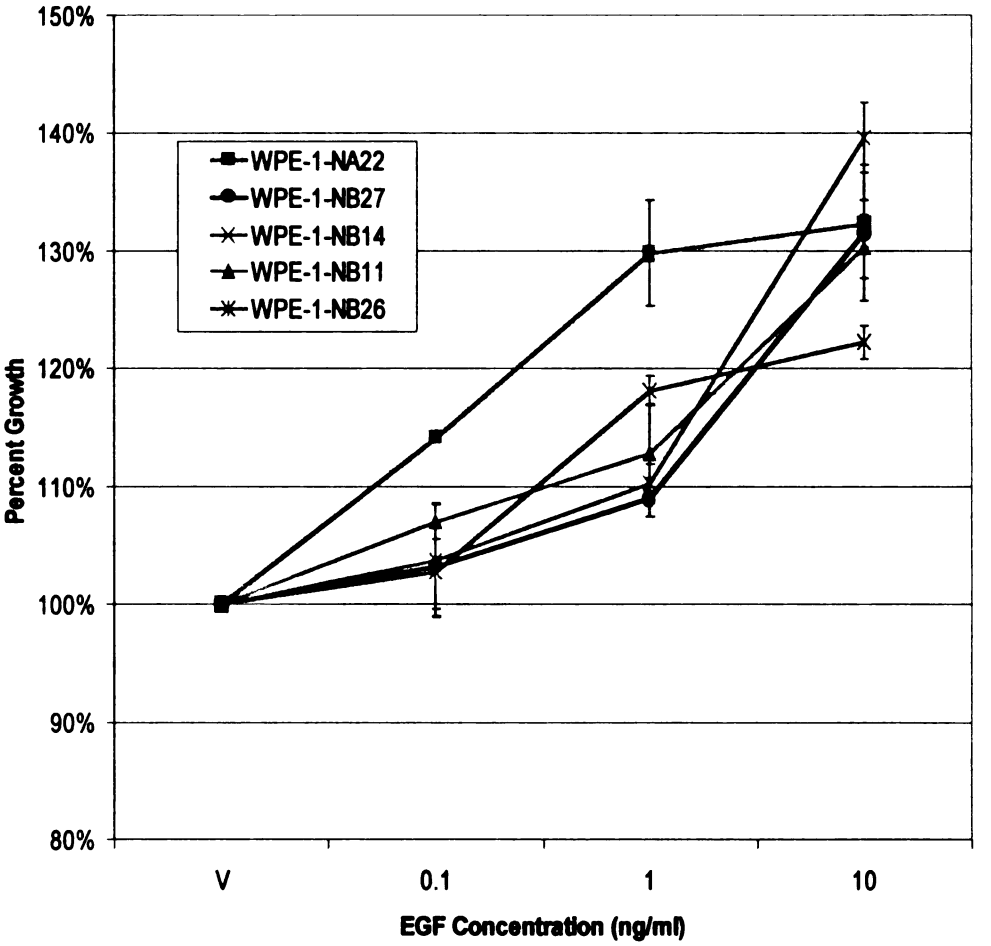

Figure 7-4: Dose Response of MNU-Transformed Cell Lines Treated with EGF. Cells were plated at a density of 5,000 cells/well in 96-well plates in complete KSFM. EGF treatment was initiated 48 h after plating. The EGF treatment medium contained only BPE. Cells were fed fresh medium every 48 hours and plates were fixed with MTT after five days of treatment. The absorbance was read at 540 nm and plotted as percentages of the vehicle control. These results represent at least two experiments for each cell line, +/- standard error of the mean.

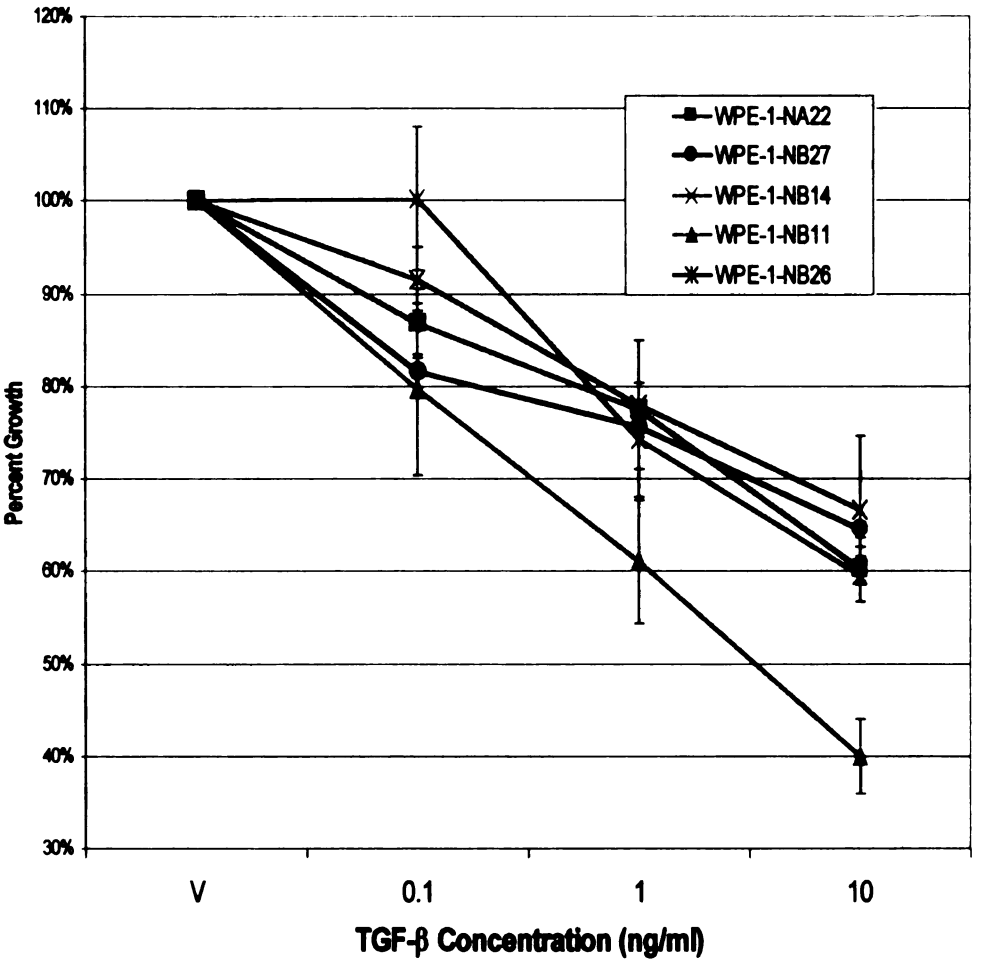

Figure 7-5: Dose Response of MNU-Transformed Cell Lines Treated with TGF-β. Cells were plated at a density of 10,000 cells/well in a 96-well plate in complete KSFM. TGF-β treatment was initiated 48 hours after plating. Cells were fed fresh medium every 48 hours and plates were fixed with MTT after five days of treatment. The absorbance was read at 540 nm and plotted as percentages of the vehicle control. These results represent at least two experiments or three wells for each cell line, +/- standard error of the mean.

Cell Morphology

The morphology of the MNU-transformed cell lines was compared by H & E staining (Figure 7-6). The inclusion of the parent RWPE-1 cell line in morphological studies is essential to determine the extent of morphological diversion and abnormalities that may exist in the MNU-transformed cell lines. The polygonal, epithelial morphology of the WPE-1-NA22 cells (Figure 7-6b) closely resembles that of the RWPE-1 parent cells (Figure 7-6a). In the WPE-1-NB27 cells (Figure 7-6c) there appears to be a mixed population of epithelial and elongated cells. The WPE-1-NB14 cells (Figure 7-6d) appear to have a morphology neither entirely epithelial nor elongated but intermediate between the two. The WPE-1-NB11 (Figure 7-6e) and WPE-1-NB26 cells (Figure 7-6f) have the most abnormal morphology with the majority of cells being elongated and fibroblast-like in appearance.

Expression of Cytokeratins and Vimentin

Epithelial origin of the MNU-transformed cell lines was established by intermediate filament expression. All cell lines displayed cytoplasmic staining for cytokeratins 8 (Figure 7-7e—7-11e) and 18 (Figure 7-7f—7-11f). Each MNU-transformed cell line also stained positive for the mesenchymal intermediate filament protein vimentin (Figures 7-7g—7-11g).

Expression of PSA and Androgen Receptor

Prostatic origin of the MNU-transformed cell lines was established by immunostaining for PSA and Androgen Receptor (AR) expression. All cell lines stained positive for PSA (Figures 7-7c—7-11c). Staining for AR varied between cell lines with WPE-1-NA22 (Figure 7-7d) and WPE-1-NB14 (Figure 7-9d) showing heterogeneous nuclear staining and WPE-1-NB11 cells (Figure 7-10d) and WPE-1-NB26 (7-11d) displaying homogeneous, strong nuclear staining for AR. The WPE-1-NB27 (Figure 7-8d) showed intermediate staining for AR. Thus, the prostatic origin of the MNU-transformed cell lines was established.

Figure 7-6: Hemotoxylin and Eosin Staining of MNU-Transformed Cells. The Cell morphology was studied by H&E staining of cells grown on coverslips. a. RWPE-1 parent cells; b. WPE-1-NA22; c. WPE-1-NB27; d. WPE-1-NB14; e. WPE-1-NB11; f. WPE-1-NB26. Bar=20μ.

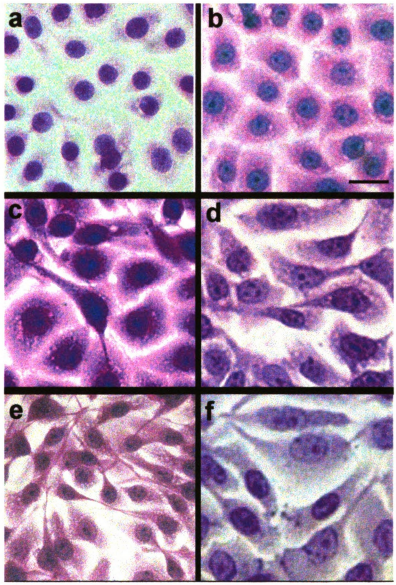
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Figure 7-7: Characterization of WPE1-NA22 Cells on the Basis of Cellular proteins. Cellular proteins were detected by immunoperoxidase staining a. haematoxylin and eosin staining; b. a control lacking primary antibody; Cells for c and d were pretreated with 5 nM mibolerone for 6 days. c. positive staining for PSA; d. positive heterogeneous staining for AR; e. positive weak staining for cytokeratin 8 (CK-8); f. positive strong staining for cytokeratin 18 (CK-18). g. positive staining for vimentin. Bar= 20μ.

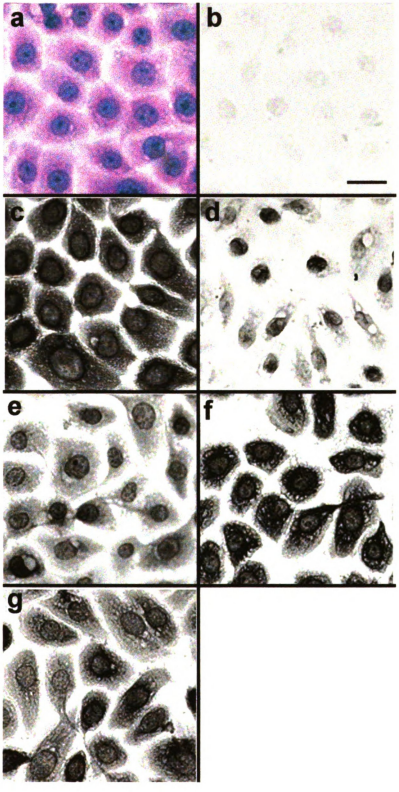
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Figure 7-8: Characterization of WPE1-NB27 Cells on the Basis of Cellular proteins. Cellular proteins were detected by immunoperoxidase staining a. haematoxylin and eosin staining; b. a control lacking primary antibody; Cells for c and d were pretreated with 5 nM mibolerone for 6 days. c. positive staining for PSA; d. positive staining for AR; e. positive staining for cytokeratin 8 (CK-8); f. positive staining for cytokeratin 18 (CK-18). g. positive staining for vimentin. Bar= 20μ.

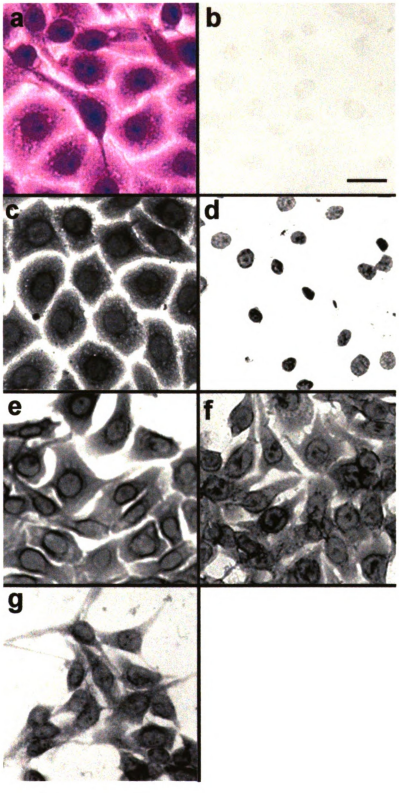
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Figure 7-9: Characterization of WPE1-NB14 Cells on the Basis of Cellular proteins. Cellular proteins were detected by immunoperoxidase staining a. haematoxylin and eosin staining; b. a control lacking primary antibody; Cells for c and d were pretreated with 5 nM mibolerone for 6 days. c. positive staining for PSA; d. positive heterogeneous staining for AR; e. positive weak staining for cytokeratin 8 (CK-8); f. positive staining for cytokeratin 18 (CK-18). g. positive staining for vimentin. Bar= 20μ.

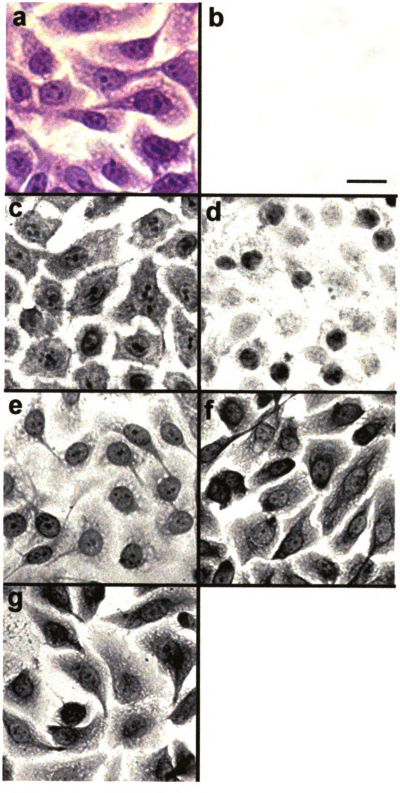
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Figure 7-10: Characterization of WPE1-NB11 Cells on the Basis of Cellular proteins. Cellular proteins were detected by immunoperoxidase staining a. haematoxylin and eosin staining; b. a control lacking primary antibody; Cells for c and d were pretreated with 5 nM mibolerone for 6 days. c. positive staining for PSA; d. positive staining for AR; e. positive staining for cytokeratin 8 (CK-8); f. positive staining for cytokeratin 18 (CK-18). g. positive staining for vimentin. Bar= 20μ.

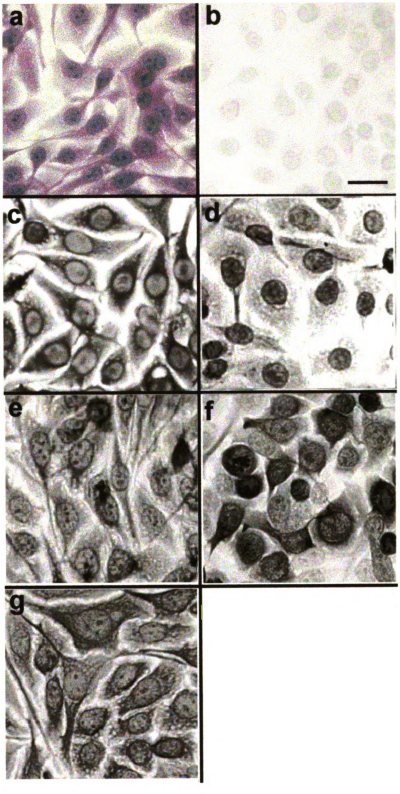
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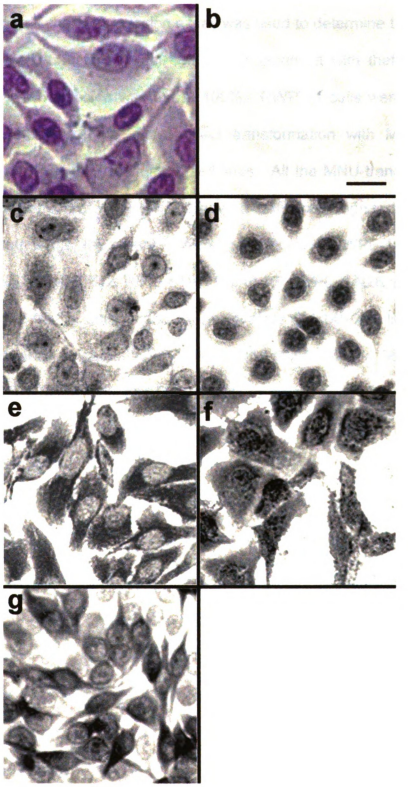
Figure 7-11: Characterization of WPE1-NB26 Cells on the Basis of Cellular proteins. Cellular proteins were detected by immunoperoxidase staining a. haematoxylin and eosin staining; b. a control lacking primary antibody; Cells for c and d were pretreated with 5 nM mibolerone for 6 days. c. positive staining for PSA; d. positive staining for AR; e. positive staining for cytokeratin 8 (CK-8); f. positive staining for cytokeratin 18 (CK-18). g. positive staining for vimentin. Bar= 20μ.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Comparison of Invasive Ability

The blind-well chamber invasion assay was used to determine the invasive ability of each MNU-transformed cell line by comparing it with that of DU-145 cells where DU-145 invasion was set at 100%. RWPE-1 cells were included in this experiment to determine what effect transformation with MNU had on the invasive ability of the transformed cell lines. All the MNU-transformed cell lines with the exception of WPE-1-NA22 have a statistically significant invasive ability greater than that of the parent RWPE-1 parent cell line, WPE-1-NB27, $p=0.0346$; WPE-1-NB14, $p=0.0442$; WPE-1-NB11, $p=0.007$; WPE-1-N26, $p=0.0275$. Figure 7-12 shows that in comparison to the invasive DU-145 cell line, WPE-1-NB26 cells show 95% invasion, WPE-1-NB11 75%, WPE-1-NB14 36%, WPE-1-NB27 30%, WPE-1-NA22 16%, and RWPE-1 9% invasion.

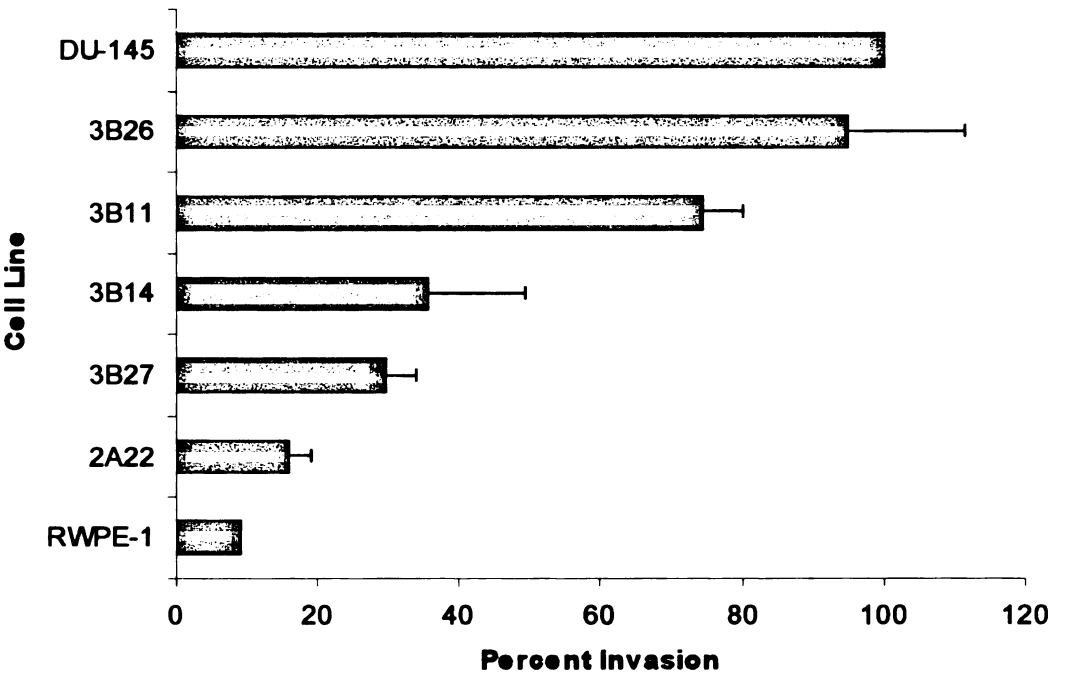

Figure 7-12: Invasive Ability of MNU-Transformed Cell Lines. For the invasion assay, 200,000 cells were added to the upper chamber of a Blind-well chamber with a nuclepore filter coated with the reconstituted basement membrane, Matrigel separating the top chamber from the bottom chamber that contained the chemoattractant. Cells were allowed to invade for 24h after which cells that had migrated to the lower chamber were quantified. DU-145 cells were used as the 100% positive control to which the MNU-transformed cell lines were compared. Results plotted above represent an average of at least two experiments for each cell line, +/- standard error of the mean.

Table 7-1 Summary of Characteristics MNU-Transformed Cell Lines

	RWPE-1	WPE-1-NA22	WPE-1-NB27	WPE-1-NB14	WPE-1-NB11	WPE-1-NB26
Prostatic Epithelial Markers	PSA, AR, CK-8 and CK-18 expression	PSA, AR, CK-8 and CK-18 expression	PSA, AR, CK-8 and CK-18 expression	PSA, AR, CK-8 and CK-18 expression	PSA, AR, CK-8 and CK-18 expression	PSA, AR, CK-8 and CK-18 expression
Doubling Time	120 h	96 h	62 h	45 h	40 h	40 h
Response to Growth Factors	Stimulated by EGF and Inhibited by TGF-β	Stimulated by EGF and Inhibited by TGF-β	Stimulated by EGF and Inhibited by TGF-β	Stimulated by EGF and Inhibited by TGF-β	Stimulated by EGF and Inhibited by TGF-β	Stimulated by EGF and Inhibited by TGF-β
Anchorage-Independent Growth	None	Very low colony forming efficiency	Very high colony forming efficiency	Intermediate colony forming effieiency	Intermediate colony forming efficiency	High colony forming effieiency
Invasive Ability	Negligible invasive ability	Low invasive ability	Low invasive ability	Intermediate invasive ability	High invasive ability	Very high invasive ability
Tumorigenicity in Nude Mice	Nontumorigenic	small	large, hollow, fluid filled, cyst-like tumors	intermediate-size tumors	intermediate-size tumors	large tumros

Discussion

It is a widely known fact that cancer cells of various origin have unique characteristics and respond in unique ways to various stimuli. It is, therefore, imperative to confirm the origin of cells before performing cell culture studies to ensure that all discoveries can confidently be associated with the correct cell type. Biopsy specimens may contain not only the cancer cells but also cells of mesenchymal origin from the surrounding tissue. Thus, the initial objectives of this study were to confirm the epithelial and prostatic origin of the MNU-transformed cell lines.

The morphology of the MNU-transformed cell lines was studied by H & E staining. There appears to be a distinct alteration in morphology in most of the MNU cell lines as compared to the parent RWPE-1 cell line (Figure 7-6). The WPE-1-NA22 cells have an epithelial morphology strongly resembling that of the RWPE-1 parent cells while the WPE-1-NB27, WPE-1-NB14, WPE-1-NB11 and WPE-1-NB26 cell lines display a progressive increasingly abnormal, elongated morphology. These differences in morphology may manifest in different characteristics for the cell lines. Increasing alterations from the original parent cell morphology suggest increasing degrees of transformation and malignancy.

Cytokeratin expression is a characteristic of epithelial cells and secretory, luminal prostatic epithelial cells are known to express cytokeratins 8 and 18 (12-15). I observed the expression of the two cytokeratins, CK-8 and CK-18, in all the MNU-transformed cell lines (Figures 7-7—7-11), thus, confirming their epithelial origin. The MNU cell lines also expressed vimentin, a mesenchymal

marker. This ostensibly anomalous phenomenon can be reconciled by the fact that often when epithelial cells are placed in culture they display vimentin expression (6,13).

Prostatic origin was established by examination of prostate-specific characteristics of prostate specific antigen (PSA) and androgen receptor (AR) expression. Expression of PSA is a function of normal, differentiated prostatic epithelial cells. With the exception of LNCaP (16), ALVA (17), and RWPE-2 (6) the MNU-transformed cell lines are the only immortalized, tumorigenic human prostatic epithelial cell lines that have been shown to express PSA (Figures 7-7—7-11). PSA expression was induced by pretreatment of the cells with the synthetic androgen, mibolerone. Mibolerone was used in lieu of 5α-DHT because of the short, ~2 hour, half-life of the natural androgen in culture medium, whereas, mibolerone is not metabolized by the cells and is, thus, able to exert a continuous hormonal effect (18). Similarly, androgen pretreatment of cells allowed the detection of AR by immunostaining in each of the MNU cell lines because androgens upregulate AR expression. With the prostatic, epithelial origin established, I was able to pursue further characterization of these cell lines.

The growth rates of the MNU cell lines were established by a 3, 6 and 9 day microplate growth assay where each cell line was initially plated at a density of 5,000 cells per well in six wells of 96-well plates. Interestingly, cell populations for each cell line displayed a unique rate of growth. All the MNU cell lines proliferate more rapidly than the parent RWPE-1 cell line (Figure 7-2). The WPE-1-NA22 cell line has the slowest mitotic rate (doubling time ~ 96h) of the MNU-

transformed cell lines followed by WPE-1-NB27 (doubling time ~ 62h), WPE-1-NB14 (doubling time ~ 45h), WPE-1-NB11 (doubling time ~ 40h), and WPE-1-NB26 (doubling time ~ 40h) having the highest rate of growth. The doubling times of the WPE-1-NB27 and WPE-1-NB26 reported need to be further examined. It is obvious that these two cell lines are the fastest growing of the MNU-transformed cell lines, based on the high absorbance readings, between days 3 and 6. By day 3 the two cell lines already exhibit a significantly higher growth rate than the WPE-1-NA22, WPE-1-NB14 and WPE-1-NB11 cell lines. Thus, the WPE-1-NB27 and WPE-1-NB26 cell lines have an increased base line from which doubling time must be measured. The plating of fewer cells or decrease in the number of days of the study (for example fixing plates on days 2, 4, and 6) may yield doubling times that more closely represent the growth rates of these two cell lines.

The different rates of growth amongst the MNU-transformed cell lines sharing the common RPWE-1 ancestor have exciting implications. High proliferative capacity is a characteristic of malignant cells and collection of cell lines displaying a range of growth rates from slow to fast growing suggests that these cells may represent varying degrees of malignancy. However, it is necessary to confirm this assertion through further experimentation.

Normal epithelial cells *in vivo* rest on a basement membrane that is required for their polarization and growth. In cell culture, normal cells (except lymphocytes) require a substrate for attachment before they undergo DNA synthesis and cell division. This phenomenon is called anchorage dependence. If normal cells are placed in suspension, such as in soft agar, they do not

proliferate. Upon malignant transformation, cells show varying degrees of loss of anchorage dependence so that if placed in soft agar they have the ability to proliferate and form colonies (19,20). This ability of cancer cells to grow in soft agar is associated with autocrine secretion of growth factors and secretion of proteases involved in invasion, during the promotional stage of carcinogenesis. Soft agar assay provides an *in vitro* method to identify and quantify the number of potentially transformed cells and to assess the efficacy of chemopreventive agents that inhibit colony formation. A strong correlation between increased cloning efficiency in agar and tumor formation has been demonstrated (21). Soft agar assays were conducted to establish the ability of MNU-transformed cell lines to grow in an anchorage-independent manner. Figure 7-3 shows that the anchorage independent growth results closely mirror those of the monolayer growth with the non-tumorigenic RWPE-1 cells unable to form colonies followed by WPE-1-NA22 forming a very small number. A surge in colony forming efficiency is seen with WPE-1-NB14 cells (1.85%), WPE-1-NB11 (2.04%), WPE-1-NB26 (2.58%) and WPE-1-NB27 (3.24%). Thus, once again the MNU-transformed cell lines exhibit a wide range of results when comparing this marker for transformation. The correlation between colony forming ability and tumorigenicity suggests that the MNU cell lines represent increasing malignant characteristics starting from the non-tumorigenic RWPE-1 parent cells at one end of the spectrum and WPE-1-NB26 and WPE-1-NB27 cell lines on the other. However, growth in soft agar is not a definitive test of malignancy and further evidence is required to prove whether the above assertion holds true.

A definitive test for malignancy is to examine the invasive potential of the cells. Whereas normal, non-invasive epithelial cells typically rest upon the basement membrane, transformed, malignant cells secrete proteolytic enzymes degrade the basement membrane, thus, allowing the cells to invade and metastasize (22). This invasive ability can be measured *in vitro* by the ability of cells to invade a reconstituted basement membrane, using a blind-well chamber assay (11). The highly invasive DU-145 cell line was used as a positive control and the MNU cell lines were plotted as percentage of DU-145 invasion. The invasion assay results were consistent with the above growth experiments confirming the increasing malignant nature of the MNU-transformed cell lines with the exception of WPE-1-NB27 (Figure 7-12). It is surprising that this cell line is amongst the most rapidly proliferating in monolayer culture and displays the greatest colony forming efficiency and yet it is amongst the least invasive (22%) with the exception of WPE-1-NA22 (16%). The WPE-1-NB26 cells are the most invasive (97%) approaching the invasive ability of DU-145 cells. WPE-1-NB11 and WPE-1-NB14 cells once again represent an intermediate degree of malignancy with invasion assay percentages of 57% and 40% respectively. The RWPE-1 cells, as expected, were least invasive (9%). The inconsistency of the WPE-1-NB27 cells cannot easily be explained. The morphology studies of WPE-1-NB27 (Figure 7-6c) suggests the coexistence of two distinct cell types; one having epithelial and the other elongated cell morphology. It is possible that these cell types interact symbiotically as positive regulators for one another under certain conditions and negative regulators in others. For example, in monolayer and anchorage independent growth, the two cell types may stimulate

one another through paracrine interaction supplying one another with growth factors necessary to promote colony formation. In invasion studies, one cell type may act as a negative regulator, thereby inhibiting release of proteases in the other required for invasion occur. These explanations require further investigation. The obvious approach would be to sub-clone the WPE-1-NB27 cell line in an attempt to separate the two cell types and proceed with characterization of these populations.

Studies of tumorigenicity in nude mice using the MNU-transformed cell lines were conducted by Dr. Kleinman. Injection of the MNU-transformed cell lines into nude mice yielded tumors in all cases but of different size. WPE-1-NB26 cells formed the largest tumors, WPE-1-NB11 and WPE-1-NB14 formed tumors of similar, intermediate size while WPE-1-NA22 cells formed the smallest tumors. WPE-1-NB27 cells formed tumors nearly the size of those formed by WPE-1-NB26 cells but were not solid and contained fluid-filled centers. These results strengthened my hypothesis that these cells represent different degrees of malignancy. The cell lines listed from least to most tumorigenic adhere to the following pattern: WPE-1-NA22, WPE-1-NB14, WPE-1-NB11, WPE-1-NB27, WPE-1-NB26. However, it should be noted that the WPE-1-NB27 tumors, although large, were not solid but hollow and fluid filled. It is, thus, difficult to determine precisely where the WPE-1-NB27 cells fit within the progression of malignancy.

Although the place of the WPE-1-NB27 cell line has yet to be determined in the scope of this cell model system, the importance of these cell lines cannot be overstated. They represent the first characterization of cell lines, where all of

the cell lines are derived from a non-tumorigenic parent and are of varying degrees of malignancy. However, this fact alone does not automatically imply that these cell lines are the best models for the study of prostate cancer. A rarely addressed but major flaw in most prostate cancer cell models is that some cell lines may bear little resemblance to the *in vivo* disease. Investigators become overly preoccupied with, and base important assertions on, such cell models neglecting to realize that these cell models may be more dedifferentiated and aggressive and would, thus, represent the more advanced prostate cancer. The expression of PSA and AR, the primary markers of prostatic origin, has not been established with certainty, in two of the most commonly used human prostate cancer cell lines DU-145 and PC-3 (5). Although LNCaP cells express AR and PSA, the AR of this cell line promiscuously responds to androgens, estrogens, and progesterone, a phenomenon known not to occur in normal or cancerous prostate. It is important to note that the MNU-transformed cell lines express both of these prostatic epithelial markers.

Alteration in growth factor response is a well-established event that occurs during transition from normal to malignant cells. The more aggressive cells grow in an autocrine manner and exhibit diminished response to exogenously added positive and negative growth regulators (22). TGF-β is known to have an inhibitory effect on normal human prostatic epithelial cells (5,6,22,23). Studies involving treatment of DU-145, PC-3 and LNCaP cells with TGF-β show that DU-145 and PC-3 cells are initally inhibited by the growth factor, but growth eventually returns to control levels despite retreatment with TGF-β while LNCaP cells were altogether unaffected by the growth factor (23).

Dose response studies for TGF-β treatment of the MNU cell lines shows that all cell lines are sensitive to this growth factor (Figure 7-5). This finding confirms that the MNU-transformed cell lines have not progressed to as highly a malignant state as the three commonly used prostate cancer cell lines. Similarly, the MNU-transformed cell lines all appear to be sensitive to EGF treatment with the cell line exhibiting the least malignant characteristics, WPE-1-NA22, being the most sensitive while the most malignant cell line, WPE-1-NB26, appears least sensitive (Figure 7-4). Taken together, these studies suggest that the MNU-transformed cell lines may represent *in vitro* cell models that most closely resemble *in vivo* prostate carcinogenesis and progression. Furthermore, the value of these cell models is enhanced by the fact that they share a common lineage. This common lineage of cells representing varying degrees of progression will allow us to gain a better understanding of tumor progression and will enable us to dissect the molecular events associated with carcinogenesis.

It is worth noting that the established prostate cancer cell lines, DU-145, PC-3 and LNCaP, have been in culture of several years while the MNU-transformed cells are relatively new. The longer the cells are in culture the greater the likelihood that they will diverge further from their *in vivo* counterparts. It is also extremely important to note that the three established prostate cancer cell lines were derived from metastatic lesions. The difficulty of treating metastasized prostate cancer is a widely accepted and unfortunate fact. Therefore, the three prostate cancer cell lines currently in use may, at best, aid in the treatment of metastatic prostatic carcinoma. Thus, these cell models are extremely limited in their realistic applicability. The MNU-transformed cell lines,

on the other hand, are a family of cell models sharing a common ancestor representing early to late stage prostate cancer. These cell models do not represent the highly progressed state of the DU-145, PC-3 and LNCaP cell lines, but are stronger representatives of early stage, localized prostate cancer. The common lineage of the MNU-transformed cell lines will provide invaluable information regarding the molecular events associated with prostate carcinogenesis and progression. The potential knowledge that may be derived from the MNU-transformed cell lines may provide the understanding needed to elucidate the etiology of prostate carcinogenesis and how it may be blocked. Further studies to dissect the differences between the MNU-transformed cell lines at the molecular level are, therefore, required.

References

1. American Cancer Society. Cancer Facts and Figures-1998.

2. Potosky AR, Miller BA, Albertsen PC, Kramer BS. The role of increasing detection in the rising incidence of prostate cancer. JAMA, 273:548-552, 1995.

3. Carter HB, Coffey DS. The prostate: an increasing medical problem. Prostate, 16:39-48, 1990.

4. Foster CS. Predictive factors in prostatic hyperplasia and neoplasia. Hum Pathol, 21:575-577, 1990.

5. Webber MM, Bello D, Quader S. Immortalized and tumorigenic adult human prostatic epithelial cell lines: characteristics and applications part 2. Tumorgenic cell lines. Prostate, 30:58-64, 1997.

6. Bello D, Webber MM, Kleinman HK, Wartinger DD, Rhim JS. Androgen responsive adult human prostatic epithelial cell lines immortalized by human pappillomavirus 18. Carcinogenesis, 18:1215-1223, 1997.

7. Greenwald P. Chemoprevention of cancer. Sci Amer, 274:96-00, 1996.

8. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science, 278:1073-1077, 1997.

9. Rhim JS, Jin S, Jung M, Thraves PJ, Kuettel MR, Webber MM, Hukku B. Malignant transformation of human prostatic epithelial cells by *N*-nitroso-*N*-methylurea. Cancer Res, 57:576-580, 1997.

10. Rhim JS, Webber MM, Bello D, Lee MS, Arnstein P, Chen LS, Jay G. Stepwise immortalization and transformation of adult human prostate epithelial cells by a combination of HPV-18 and v-Ki-*ras*. Proc Natl Acad Sci USA, 91:11874-11878, 1994.

11. Albini A, Iwamoto Y, Klienman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RNA. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res, 47:3239-3245, 1987.

12. Moll R, Franke WW, Schiller DD, Geiger B, Krepler R. The catalogue of human cytokeratins: patterns of expression in normal epithelial tumor and cultured cells. Cell, 31:11-24, 1982.

13. Traub P. Intermediate filaments: a review. New York, Springer-Verlag. Pp. 450, 1985.

14. Sherwood ER, Berg LA, McEwan RN, Paschiak RM, Kozlowski JM, Lee CJ. Two-dimensional protein profile of cultured stromal and epithelial cells from hyperplastic human prostate. J Cell Biochem, 40:201-214, 1989.

15. Hedrick L, Epstein JI. Use of keratin 903 as an adjunct in the diagnosis of prostate carcinoma. Am J Surg Pathol, 13:389:396, 1989.

16. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA. The LNCaP cell line-a new model for studies on human prostatic carcinoma. In Murphy GP (ed): "Models for Prostate Cancer." Prog in Clinical Biol Res, 37:115-132, 1980.

17. Plymate SR, Loop SM, Hoop RC, Wiren KM, Ostenson R, Hryb DJ, Rosner W. Effects of sex hormone binding globulin (SHBG) on human prostatic carcinoma. J Steroid Biochem Mol Biol, 40:833-839, 1991.

18. Webber MM, Bello D, Quader S. Immortalized and tumorigenic adult human prostatic epithelial cell lines: Characteristics and applications part 1. Cell markers and immortalized nontumorigenic cell lines. Prostate, 29:386-394, 1996.

19. MacPherson I, Montagnier L. Agar suspension culture for the selective assay of cells transformed by polynoma virus. Virology, 23:291-294, 1964.

20. Tsao MS, Earp HS, Grisham JW. Gradation of carcinogen-induced capacity for anchorage independent growth of cultured rat liver epithelial cells. Cancer Res, 45:4428-4432, 1985.

21. Shin SI, Freedman VH, Risser R, Pollack R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth *in vitro*. Proc Natl Acad Sci USA, 72:4435-4439, 1975.

22. Webber MM, Waghray A, Bello D. Prostate specific antigen (PSA), a serine protease, facilitates human prostate cancer invasion. Clin Cancer Res, 1:1089-1094, 1995.

23. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Hittmair A, Zhang J, Thurnher M, Bartsch G, Klocker H. Regulation of prostatic growth function by peptide growth factors. Prostate, 28:392-405, 1996.

24. Wilding G; Zugmeier G; Knabbe C; Flanders K; Gelmann E. Differential effects of transforming growth factor beta on human prostate cancer cells *in vitro*. Mol Cell Endocrinol, 62:79-87, 1989.

Overall Conclusions

Research on prostate cancer, using cell culture, has been difficult due to a lack of well characterized cell models. The majority of the established prostate cell models were derived from biopsy specimens of metastatic tumors, but the resulting cell lines appear to bear little resemblance to the *in vivo* disease. The initial purpose of this project was to provide additional cell models for the study of prostate cancer by chemical transformation of nontumorigenic, human prostatic epithelial cells with MNU. As many significant discoveries in science, this undertaking proved serendipitous and resulted in five cell lines that exhibit an array of morphology, suggesting phenotypes of varying malignancy. Following the establishment of prostatic epithelial origin, differences in malignant characteristics were confirmed by monolayer growth studies in which the more morphologically abnormal cells exhibited greater proliferative capacity. The pattern of anchorage-independent growth characteristics of the MNU-transformed cell lines closely resembles that of their monolayer growth. The WPE-1-NA22 cells have the slowest growth rate followed by WPE-1-NB14 and WPE-1-NB11 representing an intermediate rate, and WPE-1-NB27, and WPE-1-NB26 have the highest rate of growth. A similar pattern was established through studies of the invasive ability of the MNU-transformed cell lines, however, the WPE-1-B27 cells were among the least invasive. The reason for this disparity can only be a matter of speculation at present, but further studies on the heterogeneous nature of the WPE-1-B27 cell population may yield answers. Finally, the sensitivity of these cell lines to the growth factors, EGF and

TGF-β, confirms that the behavior of the MNU-transfc cell lines resembles early stage prostate cancer more closely than metastasized, late stage cancer. Written in increasing degree of malignancy, I believe the following order holds true: WPE-1-NA22, WPE-1-NB27, WPE-1-NB14, WPE-1-NB11, and WPE-1-B26. These cell models may, thus, serve to aid in our understanding of the early events associated with prostate carcinogenesis.

A major problem often encountered by physicians treating patients for prostate cancer is the inability to determine the malignant potential of the latent form of the disease. Currently, there is no method by which one can determine whether latent prostate cancer will develop into an invasive, metastatic form. The MNU-transformed cell models, representing different levels of tumor progression, may be applied to determine the molecular events associated with carcinogenesis thereby aiding physicians in determining which molecular abnormalities will most likely lead to an increasingly malignant state. Furthermore, elucidating the molecular mechanisms associated with prostate carcinogenesis will enable us to gain a better understanding of how the multiple steps in prostate carcinogenesis and in tumor progression may be blocked by chemoprevention. These cell lines also provide suitable cell models that can be used to identify potential cancer prevention agents.