

**Tumor Suppression
Of The DU 145 Prostate Cancer Cell Line:
Implication Of Chromosomes 8 And 12**

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***Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Biochemistry***

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Ce travail est dédié à mes parents, Odette et Réal.

ABSTRACT

Tumorigenesis results from the accumulation of genetic events which give rise to inappropriate behaviour of cells. Mutations which lead to cancer progression are generally classified in two major categories, that of oncogenes and tumor suppressor genes. The latter have been more difficult to identify due to their recessive nature. The application of somatic cell fusion and microcell fusion technologies presents a means to identify genes inactivated in tumor cells. We have used these methodologies to identify specific chromosomes that, once introduced into the DU 145 prostate cancer cell line, have the ability to suppress tumorigenicity. Chromosomes 8 and 12 were shown to encode tumor suppressor genes inactivated in this prostate cancer cell line.

To isolate the tumor suppressor gene encoded on chromosome 12, a smaller candidate region was identified by the use of radiation hybrid technology. Molecular methods subsequently led to the identification of chromosome 12 genes displaying increased expression in suppressed hybrids compared to tumorigenic hybrids. Novel genes and genes encoded by other chromosomes also demonstrated increased expression. We suggest that these genes are involved in the cellular pathway that leads to suppression in DU 145 prostate cancer cells upon the introduction of chromosome 12. Further analysis of the genes isolated in this study have the potential to serve as prognostic markers of prostate carcinogenesis.

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I would like to acknowledge the people who have contributed to the scientific content of this work. Marsha Speevak generated and analyzed the mouse/human hybrid panel (Chapter 2). She also produced the DHM hybrids (Chapter 3) and verified all the G-banded karyotypes. Dr. Henriette Gourdeau performed the whole cell fusions between DU 145 and PC3 cells. (Chapter 3) Dr. Mario Chevrette did the soft agar assays of the DP hybrids (Chapter 3) and generated the Alu-PCR differential fragments (Chapter 5). Finally, Kayvan Amjadi contributed to the mapping of the radiation hybrids (Chapter 4). We also thank Dr. S.D. Lupton for providing us with the tgCMV/HyTK double selectable marker.

LIST OF ABBREVIATIONS

β -ME	Beta-mercaptoethanol	dTTP	deoxythymidine triphosphate
^3H -dTTP	Tritiated deoxythymidine triphosphate	EDTA	Ethylenediaminetetraacetic acid
Alu-PCR FISH	Alu-Polymerase chain reaction fluorescent <i>in situ</i> hybridization	EF-1	Elongation factor 1
APC	Adenomatous polyposis coli	EGF	Epidermal growth factor
ATCC	American type culture collection	FBS	Fetal bovine serum
BLAST	Basic Local Alignment Search Tool	FCM	Flow cytometry
bp	Base pair	FISH	Fluorescent <i>in situ</i> hybridization
BPH	Benign prostatic hyperplasia	FITC	Fluorescein isothiocyanate
BRCA1	Breast cancer gene 1	GAP	G protein activating protein
BSA	Bovine serum albumin	GCV	Ganciclovir
CDK	Cyclin-dependent kinase	GNAI2	G protein α_2
cDNA	Complementary DNA	GNAI2L	G protein α_2 -like
CENP-E	Centromeric protein E	gpt	Xanthine-guanine phosphoribosyltransferase gene
CGH	Comparative genomic hybridization	GRAIL	Gene Recognition and Analysis Internet Link
CKI	CDK inhibitor	GT	Generation time
cM	Centimorgans	GTBP	GT binding protein
CO ₂	Carbon dioxide	HCl	Hydrochloric acid
dATP	deoxyadenosine triphosphate	HEPES	N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid
DCC	Deleted in colorectal cancer	HLH	Helix-loop-helix
dCTP	deoxy cytosine triphosphate	HMG I	High mobility group protein I
DDRT-PCR	Differential display reverse transcriptase PCR	HNPCC	Hereditary non-polyposis colon cancer
DEPC	Diethylpyrocarbonate	hph	Hygromycin phosphotransferase gene
dGTP	deoxyguanosine triphosphate	Hsa	Homo sapiens chromosome
DHT	Dihydrotestosterone	HSF	Human skin fibroblasts
DMEM	Dulbecco's minimum essential medium	HyTK	Hygromycin phosphotransferase /thymidine kinase fusion gene
DMSO	Dimethylsulphoxide	IGF-I-R	Insulin-like growth factor I receptor
DNA	Deoxyribonucleic acid	IGF2	Insulin-like growth factor 2
dNTP	deoxynucleotide triphosphate	IMC	Image cytometry
DOP-PCR	Degenerate oligonucleotide primed PCR	KCl	Potassium chloride
DTT	Dithiothreitol		

LB	Luria-Bertani media	RT-PCR	Reverse-transcriptase PCR
LOH	Loss of heterozygosity	SDS	Sodium dodecyl sulfate
Mb	Megabase	Taq	Thermophilus aquaticus DNA polymerase
MCC	Mutated in colorectal cancer	TCA	Trichloroacetic acid
MDM2	Mouse double minute 2 gene	TEMED	Tetramethylethylene diamine
MEM	Minimum essential medium	TFIID	TATA-binding protein- associated factor IID
MgCl ₂	Magnesium chloride	TGF	Transforming growth factor
MI	Mitotic index	TSG	Tumor suppressor gene
MOPS	3-(N-morpholino)propane- sulfonic acid	U	Unit
mRNA	Messenger ribonucleic acid	UBF	Upstream binding factor
MRP1	Motility-related protein 1	UTR	Untranslated region
MTS1	Multiple tumor suppressor 1	UV	Ultra violet
MTS2	Multiple tumor suppressor 2	VDAC1	Voltage-dependent anion channel 1 gene
NaCl	Sodium chloride	VDAC2	Voltage-dependent anion channel 2 gene
NCBI	National Center for Biotechnology Information	VHL	von Hippel-Lindau gene
neo	Neomycin phosphotransferase gene	WT1	Wilms' tumor 1 gene
NF1	Neurofibromatosis type 1 gene	Xgal	5-bromo-4-chloro-3- indolyl- β -D-galactoside
NF2	Neurofibromatosis type 2 gene	YAC	Yeast artificial chromosome
PC	Prostate cancer		
PCNA	Proliferating cell nuclear antigen		
PCR	Polymerase chain reaction		
PDGF-A	Platelet-derived growth factor chain-A		
PEG	Polyethylene glycol		
PIN	Prostatic intraepithelial neoplasia		
PMS-1	Postmeiotic segregation-1 gene		
PMS-2	Postmeiotic segregation-2 gene		
poly (A)+	Polyadenylated (mRNA)		
PSA	Prostate specific antigen		
RAR-alpha1	Retinoic acid receptor-alpha 1		
Rb	Retinoblastoma		
RDA	Representational difference analysis		
	Research Tool		
RNA	Ribonucleic acid		

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

This work is concerned with the identification of genes which influence the tumorigenic potential of prostate cancer cells. To this end, human chromosomes that suppress the tumorigenic phenotype of a prostate cancer cell line were identified, and the position of a putative tumor suppressor gene(s) was further resolved by the use of radiation hybrid technology. Novel molecular methods allowed the characterization of genes that could behave as tumor suppressors of prostate carcinogenesis. This introductory chapter provides a framework for the understanding of the mechanisms involved in tumor suppressive factors in general, and in the progression of prostate cancer. During the past decade, the powerful techniques of molecular biology and genetics have been applied to the study of human cancer. Relevant methodologies that suggest the presence of tumor suppressor genes and lead to their eventual cloning will be discussed in detail to provide background knowledge relevant to the findings described in this work.

1.1 Cancer: general concepts

1.1.1 Description of the cancer cell

Whether normal cells divide, stay dormant, or die is governed by tightly controlled interconnected processes that require perfect timing. Cells can function properly due to complex, linked pathways and therefore are readily susceptible to defects.

In cancer cells, such defects can disrupt the patterns that determine growth and division, leading to inappropriate behavior. An imbalance favoring cell division over cell loss within a tissue ensues, ultimately leading to a gradual population increase. This progressive accumulation of superfluous tissue forms a tumor mass. A benign tumor remains in the confines of the tissue, while in some instances, the tumor cells acquire new properties that allow it to invade the circulatory and lymphatic system and to colonize a foreign tissue elsewhere in the body. This process, referred to as metastasis, is usually the most life-threatening due to the interference of the secondary tumors (metastases) with the normal function of the invaded organ. Cancer cells exhibit several unusual properties in culture, such as an indefinite lifespan (transformed cells) or reduced density-dependent inhibition of growth. Other properties include anchorage-independent growth (as assayed in soft agar), tumor formation after injection into nude mice, and cell surface alterations resulting in morphological changes. Several factors can lead to formation of the carcinogenic phenotype, including dietary and environmental factors, as well as inherited and somatic mutations.

1.1.2 Cancer is a multistep process

The idea that cancer is caused by genetic factors has long been hypothesized, but only since the advent of well-developed molecular biology techniques has this been substantiated through the identification of both inherited and somatic mutations in human cancers. An accepted notion in the cancer field is that mutations in two major types of genes, namely oncogenes and tumor suppressor genes, can lead to carcinogenesis.^{1,2}

Cellular oncogenes may be considered as genes whose products normally regulate growth and differentiation in a positive fashion, while tumor suppressor genes transduce negative growth regulatory signals in the cell. The mutational inactivation of tumor suppressor genes coupled with the activation of oncogenes can result in loss of growth control and subsequent tumor formation. The nature and role of these two classes of genes are best understood in colorectal tumors.³ In adenocarcinomas of the colon, several mutations of oncogenes and tumor suppressor genes were demonstrated, and the timing of each could sometimes be correlated to particular stages of tumor development. However, as a general rule, it was observed that the accumulation rather than the order of appearance of mutations ultimately leads to tumor development. A multi-step model of carcinogenesis was postulated, describing the accumulation and interaction of the multiple genetic alterations present in colorectal tumors, including mutations of the Adenomatous polyposis coli gene (APC), the Deleted in colon carcinoma gene (DCC), the Mutated in colon carcinoma gene (MCC), the *p53* tumor suppressor genes and of the Ras oncogene.⁴ Since then, the accumulation of mutations during progression of several cancers was demonstrated, with precancerous lesions already displaying some genetic changes which are insufficient for the expression of the fully malignant phenotype. In persons with early onset hereditary cancer such as Retinoblastoma or Wilms tumor, presumably one of the key steps necessary for tumor development is inherited through the germline, resulting in higher susceptibility or predisposition for these individuals.

1.1.3 Tumor suppressor genes

A tumor suppressor gene (TSG) was described as the "*genetic element whose loss or inactivation allows a cell to display one or another phenotype of neoplastic growth deregulation.*"¹ Many types of gene products can fulfill this definition. They could be involved in any part of the extensive intracellular signaling pathways that allow cells to receive and process growth-inhibitory signal. Loss of critical elements of this signaling network leads to elimination of responsiveness to surrounding signals. More than 20 years ago, Knudson proposed a model to explain the cause of retinoblastoma, a rare childhood disease. He hypothesized that two "hits" must occur to result in tumor development.⁵ It was later shown that mutational inactivation of a particular cancer gene, called the Retinoblastoma gene (*Rb*), was the cause of tumor formation. This notion of inactivating both alleles of a gene was supported by other lines of evidence, such as somatic cell hybridizations and the generation of monochromosome-cell hybrids, observation of consistent cytogenetic abnormalities, and deletion mapping employing loss of heterozygosity (LOH).

1.1.3.a Somatic cell hybridization and microcell fusion

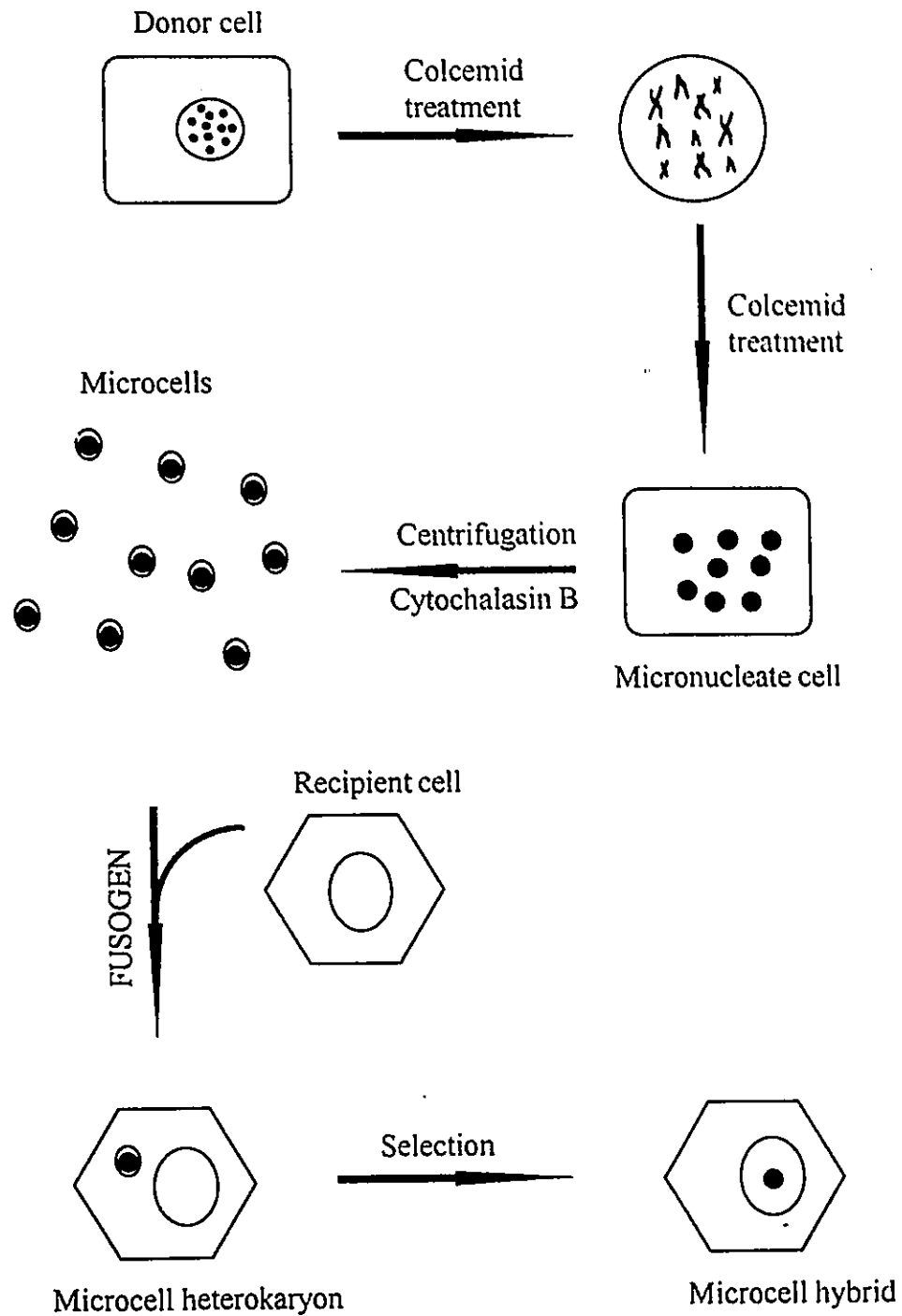
The theory that malignancy is determined by loss of genetic information was introduced by Harris et al in 1969.^{6,7} Common belief at the time maintained that transformation was mostly caused by viruses and that malignancy was a dominant characteristic. Rejecting this unanimity of opinion, Harris performed somatic cell

hybridizations between rodent tumor cells and non-malignant cells and showed that the hybrids produced very few tumors. Tumors that did appear showed long lag periods and substantial chromosome losses.⁷ His interpretation was that malignancy was initially suppressed by some chromosome(s) originating from the normal cells, and that the loss of these chromosomes from the hybrids caused the malignant phenotype to reemerge.

Since then, many studies supported the theory that normal chromosomes encode normal genes which can complement defective genes in a tumor cell.^{8,9} The main problem in the early studies was the extensive loss of chromosomes after fusion. This was partly overcome by examining intraspecific human cell hybrids which are more stable than rodent hybrids. The fusion of human cancer cells to normal human diploid fibroblasts resulted in completely non-tumorigenic hybrids. Rarely did tumorigenic segregants arise.^{10,11,12} Only after extensive culture and inoculation cycles did tumorigenic segregants appear, allowing the identification of specific missing chromosomes containing the putative tumor suppressor genes. To assess whether several tumor suppressor genes existed in different tumor types, complementation studies were performed between different tumor cell lines, sometimes resulting in completely suppressed hybrids.¹³

In conclusion, somatic cell hybridization studies provided the first indications of the existence of tumor suppressor genes and showed that cancer cells arise as a result of multiple genetic defects which can be corrected by the introduction of normal genetic

Figure 1-1: Microcell-mediated transfer. Diagrammatic representation of the steps involved in the preparation of microcell hybrids. Adapted from Lugo and Fournier.¹⁴



material. Finally, complementation studies clearly demonstrated that multiple tumor suppressor genes exist in the human genome.

One of the major drawbacks of somatic cell hybridization was the extreme complexity of the whole cell hybrids. The resolution of these studies was increased by the ability to carry out single chromosome transfers. The technique of microcell-mediated transfers, refined by Fournier et al¹⁵ in 1977, allowed the transfer of an entire intact chromosome to a recipient cell to construct simple hybrid cells with precisely defined genotypes. In brief, exponentially growing cells are treated with colcemid to initially block the cells in metaphase. However, a large number of cells will escape the block and enter G1. Each chromosome condenses with its own nuclear membrane since the mitotic spindle does not form. The cells are then enucleated by a combination of cytochalasin B and centrifugal force to produce microcells which can subsequently be fused to recipient cells (Figure 1-1).

Microcell transfer technology was used extensively to identify many chromosomes that suppress tumorigenicity of cancer cell lines (Table 1-1). In general, the transfer of a single copy of the normal chromosome was sufficient to induce growth inhibition *in vitro* and/or tumor suppression *in vivo*¹⁶, supporting the hypothesis that inactivation of both alleles of a TSG was essential for a cell to be tumorigenic. In some cases, several different chromosomes could suppress the tumorigenic phenotype in one cancer cell type.^{17,18} On the other hand, the suppressive activity of a particular chromosome was sometimes specific to only a few tumor cell types. For example, the

Table 1-1: List of microcell-mediated transfers.

Transfer of single human chromosomes via microcell fusion into various tumor cell lines. Usually, one copy of a normal chromosome is sufficient to induce tumor suppression *in vivo*. Updated from Anderson et al.¹⁹

Tumor cell line	Suppressed	Nonsuppressed	Reference
Bladder carcinoma	13		105
Breast carcinoma	6,11		20
Cervical carcinoma	11	X	21,22
Colorectal carcinoma	4,5,17,18	11,15	16, 18
Endometrial carcinoma	1,6,9	11	17
Fibrosarcoma	1,11	2,7,12	23
Glioblastoma	10	2	24
Melanoma	6		25
Neuroblastoma	1,17	11	26,27
Prostate carcinoma	del(12)(q13),13,5	3	105,106,107
Retinoblastoma	13		105
Renal cell carcinoma	3p14-q11	11	28,29
Rhabdomyosarcoma	11		22,30
Teratocarcinoma	4	7,12	31
Wilm's tumor	11p15	X,13	32

transfer of chromosome 11 in breast and cervical carcinoma cells, as well as fibrosarcoma and Wilm's tumor cells resulted in suppression of tumorigenicity. However, its transfer in colorectal, endometrial and renal carcinomas and in neuroblastoma cells had no effect on tumorigenicity (Table 1-1).

1.1.3.b Cytogenetics and allelic losses in solid tumors

In the past, cytogenetic analysis of hematopoietic tumors yielded tremendous information on the status of chromosome aberrations and was of great diagnostic value. This was mainly due to the fact that the primary chromosomal changes were constant and unique,³³ a situation not characteristic of most epithelial tumors such as breast, ovarian, prostate, colon and lung carcinomas. In these cases, primary chromosomal changes were difficult to define due to the inherent genetic instability of solid tumors. Cytogenetic examination was usually performed at an advanced stage, by which time the karyotype was too complex to allow the identification of primary alterations.

The accumulation of cytogenetic data for malignant epithelial tumors was very slow due to numerous obstacles. The cells usually displayed a low mitotic index and were difficult to grow in culture. Solid tumors much more often consisted of different cells types and cytogenetically unrelated subclones than did hematological or mesenchymal neoplasms, thus obscuring which cells represented the true *in vivo*

situation. Different methodologies could potentially establish which cells were preferentially karyotyped.³⁴

Despite these many drawbacks, the accumulation of cytogenetic analyses on epithelial tumors provided some information. In general, simple karyotypes were associated with low malignant potential, while extensive rearrangements reflected a more aggressive phenotype.³⁵ Moreover, adenocarcinomas were mainly characterized by deletions rather than rearrangements, indicating the loss of negative regulators or tumor suppressor genes.³⁶ Several deletions were discernible in the $\pm$ cancers, possibly representing the stepwise process of genetic changes leading to increased cell growth and eventually malignant transformation.³ Several chromosomal aberrations were associated with primary tumors, progression, metastasis and sometimes clinical outcome, but they seemed to differ between different adenocarcinomas, suggesting a unique and restricted cascade of genetic events in each tumor type.

In the last five years or so, technical improvements have revolutionized the field of solid tumor cytogenetics.³⁷ The advent of fluorescent *in situ* hybridization (FISH) using centromere-specific probes on interphase cells offered better detection of numerical abnormalities (such as missing or extra chromosomes) of *in vivo* conditions.³⁸ The use of interphase nuclei as hybridization targets circumvented the need for a dividing cell population and facilitated the screening of large numbers of cells, thus decreasing the effect of selection mentioned previously. It also provided access to new types of tissues, such as degenerated tissue retaining intact nuclei, and more importantly, archival

paraffin-embedded tissue sections.³⁹ Comparative Genomic Hybridization (CGH) is another novel method that detects genome-wide chromosomal regions which are over- or under-represented in tumors.^{40,41} It requires no culture or metaphase spreads and no prior knowledge of the chromosome constitution.⁴²

Another route followed in the discovery of tumor suppressor genes involved the germline or somatic mutations which inactivate one allele of suppressor genes. Such a process results in the change from heterozygosity of the chromosomal site to homozygosity of the mutant suppressor allele. Since this reduction to homozygosity usually involves the flanking chromosomal regions as well, researchers can scan the cancer cell genome to determine the frequency of losses of heterozygosity (LOH) at various loci using a large number of polymorphic chromosomal markers.⁴³ Detection of allelic losses brought some advantages compared to karyotyping methods in that it did not require metaphase spreads and it allowed the detection of smaller chromosomal deletions. These “allelotyping” techniques could detect the presence of a tumor suppressor gene in the vicinity. The precise localization and identification of the gene could subsequently be determined by molecular techniques such as positional cloning.

1.1.3.c Known tumor suppressor genes

The knowledge that loss of function can influence the process of cancer progression spurred a tremendous amount of work towards the isolation and characterization of tumor suppressor genes. The number of tumor suppressor genes identified is increasing steadily (Table 1-2). Tumor suppressors were shown to exercise

Table 1-2:

List of candidate tumor suppressor genes.

Candidate tumor suppressor genes are listed and categorized according to their putative functions in the cell. Chromosomal location of each gene is included. A detailed discussion of these genes is included in Appendix I.

Tumor suppressor gene	Chromosomal location	Function
Rb	13q14	Cell cycle: G1 arrest, apoptosis
p53	17p13	Cell cycle: G1 arrest, apoptosis, DNA repair
WAF1/Cip1/SDI1/p21	6q21	Cell cycle inhibitor
MTS1/INK4/p16	9p21	Cell cycle inhibitor
WT1	11p13	Transcription factor
VHL	3p26	Transcription elongation
MSH-2	2p16	Mismatch repair
MLH-1	3p21	Mismatch repair
PMS-1	2q31-33	Mismatch repair
PMS-2	7p22	Mismatch repair
GTBP	2	Mismatch repair
E-cadherin	16q22	Cell adhesion
α -catenin	5	Cell adhesion
APC	5q21	Cell adhesion
Plakoglobin	17q21	Cell adhesion
NF2	22q	Cell adhesion
NF1	17q11	Binds ras (GAP)
BRCA1	17q21	Development

their suppressive functions in various parts of the cell. Some genes exert their effect in the nucleus, acting at control points of the cell cycle, while others play a role at the plasma membrane in connection to cell-cell interactions. Some interact with oncogenes, such as ras and myc to modulate their transforming potential. Another group of suppressors were linked to the mismatch repair mechanism and act as safeguards of genomic integrity. Yet another role, related to transcription elongation mechanisms, was recently attributed to a known suppressor gene, VHL. Evidently, a wide variety of genes can potentially be involved in tumor suppressive activities, suggesting that many more such molecules have yet to be identified in the cell. A detailed discussion of candidate tumor suppressor genes is included in Appendix I.

1.2 Prostate cancer

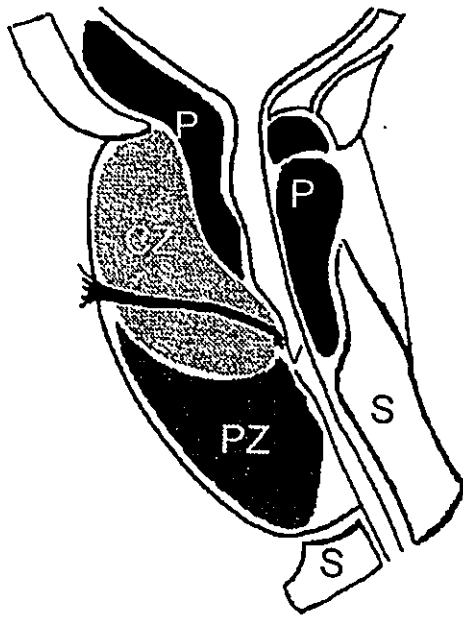
1.2.1 Anatomy and function of the prostate

The name prostate was derived from the Greek “prohistani” which means “to stand in front of”. It was attributed to Herophilus of Alexandria in 335 B.C. to describe the organ located in front of the urinary bladder.⁴⁴ Considering how long physicians have known about this complex and heterogeneous organ, adequate characterization at the structural level was very recent. Much still needs to be discovered regarding the basic aspects of prostatic structure and function. Here, it will be discussed only in a limited fashion with emphasis on what is relevant and necessary to the basic understanding of prostate malignancies.

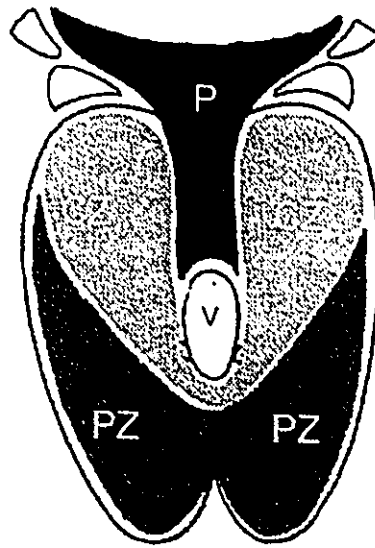
The prostate is an accessory gland of the male reproductive tract which lies underneath the urinary bladder and surrounds the urethra (Figure 1-2). Its role is to provide secretions that form the seminal plasma of the ejaculate. It is stimulated to grow and is maintained by the presence of serum testosterone. The prostate is comprised of glandular and fibromuscular tissue enclosed in an envelope or capsule. Although considerable controversy still surrounds the structure of the prostate, the McNeal zonal anatomy is now broadly accepted.⁴⁵ It describes five histologically distinct zones, termed anterior, peripheral, transitional, preprostatic, and central. The largest part is the peripheral zone (approximately 75% of the glandular component), extending around the gland from its apex to its base. It is characterized by small acinar spaces lined by tall columnar secretory epithelial cells and is embedded in a smooth muscle stroma. The second largest component is the central zone which surrounds the ejaculatory ducts and makes up the majority of the prostatic base. The glandular prostate (transition zone) comprises only 5-10% of the prostatic volume. It is separated from the other two zones by a narrow layer of muscular stroma. Benign prostatic hyperplasia (BPH), characterized by an enlargement of the prostate, frequently originates in the transition zone, while carcinoma of the prostate (PC) appears to arise predominantly, but not exclusively, in the peripheral zone.⁴⁶

The boundaries of the prostate, called “capsule” are difficult to determine but its definition becomes crucial in the light of pathological grading of prostate malignancy and determination of course of clinical action. This is often assessed by the accurate

Figure 1-2: Anatomy of the prostate gland. The morphology of the prostate (McNeal), showing the central zone (CZ) and peripheral zones (PZ) in diagrammatic midline sagittal section (A) and coronal section behind the urethra (B). P = preprostatic sphincter; S = striated sphincter of the urethra and external sphincter; V = verumontanum. Adapted from "The endocrinology of prostate tumors" (1982).⁴⁷



A



B

distinction between confined (inside the capsule area) or invasive disease (capsular penetration). For example, penetration of malignant cells out of the prostate, through the capsule and into the surrounding connective tissue elements was recognized as influencing the prognosis of patients who undergo radical prostatectomy and is a basis for recommending hormonal therapy.

Standardized grading and staging systems for PC were necessary to achieve proper assessment of treatment, good prediction of prognosis and rational comparison of different therapeutic strategies. Histological tumor grade can be a strong predictor of outcome for men with PC and has helped physicians to determine treatment options. The Gleason system of grading has achieved relatively widespread acceptance. It is based on the architectural arrangement of malignant epithelial cells. The Gleason system originally described nine identifiable patterns of malignancy, which were subsequently arranged into five grades. The grade of the two most predominant patterns in a given patient's biopsy specimen are added (sum ranging from 2 to 9) to give the histologic score or "Gleason sum". The advantage of such a system is that it accounts for tumor heterogeneity by identifying primary and secondary patterns.⁴⁸

Clinical staging was also instituted to establish the extent of progression.⁴⁹ Stage A malignancies are microscopic and are usually noted after prostatectomy. Stage B malignancies are palpable but confined to the prostate, while stage C are extending locally outside the prostate (capsular invasion). Stage D consists of the metastatic disease, typically spreading to the lymph nodes and primarily to the bone. Men with

metastatic disease generally do not survive more than two to five years following diagnosis.

1.2.2 Incidence and epidemiology

The steady increases of incidence and mortality of PC uncover the serious impact of this disease on American men. The yearly occurrence and the mortality rates have been on the rise during the last decade: from 1983 to 1995, the age-adjusted mortality has almost doubled, from 24 100 to 40 400 deaths per year in the United States. Such numbers make adenocarcinoma of the prostate the most common mortal tumor in American men after skin cancer.⁵⁰ It is estimated that 244 000 new cases will be diagnosed in 1995, up from 75 000 in 1983, thus presently accounting for 36% of the newly diagnosed cancer cases in men.⁵⁰ Put in other terms, this means that one out of six American males will develop clinical (invasive) PC during their lifetime. These projections even surpass those for lung cancer and breast cancer in women. Increased incidence can be partly attributed to improved methods of detection and heightened patient awareness. However, there is evidence suggesting a true increase in incidence and that risk factors might have an impact on the development of clinically relevant PC.

Autopsy data has revealed that clinical incidence of PC varies by region and by race.⁵¹ Although the worldwide prevalence of latent PC is somewhat constant, people who migrate from low- risk to high-risk areas become more susceptible to developing clinical PC.⁵² This indicates that initiation of the carcinogenic process is uniform, while promotion and progression to full-blown disease could be affected by environmental factors.

Theories concerning the aetiology of PC remain controversial due to the lack of conclusive data. Many risk factors such as tobacco use, cadmium exposure, sexual behaviour, vasectomy, and BPH have been suggested but could not be properly substantiated and no clear association has yet been established. Several lines of evidence implicated genetic factors in the aetiology of PC. For instance, the chance of developing PC is greater for men who have an affected brother or father, and the risk increases proportionately with the number of affected first-degree relatives.^{59,53} Supporting the concept of a genetic influence is the racial differences observed in the incidence and mortality rates of PC. Namely, black men develop PC at an earlier age, and have an increased mortality compared to white men.⁵⁴

Steroid hormones are thought to be a major factor in the genesis of PC. Testosterone and its metabolite dihydrotestosterone (DHT) can markedly accelerate PC growth and withdrawal can retard its growth. In fact, androgen ablation is routinely used as therapy for advanced stage PC. Moreover, the rate of conversion to testosterone is increased in prostatic tissue of men with PC.⁵⁵ Some evidence indicates a link between a high-fat diet and the risk of PC.^{56,57} A recent study described a two- to threefold increase in relative risk of PC in men with high amounts of linoleic acid in their plasma.⁵⁸

1.2.3 Tumor suppression in prostate cancer

The role of genetic factors in PC was alluded to by studies demonstrating familial clustering of PC,⁵⁹ which follows a pattern of mendelian inheritance.⁶⁰ Heritable PC susceptibility genes that play a role in the progression of this disease have not yet been identified. For this purpose, molecular genetic analyses have been and are still being used. They include cytogenetic characterization of human prostate tumors to identify and map any consistent chromosomal abnormalities, linkage studies to identify frequent allelic losses, chromosomal transfer studies to reconstitute loss of function and suppress tumorigenicity, and finally, characterization of the expression of known tumor suppressor genes. Advances in these areas will be discussed in detail in the following section.

1.2.3.a Cytogenetics

PC tissue samples frequently showed striking morphological heterogeneity and multifocal growth, making it one of the most difficult adenocarcinomas to study cytogenetically. The preferential growth of normal diploid epithelial cells at the expense of cancer cells in short term culture⁶¹ and the low mitotic index (MI) made it difficult to obtain enough cancer cells to analyze *in vitro*. Owing to these culturing problems, only about 200 prostatic carcinomas were studied by means of traditional cytogenetics until 1994. No one specific chromosomal change was established in PC, but there were some commonly observed alterations such as loss of chromosomes 1, 2, 5, Y, gain of chromosomes 7, 14, 20, 22 and frequent rearrangements of chromosomes 2p, 7q and 10q.

Sandberg summarized the literature on early cytogenetic findings⁶² and proposed a scheme of ordered genetic events which might be implicated in the evolution of prostatic cancer in agreement with the notion of multistep carcinogenesis.

The chromosomal changes in prostatic cell lines are very complex, displaying multiple abnormalities, high chromosomal counts, and a high number of markers.^{63,64,65,66,67,68} These changes (which are not shared between cell lines) could reflect those of the original tumors, including the primary and some secondary changes. However, it is probable that most changes do not represent the major karyotype present in the tumor, but were generated *in vitro* as a result of culture conditions.

A major obstacle of traditional cytogenetic works was the lack of a direct morphological correlation between the analyzed metaphases and the original cells. As a result, early studies found that the most common karyotype seen was a normal diploid karyotype. It was later shown that one-third of biopsies from patients with prostatic cancer used for karyotyping contained no tumor tissue in histological control sections.⁶⁹ More recent studies strived to overcome some of these difficulties by correlating the karyotypic results with histology and with the ploidy status of the tumor cells determined by means of flow cytometry (FCM) and/or image cytometry (ICM). Moreover, the advent of FISH and interphase cytogenetics allowed a more quantitative analysis of PC specimens and obviated the need for short term culture.

These studies revealed that in many cases, no aberration which might be specific for PC was observed. However, the rate of aneusomy increased significantly with histological grade.⁷⁰ Interphase FISH could miss smaller deletions in the chromosomal arms. In fact, a study using interphase FISH with 8p22 cosmid probes detected frequent loss of this region in prostate tumors in the absence of 8 cen or 8q sequence dosage alterations.⁷¹ Supporting earlier cytogenetic results, aneusomy of chromosome 7 was a common non-random alteration which was associated with poor prognosis, correlated with clinically aggressive PC. It could potentially represent a novel marker for PC progression.^{72,73} Of interest in the scope of this thesis, Brothman et al⁷⁴ performed interphase FISH analysis on 20 paraffin-embedded primary prostatic specimens (B stage) using pericentric probes for chromosomes 12, 17, X, and Y. They observed significant loss of chromosomes 12 and 17 (55% and 80%, respectively), while the loss of chromosome Y was not statistically significant. This was the first study demonstrating loss of chromosome 12 in PC, which was initially picked as a control chromosome. This illustrated how the choice of chromosomal probes is often influenced by earlier cytogenetic analyses. Therefore, other autosomes must eventually be examined by similar techniques to indicate additional chromosome changes that may be significant in PC. Comparative genomic hybridization (CGH) could be of help in this regard. It was performed by two different groups so far on primary and recurrent uncultured prostate carcinomas, to highlight several chromosomal regions that may harbor important genes

for PC tumorigenesis and progression.^{75,76} Their combined results is summarized in Table 1-3:

Table 1-3:Comparative genomic hybridization (CGH) of prostate carcinoma samples

Tissue analyzed	Chromosome gain	Chromosome loss	Reference
17 malignant	8q	8p,13q,16p,16q,17p, 17q, 20q,Y	Cher et al (1994) ⁷⁵
1 benign, 31 primary	8q	6q,8p,9p,13q,16q,18q	Visakorpi et al (1995) ⁷⁶
9 recurrent	8q,7p13, Xp11-13, Xq23-qter	5q,6q,8p,13q,16q	Visakorpi et al (1995) ⁷⁶

Of note in the above table is that 6q and 9p losses were not reported previously in PC. The critical region at 6q was 6cen-q21, indicating that this region may encode a TSG important in the development of PC. The candidate tumor suppressor gene p21/WAF1 could be involved in these cases since it was mapped to chromosomes 6q21. The putative TSG at 9p could potentially be the *MTS1* gene, which was recently identified at 9p21.⁷⁷ It is of interest that the frequency of 5q losses was over seven times higher in recurrent tumors, in light of the fact that the *APC* TSG maps to this region (5q21). Loss of chromosome 16q was common to both studies. Cher et al have recently mapped the region of common deletion to 16q13.1 by using region-specific chromosome 16 cosmid probes hybridized to interphase prostatic carcinoma nuclei.⁷⁸

1.2.3.b Loss of heterozygosity

Although cytogenetic techniques are useful, they do not detect the entire range of inactivating events such as microdeletions and homologous recombination with a defective chromatid. More accurate molecular methods should be used to screen for genetic alterations in tumors and delineate the smallest chromosomal region involved.

Loss of heterozygosity studies have served this purpose successfully in PC research to indicate the presence of unidentified TSG or implicate known TSG. Initial studies reported loss of heterozygosity on chromosomes 8p, 10q, 16q and 18q.^{79,80} All of these chromosomes were shown by cytogenetic analysis to have alterations. Deletions of chromosome 8 have since been mapped to 8p22 (near the MSR locus = macrophage scavenger receptor)⁸¹, 8p21(encompassing the NEFL locus)⁸² and to 8p12-21 (D8S87-ANK1 loci)⁸³, indicating the presence of three distinct putative TSG on this chromosome. Allelic losses at 8p were observed frequently in several human tumor types including pancreatic, gastric, bladder, breast, colorectal, non-small cell lung, and hepatocellular cancer.⁸⁴

Loss of heterozygosity of the chromosomal region 10q23-25 is a frequent event in the progression of PC. Although the *Mxi1* gene is located at 10q25, a recent report failed to detect any mutations of this gene in 23 tumors showing *Mxi1*-associated marker loss. Their results suggest the presence of another tumor suppressor gene at 10q23-24.⁸⁵

A possible candidate TSG for chromosome 18 is the Deleted in Colorectal Cancer (*DCC*) gene. In fact, it was shown that while *DCC* expression was present in normal prostatic cells, it was decreased or undetectable in several human and rat cell lines and reduced in 86% of patients studied.⁸⁶

In a linkage study of seven breast-ovarian cancer families with 17q linkage, PC was found to be the most common malignancy after breast cancer, suggesting that a gene on this chromosome may predispose to prostatic cancer in male carriers.⁸⁷ LOH

investigation in the 17q12-21 revealed a high degree of deletions at the site of the *BRCA1* gene, which is thought to be responsible for inherited breast and ovarian cancers.⁸⁸ Other regions on 17q were found to display a high degree of LOH, suggesting the presence of yet another TSG on this chromosome.⁸⁹ Deletions of chromosome 7q31.1 were recently reported to be frequent in PC tissues,^{90,91} even more so in localized tumors, suggesting a role in the early stages of PC.⁹² This gene could represent a broad range tumor suppressor, since a comparable region is implicated in several tumor types. Allelic loss of other known TSG such as *Rb*^{93,94}, *p53*^{95,96,97}, and *APC*^{94,97} were also reported in several different studies, indicating their possible influence on PC progression. Allelic loss is therefore a useful tool towards the demanding task of cloning tumor suppressor genes, but a high frequency of LOH can only serve as an indication of the presence of such genes and cannot demonstrate a tumor suppressor phenotype per se. Other methods, such as microcell-mediated transfers of particular chromosomes and transfection of specific cDNAs can better demonstrate the suppressive function of a candidate gene.

1.2.3.c Microcell-mediated transfers

The transfer of normal chromosomes into cancer cells provided further evidence of a role for tumor and metastasis suppressor genes in the genesis of PC. Much work was done with regards to genes that control metastatic potential of PC cells. Initial studies using somatic cell hybridization showed that the fusion of nonmetastatic and highly metastatic Dunning rat prostatic cancer cells resulted in hybrids that did not develop distant metastasis when injected into animals. Occasional animals developed distant

metastases upon passaging of the hybrids *in vivo*, and cytogenetic analysis demonstrated that these metastatic revertants had lost rat chromosome 2.⁹⁸ This chromosome is syntenic with portions of human chromosomes 1, 4, 5, 6, 10 and 15. Since many allelotyping studies had implicated two regions of chromosome 8 in PC, the same group subsequently showed that introduction of normal human chromosome 8 in highly metastatic PC cells (AT6.2) resulted in suppression of metastatic ability of the microcell hybrids, whereas no suppression of the *in vivo* growth rate or tumorigenicity were observed.⁹⁹ This suggested that metastatic ability of a cancer cell may be regulated independently of its growth rate or tumorigenicity. Similar results were obtained upon the introduction of human chromosome 11, which is known to contain tumor suppressor genes for Wilm's tumors, bladder, breast and hepatocellular carcinoma. Spontaneous deletion of portions of human chromosome 11 in some of the hybrids allowed the mapping of the suppressor gene to 11p13-11.2.¹⁰⁰ A candidate metastasis suppressor gene mapping to 11p11.2, *KAI1*, was recently cloned by differential *Alu*-PCR techniques. Expression of *KAI1* was reduced in human cell lines derived from metastatic prostate tumors and it may be involved in the malignant progression of prostate and other cancers.¹⁰¹ This study was a good example of the use of microcell fusion to identify a suppressor chromosome and subsequent use of the suppressed hybrids as tools to clone a novel suppressor gene. A truncated human chromosome 17(pter-q13) was also shown to suppress the metastatic potential of rat PC cells. This suppressor activity was shown to be *p53*-independent and not due to enhanced expression of the NM23 protein.¹⁰²

So far, only three studies have identified tumor suppressor genes involved in PC by microcell fusion. It was shown that the RB protein is aberrantly expressed in the DU 145 cell line.¹⁰³ When normal *Rb* gene expression was restored in DU 145 cells by retrovirus mediated gene transfer, their tumorigenicity was suppressed.¹⁰⁴ In an attempt to provide more physiological expression of the introduced gene, Banerjee et al introduced chromosome 13 to express the *Rb* gene under its natural promoter and other endogenous *cis*-acting elements. They only obtained one *Rb*-positive clone after chromosome transfer, which showed reduced, but not complete suppression of tumorigenicity. The difficulty in obtaining hybrids may illustrate a growth inhibitory effect provided by *Rb* complementation. Some of the resected tumors still expressed normal RB protein, demonstrating that this gene was not a potent inhibitor in these PC cells.¹⁰⁵

The second microcell transfer performed in PC cells will be described in detail in chapter 3 of this thesis. It is the first study of its kind to identify the location of a novel tumor suppressor gene in PC. Briefly, we introduced human chromosome 3 and del(12)(q13) into the PC cell line DU 145. While chromosome 3 had no effect on the *in vivo* tumorigenicity, three individual hybrids containing a tagged del(12)(q13) displayed complete suppression of tumorigenicity upon injection into nude mice. Loss of the tagged portion of chromosome 12 in each case led to reexpression of the malignant phenotype.¹⁰⁶ These results suggested that one or many genes on chromosome 12 act as potent tumor suppressors of prostate carcinogenesis.

Another chromosomal transfer in prostate cells was described by Ewing et al.¹⁰⁷ They introduced a normal human chromosome 5 in the PC-3 prostate cancer cell line. This cell line has a dysfunctional adhesion system by virtue of a deletion of the α -catenin gene on chromosome 5q. This gene encodes a protein which associates with E-cadherin and is necessary for its normal cell-cell adhesion function. Microcell-mediated transfer of chromosome 5 resulted in the re-expression of α -catenin and a dramatic alteration in cell morphology. When injected into nude mice, the hybrids formed slow-growing tumors or no tumors at all, demonstrating the tumor suppressive ability of chromosome 5 and possibly of the α -catenin gene.

1.2.3.d Known tumor suppressor genes

Recognition of the significance of tumor suppressor genes in human PC has emerged in the last few years. Several well-characterized tumor suppressor genes such as *Rb* and *p53* and other candidate genes have been implicated in PC by several methods, including detection of deletional events, loss of expression studies, immunocytochemistry, and antisense experiments.

The retinoblastoma gene

The *Rb* gene was examined in several PC cell lines. Only DU 145 was found to contain an abnormal RB protein translated from an *Rb* messenger RNA transcript that lacked 105 nucleotides (35 aminoacids), due to a point mutation in exon 21 (resulting from a splicing defect). Retrovirus-mediated transfer of wild-type *Rb* into DU 145 cells

resulted in the formation of smaller tumors. The latter were found to originate from revertant cells expressing only the endogenous mutated *Rb* but not the exogenous wild-type *Rb*.¹⁰⁴ Thus, similar to a previous study in retinoblastoma cells that lack normal *Rb*,¹⁰⁸ restored expression of *Rb* could suppress the tumorigenicity of the DU 145 cells. In a different investigation, *Rb* expression was detected in non-neoplastic prostatic epithelial cells and in one primary cancer, but was severely reduced or absent in two out of six metastatic specimens. In one case, the *Rb* mutation was characterized as a 103-base pair deletion in the promoter region, followed by loss of the second *Rb* allele.¹⁰⁹ Based on these results and the rate of 13q allelic loss (23%),⁷⁹ it was initially estimated that *Rb* mutations were implicated in about 20% of PCs.¹¹⁰

The p53 gene

The *p53* tumor suppressor gene was shown to be defective or missing in three prostate carcinoma cell lines. Transfection of the wild type gene into two of these cell lines resulted in decreased colony formation.¹¹¹ Infection of androgen-independent human prostate Tsu-pr1 cells lacking functional *p53* alleles resulted in high levels of p53 protein, induction of apoptosis and suppression of tumor formation in nude mice.¹¹² In the DU 145 cell line, p53 was detectable by immunoblotting, indicating that it had an extended half-life, a property often associated with mutated forms of *p53*. Effert et al¹¹³ studied *p53* in one individual with a mutant *p53* allele in a portion of the primary tumor and also in the metastatic lesion from the same patient. This implied that the metastatic cells originated from cells with mutant *p53* in the primary tumor thus suggesting a role

for *p53* in the progression of PC. Only 17% of samples showed low to high levels of *p53* immunoreactivity (suggesting mutated *p53*) in archival, paraffin embedded prostatic carcinomas, which was relatively low compared to other types of tumors. However, high-level *p53* accumulation predicted short, progression-free interval and poor survival with about a 12-fold relative risk of death as compared with *p53*-negative cases.¹¹⁴ More recently, Chi et al¹¹⁵ found that 20 out of 40 (50%) prostate tissue samples displayed *p53* gene abnormalities that would result in altered protein expression. Nucleotide base-pair transitions of A->G or T->C were the most frequent. They concluded that *p53* mutations are frequent in PC and they proposed the possible involvement of a carcinogenic agent(s). Taking all this data into consideration, *p53* could be implicated in a subset (20-50%) of PCs.

C-CAM1

C-CAM is an epithelial cell adhesion molecule belonging to the immunoglobulin family. Its expression in the prostatic epithelium was shown to follow a pattern correlating with androgen-induced differentiation.¹¹⁶ Furthermore, transfection of *C-CAM1* into an androgen-independent cell line (PC-3), which does not express C-CAM1 resulted in reduced tumorigenicity in nude mice. Down regulation of *C-CAM1* in NbE nontumorigenic rat prostate epithelial cells by transfection of the antisense RNA resulted in increased tumorigenicity *in vivo*.¹¹⁷ Further supporting a role for *C-CAM1* as a tumor suppressor gene, Kleinerman et al¹¹⁸ found that while basal cells of fetal and normal adult prostate expressed normal levels of C-CAM1, BPH and prostatic intraepithelial

neoplasia (PIN) tissues had low levels, and prostate carcinomas did not express C-CAM1. They also recently established that delivery of *C-CAM1* adenovirus was able to repress the growth of PC-3 induced tumors in nude mice for at least 3 weeks, indicating that *C-CAM1* is a likely candidate for human PC therapy.¹¹⁹

E-cadherin, catenins and the APC link

Another example of an adhesion molecule as a candidate suppressor of PC is a Ca^{2+} -dependent cell adhesion molecule called E-cadherin. The E-cadherin gene is located at chromosome 16q22.1,¹²⁰ a common region of deletion in PC cells.^{79,80} Bussemakers et al studied E-cadherin in the Dunning rat model, and found a strong correlation between the lack of E-cadherin and metastatic and/or invasive potential.¹²¹ Another study looking at E-cadherin protein levels in surgical specimens of PC established that close to half the tumors examined had reduced or no E-cadherin protein.¹²² In fact, aberrant E-cadherin staining is a powerful predictor of poor outcome, in terms of both disease progression and overall survival.¹²³ The mechanism by which E-cadherin is silenced has been unclear. Graff et al¹²⁴ showed that frequent loss of E-cadherin expression in human breast and prostate carcinomas resulted from hypermethylation of the E-cadherin promoter region. Cadherins form a complex with cytoplasmic proteins called catenins (α and β), and this complex constitutes intercellular adherens junctions and cytoskeletal components including actin fibers. Catenins were reported to bind the tumor suppressor gene *APC*,^{307,125} and this interaction possibly regulates cell adhesion or transmission of contact inhibition signals into the cell. E-cadherin, as well as α -catenin expression, was either

reduced or absent in 5 out of 6 PC cell lines. Alpha-catenin was recently mapped to chromosome 5q22,¹²⁶ and preliminary LOH analysis suggested that this locus was deleted in approximately 25% of PCs analyzed.¹²⁷ Interestingly, the loss of α -catenin in PC-3 cells was the result of a homozygous deletion of a large portion of the coding region of the gene.¹²⁸ Alpha-catenin was re-expressed in this cell line by transfection of a copy of the α -catenin cDNA under the control of a heterologous promoter or by microcell-mediated transfer of chromosome 5. In both cases, alteration of cell morphology and tumor suppression in nude mice were observed.¹⁰⁷

Deleted in colorectal cancer (DCC)

Another good candidate TSG, deleted in colon carcinoma (*DCC*) was implicated in PC. The *DCC* gene, located on chromosome 18q was partially cloned and demonstrated a high homology with the neural cell adhesion molecule (N-CAM) and other related cell surface glycoproteins of the immunoglobulin superfamily.¹²⁹ High frequency of allelic losses at chromosome 18q were detected in colorectal, gastric, pancreatic and esophageal tumors.^{130,131,132} Ramaswamy et al demonstrated that antisense *DCC*-expressing Rat-1 fibroblasts cells showed a faster growth rate, anchorage independence and tumorigenicity in nude mice, thus strongly implicating *DCC* as a tumor-suppressor gene.¹³³ In PC, 26% of tumors showed loss of heterozygosity at the *DCC* locus. Five of the tumors displaying LOH were advanced stage and clinically localized, suggesting that loss of *DCC* may influence progression in prostatic

carcinoma.⁹⁷ In agreement with these data, it was demonstrated that loss of expression of *DCC* is often observed in prostatic carcinomas.⁸⁶

KAI1

The microcell transfer of a human chromosome 11 into the highly metastatic rat AT6.1 prostate cancer cell line caused the suppression of their metastatic potential. A spontaneous deletion in the transferred chromosome in some hybrids helped to further map the putative suppressor gene to human chromosome 11p11.2-p13.¹⁰⁰ Human-specific genomic fragments distinct to the cells displaying a suppressed phenotype were obtained in the p11.2-13 region by *Alu*-PCR. After screening a cDNA library with these DNA fragments, the *KAI1* gene was isolated.¹⁰¹ Transfection of *KAI1* into the parental AT6.1 metastatic cells suppressed lung metastases of these cells in nude mice. The 2.4 kb *KAI1* transcript was detected in all the human tissues tested, and the coding sequence was highly conserved in human, monkey, dog, and rabbit.¹⁰¹ *KAI1* is a member of a family of membrane glycoproteins that includes ME491/CD63, MRP-1/CD9, TAPA-1, CD37, and CD53. All these proteins have four transmembrane domains and a large extracellular N-glycosylated domain and consequently are potentially involved in cell-cell or cell-matrix interactions.

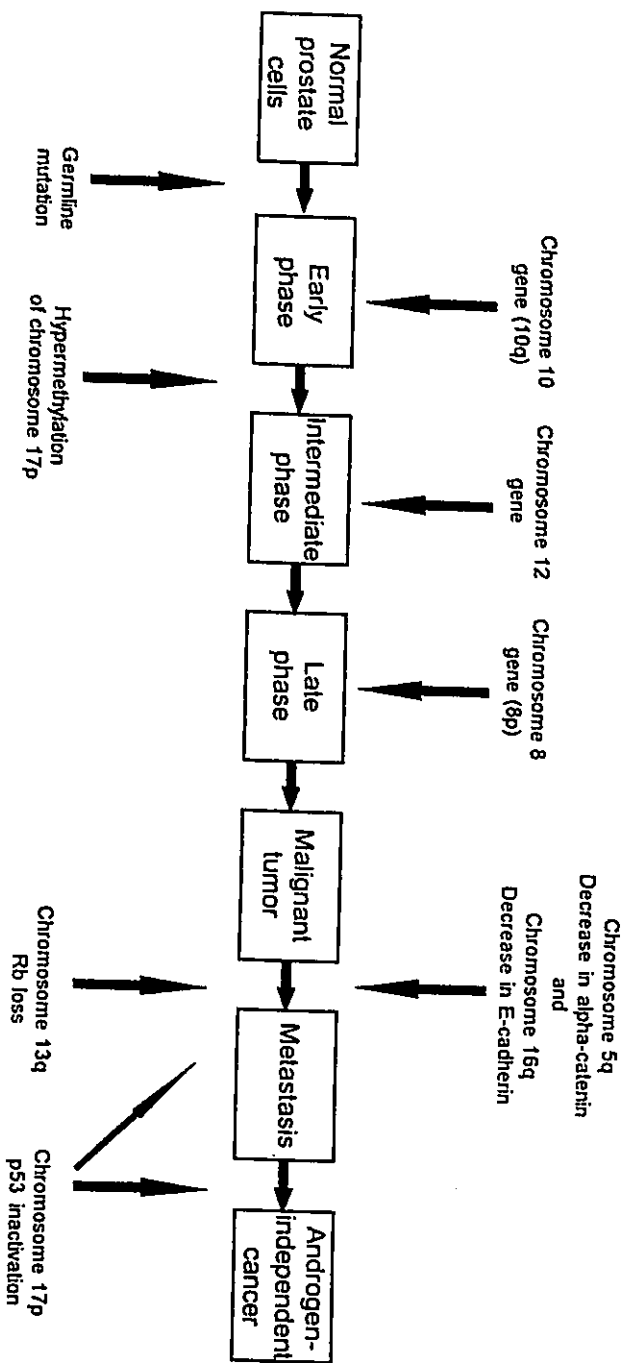
Mxi1

Deletions of chromosome 10q were often reported in prostatic cancer, suggesting the presence of a TSG in that region.^{35,134} One of the genes that map to this region is *Mxi1*(10q24-q25). The *Mxi1* gene product is thought to heterodimerize with c-Myc and

reduce its transcriptional potential by competing with active Myc-Max heterodimer formation. This proposed role is consistent with a tumor suppressor function and as a result, *Mxi1* was examined in PCs exhibiting 10q24-q25 deletions, to determine whether the remaining allele contained any inactivating mutations. Eagle et al¹³⁵ found mutations in the retained *Mxi1* alleles in four primary prostate tumors. Two tumors contained inactivating mutations leading to inability to bind the c-Myc recognition site. Two others contained an identical missense mutation, which did not abrogate DNA binding but might alter the interactions of Mxi1 protein with other transcription factors. The structural region of Mxi1 that conferred growth suppression was a 36-aminoacid amino-terminal domain that interacts *in vivo* with a mammalian homologue of the yeast transcriptional repressor Sin3.¹³⁶ Interestingly, Mad (another myc antagonist) had an analogous region and has been reported to dramatically inhibit the proliferation and tumorigenicity of human astrocytoma cells.¹³⁷

Based on the different studies discussed above, we propose a schematic for the multistep carcinogenesis of prostate cancer (Figure 1-3).

Figure 1-3: Multistep carcinogenesis of prostate cancer. Putative pathway through which prostate cancer develops. A series of mutations give rise to increasingly large tumors that eventually become malignant. Each progressive involvement of a gene or change decreases the responsiveness of the affected cell to control mechanisms, ultimately resulting in a tumor capable of invasion or metastasis. The arrows do not necessarily indicate a temporal order of events. Rather, the accumulation of mutations (in any order) results in a more aggressive phenotype. This scheme may apply to only a subtype of PC, with other schemes being necessary for other types of this neoplasm.



1.3 Perspective

There is a high degree of variability among different PCs, some being much more aggressive than others, leading to rapid invasion and metastasis. If the tumor is observed early enough while it is still localized to prostatic tissue, radical prostatectomy or radiation therapy can effectively cure the disease. However, some side effects such as impotence, incontinence, and bowel injury can significantly decrease the quality of life of these patients. Accordingly, it would seem rational to initiate aggressive screening for PC of men as they get older. Such an approach in itself brings forth problematic issues and creates a dilemma for the management of PC. This stems from the fact that most histologically localized PCs remain in the latent stage and will never progress to full blown metastatic disease. Such cases of indolent tumors which do not require intervention are at present indistinguishable from tumors which are likely to progress to a higher stage. While treatment of patients with latent PC that will not progress can cause them needless pain, discomfort and risk, “watchful waiting” can allow the cancer to invade and progress to a point where the disease has become life-threatening, and therapeutic intervention is ineffective.

Most PC arise anterior to the midline of the prostate where it is difficult to detect by digital rectal examination (DRE), the classic technique for the detection of PC. Consequently, most patients have advanced disease by the time of diagnosis. An early detection method involves the measurement of prostate-specific antigen (PSA) levels.

PSA, a kallikrein-like serine protease, is solely and constitutively secreted by human prostatic epithelial cells and is a normal component of the seminal plasma.^{138,139} It has recently been demonstrated that PSA degrades extracellular matrix glycoproteins fibronectin and laminin and may be involved in the proteolytic cascade during PC invasion and metastasis.¹⁴⁰ In general, an abnormal increase in plasma PSA levels (over 4ng/ml) correlates with increased tumor volume and clinical disease progression.¹⁴¹ However, the use of PSA for early detection is considered limited due to false-positive elevations produced by benign prostatic hyperplasia (BPH).¹⁴² **If screening for earlier detection is to be used, better diagnostic markers will be needed to identify which PCs will progress to clinical disease, and which ones will not.**

Local surgery cannot be used to cure PCs which have already invaded other tissues. Metastatic malignancies require different therapeutic approaches such as systemic androgen ablation. However, in over 50 years, this kind of therapy has not significantly reduced PC mortality. This results from the heterogeneous property of prostate tumors: it is composed of different clonal populations, some which are androgen-sensitive and others which are androgen-independent. The latter are the major cause of relapse in patients often seen after an initial positive response to treatment.

Clearly, PC poses complex medical management issues. **The problems that plague PC diagnosis and treatment can be solved in part by the development of new diagnostic methods, which can only result from the study of molecular and cellular changes associated with prostatic carcinogenesis.** The work presented in this thesis

contributed to the elucidation of the genetics of PC progression and hopefully will lead to the characterization of genes which could possibly be used as markers for diagnosis and eventual treatment of this deadly disease.

CHAPTER 2

ANALYSIS OF CELL LINES

2. ANALYSIS OF CELL LINES

2.1 Introduction

Following whole cell hybridization or microcell fusion, donor chromosomes are not retained in a stable manner and for that reason, they are progressively lost from hybrid cell lines. Some chromosomes tend to be retained more readily than others, and there is a suggestion that certain combinations of donor chromosomes are more unstable than individual chromosomes. Consequently, frequent testing of the hybrids for the presence or absence of donor chromosomes using cytological and genetic marker analysis becomes necessary. These problems can be alleviated by the use of selection systems that can be applied to retain the donor chromosomes. One possibility for hybrid biochemical selection is the introduction of single, dominant selectable genes into a donor cell population by DNA-mediated gene transfer. The integration of a selectable marker in a given chromosome is necessary to ensure transfer and retention of that chromosome. In most studies, markers such as the bacterial neomycin phosphotransferase (neo),¹⁴³ hygromycin phosphotransferase (hph)¹⁴⁴ or xanthine-guanine phosphoribosyltransferase (gpt)¹⁴⁵ genes were introduced via transfection or retroviral infection. The major disadvantage of using such markers was the incapability to select for segregant cells which have lost the tagged chromosome. Consequently, the microcell hybrids had to be

cultured in the absence of selection until the tagged chromosome was randomly eliminated from the cells. Segregants are useful to demonstrate that the change in phenotype caused by the presence of the tagged chromosome can be reverted by the loss of that same chromosome.

We have developed a novel system which facilitates the isolation of segregant clones by introducing the tgCMV/HyTK plasmid¹⁴⁶ into the DNA of donor chromosomes. This marker provides a means of selecting for the retention of the tagged chromosome with hygromycin B, while allowing negative selection of this chromosome through sensitivity to ganciclovir (GCV). The transfer of whole or fragmented chromosomes into cancer cells, resulting in suppression of their neoplastic phenotype, followed by the negative selection of the same chromosome to restore tumorigenicity, represents an effective strategy for the identification of chromosomal loci involved in tumor suppression.

To allow the selection of hybrid cells following fusion experiments (described in chapter 3), the cell lines were tagged with various selective markers. The generation of the DP whole cell hybrids was feasible upon the introduction of the hph gene into DU 145 and of the neo gene into PC3 cells prior to their fusion. Similarly, human skin fibroblasts were tagged with the tgCMV/HyTK plasmid, and the neo gene was introduced into DU145 cells. This allowed the transfer by microcell fusion of normal human chromosomes into prostate cancer cells.

The segregation and “immortalization” of normal human tagged chromosomes was achieved by their transfer into a mouse melanoma cell line, B78. As a result, an extensive panel of hybrids, acting as a permanent “library” of tagged human chromosomes was analyzed and made available for fusions into any recipient cell line.¹⁴⁷ One particular mouse/human hybrid (B78MC9) was used to specifically transfer human chromosome 12 into the DU145-N19 cell line.

Finally, thorough analysis of the DU 145 cell line and the B78MC9-5 mouse/human hybrid was necessary to determine their suitability as recipient and donor cell lines, respectively, for microcell fusion experiments. Chromosomal content, growth and tumorigenic properties were assessed in preparation of fusion experiments described in chapter 3.

All these preliminary steps established a controlled system in which the generation and analysis of the whole cell or microcell hybrids were crucial to the success of subsequent studies.

2.2 Materials and methods

The source of all chemicals used are listed in appendix III.

Cell lines and culture conditions: The DU145 and PC3 cell lines (American Type Culture Collection, Rockville, MD) and all resulting cell lines were maintained as monolayer cultures at 37°C under 5% CO₂ in minimal essential media (MEM)

supplemented with 10% FBS. Human fibroblasts were established from foreskins and grown in Dulbecco's modified Eagle medium/Ham's F-12 (1:1) medium supplemented with 10% FBS. B78 mouse melanoma cells (a gift from M. Thayer, Oregon Health Sciences University, Portland, OR) were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS. B78MC microcell hybrids were cultured as B78 cells but in presence of hygromycin B (400 µg/ml).

Electroporation: The minimal concentration of G418 or hygromycin B required in the medium to kill all the non-resistant cells efficiently was determined. DU 145 or PC-3 cells were grown in petri dishes (2×10^5 cells in 10 ml media) at different concentrations of G418 (100, 250, 500 and 1000 µg/ml) or hygromycin B (10, 50, 100, 200 and 400 µg/ml). The number of colonies were counted after 21 days. Prior to the transfer, pSV2-neo was linearized with Eco RI whereas pSV2-hygro was linearized with Sal I. Following a phenol/chloroform extraction, the plasmid DNA was resuspended in sterile Tris-EDTA to obtain a final concentration of 1 µg/µl. Before electroporation, the cells (usually from two confluent 150 cm² flasks) were washed, trypsinized and resuspended in 20 ml of MEM supplemented with 10% FBS. An aliquot of 0.1 ml was counted with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). One million cells were centrifuged and resuspended in HEBS buffer [21 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose]. The cells and the plasmid DNA were

added to the electroporation chambers (Cell-Porator, BRL Life Technologies, Inc.) and electroporated according to Table 2-1. The hygromycin B-resistant HSF clones obtained were pooled in two different batches, designated as HFHT2 (249 clones) and HFHT4 (483 clones).

Table 2-1:
Electroporation conditions for the transfer of selective markers into different cell lines.

Cell lines	Plasmid name	Plasmid (μ g)	Number of cells ($\times 10^6$)	Capacitance (μ Fa)	Voltage (Volts)	Selection (μ g/ml)
DU145	pSV2hygro	5	4.0	800	250	400 (Hygro)
DU145	pSV2neo	5 or 10	2 or 4	800	250	800-1000 (G418)
PC3	pSV2neo	5 or 10	2 or 4	800	250	1000 (G418)
HSF	tgCMV/HyTK	2	2.5	960	300	200 (Hygro)

Cell growth studies: To determine the doubling time, 2×10^5 cells were seeded and the cells counted every day for 5 or 6 days. Doubling times were determined during log phase growth, using values obtained from three experiments. To determine the anchorage-independent growth, 5×10^4 cells were plated in agar (top layer: 3 ml, 0.3% noble agar; bottom layer: 3 ml, 0.6% agar; both in MEM with 10% FBS) in 60 cm² tissue culture dishes. Dishes were fed at weekly intervals with 1 to 2 ml of MEM supplemented with 10% FBS. Colonies of cells growing in soft agar were scored after 21 days in culture. The values are means of three dishes.¹⁴⁸

Nude mice injections.: The nude mice used in this study were 2 to 4-week-old males obtained from Charles River. The mice were housed in a specific-pathogen-free isolation facility, and kept in a room separate from all other experimental animals. Cages, litter,

food, and water were all sterilized prior to use, and filter tops were used for each cage. Handling of the animals was accomplished while wearing sterile gowns, gloves, caps, and masks.

1×10^7 DU145-N19 cells were resuspended in 0.5 ml sterile saline (0.9% NaCl) in sterile 1.5 ml Eppendorf tubes, and 0.3 ml (6×10^6 cells) were injected per site with a 1 ml syringe fitted with a 27-gauge needle, in the shoulders and thighs of nude mice. For B78 and B78MC9-5 cells, 1.5×10^6 cells in 0.2ml sterile saline were injected at four different sites in two different mice. Tumor formation was monitored once or several times a week. For some injections, tumor volume was measured using a sterilized caliper under sterile conditions. The length, width, and height of the tumors were recorded. These values were used to estimate tumor volume, using a formula which makes the assumption that the tumors are hemiellipsoids. These calculations also take into account skin thickness (0.4mm) and the high rate of variation in the height measurements.¹⁴⁹

$$V = \frac{(4\pi/3)(l/2)(w/2)h}{2} \frac{(1-0.8)(w-0.8) \sqrt{(1-0.8)(w-0.8)/4 + (h-0.4)^2}}{4}$$

Where:

V = volume (mm³)

l = length (mm)

w = width (mm)

h = height (mm)

A BASIC program was written to calculate tumor volumes according to the above equation. Graphs were generated from the average of several tumors (usually 4).

G-banding analysis: Metaphase chromosome spreads were prepared according to standard protocols. The cells were exposed to 0.05 µg/ml KaryoMAX colcemid for 90 min. After a 20 min hypotonic treatment in 1% sodium citrate, the cells were fixed with a mixture of glacial acetic acid and methanol (1:3). Slides were presoaked in 95% ethanol and rinsed in cold water. With a pasteur pipette, two drops of fixed cells were dropped on each slide and warmed over a steaming bath. After aging at 40°C on a slide warmer (Fisher Scientific) for several days, the slides were banded using Seabright's trypsin-Giemsa technique.¹⁵⁰ The slides were passed through a series of solutions in coplin jars, including 0.15M NaCl containing 1 ml of trypsin stock solution for 30-120 sec. The trypsin was a crude preparation dissolved in 10 ml of distilled water and stored frozen in 1 ml aliquots. The slides were then incubated in 0.01M phosphate buffer, pH6.8 containing 1.5 ml of concentrated Giemsa for 1 to 2 min followed by a brief rinse in distilled water. The slides were then placed on end on a paper towel to drain and dry and aged on a slide warmer.

Fluorescence *in situ* hybridization. Hybridization and amplification of chromosome painting probes were done according to manufacturer's protocol (Oncor). The *Alu9* probe (specific for human chromosome 12) was generated upon PCR amplification of B78MC9 DNA with four primers directed to the *Alu* consensus sequence (*Alu* primers 450, 153, 154, and 451).¹⁵¹ The composition of the PCR reaction buffer was: 10 mM Tris-HCl pH 8.3; 50 mM KCl; 0.01% gelatin; 1.5 mM MgCl₂; 200 mM of each dNTP; and 250 ng of consensus

Alu primer. The reactions were carried out in a final volume of 50 µl with 1U of AmpliTaq DNA polymerase and 100 ng of genomic DNA, using a Pharmacia thermal cycler under the following conditions: 1 cycle 94°C, 3 min; 60°C, 1 min; 72°C, 1 min, followed by 35 cycles of 92°C, 45s; 60°C, 1 min; 72°C, 1 min, with an increment of 6s per cycle (35 cycles), and 1 cycle of 92°C, 45s; 60°C, 1 min; 72°C, 10 min. The four different PCR products were pooled and precipitated. The DNA was labeled by random priming with biotin-14-dCTP (BRL BioPrime DNA labelling kit) and 2mM ³H-dTTP. Unincorporated nucleotides were eliminated by two ethanol precipitations. The level of biotin incorporation was determined by calculating the incorporation of ³H-dTTP. Immediately before use, slide preparations were passed through an ethanol series (70-90-100%), denatured at 70°C in 70% formamide; 2X SSC (1X SSC = 150 mM NaCl; 15 mM sodium citrate pH 7.0) for 2 min, and passed through another ethanol series (70-90-100%). The probe mixture (50% formamide, 10% dextran sulfate, 2X SSC, 90 to 100 ng of biotinylated probe, 1 to 3 mg of human Cot1 DNA, 1mg of salmon sperm DNA) was denatured at 75°C for 10 min and then preannealed at 37°C for 30 to 60 min. The hybridization mixture (10µl) was applied to each slide preparation under a 22x22-mm coverslip, sealed with rubber cement, and incubated at 37°C for 16 to 20h in a humidified chamber. The slides were washed at 45°C in 2X SSC; 50% formamide for 10 min; 2X SSC for 10 min; 4X SSC, 5% Triton-X 100 for 5 min. Avidin-FITC detection was carried out as outlined in the Oncor Chromosome *in situ* Hybridization System. The slides were mounted with propidium iodide/antifade mixture. Results were

analyzed using an 09 filter combination on a Zeiss axioskop microscope equipped with epifluorescence.

2.3 Generation of selectable cell lines

For whole cell and microcell fusions, selectable markers were introduced into DU145 and PC-3 cell lines and into the primary human foreskin fibroblasts (HSF).

2.3.1 DU 145

DU 145 is a long-term tissue culture cell line derived from a human prostate adenocarcinoma metastatic to the brain.⁶⁵ The primary tissue was obtained from a 60-year-old Caucasian male with widespread metastatic carcinoma of the prostate and a 3-year history of lymphocytic leukemia. The cells are epithelial, grow in isolated islands on plastic Petri dishes, and form colonies in soft agar suspension culture. They are not detectably hormone sensitive or dependent.

We proceeded to insert pSV2neo and pSV2hygro plasmids in the DU 145 cells (as described in the materials and methods section) to allow selection with the drug G418 and hygromycin B, respectively. This was done to allow proper selection of the hybrids after whole cell or microcell fusions. The pSV2neo plasmid encoding the enzyme neomycin phosphotransferase was transferred into the cells by electroporation. Several G418-resistant clones were picked and expanded. Clone DU145-N19 was chosen for use in further experiments.

2.3.2 PC-3

The PC-3 cell line was originally established from a human prostatic adenocarcinoma metastatic to bone.¹⁵² These cells show anchorage-independent growth in both monolayers and in soft agar suspension and produce subcutaneous tumors in nude mice. Karyotypic analysis revealed the cells to be completely aneuploid with a modal chromosome number in the hypotriploid range. We electroporated these cells with the pSV2neo or the pSV2hygro plasmids. In each case, several clones were picked, expanded, and frozen for future use in whole cell fusion experiments.

2.3.3 Human skin fibroblasts

Primary human skin fibroblasts (HSF) were established from human male foreskins. While still at low passage, HSF cells were electroporated with the tgCMV/HyTK plasmid. This plasmid contains the hygromycin phosphotransferase gene fused in-frame with the herpes simplex virus type 1 thymidine kinase gene.¹⁴⁶ The resulting HyTK fusion protein confers resistance to hygromycin B and sensitivity to ganciclovir (GCV). More than 650 hygromycin-resistant fibroblast clones were pooled in two batches named HFHT2 and HFHT4. These tagged fibroblasts were used as donors of normal tagged human chromosomes in microcell fusions directly into the DU145-N19 cell line. They were also fused to an immortal rodent cell line to provide a permanent panel of mouse-human hybrids. The latter is more easily analyzed and allows the

identification of the tagged chromosome prior to microcell transfer into another cell line (see section 2.5).

2.4 Analysis of DU145-N19

2.4.1 Karyotype and FISH

DU 145 cells were previously described as hypotriploid to tetraploid with abnormalities including breaks, dicentrics, minutes and large telocentric markers. The modal chromosomal number was 64 (ranging from 46-143 chromosomes) at passage 59. The heterogeneous nature of cells in culture made it mandatory to analyze the particular subclone to be used in fusion experiments. The DU145-N19 subclone was karyotyped to facilitate the identification of introduced chromosomes after microcell fusion (Figure 2-1). Several spreads were analyzed, and the different chromosomal contents demonstrated that the subclone was already changing in culture. The average number of chromosomes was 60 (Table 2-2). The average karyotype corresponds very closely to the originally described karyotype of DU 145 cells.

2.4.2 Tumorigenicity assays

Nude mice were first described in detail by Flanagan in 1966.¹⁵³ The hairless phenotype results from an inherited developmental defect which is determined by an autosomal recessive mutation. This mutation arose in a closed but not deliberately inbred stock of albino mice. It was later found that the homozygous nude mice also

Figure 2-1: G-banded metaphase chromosome spread of the DU145-N19 subclone. Metaphase spreads were obtained upon colcemid and hypotonic treatment of the cells and G-banded using Seabright's trypsin-Giemsa technique,¹⁵⁰ as described in the materials and methods section. The complex cytogenetic pattern corresponds to the originally described karyotype of DU 145 cells.⁶⁵

DU145-N19

1	2	3	4	5
6	7	8	9	10
11	12	13	14	15
16	17	18	19	20
21	22	X	M	

Table 2-2: Karyotype analysis of different DU145-N19 cells. Several cells were analyzed by G-banding and the number of each chromosome was counted. Marker chromosomes which could be partly recognized as a known chromosome were included in the count of that chromosome. Different spreads analyzed were designated a, b, c, d, e, f, and g. N19 = DU145-N19 spreads; chr# = Total number of chromosomes in the cell; M = Marker chromosomes.

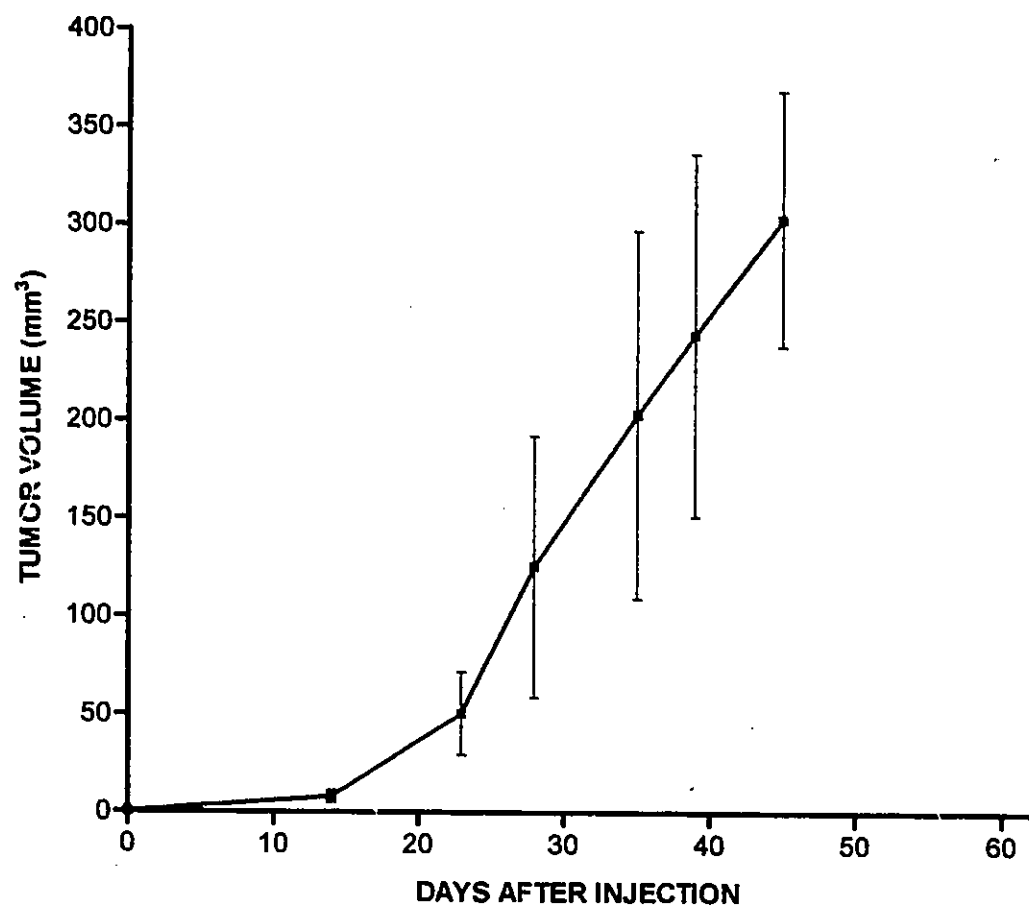
N19	Chr #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	M
a	60	2	4	3	1	3	3	2	2	1	3	2	2	3	3	2	3	3	2	2	1	2	2	2	7
b	60	2	4	3	1	3	3	3	2	3	1	3	2	2	3	4	3	3	1	2	1	1	2	1	8
c	61	3	3	3	1	1	3	3	2	3	1	3	2	3	2	5	3	3	2	2	1	2	2	2	6
d	60	3	4	2	1	3	3	3	2	1	3	2	2	4	2	3	3	3	2	2	2	2	2	1	6
e	58	2	3	3	1	3	3	2	2	4	1	3	2	4	3	3	3	3	1	2	1	1	2	1	6
f	63	3	3	2	1	3	5	2	3	3	1	3	2	3	2	4	3	3	2	2	1	2	2	2	6
g	60	3	4	3	1	3	3	3	2	3	1	2	2	3	3	3	3	3	2	2	1	1	2	1	5
Modal #	(60)	3	4	3	1	3	3	3	2	3	1	3	2	3	3	3	3	3	2	2	1	2	2	1	6

suffered from a congenital failure to develop a normal thymus gland, resulting in a complete deficiency in thymus-dependent (T-cell) immunological functions.¹⁵⁴ For the general purpose of cellular tumorigenicity assays of cultured animal cells, nude mice offer the best system presently known.

DU 145 cells possess the ability to form solid tumors when injected subcutaneously (s.c.) into nude mice. The injection of 6×10^6 DU145-N19 cells per site resulted in the formation of a tumor with a latency period of 2 weeks. After approximately 2 months, the tumors reached a size ($>1\text{cm}^3$) which could be uncomfortable for the mice. The latter were consequently euthanized at that time. In initial experiments, 6 tumors were formed out of 6 injection sites. In a second set of injections of cells at later passage, 4 tumors were formed out of 4 injections. Tumor growth rates were assessed by measurement of the tumor sizes using a caliper (Figure 2-2). DU145-N19 cells also demonstrated anchorage-independent growth capacity, as assayed by growth of cells in 0.3% soft agar. As shown in Table 3-1 (chapter 3), an average $2.24 \pm 0.42\%$ foci were formed per petri dish.

These results demonstrated that DU145-N19 cells are tumorigenic in nude mice in a relatively short period of time and that they display anchorage-independent growth. We concluded that DU145-N19 cells were satisfactory recipients for microcell fusions to assess the suppressive effects of transferred normal human chromosomes.

Figure 2-2: Tumor growth curve of DU145-N19. Each point on the graph represents the average volume of 4 tumors produced upon the injection of 6×10^6 cells in either the shoulders or thighs of athymic nude mice. Tumor volumes were calculated according to Dethlefsen et al,¹⁴⁹ using the assumption that the tumors were hemiellipsoids and taking into account skin thickness.



2.5 Generation of selectable mouse/human hybrid panel

A panel of mouse/human microcell hybrids was constructed, containing individual human chromosomes tagged with a dual selectable marker (tgCMV/HyTK) conferring hygromycin B resistance and GCV sensitivity.¹⁴⁷ This panel provided the access to specific tagged normal chromosomes in a background of immortal cells. These hybrids also have the advantage of ease of manipulation and analysis.

Briefly, microcells were produced from the HFHT4 pool of tagged fibroblasts (see section 2.3.3) and subsequently fused to B78 mouse melanoma cells in a confluent monolayer. They were refed with selective media containing hygromycin B and 3 μ M ouabain to ensure sole survival of tagged hybrids. Over 500 independent microcell hybrids (B78MC) were generated and more than 200 were individually picked and expanded. The human chromosome content of several B78MC hybrids has been determined and almost every human chromosome is represented in this panel.

2.6 Analysis of B78MC9-5

2.6.1 Identification of human content

Based on G-banded chromosomes spreads of the B78MC9 mouse/human hybrid, the latter was initially thought to contain chromosome 16. Since this chromosome was implicated in PC progression,^{79,80} we immediately transferred the tagged chromosome

into the DU145-N19 prostate cancer cells (see chapter 3). While waiting for the tumorigenicity results, this hybrid was further analyzed to validate the fact that chromosome 16 was indeed present. The heterogeneity displayed by DU145-N19 cells and the presence of numerous marker chromosomes rendered the identification of extra fragments of chromosomes by G-banding impossible. Therefore, we transferred the human tagged chromosome present in B78MC9 to PA1 teratocarcinoma cells which displayed a stable diploid female karyotype with a single, balanced translocation between chromosomes 15 and 20.¹⁵⁵ One of the clones obtained (PAB9-3), was analyzed by FISH using the chromosome 16 painting probe. Hybridization was only observed on the pair of chromosome 16s of the PA1 cells (Figure 2-3) Hence, B78MC9 cells contained a human chromosome distinct from chromosome 16.

To determine the human chromosome content of B78MC9, we performed Southern analysis using probes specific for chromosomes known to be involved in PC or on chromosomes known to contain tumor suppressor genes. Probes specific for chromosomes 7p, 8p, 12q, 17p (*p53*), and 13q (*Rb*), were hybridized to genomic DNA of DU145-N19 (positive control), B78 (negative control), and B78MC9. The probe localized on 12q13 was the only one able to hybridize (faintly) to B78MC9 DNA (Figure 2-4).

The novel method of *Alu*-PCR fluorescent *in situ* hybridization (*Alu*-PCR FISH) became available at the time and a protocol was adapted from Dorin et al.¹⁵¹ This

Figure 2-3: FISH of PAB9-3 with a chromosome 16 painting probe. The tagged human chromosome from B78MC9 was transferred by microcell fusion into the PA1 teratocarcinoma cell line. A chromosome 16 painting probe (Oncor) was hybridized to one of the hybrids, PAB9-3. Only the endogenous pair of chromosome 16 was painted.

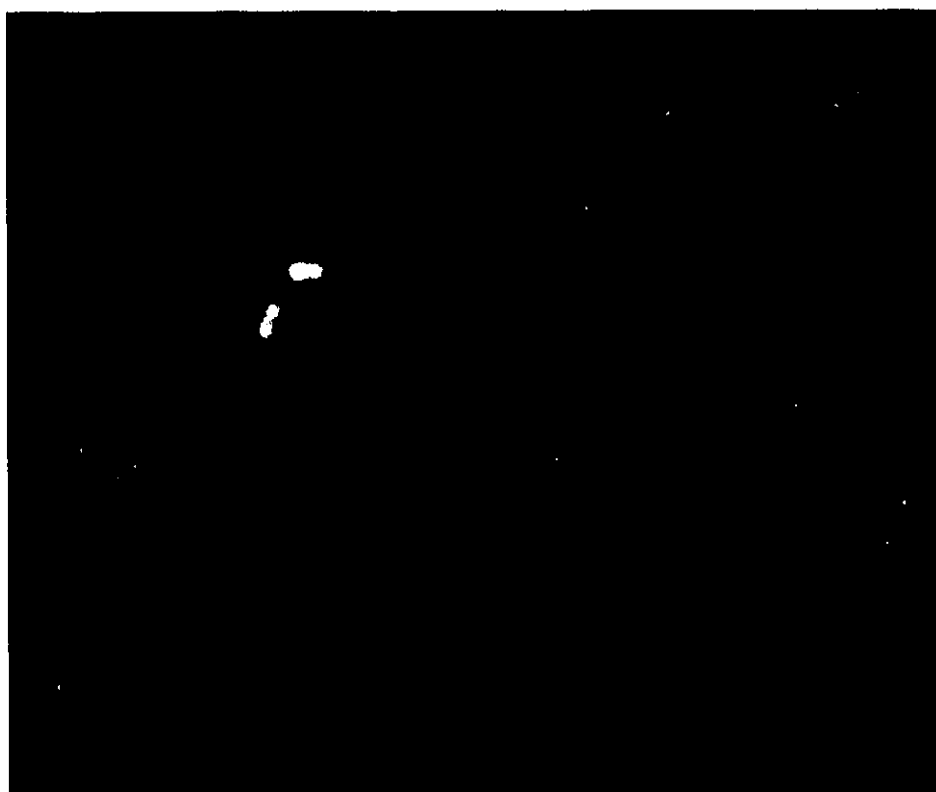
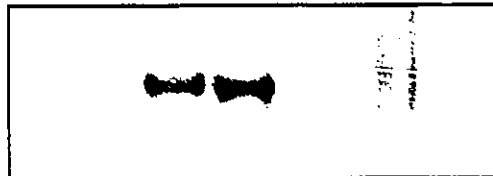


Figure 2-4: Southern analysis of B78MC9 using human chromosome-specific probes. Genomic DNA (10 μ g) from the following cells was digested with EcoRI and probed with different human chromosomal markers (indicated on the right): (a) Human PA1; (b) Human DU145-N19; (c) Mouse B78; (d) Mouse/human hybrid B78MC9. The arrow indicates a faint hybridization signal observed with the 12q-specific marker.

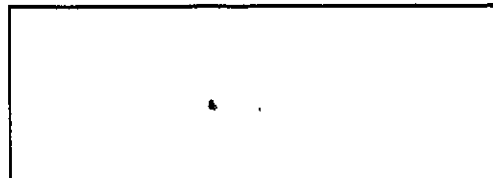
a b c d



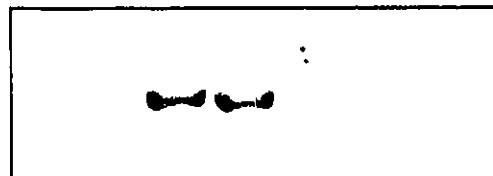
17p (p53)



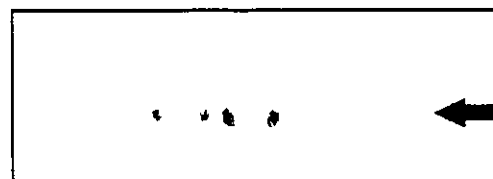
13q (Rb)



7p



8p21



12q13

Figure 2-5: PCR analysis of B78MC9 using chromosome 8 and 12-specific primers. Genomic DNA (10ng) from each of the following cell lines were amplified using the polymerase chain reaction (PCR) with human primers specific to either chromosome 8 (PENK) or chromosome 12 (D12S43). The amplicons were resolved on a 2% NuSieve agarose gel along with the 100bp ladder molecular weight markers. The arrows indicate the approximate amplicon sizes expected for each primer set.

Hsa 8

Hsa 12

100 bp ladder

PA1N5

DU145-N19

B78

B78MC9

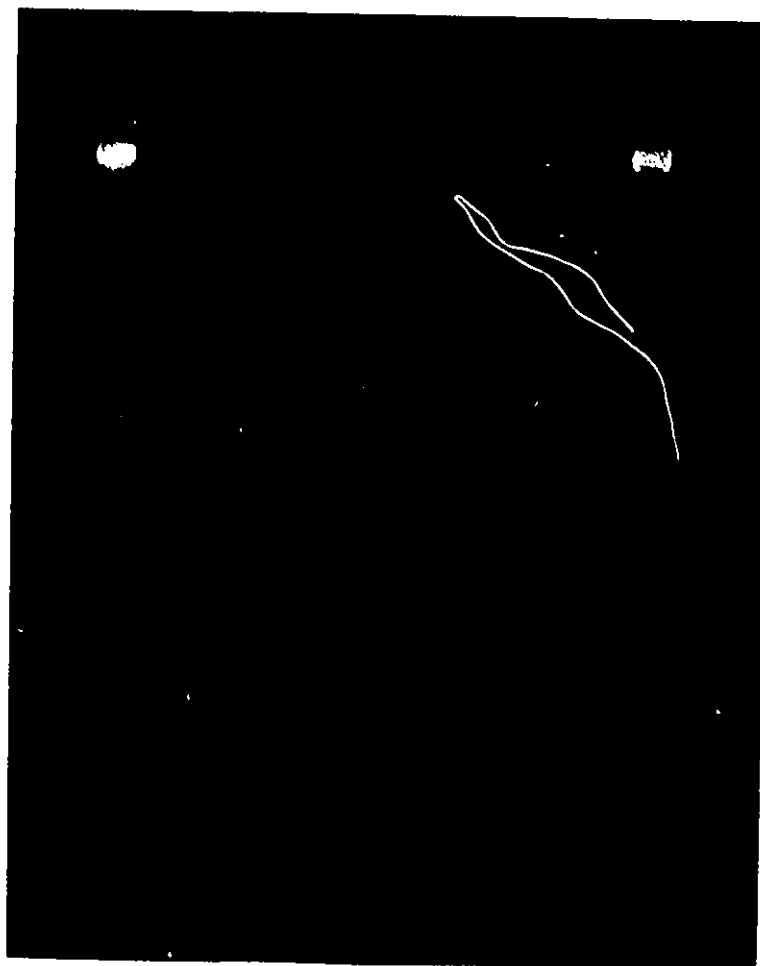
PA1N5

DU145-N19

B78

B78MC9

100 bp ladder



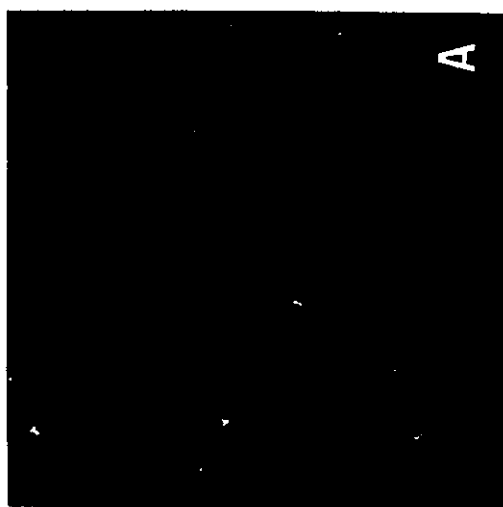
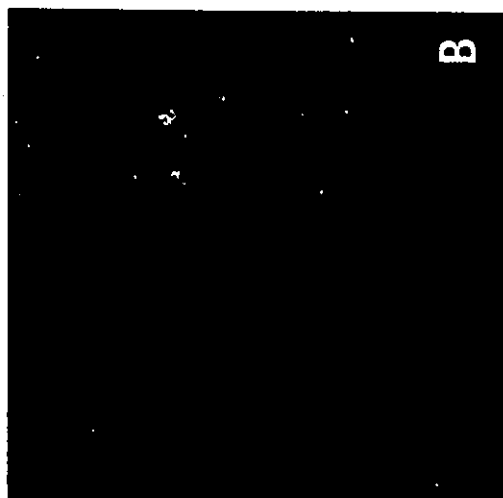
method allowed the specific amplification of human DNA from B78MC9 cells using *Alu*-specific primers and the subsequent hybridization of the biotinylated products to normal metaphase chromosome spreads. We observed painting along the entire length of a single chromosome pair with the morphology of either chromosome 8 or 12. To resolve between the two chromosomes, PCR analysis was performed using chromosome 8 or 12-specific primers and confirmed that the human component of B78MC9 was chromosome 12 (Figure 2-5).

2.6.2 Production and mapping of the B78MC9-5 subclone

Although *Alu*-PCR FISH revealed that B78MC9 contained a whole chromosome 12, G-banding analysis suggested that the tagged chromosome was much shorter. To elucidate this discrepancy, we performed FISH on B78MC9 with a total human DNA probe. This analysis established that two thirds of the cells contained a small piece of centromeric human chromosome (Figure 2-6A), while an additional acentric human element was also present in the remaining one third of the cells. These findings suggested that B78MC9 is a heterogeneous population of cells. All the cells contained a centromeric tagged portion of chromosome 12, and a minority of the population also contained an additional acentric (untagged) fragment of chromosome. To obtain a population consisting solely of cells containing the tagged piece of chromosome 12, B78MC9 cells were seeded sparsely enough to pick individual clones (B78MC9-1 to B78MC9-10). Ten subclones were screened by PCR analysis using four different CA

Figure 2-6: Analysis of the mouse/human hybrid subclone B78MC9-5.

(A) Biotinylated total human DNA was hybridized to a chromosome spread of the mouse/human hybrid subclone B78MC9, demonstrating the presence of only one tagged human chromosome in that hybrid. (B) FISH of *Alu*-PCR products of B78MC9-5 to a normal chromosome spread. B78MC9-5 genomic DNA was amplified using human-specific *Alu* primers. The human-specific amplicons were biotinylated and subsequently hybridized to normal chromosomes. The hybridization signal corresponds to 12pter-12q13.



repeat microsatellite primers (MapPairs) specific for different regions along chromosome 12. Six out of the ten subclones lacked part of the q arm, indicating that the acentric untagged chromosome 12 elements, had been lost in these cells. The remaining four subclones contained all four regions, suggesting that they contained both pieces of chromosome 12. One of the subclones lacking the acentric q arm element but retaining the tagged centromeric fragment (B78MC9-5) was chosen for further analysis. Hybridization of biotinylated *Alu*-PCR B78MC9-5 amplicons to normal human male spreads, and G banding analysis demonstrated that the tagged piece of chromosome 12 present in B78MC9-5 corresponds to 12pter--12q13 (Figure 2-6B). This was confirmed by PCR analysis using chromosome 12-specific primers. The isolation of B78MC9-5 shows that the B78MC hybrids can be purified through passaging and subcloning to allow the emergence of the tagged human chromosome as the only human element in the hybrid.

2.6.3 Confirmation of dominant negative selectability

Retention of the tagged human chromosome in the B78MC9-5 hybrid was achieved through dominant positive selection with hygromycin B during routine cell culture. To verify that the hybrid is susceptible to dominant negative selection as well, its sensitivity to GCV was assessed. In the presence of GCV, most cells died rapidly, leading to the emergence of rare colonies displaying resistance to GCV. To determine if the resistant colonies had lost the tagged human chromosome or had simply emerged

from the inactivation of the HyTK fusion gene, several colonies from GCV-treated cultures of B78MC9 were picked and expanded (B78MC9-R). PCR amplification of B78MC9-R hybrids using chromosome 12 specific primers (vWF locus) showed that this locus was no longer detectable. Complete removal of the tagged portion of chromosome 12 from the B78MC9-R subclones was confirmed by total human DNA FISH analysis. In all cases analyzed, GCV selection resulted in the specific loss of the tagged human chromosome from the hybrid cells. These results suggested that the segregation of the tagged chromosome would also be possible in the DU145-N19 background.

CHAPTER 3
CHROMOSOME TRANSFERS INTO DU145-N19

3. CHROMOSOME TRANSFERS INTO DU145-N19 CELLS

3.1 Introduction

The introduction of normal chromosomes into tumor cells by whole-cell or microcell fusion-mediated transfers are powerful techniques to identify putative tumor suppressor genes. In a set of preliminary experiments, we have performed whole cell fusions between two prostate cancer cell lines, PC3 and DU 145. This was done to determine whether complementation of defective genes could occur between these two metastatic cell lines and hence suggest the presence of more than one tumor suppressor gene. Due to the karyotypic complexity of the hybrids, such studies yield limited information and cannot easily reveal the identity of the chromosomes involved in the complementation.

To increase the resolution of whole cell fusion studies, we next performed several microcell fusions to independently transfer human chromosomes 3, 8 and 15, and del(12)(q13) into the human prostate cancer cell line, DU145-N19. We showed that while an extra copy of chromosome 3 or chromosome 15 had no effect on the *in vivo* tumorigenicity of these cells, microcell hybrids in which chromosomes 8 or del (12)(q13) was transferred exhibited marked suppression of tumorigenicity in athymic nude mice.

The presence of a dual selectable marker facilitated the selection for cells having segregated chromosomes 8 or del(12)(q13). Loss of these chromosomes in various "revertants" led to re-expression of the tumorigenic phenotype. These results demonstrated

that genes on human chromosomes 8 and 12 function as tumor suppressors of prostate carcinogenesis.¹⁰⁶

3.2 Materials and methods

Cell lines and culture conditions. The DU 145 cell line (obtained from the American Type Culture Collection, Rockville MD, USA) and all other cells were maintained in monolayer culture at 37°C under 5% CO₂ in media supplemented with 10% fetal bovine serum (FBS). DU145-N19, obtained upon electroporation with pSV2neo plasmid, was grown in minimum essential medium (MEM) containing 800 µg/ml G418 and used as recipient for all microcell fusions. Human skin fibroblasts were established from foreskins and grown in Dulbecco's modified Eagle's medium/Ham's F12 (DME/F12 1:1 medium). 2x10⁷ cells were electroporated with plasmid tgCMV/HyTK which confers resistance to hygromycin B and sensitivity to ganciclovir.

Whole-cell fusions. To generate the DP1 whole cell hybrids, 1.5x10⁶ cells each of PC3-N5 (G418^r) and DU145-H58 (hygromycin^r) were mixed and plated in six 25cm² flasks. Fusion was performed 24 hours after plating, using six different conditions, including 48%, 50% and 52% polyethylene glycol (PEG) with or without 10% DMSO for 1min. The cells were rinsed three times with serum-free MEM and fed with MEM containing 10% FBS. The day following the fusion, each flask was split into two 75cm² flasks and fed with selective media (MEM + 10% FBS + hygromycin B (400 µg/ml) + G418 (800 µg/ml). DP1

clones were picked from the best fusion conditions which were 50% PEG/10% DMSO and 52% PEG without DMSO. To generate the DP2 hybrids, 1.9×10^6 cells of each PC3-N14 and DU145-H53 were mixed and plated in six 25cm² flasks. The cells were fused 24 hours later using different conditions including 50%, 52% and 54% PEG for 90 or 120s. The next day, each fusion was split into three 75cm² flasks fed with selective media (same as with DP1). Colonies were picked and expanded for further analysis.

Microcell-mediated chromosome transfer. All microcell fusions were performed as described by Fournier.¹⁵ To transfer human chromosome 12, microcells were produced upon colcemid treatment (0.06mg/ml) of the mouse/human hybrid B78MC9 (containing a tagged portion of chromosome 12). These microcells were fused (90s in 54% PEG) to a monolayer of DU145-N19 cells. Microcell hybrids were selected in MEM containing G418 (800 µg/ml) and hygromycin B (400 µg/ml). After 24 days, thirteen hybrid clones were picked and expanded. Chromosome 3 was transferred into the DU145-N19 cells by fusing microcells obtained from the tagged human skin fibroblasts population (90s in 54% PEG).

Cell growth studies: To determine the doubling time of the hybrids and segregants, 2×10^5 cells were seeded and the cells counted every day for 5 or 6 days. Doubling time was determined during log phase growth, using values obtained from three experiments. To determine the anchorage-independent growth, trypsinized cells (5×10^4) were plated in agar (top layer: 3 ml, 0.3% noble agar; bottom layer: 3 ml 0.6% agar; both in MEM with 10% FBS) in 60 cm² tissue culture dishes. Cells were fed at weekly intervals with 1 to 2 ml of

MEM supplemented with 10% FBS. Colonies of cells growing in soft agar were scored after 21 days in culture. The values are means of 3 dishes.

Nude mice injections. Injections and measurements of tumor volumes were performed essentially as described in chapter 2. Suspensions of $4 \text{ to } 6 \times 10^6$ cells in a volume of 0.3 ml of sterile saline were injected subcutaneously into 4-6 week-old male athymic nu/nu mice. All animals were monitored on a weekly basis up to 6 months after injection. Cells were considered to be non-tumorigenic if no tumors were seen by 3 to 6 months after inoculation.

Fluorescence *in situ* hybridization. FISH analysis was performed as described in chapter 2.

3.3 Whole cell fusions

3.3.1 PC-3 and DU 145 fusions

To determine whether more than one tumor suppressor gene is inactivated in prostate cancer, two different prostate cancer cell lines known to form tumors in nude mice were fused to generate whole cell hybrids. Loss of the tumorigenic phenotype of resulting hybrids could be interpreted as evidence that at least two different tumor suppressor genes are inactivated in each cell line. Complementation is occurring to eliminate the effect of the missing genes. Fusions between DU145-H58 and PC3-N5 yielded 18 different whole cell hybrids called the DP1 hybrids, of which only six

survived. Fusions between DU145-H53 and PC3-N14 yielded 16 different DP2 hybrids, of which only 6 survived.

3.3.2 Analysis of DP1 and DP2 hybrids

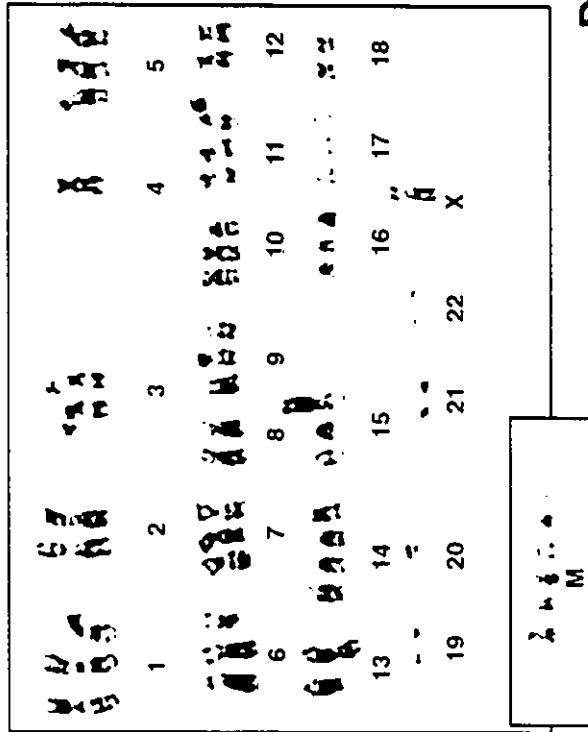
Karyotype analysis of DP2-20 confirmed that the DP hybrids are authentic whole cell hybrids. It contained a specific marker chromosome and a der(5) originating from PC3 cells and a normal chromosome 12 found in DU145 cells but not in PC3 cells (Figure 3-1).

To determine the anchorage-independent growth properties of the DP1 and DP2 hybrids, the cells were plated on soft agar. The parental cell lines could form colonies effectively, ranging from 0.1% to 3.4% (Table 3-1). The majority of whole cell hybrids could not form colonies in the same conditions, suggesting that they had lost their anchorage-independent growth ability. Some exceptions, such as DP2-9, DP2-24 and DP1-9 could be explained by the fact that some chromosomes which influence anchorage-independent growth may have been lost from these hybrids, a phenomenon commonly observed in whole cell hybrids. These results suggested that complementation of genes involved in anchorage-independent growth and possibly tumorigenicity occurred in the DP hybrids.

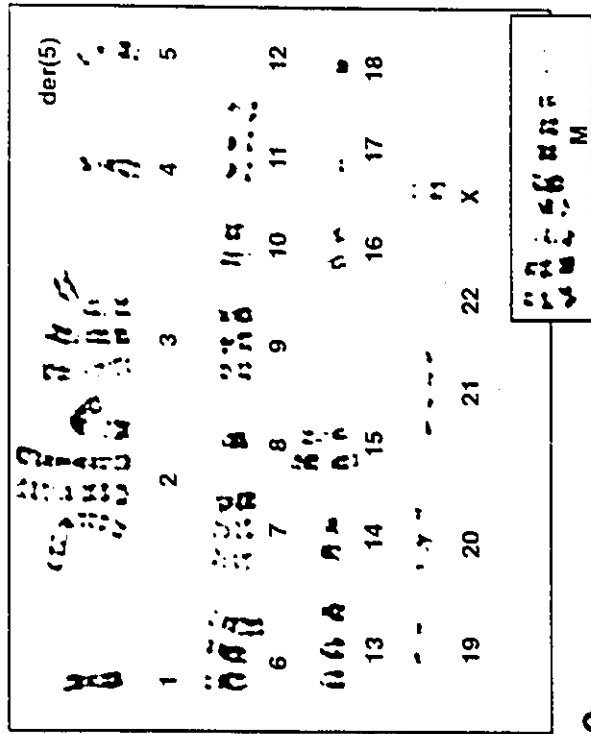
Upon injection of the DP hybrids in nude mice, only DP2-24 and DP2-9 exhibited the ability to form tumors. Thus, most DP hybrids lost the original tumorigenic potential

Figure 3-1: G-banded chromosomes of the DP2-20 whole cell hybrid. Metaphase chromosomes from the parental cells DU 145 and PC3 and of the DP2-20 whole cell hybrid were G-banded according to Seabright's method.¹⁵⁰ Arrows indicate a der5 chromosome originating from PC3 and a normal chromosome 12 originating from DU 145, demonstrating that genetic material from both parental cell lines is represented in this hybrid.

DU 145



PC 3



DP 2-20

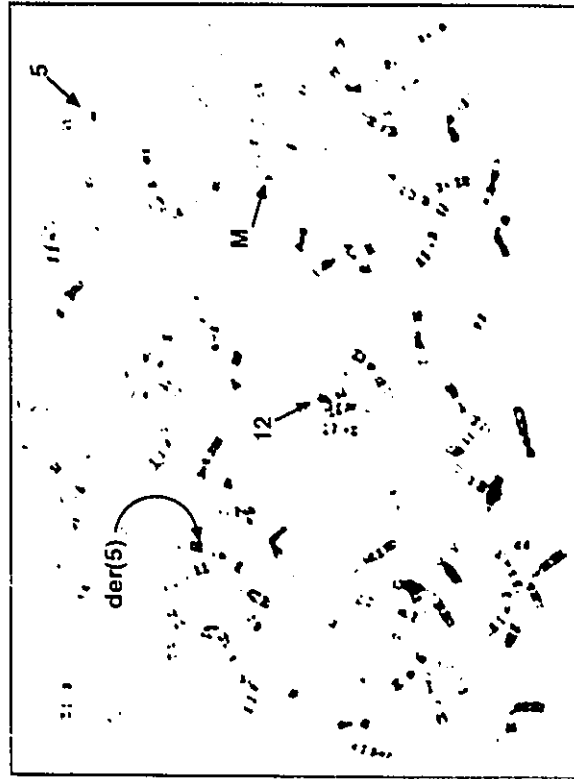


Table 3-1: Soft agar assay and tumorigenicity of DP hybrids.

Cell Line	Hsa #^a	% Growth in Soft Agar^b	Tumorigenicity^c
DU145-H53	58-60	0.1	N.D.
PC3-N14	57-62	0.6	N.D.
DP2-9	110-120	<0.1	Yes
DP2-14	95-100	0	No
DP2-20	100-110	0	No
DP2-24	91-100	0.8	Yes
DU145-H58	59-63	3.4	N.D.
PC3-N5	53-57	0.7	N.D.
DP1-4	150-160	0	No
DP1-9	90-95	1.0	No
DP1-10	75-80	0	No
DP1-12	100-110	0	No
DP1-14	100-110	0	No
DP1-17	90-100	0	No
DP1-20	90-100	0	No

^a Range of total number of chromosomes in cells of the parent or hybrid cells

^b Number of colonies divided by 50 000 cells expressed as a percentage

^c 4×10^6 cells were injected subcutaneously. The appearance of any size tumor was considered as tumorigenic. Mice were kept for nine months after the injection.

N.D. Not determined.

originally displayed by parental prostate cell lines, suggesting the presence of at least two complementing tumor suppressor genes in this system.

Although this fusion experiment demonstrated the presence of tumor suppressor genes acting in prostate cancer cell, it was not useful to identify specific chromosomes involved in suppression. Because they contained so many human chromosomes, the DP hybrids were highly heterogeneous, as several chromosomes were lost during passaging of the cells. Due to the complexity of the DP whole cell hybrids, microcell hybrids were produced to increase the resolution of the search for tumor suppressors.

3.4 Microcell-mediated transfers

3.4.1 Chromosomes 3, 8 and 15

In the first series of microcell transfers, the donor cells consisted of human skin fibroblasts (HSF) tagged with the tgCMV/HyTK plasmid. Microcells produced from these cells contained normal human tagged chromosomes. These microcells were subsequently fused to the DU145-N19 (G418^r) cancer cells. The resulting microcell hybrids, named DHM hybrids, were analyzed by karyotyping and FISH methods. Their anchorage-independent growth ability and their tumorigenic potential in nude mice were also assessed to determine whether the transferred chromosomes encode tumor suppressive elements.

Figure 3-2: G-banding analysis of DHM-5 and DHM-6 microcell hybrids. Metaphase chromosomes were G-banded according to Seabright's method.¹⁵⁰ (A) Chromosome spread of the DHM-5 microcell hybrid. The arrow indicates the presence of an introduced tagged normal chromosome 3 in this hybrid. (B) Chromosome spread of the DHM-6 microcell hybrid. The arrows indicate the presence of introduced chromosomes: two copies of chromosome 8 and one copy of chromosome 15.

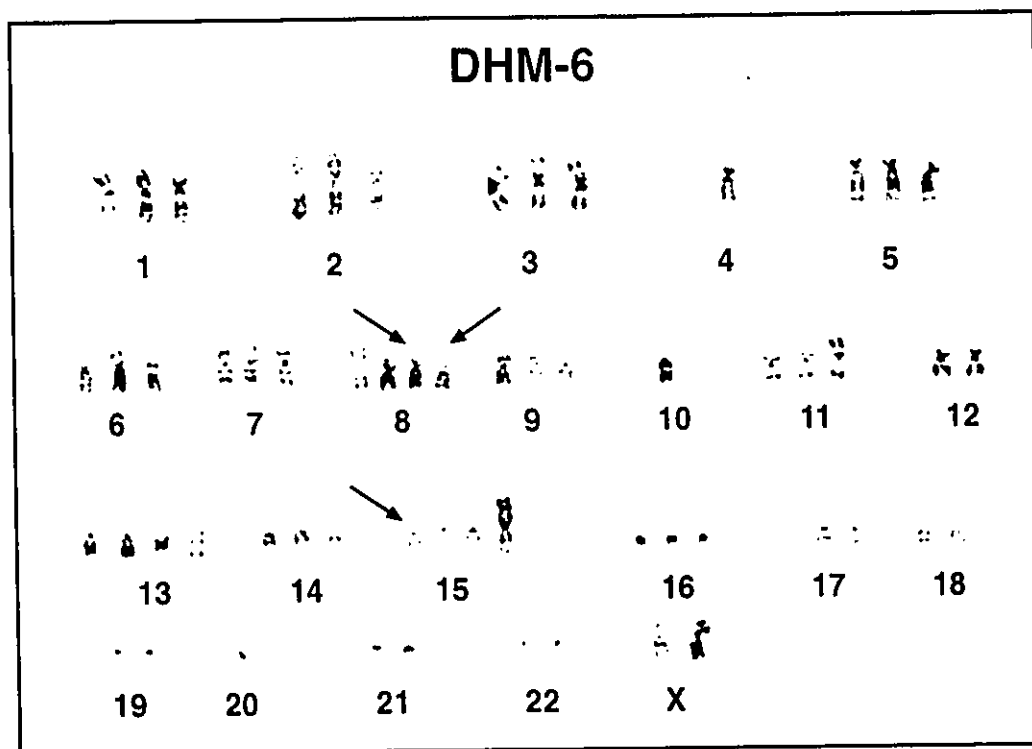
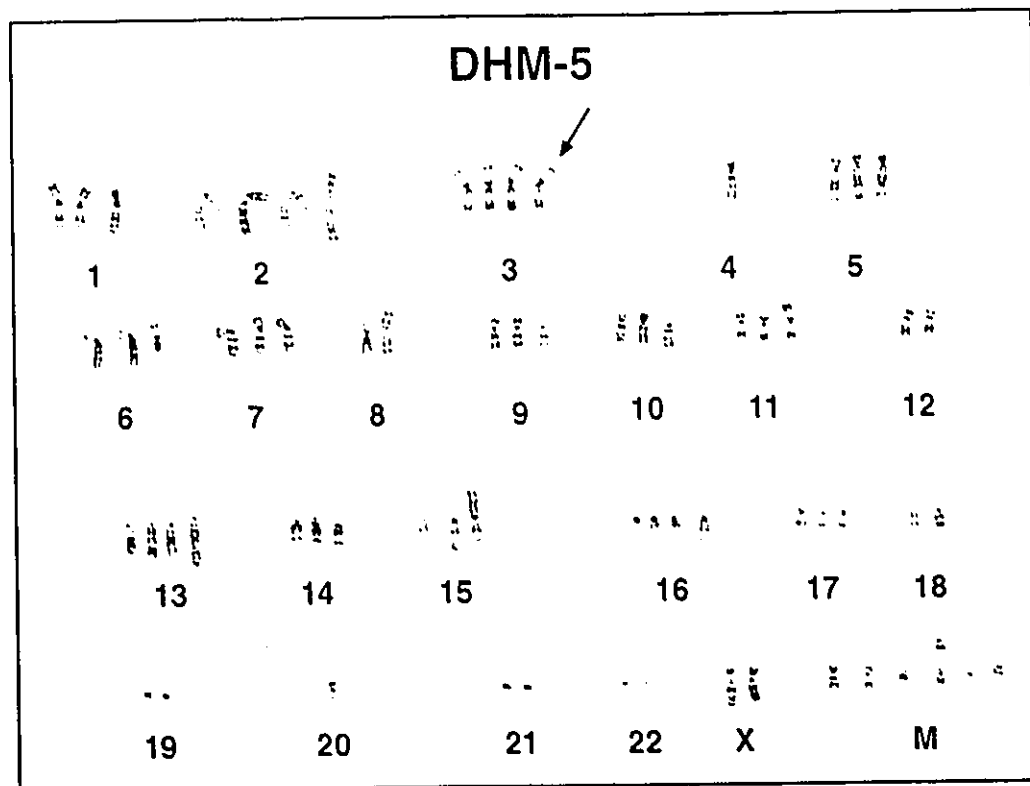


Figure 3-3: Chromosome 8 FISH analysis of DHM-6 hybrids. A chromosome 8-specific painting probe was hybridized to metaphase chromosome spreads of (A) DU145-N19; (B) DHM-6 and (C) DHM-6R1. In panel B, arrows indicate the two putative extra chromosomes 8 (identified only by morphology) that have been transferred into the DHM-6 hybrid..

3.4.1.a Validation of transfers

To determine which normal human tagged chromosome was transferred in each DHM hybrid, G-banded metaphase spreads were analyzed and compared to the karyotype of the parental DU145-N19 cells. In the DHM-4 and DHM-5 hybrids, the only karyotypic difference observed was the presence of an extra copy of chromosome 3 (Figure 3-2A). In the DHM-6 hybrid, however, two extra copies of chromosome 8 and an extra copy of chromosome 15 were observed (Figure 3-2B). The presence of the two extra normal chromosome 8 was confirmed by FISH analysis using a chromosome 8 painting probe (Figure 3-3).

3.4.1.b Generation of segregant clones

To determine which chromosome was tagged in the DHM-6 hybrid, these cells were exposed to GCV in culture. This selection allowed the growth of cells which had lost the tagged chromosome. Four segregant clones were obtained in this manner, and were named DHM6-R1, DHM6-R3, DHM6-R4 and DHM6-R6. Each of these segregant clones was analyzed by karyotypic and FISH analyses. Segregant clones R3, R4, and R6 lost one copy of chromosome 8 while the extra chromosome 15 was retained. Segregant clone R1 lost either one or two copies of chromosome 8 but still contained the extra

chromosome 15. These results suggested that one of the extra chromosome 8 in DHM-6 was tagged.

3.4.1.c Tumorigenicity in nude mice

The tumorigenic potential of the DHM hybrids was assessed by subcutaneous injections in nude mice (Table 3-2). While DHM-4 and DHM-5 hybrids (containing an extra chromosome 3) developed tumors in nude mice, the DHM-6 hybrid did not. The DHM-6R segregant clones were also injected. The only segregant clone that regained the ability to form tumors in nude mice was the DHM-6R1 hybrid which had lost one or both copies of chromosome 8.

**Table 3-2:
Tumorigenic properties of DHM hybrids**

Hybrid	Tumorigenicity ^a	Introduced fibroblast chromosomes ^b
DHM4	3/4	Hsa3
DHM5	4/4	Hsa3
DHM-6	0/4	2(Hsa8) + Hsa15
DHM-6R1	4/4	Hsa15
DHM-6R3	0/5	1(Hsa8) + Hsa15
DHM-6R4	0/3	1(Hsa8) + Hsa15
DHM-6R6	0/7	1(Hsa8) + Hsa15

^a Number of tumors formed / number of sites inoculated.

^b As determined by G-banding analysis and/or FISH

These results suggested that normal chromosome 8 acts to suppress tumorigenicity in the DHM-6 hybrid while its loss in DHM-6R1 causes a reversion to the

original tumorigenic phenotype of DU145-N19 cells. While chromosomes 3 and 15 cannot suppress tumorigenicity, chromosome 8 could encode a tumor suppressor inactivated in prostate cancer.

3.4.2 Chromosome del(12)(q13)

3.4.2.a Transfer

As described in chapter 2, the B78MC9 mouse-human hybrid contains a tagged portion of human chromosome 12 designated del(12)(q13). Microcell fusion was used to transfer chromosome del(12)(q13) into DU145-N19. The presence of tagged chromosomes in three independent microcell hybrids (DBM9-4, DBM9-7 and DBM9-10) was determined by Southern analysis using tgCMV/HyTK as probe (Figure 3-4, lanes d, f, and h).

Alu-PCR FISH using the *Alu9* probe (chromosome 12-specific) on DU145-N19 chromosome spreads demonstrated that DU145-N19 cells contain two apparently normal copies of chromosome 12, and a marker chromosome consisting of the long arm of chromosome 12 translocated to part of chromosome 11 (Figure 3-5B). This particular chromosome marker is also present in DU 145 (Figure 2-1). The hybridization pattern obtained for DBM9-4 (Figure 3-5C) and DBM9-7 (Figure 3-5E) microcell hybrids is similar to that of DU145-N19, except that an additional centromeric segment of chromosome 12 is present in all the cells analyzed. Similar results were obtained for DBM9-10, except that the extra piece transferred has been translocated to a chromosome of the recipient cell line.

Figure 3-4: Southern blot analysis of the hybrid cell lines and segregants. Genomic DNA (10 μ g) from the following cells was digested with Eco R1 and probed with the tgCMV/HyTK plasmid DNA: (a) mouse B78, (b) B78MC9, (c) DU145-N19, (d) DBM9-4, (e) DBM9-4R, (f) DBM9-7, (g) DBM9-7R, (h) DBM9-10, (i) DBM9-10R. The arrow indicates the tgCMV/HyTK internal 1.85kb Eco R1 fragment.

a b c d e f g h i

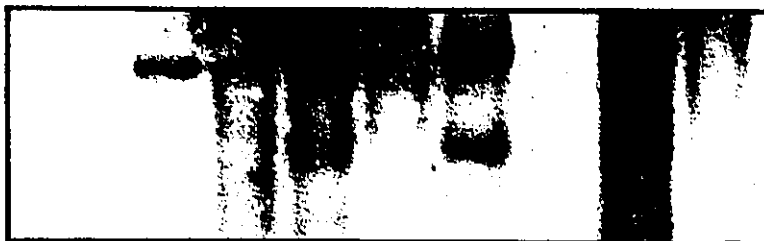
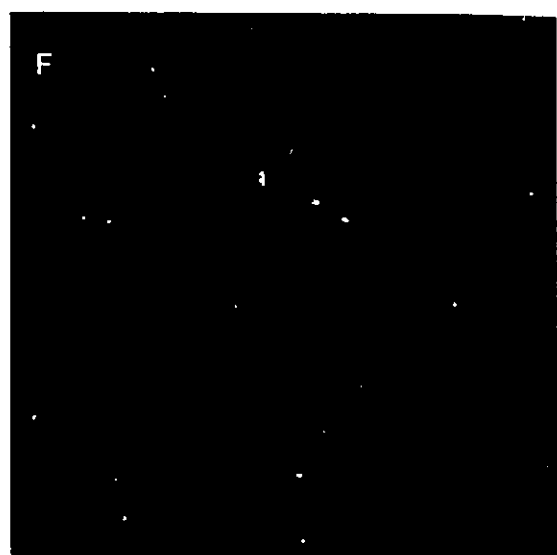
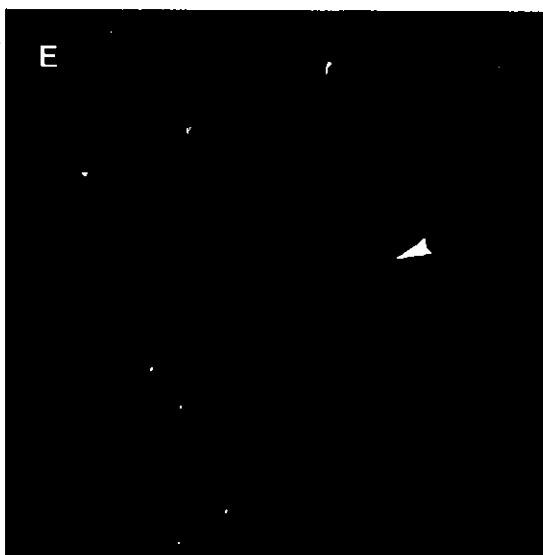
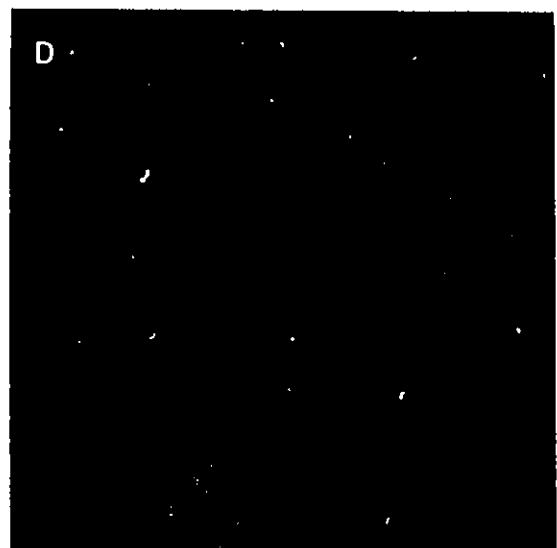
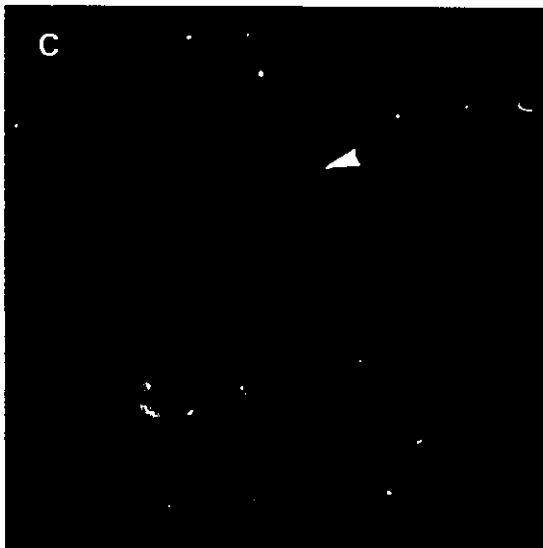


Figure 3-5: Chromosome 12 FISH analysis of hybrid cells and segregants. The *Alu9* probe (specific for human chromosome 12) was hybridized to metaphase spreads of the following cells: (A) DU145-N19; (C) DBM9-4; (D) DBM9-4R; (E) DBM9-7; (F) DBM9-7RTD. The arrows in panels (C) and (E) indicate the chromosome del(12)(q13) transferred by microcell fusion. Panel (B) depicts G-banded chromosome 12 material specific to DU145-N19. The portion above the arrow represents part of chromosome 11 and the portion below the arrow represents part of 12q.



B



Even though chromosome del(12)(q13) might have been further fragmented during the transfer and has not been completely characterized in each DBM9 hybrid, we will continue to refer to the tagged transferred portions as del(12)(q13) since they were derived from that region.

3.4.2.b Generation of segregant clones

To confirm that the introduction of chromosome del(12)(q13) was the cause for the observed suppression (and to show that the suppressed phenotype was not due to the transfer of a mouse chromosome), the DBM9 hybrids were challenged with media containing 10 μ M GCV, which killed cells expressing the tgCMV/HyTK marker, thus allowing selection for cells having lost chromosome del(12)(q13). Three segregants obtained in this manner (DBM9-4R, DBM9-7R and DBM9-10R) reverted to complete hygromycin sensitivity (Table 3-3). Southern blot analysis using tgCMV/HyTK probe (Figure 3-4, lanes e, g, and i), confirmed that the tag present in the DBM9 hybrids was selectively lost in the respective segregants. *Alu*-PCR FISH on the DBM9-4R segregant with the *Alu9* probe demonstrated that the introduced chromosome del(12)(q13) was in fact lost in 100% of the cells analyzed (Figure 3-5D). Similar results were obtained for DBM9-7R, although 30% of the cells analyzed still retained the introduced chromosome del(12)(q13).

3.4.2.c Tumorigenicity in nude mice

The anchorage-independent growth, as assayed by growth in soft agar, was decreased in the hybrids, although no difference is apparent between the hybrids and their segregants (Table 3-3). Upon initial subcutaneous injections in athymic mice, the DBM9-4, DBM9-7 and DBM9-10 hybrid cells showed complete loss of their tumor-forming ability. Whereas the DU145-N19 cells showed tumor formation within a month after injection (6 tumors out of 6 injection sites), no tumors were observed upon injection of the DBM9 hybrids (0 tumors out of 13 injections), even 3 to 6 months after being inoculated (Table 3-3). These results indicate that the presence of the introduced portion of chromosome 12 eliminates the tumorigenic phenotype of DU145-N19 cells. In a second set of injections of the hybrids at higher passage, significant, although not complete suppression of tumorigenicity was observed, with DBM9-7 displaying the highest degree of suppression (Figure 3-6).

Unlike the DBM9 hybrids, the three segregants formed tumors (11 tumors out of 13 sites inoculated) within a month after injection into nude mice (Table 3-3). One of the tumors formed upon injection of DBM9-7R cells was resected and cultured *in vitro*. We had previously mentioned that DBM9-7R was not a complete revertant, since 30% of the cells analyzed by *Alu*-PCR FISH still retained the introduced chromosome del(12)(q13). Analysis of the tumor-derived cells (DBM9-7RTD) by *Alu*-PCR FISH using the *Alu9* probe

Table 3-3:
Growth and tumorigenic properties of DBM9 hybrids

Cells	Growth in Hyg B	CGT $\pm$ SEM (hours) ^a	% Soft agar growth $\pm$ SEM ^b	Tumorigenicity ^c	Latency period (months)	Presence of tag ^d
DU145-N19	-	35.6 $\pm$ 4.2	4.27 $\pm$ 0.42	6/6	<1	-
DBM9-4	+	35.6 $\pm$ 2.6	0.03 $\pm$ 0.03	0/5	6	+
DBM9-4R	-	31.3 $\pm$ 2.2	0.53 $\pm$ 0.23	4/4	<1	-
DBM9-7	+	42.7 $\pm$ 1.0	1.43 $\pm$ 0.23	0/4	6	+
DBM9-7R	-	51.3 $\pm$ 5.7	1.57 $\pm$ 0.45	3/5	<1	+/-
DBM9-7RTD ^e	-	32.8 $\pm$ 5.3	N.D.	N.D.	N.D.	-
DBM9-10	+	36.3 $\pm$ 1.1	0.45 $\pm$ 0.13	0/4	6	+
DBM9-10R	-	25.8 $\pm$ 3.0	0.49 $\pm$ 0.12	4/4	<1	-
DHM-5	+	N.D.	N.D.	4/4	<1	introduced chromosome 3

N.D. Not determined.

^a Cell generation time determined during log phase of growth, from triplicates.

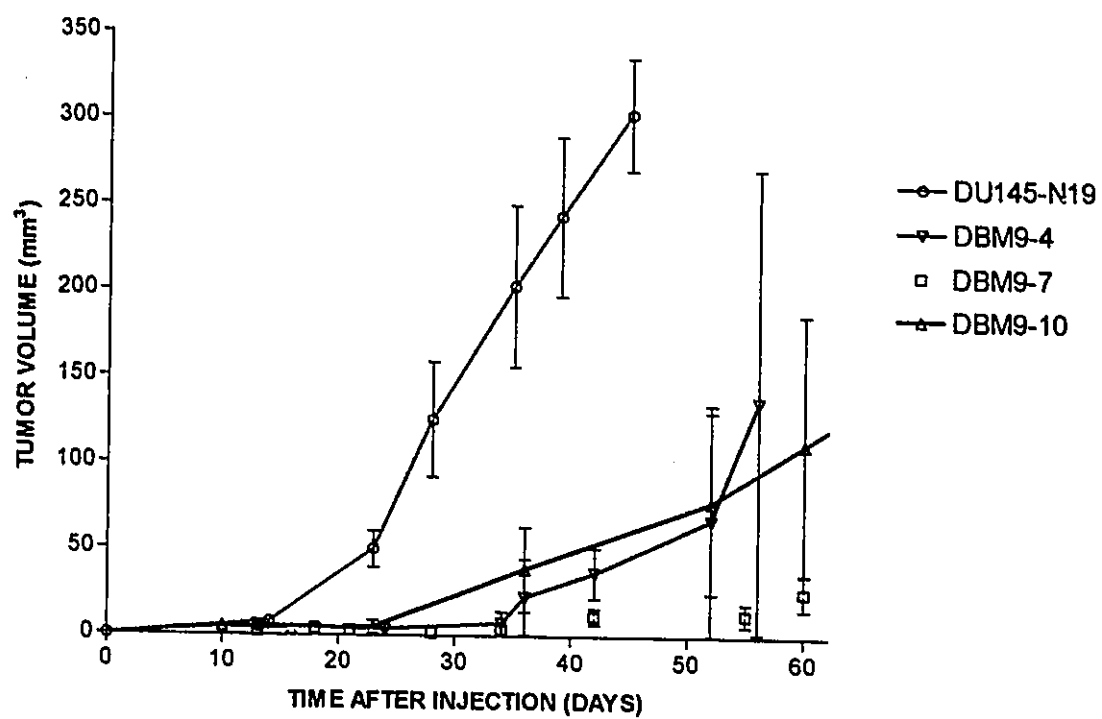
^b Number of colonies obtained divided by 50000 cells plated, expressed as a percentage.

^c Number of tumors formed / number of sites inoculated.

^d As determined by Southern blot analysis and/or A/ μ -PCR FISH.

^e Cells were resected from a tumor derived from DBM9-7R cells.

Figure 3-6: Tumor growth curve of DBM9 hybrids Each point on the graph represent the average volume of 4 tumors produced upon the injection of 6×10^6 cells in either the shoulders or thighs of athymic nude mice. Tumor volumes were calculated according to Dethlefsen et al,¹⁴⁹ using the assumption that the tumors were hemiellipsoids and taking into account skin thickness.



revealed that 100% of the cells analyzed had lost chromosome del(12)(q13), suggesting that the tumor originated from cells having lost the tagged chromosome (Figure 3-5F).

We conclude that selective loss of the introduced chromosome del(12)(q13) in three distinct microcell hybrids restored the original tumorigenic potential of DU145-N19 cells, demonstrating that suppression of tumorigenicity was in fact caused by the introduction and retention of chromosome del(12)(q13).

3.5 Discussion

A growing body of evidence suggests that multiple tumor suppressor genes are lost or inactivated in a given tumor. Frequent losses of heterozygous alleles have been reported for multiple chromosomes in a wide array of tumor types. Recently, several putative tumor suppressor genes have been implicated in the progression of PC, supporting the idea that, as in colorectal cancer, numerous tumor suppressor loci are inactivated during prostate carcinogenesis. Not surprisingly, in humans, both types of cancer occur after a long incubation period, indicating that many events are necessary for the development of the full tumorigenic phenotype.

The data described in this chapter suggest the presence of other putative tumor suppressor loci, located on chromosomes 8 and 12, which completely suppress the tumorigenicity of DU 145 prostate cancer cells. Upon selection for the loss of these chromosomes in the suppressed hybrids, the cells regained their original tumor-forming ability, demonstrating that the presence or absence of these chromosomes in the hybrids

correlates with their tumorigenic phenotype. This dual selection confirms that the presence of loci on chromosome 8 or del(12)(q13) are responsible for the tumor suppression. It also makes it unlikely that the effects on tumorigenicity in different independently obtained hybrids and the corresponding revertants are due to the transfer and subsequent removal of an untagged mouse chromosome encoding a tumor suppressor gene.

The transfer of chromosome 8 into DU145-N19 had some effect on the morphology of these cells, rendering them flatter and less refractile. This could imply an effect of the tumor suppressor gene on cell adhesion. Loss of heterozygosity studies have already implicated chromosome 8 in PC progression. Deletions in PC samples have been mapped to three distinct regions of the short arm (8p22⁸¹, 8p21⁸² and 8p12-21⁸³), indicating the presence of three distinct putative TSG on this chromosome. Allelic loss at 8p has also been observed frequently in several human tumor types including pancreatic, gastric, bladder, breast, colorectal, non-small cell lung, and hepatocellular cancer.⁸⁴

The introduction of a tagged chromosome del(12)(q13) did not affect the morphology of the cells, nor did it alter their anchorage-dependent growth except maybe in the DBM9-7 hybrid. The observation that the DBM9-4 and DBM9-10 hybrids have similar doubling times *in vitro* as the parental cells suggests, as it has been previously proposed, that *in vitro* growth behavior and tumorigenicity are under separate genetic control. A similar phenomenon has been observed upon transfer of chromosome 13 into DU 145¹⁰⁵.

The suppressive effect of chromosome 12 on tumorigenicity in other tumor models has not yet been demonstrated. For example, microcell fusion has previously been used to

transfer a human chromosome 12 in human fibrosarcoma cells (HT1080) without affecting tumorigenicity, suggesting some level of specificity for prostate cancer cells. In our own studies however, mouse-human B78MC hybrids containing chromosome 12 material consistently displayed reduced tumorigenicity in nude mice. One study by Brothman et al⁷⁴ did identify frequent loss of chromosome 12 material near the centromere using FISH probes on early stage PC samples. However, allelotyping studies have not yet linked chromosome 12 with PC. This could be explained if the inactivation in prostatic tumors is due to small deletions or point mutations rather than gross rearrangements. A more careful analysis, as it was done for the FCC locus (located on human chromosome 2) associated with hereditary nonpolyposis colorectal cancer¹⁵⁶ might help to implicate this chromosome 12 region in the development of human prostate cancer. Chromosome 12 aberrations detected by cytogenetic analyses have been reported in a small subset of cancers, namely in human germ cell tumors, acute myeloid leukemia, lipomas, and pleomorphic adenomas of the salivary gland.¹⁵⁷ Such rearrangements may cause the inactivation of genes on chromosome 12 which are involved in the etiology of these tumors..

Some evidence suggests that tumor suppressor genes such as *Rb* and *p53* may be involved in the progression of PC.¹⁰⁹ Due to a splicing mutation, the *Rb* gene is inactivated in DU 145 prostate cancer cells.¹⁰³ Transfer of a wild type *Rb* gene into these cells by cDNA retroviral infection effectively suppressed their tumorigenicity.¹⁰⁴ Similarly, the transfer of chromosome 13 containing a normal *Rb* allele markedly, but not completely, suppressed the tumorigenicity of DU 145 cells.¹⁰⁵ Both alleles of the *p53* gene are mutated

in the three prostate cell lines PC-3, Tsu-PR1 and DU 145. Transfection of the wild-type *p53* gene versus a mutated *p53* gene into PC-3 and Tsu-PR1 results in reduced colony formation.¹¹¹

Although these results suggest a role for *Rb* and *p53* in the progression of PC, these suppressor genes are only implicated in a subset of prostatic tumors, therefore indicating the involvement of other, as yet unidentified, tumor suppressor genes. It is interesting that the locus (or loci) present on chromosomes 8 and 12 effectively suppress tumorigenicity of the DU145-N19 cells despite the fact that both the *Rb* and *p53* genes are also mutated in this cell line. This implies that the putative suppressor genes can compensate for the absence of two well-established tumor suppressors, and suggests a role for the gene product in the same signal transduction pathways as P53 and RB or in a parallel redundant pathway. It is also possible that a change in gene dosage rather than complementation of inactivated alleles is responsible for the loss of tumorigenic characteristics. Regardless of the mechanism underlying the tumor suppression, this report adds support to the hypothesis that compounded losses of tumor suppressor genes contribute to prostate carcinogenesis.

CHAPTER 4

MAPPING THE TUMOR SUPPRESSOR REGION

4. MAPPING THE TUMOR SUPPRESSOR REGION

4.1 Introduction

Once a gene is assigned to a chromosome, the next step in the mapping procedure is to localize the gene to a region within the chromosome. In 1975, Goss and Harris introduced a method for the generation and transfer of chromosomal fragments.¹⁵⁸ This protocol, based on irradiation and transfer via cell fusion, involves the use of lethal doses of X-rays to break the human chromosome of interest into several fragments. These broken chromosomal fragments are rescued by fusion to a non-irradiated recipient cell line. The resulting radiation-reduced hybrids have proved an extremely powerful source both for targeted derivation of markers using *Alu*-mediated PCR amplification and for subregional high-resolution mapping. This method has been used extensively to isolate fragments of different human chromosomes in rodent backgrounds but was only fully developed as a mapping tool in 1990 by Cox et al.¹⁵⁹ By measuring the frequency with which pairs of linked genes are cotransferred after irradiation, it is possible to determine the linear order of groups of genes and to estimate the distance between them.

Irradiation and fusion gene transfer is a technique that spans the gap between limitations of molecular methods and somatic-cell genetics, allowing the separation of DNA fragments between 0.25 and 30 Mb in size. It is conceivable to use such methods in the present study to construct a panel of prostate cancer cells containing small fragments of normal chromosome 12. This panel of radiation-reduced hybrids could be screened for the cancer phenotype and the latter correlated to the presence or absence of

specific regions of that chromosome. The location of the tumor suppressor gene can be determined to be included or excluded from various regions of the chromosome by the pattern of phenotypic results obtained. Such an analysis has the potential to significantly increase the resolution of the disease gene map.

One uncertainty is apparent before attempting such a study: whether enough polymorphism exists between the chromosome 12 material which was introduced and that which was endogenous to the prostate cancer cells. Lack of polymorphism did hamper the mapping of the chromosome 12 fragments in the radiation hybrids. However, the problem was effectively circumvented by the microcell transfer into a rodent background of different tagged chromosome 12 fragments with predetermined phenotypic effects on the prostate cancer cells. The final map obtained after correlating both presence and absence of genetic markers and the tumorigenic phenotype revealed the location of a gene with tumor suppressive effects on prostate cancer cells.

4.2 Materials and Methods

Cell lines and cell culture. DU145-N19 is a G418-resistant prostate cancer cell line metastatic to the brain. DBM9-7 is a microcell hybrid generated previously from DU145-N19, containing an extra normal human del(12)(q13) tagged with the tgCMV/HyTK marker which allows positive and negative selection with hygromycin B and ganciclovir (GCV), respectively. All human cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum. All B78-derived hybrid cells were cultured in

Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FBS. Selection of tagged pieces of human chromosome 12 in DBM9-7 cells, the DU12RH radiation hybrids and the BRH mouse/human microcell hybrids was accomplished by growth in media supplemented with 400 µg/ml of hygromycin B. Cells were monitored regularly and found to be free of mycoplasma infection.

Production of radiation-reduced hybrids. The radiation hybrids were constructed as described by Cox et al.¹⁶⁰ Before confluency, DBM9-7 cells were trypsinized and 5×10^6 cells were resuspended in serum-free medium in a 15 ml conical tube. Cells were lethally irradiated at room temperature using a Cesium¹³⁷ source set at 113.7 rads/min. (Gammacell 44 - Atomic Energy of Canada Limited, Radiochemical Company) for amounts of time calculated to deliver radiation doses of 2000, 4000, 6000 and 8000 rads. The irradiated DBM9-7 cells were then mixed with 2.5×10^6 DU145-N19 cells in suspension and were allowed to attach to 75 cm² culture flasks overnight. The cells were fused for 90 seconds in 2ml of 54% polyethylene glycol (PEG 1500) prewarmed at 37°C in serum-free media. The next day, each 75 cm² flask was then trypsinized and redistributed in ten 25 cm² flasks. Selective media (400 µg/ml hygromycin B) was added 24 hours later and maintained for 19 days or more until individual colonies could be picked and expanded. Using cloning rings, a total of 44 clones were isolated, which originated from donor cells irradiated at 4000, 6000 and 8000 rads.

Screening of hybrids. The hybrids were screened mainly by their morphological properties and chromosome contents. Cells obtained from the 4000 and 6000 rads experiments were larger and flatter in appearance than the parental DU145-N19 cells and some became

growth-arrested or died when replated into a 25 cm² flask. Metaphase spreads (see below) obtained from some viable larger hybrids were viewed with an inverted light microscope (Bausch & Lomb) and found to contain much more than the usual chromosome count of 60-64 per DU145-N19 cell. Six hybrids obtained from an irradiation dose of 8000 rads were morphologically similar to DU145-N19 and were chosen for further analysis.

Fluorescence In Situ Hybridization (FISH): Cells close to confluence in a 75mm² flask were treated with KaryoMAX colcemid at a final concentration of 0.05 µg/ml for 1 hour at 37°C, and harvested according to conventional cytogenetic methods, using 20 ml of 1% sodium citrate as the hypotonic agent. After 2 to 3 rounds of fixative treatment (3:1 methanol to acetic acid) and centrifugation, the cells were dropped onto glass slides held over a steaming water bath. Generation and biotinylation of probes specific for chromosome 12 or del(12)(q13) (*Alu9* and *Alu9-5*, respectively) was previously described in chapter 2. Denaturation of probes and chromosomal DNA, hybridization, posthybridization washes, and staining were carried out essentially as before. The biotinylated probe was pre-hybridized to 0.18µg/µl human Cot-1 DNA and then hybridized to metaphase spreads overnight at 37°C in a humidified chamber. Fluoresceinated avidin was then bound to the probe and the signal amplified with biotinylated goat anti-avidin antibodies and a second application of fluoresceinated avidin, as described in the manufacturers' instructions. Propidium iodide in an antifade solution was used as counterstain. The chromosomes were viewed and photographed on a Zeiss axioskop epifluorescence photomicroscope using an 09 filter set and Kodak Ektachrome ASA 400 color film.

Tumorigenicity and growth assays: DU145 cells are in log phase between 3 days and 5 days after plating 20 000 cells in a 60mm dish. For each radiation-reduced hybrid, 20 000 cells were plated in triplicate 60mm plates. Cells not counted after 3 days were refed. Cells were counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) at the same time of day, except for DU12RH-6, where $T = 43.25$ hours instead of 48 hours. The generation time (GT) was determined as follows: $GT = 48 \text{ hours} / (\log D5 - \log D3) 3.32^{161}$, where GT is the generation time, and D3 and D5 are the cell counts after 3 days and 5 days, respectively. To assess the tumorigenic potential of the radiation hybrids, 1×10^7 cells were spun down and resuspended in a total volume of 0.5 ml of sterile saline, of which 0.3 ml (6×10^6 cells) was injected subcutaneously into the flanks or shoulders of 2- to 4-week old male athymic *nu/nu* mice (Charles River). Tumor growth was monitored weekly for a period of 90 days or until tumor volume was too great and the mouse had to be euthanized. In a second round of injections, the length, width and height of the tumors was measured using a sterile caliper under sterile conditions. These values were used to calculate tumor volume, assuming that the tumor shape is hemiellipsoidal. Corrections were made for skin thickness, particularly with measurements of small tumors, and the height measurements were adjusted to reduce errors in volume, as proposed by Dethlefsen et al.¹⁴⁹

Microcell fusion: The donor cells (either DU12RH-3, DU12RH-4 or DU12RH-5) were treated with Colcemid at a final concentration of 0.02 $\mu\text{g/ml}$ for a period of 16 hours. Microcells were obtained as previously described by Fournier et. al.¹⁵, and fused to recipient B78 mouse melanoma cells in a 25 cm² flask, in 50% polyethylene glycol (PEG 1500) for 1 minute. The cells were washed four times with serum-free medium and grown in complete

medium for 24 hours, at which point each 75 cm² flask was redistributed into three 150 cm² flasks. Hygromycin B(400 µg/ml) and ouabain (3 µM) were added to the culture media 48 hours post-fusion to selectively kill parental cells. Two weeks following fusion, ten to twelve individual clones of each BRH hybrid experiment were isolated using cloning rings. All the BRH hybrids were resistant to hygromycin B.

Microsatellite mapping: The DU12RH radiation-reduced hybrids were screened by PCR using highly polymorphic microsatellite markers specific for human chromosome 12. DNA (75 ng) from B78MC9-5, DU145-N19 and the different radiation hybrids was amplified in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.5 mM MgCl₂, 200 µM each dTTP, dATP, dGTP, 25 µM dCTP, 0.027 µM ³²PdCTP, 0.4 µM each MapPair primer, and 2.5U AmpliTaq (Perkin Elmer) for 35 cycles at 94°C for 40 sec and the optimal annealing temperature for 30 sec. The annealing temperatures used varied between 50°C and 65°C for different primer pairs, depending on their calculated melting temperatures. Two different thermal cyclers were used: a Pharmacia LKB Gene ATAQ Controller and a Perkin Elmer DNA thermal cycler 480. The PCR products were ethanol-precipitated and resuspended in 5 µl H₂O and 5 µl formamide loading buffer. The products were run on a 6% denaturing polyacrylamide gel, which was subsequently dried at 80°C for 1 hour and exposed to Kodak XAR film for 1 hour.

PCR mapping of the BRH mouse-human hybrids: DNA (50 ng) isolated from different BRH hybrids was amplified using PCR as described above, except that the labelled dCTP was replaced by 200 µM cold dCTP and that 0.4 µM each chromosome 12 specific

MapPair primer were used. The amplicons were resolved on 2% NuSieve GTG agarose gels stained with ethidium bromide (0.5 µg/ml).

4.3 Radiation hybrids

4.3.1 Irradiation and fusion

The aim in this section was to collect a range of chromosome 12 fragment sizes centered around the selectable marker *tgCMV/HyTK*. The analysis of the DBM9-7 hybrid was performed prior to the generation of the radiation-reduced hybrids. In chapter 3, it was shown that DBM9-7 contained a transferred tagged *del(12)(q13)* which could suppress the tumorigenic phenotype of DU145-N19 cells.

The irradiation step has two functions. First, a lethal dose of radiation is necessary to kill the donor cells and ensure that any surviving cells are true hybrids. Lethal doses tend to differ slightly between cell lines but most researchers have found that doses of >1.5 krads are sufficient to kill cells.^{162,163} Second, irradiation causes double-strand breaks in DNA and fragments the chromosomes in the cell.

The DBM9-7 cells were subjected to different doses of γ -irradiation and subsequently fused to the DU145-N19 cells. Donor cells are killed by the irradiation, and a selection scheme (in this case, hygromycin B) was used to kill the nonfused recipient cells (DU145-N19) to permit the outgrowth of the hybrid cells. At 2000 rads, the flasks were covered with colonies of cells, indicating that this irradiation dose was not sufficient to completely kill the DBM9-7 donor cells. Hybrids obtained at 4000 and 6000 rads were abnormally large and started to die in the presence of selective media after a few

passages. Metaphase spreads of some of the abnormal-looking hybrids were analyzed and found to contain more than the normal 60-64 chromosome content characteristic of DU145-N19 cells. Hybrids obtained at 8000 rads and picked within a month following fusion had a DU145-like morphology and readily grew in the presence of hygromycin B. Six of these radiation hybrids were further analyzed. They were designated as: DU12RH-3, DU12RH-4, DU12RH-5, DU12RH-6, DU12RH-19 and DU12RH-20.

4.3.2 Validation of transfers

To verify that the radiation/fusion method had been successful, each radiation hybrid obtained was analyzed by FISH using a chromosome 12-specific painting probe (Figure 4-1). The results showed an extra piece of chromosome 12 material present in each hybrid. In each case, the transferred portion had been translocated to another human chromosome belonging to DU145-N19. We concluded that $\text{del}(12)(q13)$ was indeed fragmented and successfully transferred into a DU145-N19 background.

4.3.3 Growth and tumorigenic properties

Since no significant difference in cell generation times was observed in the DBM9 hybrids compared to DU145-N19, no growth effects were expected in the radiation hybrids. The anchorage-dependent rate of growth of each radiation hybrid was measured and no major deviance from that of DU145-N19 was detected (Table 4-1). A p value of 0.76 was calculated by a one-way analysis of variance (ANOVA), indicating that the difference between each hybrid and DU145-N19 cells is not significant. These results confirmed that the gene responsible for tumor suppression acts independently from

Figure 4-1: Chromosome 12 FISH analysis of radiation hybrids. The *Alu9* probe (specific for chromosome 12) was hybridized to metaphase chromosome spreads of each DU12RH radiation hybrid. The arrows indicate the irradiated fragments of chromosome 12 which were transferred in each hybrid. Each chromosome 12 fragment was translocated in endogenous chromosomes of DU145-N19 presumably for stabilization purposes.

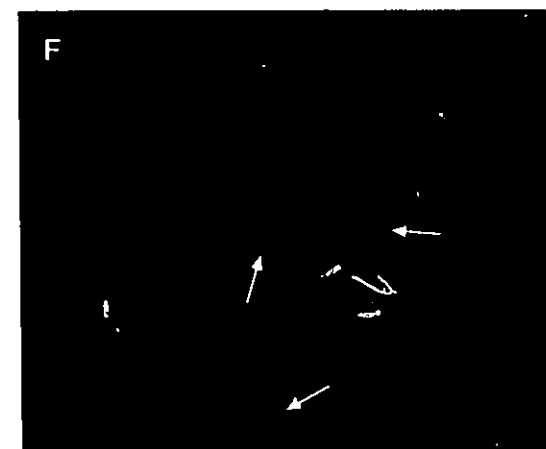
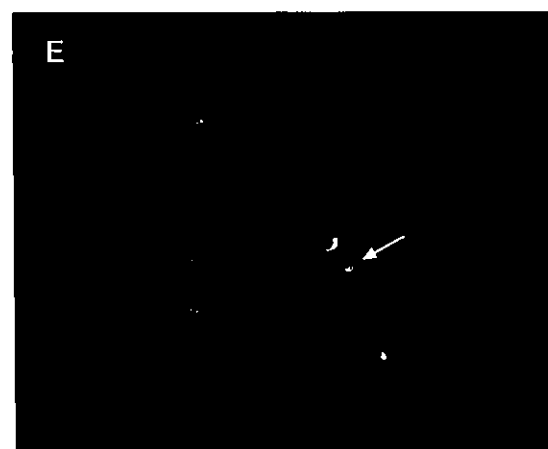
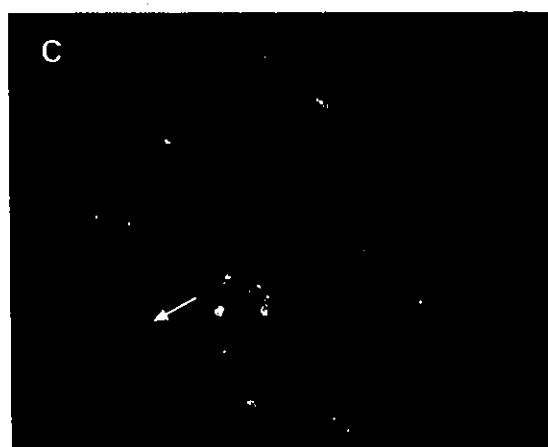
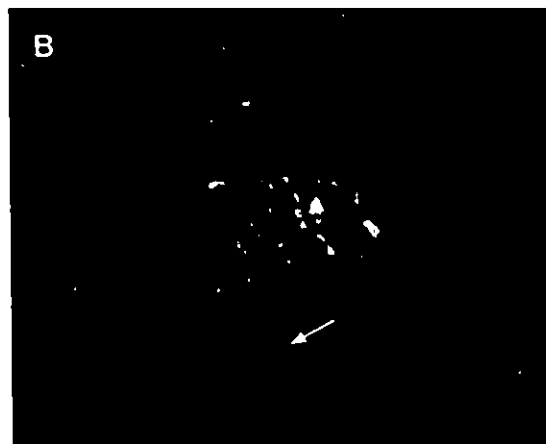


Table 4-1:
Growth and tumorigenic properties of radiation hybrids

Radiation hybrid	Growth in 10μM GCV	Growth 400μg/ml Hyg B	In CGT $\pm$SEM^a	Tumor growth^b
DU145-N19	+	-	35.6 $\pm$ 4.2	6/6
DU12RH-3	-	+	40.6 $\pm$ 0.9	8/8
DU12RH-4	-	+	50.6 $\pm$ 3.2	7/8
DU12RH-5	-	+	43.0 $\pm$ 3.8	5/8
DU12RH-6	-	+	37.9 $\pm$ 0.2	2/10 ^c
DU12RH-19	-	+	42.0 $\pm$ 1.7	9/12 ^d
DU12RH-20	-	+	43.8 $\pm$ 14.9	7/8
DU12RH-6R4	+	-	42.8 $\pm$ 1.8	0/4

^a Cell generation time determined during log phase of growth from triplicates.

^b Number of tumors formed per number of sites inoculated.

^c First set 0/4; Second set 2/6

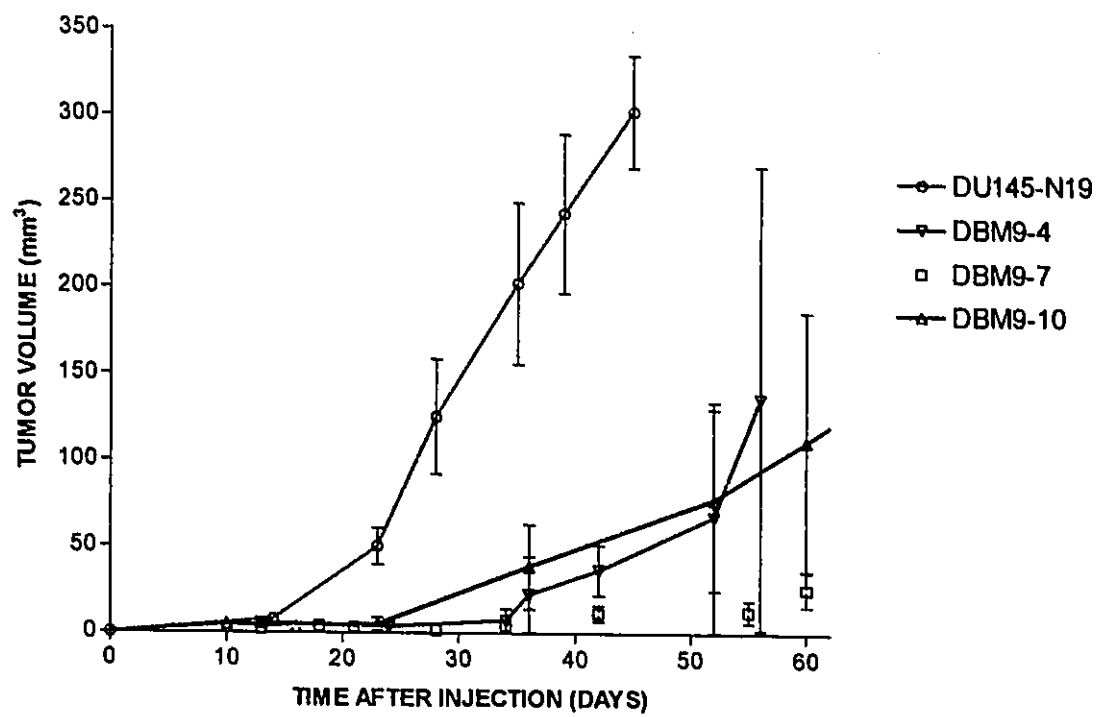
^d Four tumors regressed after 5 months.

growth control signaling pathways. The anchorage-independent growth properties of the radiation hybrids were not measured since this characteristic did not correlate with tumorigenicity in the DBM9 hybrids (Chapter 3).

To design a map of the tumor suppressor region, each radiation hybrid was injected subcutaneously in nude mice to assess the effect of each transferred tagged piece of chromosome 12 on the tumorigenicity of DU145-N19 cells. In a first set of injections, all the radiation hybrids formed tumors which appeared within 2 weeks to a month after injection. One exception was DU12RH-6 which exhibited significant suppression (0/4 and 2/6 tumors per injection sites). At early passage, DU12RH-6 cells were exposed to GCV in culture to isolate cells having lost the tagged piece of chromosome. Two revertants were obtained, named DU12RH-6R2 and DU12RH-6R4, which unexpectedly still demonstrated complete suppression (Table 4-1). Considering these results, we attempted to determine if a small portion of the introduced chromosome 12 was present in these hybrids. However, FISH or PCR amplification using microsatellite mapping failed to detect any chromosome 12 element.

In a second set of injections, the rate of tumor growth was monitored and compared to the parental tumor growth curve (Figure 4-2). The cells injected were at a later passage compared to the cells in the first set of injections. In this case, all the radiation hybrids (including DU12RH-6) were tumorigenic, sometimes even more so than DU145-N19. The revertant DU12RH-6R4 was still suppressed.

Figure 4-2: Tumor growth curve of radiation hybrids. Each point on the graph represent the average volume of 4 tumors produced upon the injection of 6×10^6 cells in either the shoulders or thighs of athymic nude mice. Tumor volumes were calculated according to Dethlefsen et al,¹⁴⁹ using the assumption that the tumors were hemiellipsoids and taking into account skin thickness.



4.3.4 Microsatellite mapping

The generation of a map to locate the position of the tumor suppressor gene cannot be established without knowing which part of chromosome 12 is represented in each hybrid. Each region can then be correlated to the presence or absence of the tumorigenic phenotype in each hybrid. Usually, radiation hybrid mapping is done in a rodent background with the use of human-specific markers. However, in our system, the background is human, bringing about a requirement for markers displaying polymorphism between the introduced fragments of del(12)(q13) and the chromosomes 12 already present in the recipient cell, DU145-N19.

PCR can be used to demonstrate a high level of polymorphism or allelic variation in the repeat number for simple sequence tandem repeats known as microsatellites. The latter consist of around 10-50 copies of motifs from 2 to 6 bp that occur frequently and randomly in all eukaryotic DNAs except yeast.¹⁶⁴ Since they are usually less than 300bp long and are embedded in DNA with unique sequence, they can be amplified *in vitro* using PCR. Microsatellites display considerable polymorphism due to variation in the number of repeat units.^{164, 165, 166}

We have tested human chromosome 12-specific dinucleotide microsatellite markers on B78MC9-5 and DU145-N19 DNA to determine whether (a) they detected a polymorphism and (b) if that polymorphism was informative. Several problems were encountered in this analysis. First, there is a difficulty in scoring dinucleotide

microsatellites because of the artefactual bands generated by the PCR, commonly 2bp shorter than the most intensely staining band. Secondly, most of the markers specific for chromosome 12 were not polymorphic and informative (15 out of 18 tested on the q arm). Hence, the majority of markers could not be used to map the regions of del(12)(q13) introduced in each radiation hybrid. Three q arm markers did demonstrate polymorphism and were used to amplify DNA isolated from each radiation hybrid (Table 4-2).

Table 4-2: Microsatellite mapping of radiation hybrids.

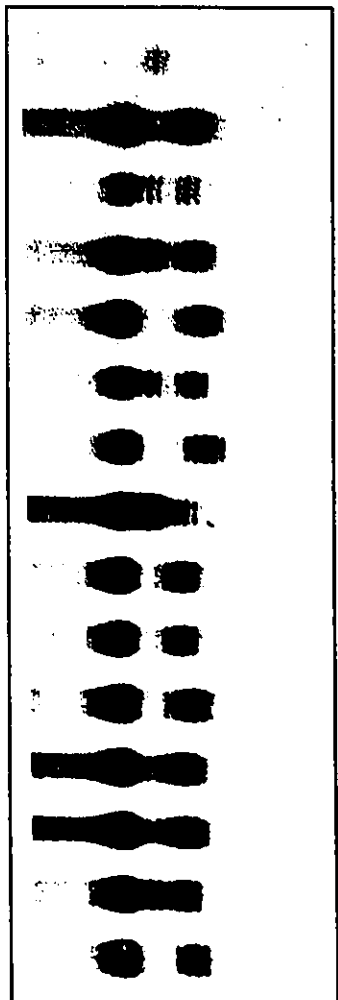
Cell lines	Microsatellite marker		
	D12S90	D12S325	D12S368
B78MC9-5	+	+	+
DU145-N19	-	-	-
DBM9-7	+	+	+
DU12RH-3	-	-	-
DU12RH-4	-	+	-
DU12RH-5	-	+	-
DU12RH-6	-	+	-
DU12RH-19	-	-	-
DU12RH-20	+	+	+
DU12RH-6R4	-	+	-

A typical autoradiograph from one of these experiments, using the D12S368 marker is shown in Figure 4-3. This marker was positive for the three DBM9 hybrids but not their revertants. It also amplified DU12RH-20 DNA. The D12S90 marker was positive for DU12RH-20 as well. The D12S325 marker was positive for several radiation hybrids. Since these radiation hybrids were not suppressed for the tumorigenic phenotype, these regions of 12q *are not* involved in the tumor suppressive mechanism. However,

Figure 4-3: Autoradiograph of radiolabeled PCR products obtained from the amplification of DBM9 and radiation hybrid DNA. PCR amplification of DU145-N19-derived hybrids using the D12S368 marker. The arrow indicates amplification products obtained from the tagged chromosome 12 DNA.

CELL LINES

MARKER^a



^a Presence (+) or absence (-) of D12S368 in the cell lines

such a low number of informative markers was not enough to produce a high resolution map of the radiation hybrids.

4.4 BRH hybrids

To circumvent the problems encountered with microsatellite mapping, the tagged fragments from DU12RH-3, DU12RH-4 and DU12RH-5 were transferred by microcell fusion into the B78 mouse melanoma cells. The mouse/human microcell hybrids obtained were designated as BRH-3, BRH-4 and BRH-5, respectively. All the BRH hybrids were able to grow in media containing hygromycin B, indicating that the tagged fragments of chromosome 12 from each radiation hybrid had been successfully transferred into a mouse background. Moreover, PCR amplification with a p arm polymorphic microsatellite marker (D12S59) confirmed that chromosome 12 material present originated from B78MC9-5 and not from DU145-N19. Transfer into a mouse background eliminated the need for polymorphic markers. In the BRH hybrids, all the human chromosome 12-specific MapPair primers were informative and could be used to map the regions of each tagged fragment. A technical advantage was also gained in that the results could be viewed directly on an agarose gel stained with ethidium bromide instead of the more labor-intensive polyacrylamide gels. No radio-labeled nucleotides were necessary in this system.

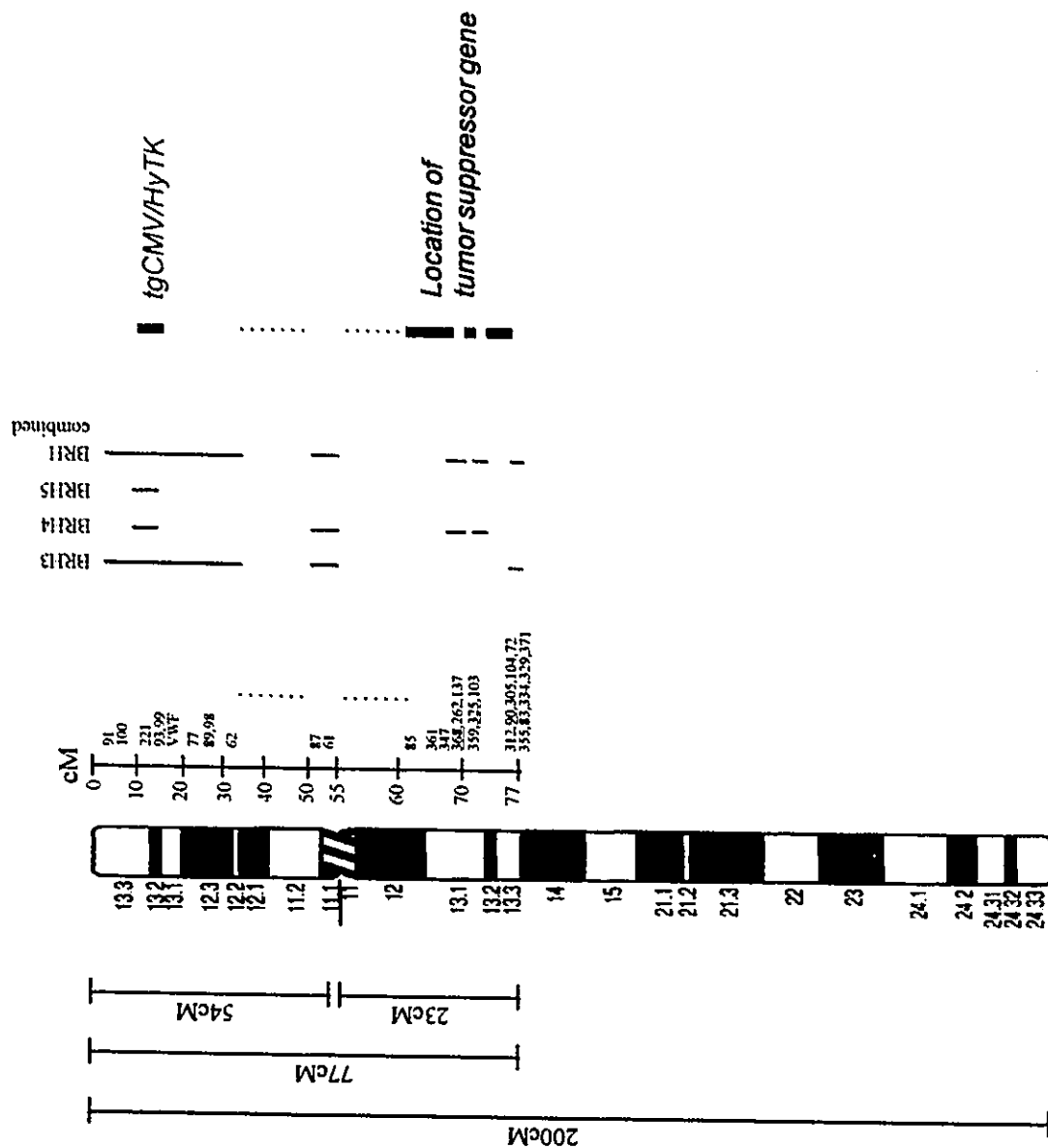
Several clones were picked and expanded for each BRH-3, BRH-4 and BRH-5 hybrids. Differences in chromosome 12 contents were observed between different BRH subclones, reflecting further breakage of the chromosome 12 upon transfer into B78 cells

Table 4-3:
Fine mapping of BRH subclones

Marker	BRH subclones								BRH-Combined	MC9-5
	3-3	4-1	4-8	4-9	4-10	5-1	5-3	5-4		
TEL-p ARM	TEL	TEL	TEL	TEL	TEL	TEL	TEL	TEL	TEL	TEL
D12S91	+		-		-		-	-	+	+
D12S100	+	+	-	+	-	+	+	-	+	+
D12S221	+	+	+	+	-	+	+	+	+	+
D12S93	+	+	+	+	-		+	-	+	+
D12S99	+	-	-	-	+	+	+	-	+	+
VWF	+		-		-		-	-	+	+
D12S77	-		-		-		-	-	-	-
D12S89	+		-		-		-	-	+	+
D12S98	+		-		-		-	-	+	+
D12S62	+		-		-		-	-	+	+
D12S87	+		-		+		-	-	+	+
D12S61	+		-		+		-	-	+	+
CEN	CEN	CEN	CEN	CEN	CEN	CEN	CEN	CEN	CEN	CEN
D12S85	-		-	-	-		-	-		+
D12S361	-		-	-	-		-	-		+
D12S347	-		-	+	-		-	-	+	+
D12S368	-		-	+	-		-	-	+	+
D12S262	-		-	-	-		-	-		+
D12S137	-		-	-	-		-	-		+
D12S359	-		-	-	-		-	-		+
D12S325	-		-	-	-		-	-		+
D12S103	-		+	-	-		-	-	+	+
D12S312	-		-	-	-		-	-		+
D12S90	-		-	-	-		-	-		+
D12S305	-		-	-	-		-	-		+
D12S104	-								-	-
D12S72	-		-	-	-		-	-		+
D12S355	+		-	-	-		-	-	+	+
D12S83	-			-			-	-		+
D12S334	-								-	-
D12S329	-		-	-	-		-	-		+
D12S371	-		-				-	-		+
D12S335	-								-	-

Note: Shaded boxes indicate putative location of tumor suppressor gene
 *D12S325 is present in DU12RH-6R4, a suppressed radiation-hybrid revertant.

Figure 4-4: Schematic representation of compounded PCR results from different BRH subclones. The distances in centimorgans (cM) are indicated at the left of the idiogram of chromosome 12. The chromosome 12-specific markers used for the mapping are shown to the right. Black lines: regions mapped to BRH hybrids. Blue lines: regions of tumor suppressor gene. Red line: region of tgCMV/HyTK integration. Dotted blue lines: regions lacking markers at the time of analysis. Underlined markers are polymorphic. Distances in cM and order of markers were taken from Krauter et al (1995) Nature 377Suppl:321-333.



(Table 4-3). The results from all the subclones were combined to yield a picture of the fragment initially present in the donor DU12RH cells (Figure 4-4). Some markers were consistently present in almost all the BRH subclones analyzed (D12S100, D12S221, and D12S93), indicating that the tag is probably located in the 12p13.2-13.3 region.¹⁶⁷ Considering that the DU12RH-3, 4 and 5 were still tumorigenic in nude mice, the p arm and small regions of the q arm can be eliminated as a candidate region for the tumor suppressor. Consequently, the tumor suppressor gene must be located in the remaining regions not identified in the BRH hybrids i.e. in subregions between the centromere and band q13 (Figure 4-4).

4.5 Discussion

A mapping panel is a collection of cell hybrids that subdivides the donor chromosome in such a way that allows unambiguous map assignments to be made. We employed a radiation-induced method to generate prostate cancer somatic cell hybrids containing diverse fragments of del(12)(q13) in order to locate a tumor suppressor gene. The DBM9-7 hybrid was chosen as donor in these experiments because it displayed the highest degree of tumor suppression when injected in nude mice at high passage (Figure 3-6).

It was previously shown that donor chromosomes under selection in foreign, recipient cells undergo stabilization by translocation to a recipient chromosome. Human fragments must acquire the chromosomal elements needed for relocation and stable mitotic segregation. Centromeres and telomeres can be obtained either by integrating into

the chromosomes of the recipient cell lines or by combining with suitable centromere and telomere containing fragments from the irradiated cell line. The DU12RH hybrids containing a small tagged fragment most often lacked a centromere and therefore could only survive if a translocation occurred, leading to stabilization and proper replication of that fragment. Since the only viable hybrids obtained had lost their centromere, it can be deduced that the tgCMV/HyTK was inserted at a good distance from the centromere. This was later confirmed by the mapping studies, in which almost all BRH subclones contained the region at 12p13.2-13.3 including the D12S100, D12S221, and D12S93 markers. This was most evident in the BRH5 hybrids, where this was the only region transferred (Table 4-3 and Figure 4-4). These translocations, although inevitable, are worrisome since they could disrupt or influence the expression of other genes at or near the site of translocation. This problem could be the cause of the observed tumor growth rates of several radiation hybrids, which exceeded that of the control parental cell line.

The tumorigenicity results regarding DU12RH-6 and its revertants are unusual but can be rationalized in the following manner. We hypothesize that two distinct fragments of del(12)(q13) were transferred in the DU12RH-6 radiation hybrid, one containing the tag (12p13), and one containing the tumor suppressor gene (12cen-q13). The tagged fragment could have been translocated, allowing continuous resistance to hygromycin B. The second fragment might not have been translocated and thus was lost as the cells were cultured, thus explaining the loss of suppression observed at higher passage of the cells. The revertants, however, were generated from low passage DU12RH-6 cells and the fragment containing the tumor suppressor gene could have been translocated and

stabilized in the DU12RH-6R4 revertant cells. Consequently, these cells remained suppressed for tumorigenicity. Because this translocated fragment was not tagged, it could not be transferred back in the mouse B78 cells and therefore could not easily be mapped.

Radiation hybrids can be analyzed in several ways, including enzyme assays and Southern blot hybridization to DNA from different hybrids. However, PCR is by far the most efficient way to analyze markers within radiation hybrid panels. Limiting amounts of DNA, often a problem with Southern blot analyses, is not a difficulty with PCR. Due to the lack of genetic markers at the time of analysis, a 7cM region below the centromere and a 5cM region at bands p13.2-p13.3 could not be evaluated for their presence in the BRH hybrids. The availability of such primers will be necessary to pinpoint the region containing the tumor suppressor gene. The few results obtained with the polymorphic microsatellite markers were informative. The D12S368 and D12S90 markers were present in DU12RH-20 (Table 4-2), a tumorigenic radiation hybrid (Figure 4-2), eliminating these regions as containing the tumor suppressor gene. This was confirmed by the presence of this region (D12S368 and D12S347) in the BRH4-9 hybrid (Table 4-3). The result with the D12S90 primer could not be disproved or confirmed in the BRH hybrids since this region was never transferred into BRH hybrids (Table 4-3). Marker D12S325 at 71cM was present in several radiation hybrids (Table 4-2), suggesting that this region does not contain the tumor suppressor gene. However, it was also present in the DU12RH-6R4 revertant hypothesized to carry a small fragment containing the tumor suppressor gene. This suggests that this marker is close to the tumor suppressor gene.

However, we have not ruled out the possibility that DU12RH-6R4 contains more than one small translocated fragments of chromosome 12. Marker D12S103, also at 71cM, was positive in BRH4-8, suggesting that the gene lies between markers D12S368 (68cM) and D12S103 (75cM). The analysis of other polymorphic markers around this region will be necessary to confirm this hypothesis. This region contains several genes, including a keratin gene cluster, the retinoic acid receptor gamma gene, the *SP1* gene and the *HOXC5* gene.¹⁶⁷

This study with the available markers, helped to achieved a 10 fold increase in resolution compared to the full length chromosome 12 (from 200cM to 20cM). The analysis of recently available markers might improve the resolution even more. Since chromosome 12 represents 4.5% of the human genome and that the latter has 3×10^9 base pairs (bp), it is estimated that chromosome 12 measures 135Mb. The candidate region therefore measures approximately 13.5Mb. We conclude that the production of radiation hybrids helped to outline the location of the tumor suppressor gene. The transfer of the tagged fragments into a mouse background subsequent to the phenotypic assessment in the human background proved to be an effective and fast method to map the location of the gene.

CHAPTER 5

CLONING CANDIDATE GENES

5. CLONING CANDIDATE GENES

5.1 Introduction

The progression from the identification of a suppressor chromosome to a specific chromosomal region provided indispensable tools to carry through the chief aim of this search: the isolation of candidate genes involved in the suppression of tumorigenicity. The question remains: How can we use these tools most efficiently to achieve this goal? The rapid evolution of molecular methods offers many options presenting various advantages and disadvantages. Traditional approaches of gene isolation have included identification of CpG islands and conserved sequences, as well as direct screening of cDNA libraries, techniques which can be effective but overly laborious. A second generation of techniques has emerged that can be roughly divided in three major categories: cDNA screening methods, subtractive hybridization methods, and differential methods.

5.1.1 cDNA screening methods

The recent successes of positional cloning to isolate disease genes has spurred new interest in assembling large scale transcriptional maps. To achieve this goal, several cDNA selection methods were developed and refined. They usually entail the use of large genomic regions packaged in yeast artificial chromosomes (YACs) or in cosmids, to “fish out” cDNAs encoded in a specific region of the genome. Many strategies exist to

accomplish this task, based on either the structure or the expression of cDNAs. In the first category, exon amplification (trapping) techniques were often employed to isolate exon sequences from cloned genomic DNA by virtue of selection for functional splice sites.^{168,169} However, this method is technically demanding and many false positives arise as a result of incorrect splicing. Alternatively, a neural network computer program, GRAIL, can be used to identify coding exons based on sequence patterns in genomic DNA.¹⁷⁰ A disadvantage of GRAIL is the requirement for the complete sequence of the target DNA. A third technique takes advantage of HTF islands, which are regions of high concentration of unmethylated CpG nucleotides found in 60% of gene promoters.¹⁷¹ Sequences flanking HTF islands can be subcloned, sequenced and/or used as hybridization probes to screen cDNA libraries.¹⁷²

Techniques which depend on expression in a given tissue, such as direct cDNA selection, encounter fewer verification problems. In such methods, the target DNA from the region of interest is either immobilized or biotinylated, and subsequently hybridized to *Alu*-repeat-depleted cDNA pools from different sources. The resulting hybrids are recovered, subcloned and sequenced.^{173, 174} One impediment encountered in this case is the expression level bias of certain genes, which increases the uncertainty of identification of genes expressed at less than 0.01%.

The microcell hybrids and the radiation hybrids described in chapters 3 and 4 established a region of interest of approximately 13.5Mb thought to encode a tumor suppressor gene. YAC contigs are now available in that region¹⁶⁷ and the length is

adequate to attempt cDNA screening methods. However, interpretation of the data only became possible in light of recent publications. Due to the uncertainty of marker assignment and gaps in the map before this new information became available, it was deemed unwise to attempt cDNA screening. Moreover, it was possible to altogether miss the gene of interest since there are no guarantees that all the genes in the region will be identified by this method.

5.1.2 Subtractive hybridization methods

Subtractive approaches usually involve several rounds of hybridization of excess DNA or RNA from the cells lacking a desired sequence (driver) with DNA or RNA from the cells containing that sequence (tester), followed by the elimination of unwanted sequences. During subtractive hybridization of RNA populations, a large excess of driver is used to remove sequences common to a biotinylated tester. After repeated subtractive hybridization cycles, the tester is separated from the driver by avidin/biotin affinity chromatography. Single-stranded targets are enriched for the desired sequence and amplified by PCR.¹⁷⁵ This methodology was instrumental in identifying genes involved in PC progression, namely calbindin,¹⁷⁶ and HMG I(Y).¹⁷⁷ This method was shown to be quite sensitive and to detect fairly rare mRNAs. However, it is labor-intensive, and can select for genes in only one direction at a time during a two-way comparison. Because hybridization is a kinetic reaction and thus never reaches completion, gene recovery can be incomplete.

A method for PCR-based subtraction of genomic DNA called RDA (representational difference analysis) was introduced in 1993 by Lisitsyn et al.¹⁷⁸ It allowed enrichment for the desired sequences 10^5 - to 10^6 -fold after a small number of subtraction rounds and thus could be used to detect very small insertions or deletions in a complex genome. The complexity of the two genomes to be analyzed is first decreased by amplifying a relatively small fraction of each genome by PCR amplification. After ligating adaptor strands to the tester amplicons only, the “representations” of tester and driver genomes are used in a subtractive hybridization step. By using excess driver DNA (which does not contain the gene of interest), sequences common to the two genomes form double stranded hybrids. PCR amplification is then performed with primers that allow the exponential amplification of only double-stranded tester segments, since non-target tester strands anneal to driver stands and these hybrids are consequently not amplified. Single-stranded DNA is then removed by a simple nuclease digestion step. Additional rounds of the same treatment generate a few discrete DNA fragments specific to the tester DNA. We have attempted to use this strategy in our system but the DNA segments obtained did not prove to be specific to the tester DNA upon Southern analysis.

5.1.3 Differential methods

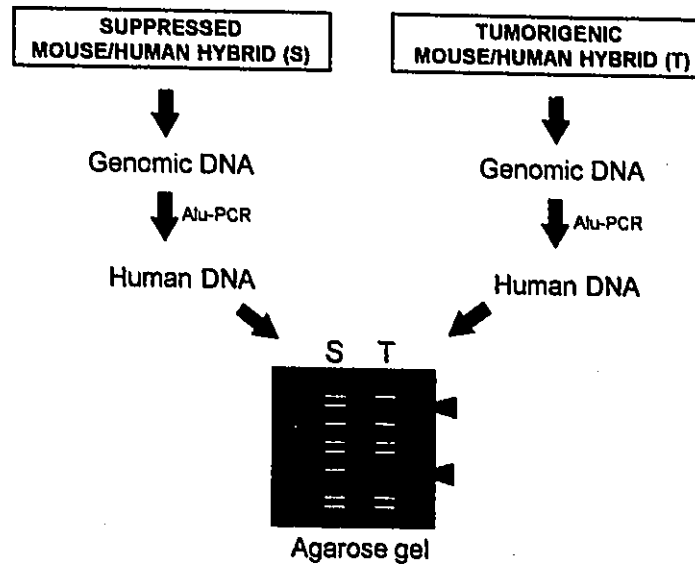
Several differential methods have appeared in the literature in the last decade, which are based on the comparison of different genomes at the DNA or at the RNA level. These technologies help to detect what is different between two or more cell populations.

Differential methods involving the comparison of DNA or RNA populations after PCR amplification have proven to be very effective. One way to identify different sequences in two or more genomes involves *Alu*-PCR amplification of genomic DNA from different sources. The products obtained can be simply and easily compared on agarose gels, and the differential bands are excised and sequenced to identify coding regions (Figure 5-1A). This method proved successful to clone the metastasis suppressor gene KAI1 with the use of microcell hybrids containing different portions of human chromosome 11 in a rodent background.¹⁰¹ In this study, a rodent/human hybrid containing an extra portion of human chromosome 11 exhibited decreased metastatic potential. An inter-*Alu* fragment amplified solely from this hybrid was identified and shown to encode the novel gene. The hybrid system generated in this thesis is similar to that used in the above study, except that the assessment of the tumor suppressive phenotype was assessed in the DU 145 human cells, instead of the rodent cells. We have used an *Alu*-PCR differential technique to compare the amplification between B78MC9-5 (Chromosome 12 TSG+) and BRH3 (Chromosome 12 TSG-) and isolate human genomic DNA fragments specific to the tumor suppressor region on human chromosome 12.

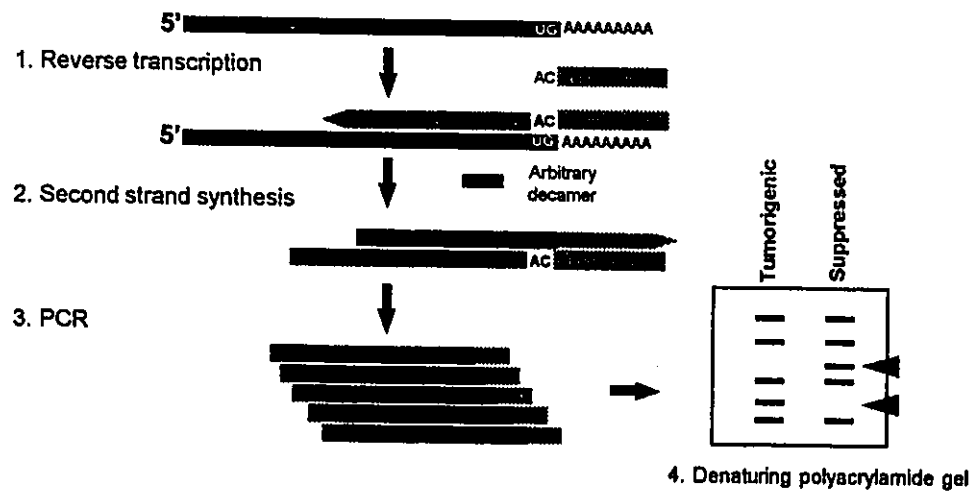
Finally, a differential expression cloning technique was developed to compare mRNA populations, and was designated differential display reverse transcriptase PCR (DDRT-PCR) or RNA fingerprinting. Since first introduced by Liang and Pardee in 1992,^{179,180} DDRT-PCR has been extensively used as a powerful tool for the identification of differentially expressed genes in various biological systems. The procedure involves

Figure 5-1: Schematic representation of differential methods used for cloning cDNAs. (A) Differential *Alu*-PCR method. (B) Differential display reverse transcriptase PCR method.

A- Differential Alu-PCR



B- Differential display RT-PCR



the reverse transcription of total RNA, followed by amplification of the cDNA at low annealing temperatures. The amplification step uses degenerate double-anchored oligo(dT) primers [i.e. 5'-(dT₁₁)AC-3'] that anneal to the start of the poly(A) tails present in most eukaryotic mRNA species, and an arbitrary decamer that anneals in the 3' region of the mRNA, in the presence of labeled nucleotides. The resulting radioactively labeled cDNA populations are displayed in adjacent lanes and compared on a denaturing polyacrylamide gel (Figure 5-1B). By changing primer combinations, most of the mRNA species in a cell (about 10 000 to 15 000) can be represented.¹⁸⁰ Differentially expressed cDNAs are excised from the gel, re-amplified, subcloned and sequenced. Some advantages of the method include the need for minimal quantities of mRNA per experiment and a reduced number of steps during which the gene of interest might be lost. It is also less time-consuming than subtractive hybridization methods since clone purification and rescreening steps are not required. One attractive feature of the method is that in the same display experiment, genes which are either enriched or underexpressed in multiple cell populations can be simultaneously visualized (Figure 5-1B). A limitation of DDRT-PCR is the inability to detect posttranslational differences between different cell populations. We have successfully used this technique to isolate genes which are enriched or down-regulated during the suppression of tumorigenicity bestowed by chromosome del(12)(q13) on prostate cancer cells.

5.2 Materials and methods

***Alu*-PCR amplification:** Genomic DNA from B78, B78MC9-5 and BRH3-6 were amplified by PCR using different *Alu* primers, independently [Alu153, Alu154, Alu451¹⁵¹, and TC65¹⁸¹].

Alu153: 5'-GGGATTACAGGCGTGAGCCAC-3'
Alu154: 5'-TGCACTCCAGCCTGGGCAACA-3'
Alu451: 5'-GTGAGCCGAGATCGCGCCACTGCACT-3'
TC65: 5'-AGGTCGCGGCCGCTTGCAGTGAGCCGAGAT-3'

The reaction mixtures consisted of 50ng genomic DNA, 0.2mM dNTPs, 2μM *Alu* primers and 2.75U AmpliTaq polymerase in a total volume of 100μl. The samples were overlaid with mineral oil and subjected to the following temperature cycles in a PE480 thermal cycler. For Alu153, Alu154 and Alu451, the samples were incubated at 95°C for 2 min and for 35 cycles at 94°C, 30 sec.; 67°C, 15 sec.; 72°C, 2 min, followed by a final step at 72°C for 10 min. For the TC65 primer, the annealing step was performed at 63°C instead of 67°C and a hot start with a primer/dNTP mix was necessary due to a high melting temperature and possible hairpin formation with this primer. The amplification products were analyzed on a 1.5% agarose gel and the observed differential bands were cut out of the gel. The DNA was extracted from the agarose by electroelution as follows. The gel slices were subjected to 150V for 35 min. in a chamber containing 0.1X TBE [8.9mM Tris-Cl, 8.9mM boric acid, 20mM EDTA pH 8.0]. The DNA was retained by

the electroelution buffer [50mM Tris-Cl pH 8.0, 2mM EDTA, 3M NaCl, 10% glycerol and bromophenol blue] which is a high molarity salt solution. Using a cut multiflex tip, the DNA in electroelution buffer was withdrawn from the apparatus and diluted to 400 μ l with TE⁻⁴ [10mM Tris, 0.1mM EDTA, pH 8.0]. To precipitate the DNA, 1 μ l of glycogen (20mg/ml) and 800 μ l ethanol (99%) were added and incubated on dry ice for 15 min. After centrifugation, the pellet was washed with 80% ethanol and resuspended in 20 μ l TE⁻⁴.

DNase I digest: To remove all traces of DNA from RNA samples that will be used for reverse transcription, each RNA sample was treated with DNase I. The DNase I enzyme was diluted to 10U/ μ l in storage buffer [20mM sodium acetate, pH 6.5; 5mM CaCl₂; 0.1mM PMSF; 50% (v/v) glycerol]. Each RNA sample (50 μ g) was digested with 0.2U/ μ l DNase I, 10mM Tris-Cl, 50mM KCl, and 1.5mM MgCl₂ for 30 min. at 37°C. After adding 100 μ l of diethyl pyrocarbonate-(DEPC) treated water, the samples were treated with an equal volume of phenol/chloroform/amyl alcohol [24:24:1]. The aqueous layer was transferred to a clean tube and the RNA was precipitated with 15 μ l sodium acetate (3M) and 375 μ l ethanol (99%) at -20°C for 30 min. After centrifugation, the supernatant was removed and the pellet dried and resuspended in 25 μ l DEPC-treated water to obtain a final concentration of 2 μ g/ μ l. Aliquots of 1 μ l were stored at -80°C.

Reverse transcription: Aliquots of RNA were thawed on ice and diluted to 0.2µg/µl with DEPC-treated water. RNA (0.4µg) was incubated in 1X First Strand buffer, 10mM dithiothreitol (DTT), 20µM dNTPs, 1µM T₁₁MN primer (20pmol) and 0.5U/µl human placental RNase Inhibitor at 65°C for 5 min. and then 37°C for 10 min., to let the primers anneal to the RNA. SuperScript™ Reverse Transcriptase (200U) was then added to each sample and the reverse transcription reaction was performed at 37°C for 50 min. The enzyme was inactivated at 95°C for 5 min. The T₁₁MN primers consisted of four sets of degenerate anchored oligo (dT) primers, where M can be G,C or A and N can be G, C, A, or T. The four sets of primers are therefore T₁₁MG, T₁₁MC, T₁₁MA, and T₁₁MT.

PCR amplification: cDNA (3µl of the reverse transcription) prepared on that day was amplified using 1µM T₁₁MN primers (20pmol) and 0.25µM arbitrary decamer¹⁸² (DDX; 5pmol) in the presence of 1X Perkin Elmer buffer, 2µM dNTPs, 0.5µCi/µl [α -³⁵S] dATP (3000Ci/mmol), and 0.075U/µl AmpliTaq Polymerase. The mixture was overlaid with paraffin oil and subjected to the following temperature changes in a Pharmacia Thermal Cycler: 94°C for 30s, 40°C for 2min., 72°C for 30s for 40 cycles; 72°C for 5 min. The radioactively labeled PCR products represented a subpopulation of mRNAs defined by the given primer set.

Differential display: The PCR samples were separated from the mineral oil by spotting onto parafilm and retransferring into a clean 0.5 ml tube. After adding 10µl of formamide loading buffer, 4 to 6 µl of denatured samples (95°C, 5min, ice) were loaded

onto a 6% denaturing polyacrylamide gel (0.4mm thick). The gels were run at 1800V, 60watts, 30 amps for 2 hours on a Model S2 sequencing gel electrophoresis apparatus (Gibco), using a Model 4001 electrophoresis power supply (Gibco). The gels were transferred to Whatman 3MM papers, covered with plastic wrap and dried at 80°C for 1 hour in a Model 583 gel dryer (Biorad) on the sequencing gel cycle. The dried gels were exposed to XAR Kodak film overnight or longer if necessary.

Isolation and re-amplification of differential bands: After positioning the autoradiograph and the gel, the differential bands were excised using a clean razor blade and transferred to a clean tube. They were incubated at room temperature for 10 min. in 100µl water after which they were boiled for 15 min. The samples were spun in a microcentrifuge for 2 min. and the supernatant was transferred to a clean tube. The products were precipitated by adding 10µl sodium acetate (3M), 5µl glycogen (20mg/ml), and 400µl ethanol (99%) and incubated for 30min. at -80°C. The samples were centrifuged for 10-15min. at 4°C, and rinsed once with 500µl cold ethanol (85%). The pellet was dried and resuspended in 10µl water. At this point, the samples were stored at -20°C or re-amplified immediately. To re-amplify, cDNA (4µl) isolated from the gel was mixed with 1X Perkin Elmer buffer, 25µM dNTPs, 1µM T₁₁MN primers, 0.25µM arbitrary decamer (DDX) and 0.025U AmpliTaq polymerase. The mixture was overlaid with mineral oil and subjected to the same temperature cycles as described in the PCR amplification section. After amplification, 10µl of each sample was resolved on a 2%

NuSieve GTG (3:1) agarose gel to verify that the size of the re-amplified DNA corresponded to the size of the band cut out of the polyacrylamide gel.

Subcloning the differential fragments: The differential bands re-amplified after isolation from the polyacrylamide gel were quantified (1µl out of the remaining 30µl) using a TK-100 fluorometer (Hoefer). The PCR products were subcloned into the pCRTMII vector using the "Original TA Cloning Kit".¹⁸³ The following formula was used to estimate the amount of PCR product needed to ligate with 50ng (20 fmoles) of pCRTMII vector:

$$\text{X ng PCR product} = \frac{(\text{Y bp PCR product})(50 \text{ ng pCR}^{\text{TM}}\text{II vector})}{(\text{size in bp of the pCR}^{\text{TM}}\text{II vector: 3900})}$$

where X ng is the amount of PCR product of Y base pairs to be ligated for a 1:1 (vector: insert) molar ratio. The ligation reaction consisted of 1X ligation buffer [6mM Tris-HCl, pH 8.3, 6mM MgCl₂, 5mM NaCl, 0.1mg/ml bovine serum albumin, 7mM β-mercaptoethanol (β-ME), 0.1mM ATP, 2mM DTT, 1mM spermidine], 50ng pCRTMII vector, and 4.0 units of T4 DNA ligase, in a total volume of 10µl. It was incubated overnight at 14°C. One ShotTM cells (INVαF' competent cells) were transformed with the ligation mixture. For each transformation reaction, a 50µl vial of frozen One ShotTM competent cells was thawed on ice, after which 2µl of 0.5M β-ME and 2µl of the ligation reaction were added directly into the competent cells and mixed gently. The cells were

incubated on ice for 30 min. and then heat shocked for exactly 30 sec. at 42°C and placed on ice for 2 min. 450µl of SOC medium [2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgSO₄, 20mM glucose] were added to each tube, and the latter were incubated at 37°C for one hour, in an horizontal shaker. The transformed cells were then put on ice. 50µl and 200µl of each transformation vial were spread on separate, Luria-Bertani (LB) agar plates containing 50µg/ml of ampicillin and 1.6mg X-Gal [40µl of stock (40 mg/ml in dimethylformamide)]. The plates were inverted and placed overnight at 37°C. Colonies were picked for plasmid isolation¹⁸⁴ and analyzed with Eco RI restriction enzyme (which liberates the insert). Plasmid DNA from colonies containing an insert of the appropriate size were grown overnight at 37°C in 2ml LB broth [1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0] containing 50µg/ml ampicillin. Glycerol stocks were made [500µl culture + 300µl sterile glycerol] and stored at -80°C.

Northern analysis: Total RNA or poly A⁺ RNA were isolated from different cell lines and stored at -80°C. The samples (RNA in 1X MOPS [40mM MOPS, 10mM NaOAc, 1mM Na₂EDTA]; 2.2M formaldehyde; 50% formamide) were heated for 15 min. at 55°C and quenched on ice before adding 0.1 volume of 10X loading buffer. The denatured samples were separated for 3 hours at 54V on a 1.2% agarose gel containing 1X MOPS and 1M formaldehyde, using 1X MOPS as the running buffer. For total RNA blots, 15µg to 20µg were loaded per lane while for poly A⁺ RNA blots, 200 to 500ng were loaded per lane. Total RNA (1µg) with 2µl of 400µg/ml stock ethidium bromide was used as RNA

marker. After migration, the gel was photographed under UV with a ruler on the side to view the migration distance of the 28S and 18S transcripts in the marker lane. The RNA from the gel was transferred overnight in 10X SSC onto a nylon membrane [Zetabind or Pall Biodyne] by capillarity.¹⁸⁵ After transfer was completed, the RNA was immobilized by UV-crosslinking in the UV Stratalinker 2400 (Stratagene) at the "Autocrosslink" setting. The membranes were prehybridized from 15 min. to 4 hours in hybridization tubes (Mandel Scientific) in prehybridization solution [50% formamide, 0.5M NaHPO₄, pH 7.2; 1mM EDTA; 1% BSA; 5% SDS; 0.08 mg/ml yeast tRNA]. The denatured labeled probe (see next section) was added and hybridization was performed at 42°C for 18-36 hours. The membranes were then washed twice for 15 min. at room temperature in 2X SSC;0.1% SDS, and for 30 min. at 65°C in 0.1X SSC; 0.1% SDS. The washed membranes were covered with plastic wrap and exposed to Kodak XAR-5 film with intensifying screens or, alternatively, exposed to a Phosphor Screen (Molecular Dynamics) subsequently processed with a PhosphorImager (Molecular Dynamics). To remove the probe, the membrane was incubated in boiling 0.1% SDS for 2 to 10 min.

Probe preparation: The probes for Northern analyses were prepared in one of two ways. For larger size DNA fragments, 20 to 50ng of DNA template was labeled with $\alpha^{32}\text{P}$ -dCTP using the Prime-ItTM II Random Primer Labeling Kit (Stratagene), according to manufacturer's instructions. The labeled probe was diluted by adding 170 μl of TE⁻⁴ and the specific activity was assessed by performing TCA precipitation on 1 μl out of 220 μl of

the probe mixture as follows. The probe (1 μ l) was added to 2ml 5% TCA and 0.1ml 0.2% BSA and left on ice for 10 min. The mixture was poured onto a Whatman 2.4cm filter under vacuum. The filter was rinsed with 10ml of 5% TCA and 10ml ethanol (99%). The filters were transferred into scintillation vials containing 5ml Ready Safe liquid scintillation cocktail and counted using a bench-top microprocessor-controlled spectrometer, model LS 1801 (Beckman). The remaining probe was precipitated by adding 4 μ l native salmon sperm DNA (10mg/ml), 4 μ l 0.1M spermine-4HCl, pH 7.2. After a 10 min. incubation on ice, the samples were centrifuged for 10 min. and the supernatant was removed. The DNA pellet was resuspended in 135 μ l of TE⁻⁴, 15 μ l of 5M NaCl and 15 μ l of 4N NaOH, denatured at 65°C for 5 min. and quenched on ice for 2 min. For smaller DNA fragments, the labelling was done simultaneously with the PCR reaction. The template DNA was mixed with 2 μ l 10X Perkin Elmer buffer, 2 μ l of 25 μ M dNTP (-dCTP), 2 μ l T11MN primer (10 μ M), 2 μ l arbitrary decamer (2 μ M), 0.2U of AmpliTaq polymerase, and 10 μ l of α -³²PdCTP (3000 Ci/ μ l; Amersham), in a total volume of 20 μ l. The samples were overlayed with mineral oil and subjected to the same temperature cycles as described earlier. The probe was precipitated and counted as described before.

Reverse Northern analysis: Reverse Northern analyses were performed essentially as described by Mou et al.¹⁹⁰ Plasmid DNA (1ng) of different subclones of differential fragments was transferred onto Pall Biotrans nylon membranes in duplicate using the

Hybri-Slot Manifold (Gibco). The DNA samples were diluted in 0.4N NaOH and 10mM EDTA, and heated at 95°C for 10 min. After transfer, the slots were rinsed with 500µl of 0.4N NaOH. The apparatus was dismantled and the membrane rinsed in 2X SSC. The DNA was immobilized by UV-crosslinking as described before. The membranes were prehybridized overnight in 10ml hybridization solution [10% PEG; 1.5X SSPE (20X SSPE = 3M NaCl, 0.174NaH₂PO₄-H₂O, 20mM Na₂EDTA-2H₂O); 7% SDS] in hybridization chambers (Mandel Scientific). Complementary DNA generated from DU145-N19 and DBM9-7 RNA was used as probe on duplicate slot blots. DU145-N19 and DBM9-7 RNA (5µg each; DNase I-treated) was reverse transcribed, using 1X First Strand buffer, 10mM DTT, 20µM dNTPs, and 200U SuperScript Reverse Transcriptase, as described in the "Reverse transcription" section. The resulting cDNA was labeled by random priming using the Prime It™ II Labelling Kit as described in the "Probe preparation" section, except that the reaction was incubated at 37°C for 30 min. instead of 10 min. The total number of incorporated counts was determined by TCA precipitation and 4x10⁶ cpm/ml hybridization solution (total of 10ml used, containing 50µg/ml denature salmon sperm DNA) were used for the hybridization step performed at 60°C for 20 hours. The membranes were washed at room temperature for 5 min. in 2X SSC; at 65°C for 30 min. in 2X SSC/1% SDS; at 65°C for 30 min. in 0.2X SSC/0.1% SDS. Detection and probe removal were performed as described in the "Northern analysis" section.

Sequencing: Plasmid inserts (from 1 to 2 μ g) representing differential fragments were sequenced using the Sequenase Version 2.0 DNA Sequencing kit, according to the manufacturer's instructions. Loading buffer [20mM EDTA, 95% formamide, 1mg/ml xylene cyanol, and 1mg/ml bromophenol blue] was added to each sample and the latter was denatured at 80°C for 5 min. before loading on the gel. The polyacrylamide gel was made by mixing together 33.6g urea, 9.6ml of 10X TBE [0.89M borate, 0.89M Tris, 20mM EDTA, pH 8.3], 9.6ml Long Ranger hydrolink gel solution, and 80ml H₂O. The mixture was filtered and 40 μ l TEMED and 400 μ l 10% ammonium persulfate were added to start polymerization. The gels were run as described in the "Differential display" section. The sequences obtained were analyzed over the Internet using the program Basic Local Alignment Search Tool (BLAST), developed by NCBI.¹⁸⁶ BLASTN is used to search a nucleic acid sequence against a nucleic acid database, and BLASTX takes a nucleic acid input sequence and translates it in all six reading frames before searching against a protein-sequence database.

5.3 Differential screening

5.3.1 Differential *Alu*-PCR

We have established in chapter 3 that a portion of human chromosome 12 suppressed the tumorigenic phenotype of the DU 145 human prostate cells. Unfortunately, analysis of the human suppressor region in a human background presents many technical difficulties, as explained in chapter 4. For this reason, we opted to

analyze portions of chromosome 12 that contain or not the tumor suppressor gene in a rodent background. The availability of mouse/human microcell hybrids containing different portions of chromosome 12 presented the opportunity to analyze the tumor suppressor region with more ease. However, the expression of genes analyzed in these hybrids had to be verified in the DU 145 hybrids, since the suppression of tumorigenicity was initially observed in that cell line.

Since the mouse/human B78MC9-5 cells contains the tagged del(12)(q13) region encoding the tumor suppressor gene (TSG+), and that BRH3 contains a big portion of the 12p arm lacking the tumor suppressor gene (TSG-), we attempted to isolate DNA segments present in the former but not in the latter. For this purpose, we took advantage of a unique property of *Alu* sequences, which are distinct to human DNA. Genomic DNA isolated from each cell population was amplified with different *Alu* primers and the products compared side-by-side on an agarose gel (Figure 5-2A). Several fragments specific to B78MC9-5 were excised from the gel and electroeluted. Table 5-1 lists the differential fragments obtained.

These fragments were analyzed by reverse Northern. Duplicate slot blots containing each *Alu* fragment were probed individually with cDNA populations obtained from DU145-N19 or DBM9-7 RNA. Since the inter-*Alu* sequences isolated represented chromosome 12 material in a very large region of interest, not all of these fragments were expected to encode genes differentially expressed between DU145-N19 and DBM9-7. In fact, only three out of twenty-two fragments encoded mRNAs which were enriched in the

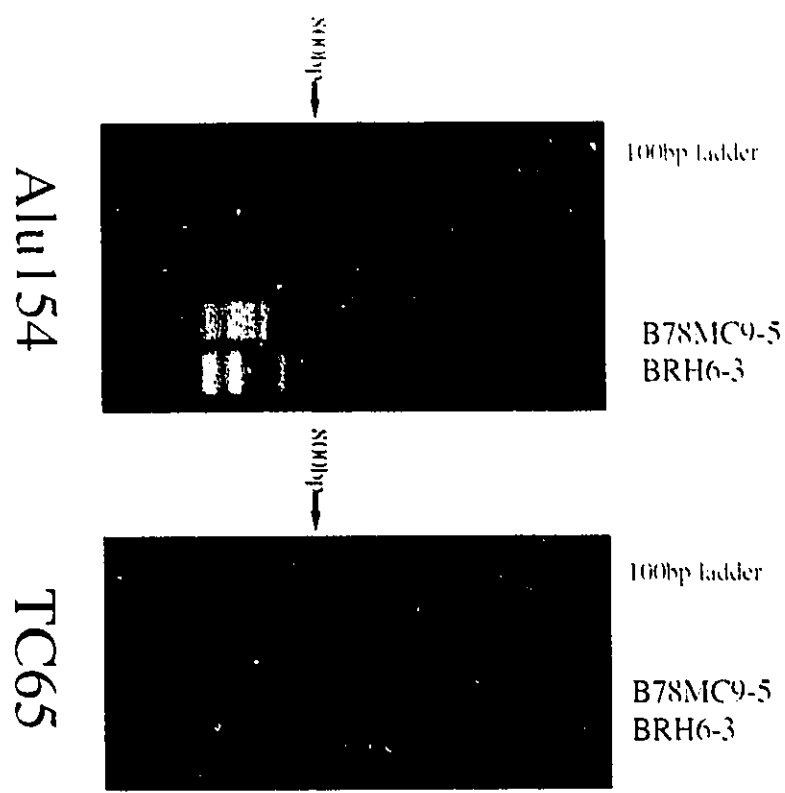
DBM9-7 suppressed hybrid compared to the DU145-N19 tumorigenic cells (Figure 5-2B). Consequently, 12TC65-1, 12TC65-4 and 12TC65-10 are chromosome 12-specific DNA sequences which encode for genes overexpressed during suppression. These should represent candidate tumor suppressor genes. Sequence analysis of these fragments will give insight as to their identity.

Table 5-1:
List of *Alu* fragments differentially amplified between B78MC9-5 and BRH3.

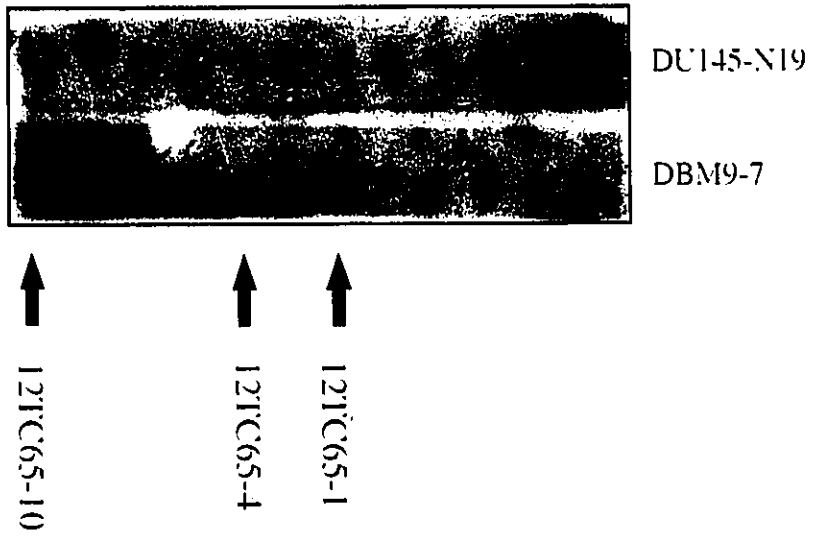
<i>Alu</i> primer	Fragment length (bp)	Fragment name
<i>Alu</i> 153	1400	12A153-1
<i>Alu</i> 153	1100	12A153-2
<i>Alu</i> 153	500	12A153-3
<i>Alu</i> 153	450	12A153-4
<i>Alu</i> 153	400	12A153-5
<i>Alu</i> 153	225	12A153-6
<i>Alu</i> 154	1050	12A154-1
<i>Alu</i> 154	925	12A154-2
<i>Alu</i> 154	800	12A154-3
<i>Alu</i> 154	600	12A154-4
<i>Alu</i> 154	550	12A154-5
<i>Alu</i> 154	500	12A154-6
<i>Alu</i> 154	300	12A154-7
<i>Alu</i> 451	700	12A451-1
<i>Alu</i> 451	600	12A451-2
TC65	2000	12TC65-1
TC65	1300	12TC65-3
TC65	1100	12TC65-4
TC65	1000	12TC65-5
TC65	900	12TC65-6
TC65	600	12TC65-8
TC65	150	12TC65-10

Figure 5-2: Differential *Alu*-PCR results. (A) Display of inter-*Alu* human DNA fragments amplified from B78MC9-5 and BRH3. Genomic DNA obtained from both mouse/human hybrids was amplified as described in materials and methods. The resulting inter-*Alu* human DNA amplicons were resolved on a 1.5% agarose gel and viewed under UV. (B) Reverse Northern of chromosome 12 inter *Alu* sequences probed with DU145-N19 and DBM9-7 cDNA.

A



B



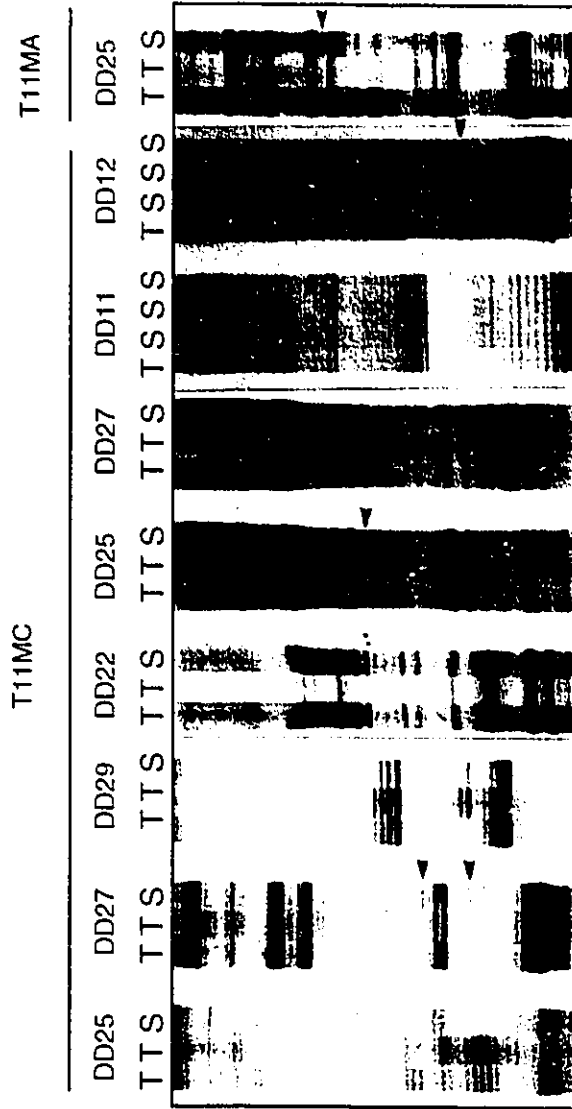
5.3.2 Differential display (DDRT-PCR)

To identify differences between tumorigenic and suppressed hybrids, we have displayed PCR-amplified cDNA populations generated from suppressed and tumorigenic hybrids side by side on polyacrylamide gels and isolated differential bands. Figure 5-3A depicts examples of differential display gels. The re-amplified bands were usually distinct, as demonstrated in figure 5-3B. The average number of bands per lane varied between 100 and 150, which corresponds to what has been reported.¹⁷⁹

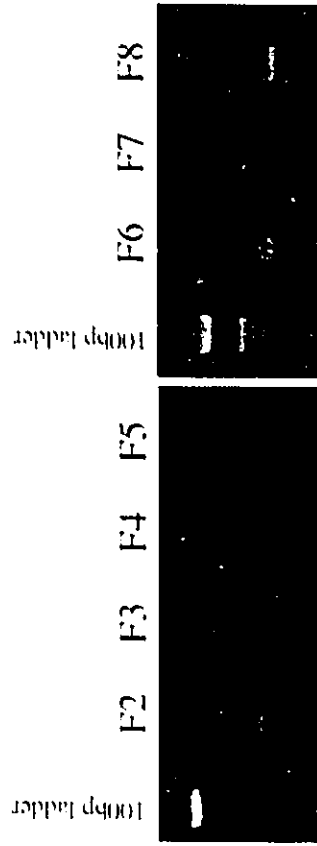
The decamers used in the first set of experiments were distinct from those used in the second set. In a first set of experiments, the tumorigenic DU145-N19 cells (T) were compared to DBM9-7, DU12RH-6R2, and DU12RH-6R4 which both displayed suppressed phenotypes (S) upon injection into nude mice (Figures 3-6 and 4-2). In a second set of experiments, the tumorigenic DU145-N19 and DU12RH-3 cells (T) were compared to the suppressed DBM9-7 cells (S). The inclusion of DU12RH-3 in the analysis avoided the isolation of genes located in regions of the p arm of chromosome 12 which do not contain the tumor suppressor gene. The use of multiple displays provided a built-in internal control for distinguishing noise from true differences. Nevertheless, not all the differences seen initially were reproducibly differential after rescreening.

A total of 13 cDNAs were isolated in the first set of experiments while 10 cDNAs were obtained in the second set, two of which were down-regulated in the cells displaying

Figure 5-3: Differential display RT-PCR results. (A) cDNAs from tumorigenic (T) or suppressed (S) prostate cells were amplified by PCR using different arbitrary decamers, as described in the materials and methods section. The amplicons were resolved on a 6% denaturing polyacrylamide gel. For decamers DD11 and DD12, T = DU145-N19 and S = DU12RH-6R2, DU12RH-6R4 and DBM9-7. For all other decamers, T = DU145-N19 and DU12RH-3 while S = DBM9-7. The arrows indicate amplified portions of cDNAs only visible in the suppressed (S) hybrids. (B) Examples of differential fragments which were excised from the polyacrylamide gels, re-amplified, and resolved on a 2% NuSieve agarose gel and photographed under UV.



A



B

a suppressed phenotype (DR1 and DR2). Two out of the 23 fragments excised from the gels could not be re-amplified successfully. After an initial screening using Northern analysis, 16 of the fragments were subcloned into the pCRTMII vector.

5.4 Analysis of candidate cDNAs

To determine if the cDNA fragments isolated were truly differentially expressed between tumorigenic and suppressed hybrids, the fragments were labeled by PCR amplification before or after subcloning, and hybridized to Northern membranes. In cases where the fragments had not been subcloned, hybridization to several RNA species could be observed, indicating that several cDNAs were extracted from the polyacrylamide gel (Figure 5-4 B and E). Several hybridization signals were observed (usually 1 to 4), but in many cases, at least one mRNA displayed increased expression in DBM9-7 (suppressed) compared to DU145-N19 (tumorigenic). To determine which cDNA was differentially expressed, the amplified products were subcloned and each subclone was analyzed individually. The fact that a cDNA recovered from what appeared to be a unique band on the polyacrylamide gel consisted of additional undetectable overlapping or unresolved cDNAs explained why the Northern probes with subcloned cDNA fragments frequently gave no signal or no difference in message expression.

To identify interesting candidates, several subcloned fragments were sequenced (see details in Table 5-2). Nine subclones matched to human cDNA sequences of unknown function. Five sequences obtained were novel, that is, they did not have

Table 5-2: List of differential products and their subclones obtained by DDRT-PCR.

cDNA ^a	Primers	Insert Size ^b (bp)	Expression ratio ^c	Homology ^d	Identity ^d
F1	T11MC-DD2		No hybridization		
F1h		200	1.0		Human cDNA (N)
F2	T11MC-DD12		2.9 at 1.5kb Fig.5-4A		
F2a		173		Vector (N)	
F2c, f		152	2.9		GNAI2L (N), 1.5kb
F2e		137			195-1 (N,X), 4.7kb
F2h		N.D.			
F2o		N.D.			
F3	T11MC-DD11	not subcloned	1.3 at 1.4kb		
F4	T11MC-DD12	not subcloned	1.7 at 3.1kb		
F5	T11MC-DD12		2.8 at 2.4kb 3.1 at 2.8kb Fig.5-4B		
F5d		183		Mouse gamma interferon receptor (X)	
F5m		228		Laminin B1 (X)	
F5o		230			Human cDNA (N)
F5g		N.D.		N.D.	N.D.
F6	T11MC-DD7		8.0 at 0.5kb Fig.5-4C		
F6h		167		Vector (N)	
F6i		197		Ca ²⁺ sensing receptor (X)	
F6k		196		Vector?(N)	
F6p		201		Vector?(N)	
F6i		N.D.	2.8 (RN)	N.D.	N.D.
F7	T11MC-DD7		N.D.		
F7a		202	0.3 at 2.6kb Fig.5-4D		VDAC2-like (N)
F7c		N.D.		N.D.	N.D.
F7e		221	6.0 (RN)	Kin1(X)	
F7g		221		None	None
F7h		208			Human cDNA (N)
F8	T11MC-DD7		3.1 at 1.4kb 4.3 at 3.0kb Fig.5-4E		
F8a,F8c		298			Rat PI transfer protein(N,X),4.7kb
F8f		295		Drosophila fat tumor suppressor gene (X)	Human cDNA (N)
F8g		297	3.1		CTD9/MRP1(N,X), 1.4kb
F8h		296			Human cDNA (N)
F12	T11MC-DD27	not subcloned	1.4		
F13	T11MC-DD42	not subcloned	1.3		
F14	T11G-DD22		Fig.5-4F	1.4 at 1.6kb	
F14b,h		320		Pou domain (X)	
F14c		320		None	None
F16A	T11MC-DD22		N.D.		
F16A,f,i		129	1.83, 1.49 (RN)	None	None
F16B	T11MC-DD22		N.D.		
F16Bh,d		146	1.92, 1.31(RN)		Human cDNA
F16Bj		166	0.86 (RN)		Human cDNA
F17	T11MC-DD27		N.D.		
F17Ba,g		215		CENP-E (N,X)	
F17Bc,d		217			Human cDNA
F17Be		N.D.		N.D.	N.D.
F17Bf		204		None	None
F17Bh		167		None	None
F18	T11MC-DD30	not subcloned	4 mRNAs not differential		
F19	T11MC-DD44		N.D.		
F19c		170		Rab5b (N,X)	
F20	T11MC-DD27		N.D.		
F20b		250		N.D.	N.D.
F21	T11MA-DD25		N.D.		
F21a				Mitochondrial gene (N)	
F21b,e,f,h,i		N.D.		N.D.	N.D.
DR1	T11MC-DD21	not subcloned	N.D.		
DR2Ac	T11MC-DD44	320		N.D.	N.D.
DR2Bc	T11MC-DD44	220	<1.0 (rn)		Human cDNA

^a Bold designation represents the fragments before subcloning.^b Size of fragment as deduced from the sequenced subclones.^c Expression ratio as determined by Northern analysis or Reverse Northern (indicated as RN) between DU145-N19 and DBM9-7.^d As determined upon sequence analysis using the BLASTN (nucleotide level) or the BLASTX (amino acid level) programs

significant homology to any sequences in the databanks. 13 sequences demonstrated homology or identity to DNA or aminoacid databases. Thirteen subclones have not been sequenced yet.

Decreased expression:

Subclone F7a hybridized to three distinct transcripts by Northern analysis, indicating that either it was alternatively spliced or that it belongs to a gene family (figure 5-4D). Its sequence corresponded to a portion of the 3' UTR of a voltage-dependent anion channel 2 (VDAC2)-like gene. While VDAC1 and VDAC2 transcripts corresponded to the two smaller transcripts (2kb and 1.3kb, respectively), the third transcript that we observed at 2.6kb has not yet been reported in the literature. The latter was markedly downregulated in DBM9-7 compared to DU145-N19, displaying an expression ratio of 0.3. DR1 and DR2 (A and B) are also downregulated fragments. One subclone of DR2B was obtained which was shown to be downregulated by reverse Northern. It has not yet been sequenced.

Marginal or no differential expression:

Several fragments displayed very low or no differential expression between DU145-N19 and DBM9-7. For example, F3, F4, F12, F13 and F18 showed between 1.0- to 1.7-fold enrichment and hence were not subcloned or analyzed further. Fragment F16A was not analyzed by Northern before subcloning. The two subclones obtained (F16Ai and F16Af) were the same novel sequences which showed a slight increase in expression on a reverse Northern (1.83 and 1.49-fold, respectively). Subclones F16Bb

and F16Bd encoded a human cDNA of unknown function which displayed differential expression on a reverse Northern. Subclone F16Bj also encodes a human cDNA of unknown function but was not differentially expressed by reverse Northern.

Notable expression increase:

Fragment F5 hybridized to several transcripts on Northern, two of which (2.4 and 2.8 kb) were upregulated 2.77 and 3.12-fold, respectively, in DBM9-7 compared to DU145-N19 (Figure 5-4B). Subclone F5d had homology to the mouse interferon gamma receptor on chromosome 6q, while subclone F5m had homology to laminin B1 at the amino acid level. Fragment F6 hybridized to only one transcript by Northern analysis. An 8-fold increase in intensity was observed for the 0.5kb transcript in DBM9-7 compared to DU145-N19 (Figure 5-4C). This fragment was subcloned and sequenced. The F6i subclone corresponded to a novel transcript with mild homology to calcium-sensing receptors at the protein level. F6t has not been sequenced, but was shown to be expressed 2.8 fold more in DBM9-7 than in DU145-N19 by reverse Northern analysis.

Fragment F14 hybridized to two transcripts on a Northern and the longer transcript was differentially expressed between DU145-N19, two of the suppressed hybrids, and their respective revertants (figure 5-4F, only longer transcript shown). F14b and F14h subclones both encoded the same novel sequence with homology to Pou or helix-loop-helix (HLH) domains at the protein level, but differential expression of these subclones has not yet been verified.

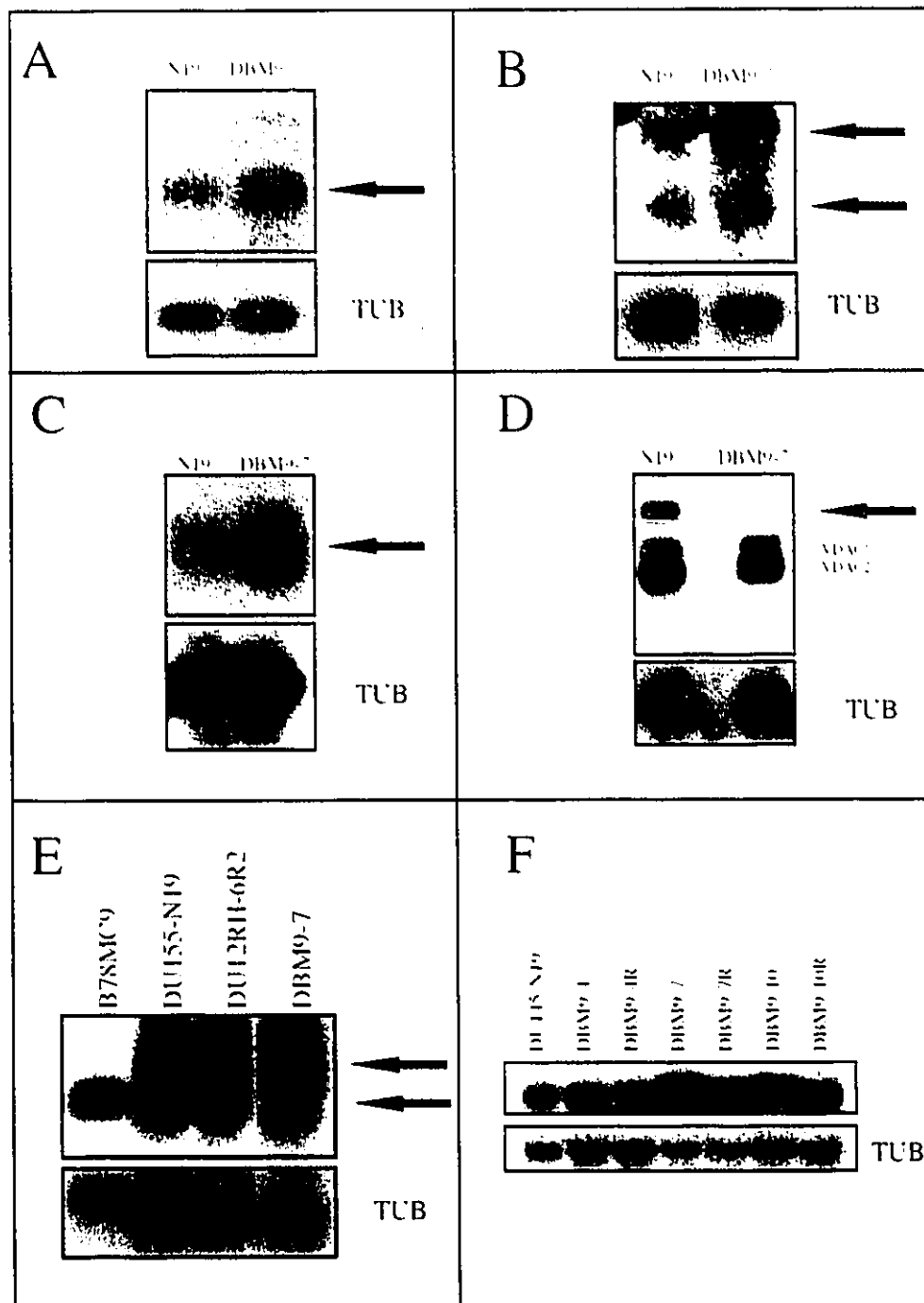
Chromosome 12 genes:

A subclone of fragment F19 demonstrated homology to the Ras-related protein Rab5B gene located on chromosome 12q13. Since Northern analysis with this fragment was unsuccessful, we have not been able to validate an increase in expression in DBM9-7 compared to DU145-N19.

The F2c and F2f subclones were identical and corresponded to the 3' untranslated region of the guanine nucleotide binding protein (G protein) α inhibiting activity polypeptide 2-like (GNAI2L) gene located on chromosome 12p13. The GNAI2L cDNA was reported to be 1.5kb and thus corresponds to the upregulated transcript revealed by Northern analysis (2.9 fold increase of a 1.5kb band indicated by the arrow in figure 5-4A). There was also a longer transcript at 2.8kb which is only present in DBM9-7. This transcript cannot correspond to EF-1, (subclone F2e) which has an mRNA transcript of 4.7kb, which was not visible on the Northern. Hence, the nature of the upregulated 2.8kb transcript is still unknown.

Fragment F8 hybridized to three different transcripts on a Northern membrane (Figure 5-4E). The lower transcript of 1.4kb (3-fold increase in DBM9-7 compared to DU145-N19) also hybridized to B78MC9-5 mRNA, indicating that it is encoded on chromosome 12. In fact, sequence analysis of subclone F8g revealed that it is identical to the coding region of the CD9/MRP1 gene on 12p13. Interestingly, this gene (mRNA of 1.4kb) has been implicated in suppression of cell motility and belongs to the same gene

Figure 5-4: Expression of various differential fragments. Northern analysis performed on DU145-N19 and DBM9-7 poly A⁺ RNA (0.5µg) with uncloned DDRT-PCR fragments (F2, F5, F6, F8 and F14) and a subcloned fragment of F7 (F7a). F14 hybridization was performed using 15µg of total RNA. All membranes were also probed with a tubulin sequence (TUB) to control for loading differences. Arrows indicate mRNA of interest. (A) F2; (B) F5; (C) F6; (D) F7a; (E) F8; (F) F14.



family as the metastasis suppressor KAI1. The middle transcript at 3kb showed a 4 fold increase in DBM9-7 compared to DU145-N19 but could not be linked to any of the subclones. Two of the subclones analyzed, F8a and F8c demonstrated complete homology to a human cDNA and about 80% homology to the rat phosphatidylinositol transfer protein.¹⁸⁷ Strong sequence similarity (88% and 84%) in the portions of the 5'- and 3'-UTR between the rat and the human mRNAs has previously been reported.¹⁸⁸ However, subclone F8f is a good candidate since it displayed a 2-fold increase by reverse Northern and showed sequence homology to a human cDNA with similarity to the *Drosophila* fat cadherin motif in the coding region of the *Drosophila* fat tumor suppressor gene.¹⁸⁹ The last subclone, F8h, had sequence homology to a human cDNA of unknown function.

Others:

Two subclones of fragment 17 were partly homologous to the centromeric protein E (CENP-E), a kinetochore-associated protein involved in chromosome movement located on chromosome 4q24-q25.¹⁹⁰ Interestingly, this exact sequence was also isolated in an independent study in the laboratory due to its increased expression in suppressed teratocarcinoma cells with an introduced normal human chromosome 4. These results indicate that this novel gene with sequence homology to CENP-E might be involved in general suppression mechanisms.

5.5 Discussion

In the past, the best method of differential expression cloning was subtractive hybridization, but due to technical difficulties presented by this method, we have turned to recently designed differential methods. They involve *Alu*-PCR or RT-PCR (of DNA or RNA, respectively) of suppressed and tumorigenic prostate cell hybrids, followed by display in adjacent lanes on agarose or polyacrylamide gels to detect differences at either the genomic or expression level. Each cell type can contain from 10 000 to 15 000 mRNA species. The tumor suppressor gene on chromosome del(12)(q13) might lead to a cascade of alterations in the regulation of many downstream genes, which ultimately determine the suppressed phenotype. Therefore, the detection of several differential genes is expected, which become either enriched or down-regulated during suppression. The DDRT-PCR method was ideally suited to these circumstances for several reasons. By using several primer combinations that yield 100 to 150 bands per lane, most of the mRNA population could be screened and compared efficiently. Also, the simultaneous display of mRNA populations allowed the identification of genes which are upregulated and downregulated in different states of the cell. Importantly, we could prevent the isolation of false-positives that arise from clonal variations by (A) comparing the display of almost identical cells (i.e. DU 145-derived cells) and (B) comparing more than two hybrids with known phenotypes.

The differential display method has been useful in many instances to clone cancer-related genes. The alpha 6 integrin was implicated as a tumor suppressor gene by this method in a breast cancer model system.¹⁹¹ Another tumor suppressor gene, Maspin, was linked to human breast cancer progression by differential display. The maspin gene maps to chromosome 18q21.3¹⁹² and encodes a protein related to the serpin family of protease inhibitors. It is expressed in normal mammary epithelial cells but not in most mammary carcinoma cell lines. Transfection of mammary carcinoma cells with the maspin gene reduced the cells' ability to induce tumors and metastasize in nude mice and to invade through a basement membrane matrix in vitro.¹⁹³ Finally, the mouse tissue inhibitor of metalloproteinases-3 (TIMP-3) gene was shown to be differentially expressed in preneoplastic or neoplastic JB6 mouse epidermal cells.¹⁹⁴ TIMP-3 is one of a family of genes implicated in tumorigenesis and tumor invasion.

These few examples demonstrate that differential display represents a reliable technique to clone tumor suppressor genes. The system that we used should be even more specific since the RNA populations we are comparing are essentially identical except for the presence of extra normal chromosome 12 material in the suppressed hybrids. This situation should limit the number of irrelevant differences that could be obtained in systems where the populations compared are more distantly related. In fact, chromosome 12 genes were identified, such as GNAI2L, CD9 and Rab5b, demonstrating the specificity of the system.

Some technical problems were encountered with DDRT-PCR, especially with the re-amplification of the excised bands. In some cases, the cDNA could not be re-amplified at all, while in some instances, the DNA re-amplified consisted of several overlapping or unresolved cDNAs. This problem was described in the literature,¹⁹⁵ and several methods have been suggested to determine which subclone is differentially expressed. These include cutting the RNA species that displays difference in expression directly from the probed Northern membrane and the re-amplification of the labeled probe fragments.¹⁹⁵ Alternatively, the use of the reverse Northern method has been reported to screen different subclones. This procedure allows the analysis of several subclones in one experiment where the plasmid DNA is transferred to a nylon membrane in duplicate and probed with labeled cDNA from either suppressed or tumorigenic cells. This proved to be an excellent screening method to pinpoint the most differentially expressed subclones. We have sometimes turned to reverse Northern analysis when no signals were obtained by direct Northern analysis.

Several known cDNAs have been identified so far using differential display (Table 5-2 and Table 5-3). We have cloned the guanine nucleotide-binding protein, alpha-inhibiting, polypeptide-h, usually designated as GNAIH; GNA, GNAI2A or GNAI2L (F2c, F2f). This gene was originally identified in the course of cloning the alpha(i) G-protein cDNA.¹⁹⁶ The GNAI2L gene was shown to encode an extremely similar but distinct G protein. Whereas the gene for alpha(i) was found to be on chromosome 7, the gene for GNAI2L was assigned to chromosome 12, in the 12p13-p12

region by *in situ* hybridization.¹⁹⁷ GNAI2L is a related sequence to the GNAI2 gene encoded on chromosome 3p21, a region frequently involved in rearrangements in various human tumors. GNAI2 has been linked to calcium activation of phospholipase A2 (cPLA2): a dominant negative GNAI2 in Chinese hamster ovary cells could inhibit ATP-dependent receptor activation of cPLA2.¹⁹⁸ GNAI2 also inhibited the c-fos promoter activity through the Rb control element (RCE) located in the c-fos promoter region. The effect of GNAI2 on RCE was shown to be mediated by the Rb gene product. It also augmented the underphosphorylated active form of pRb by promoting pRb expression and by affecting the phosphorylation state of pRb.¹⁹⁹ The properties displayed by GNAI2 suggest that the GNAI2L isoform on chromosome 12 might display similar functions.

We have also cloned a portion of exon 8 of CD9/MRP1/p24 (F8g), a gene mapping to chromosome 12p12.3-p13.2.^{200,201} The CD9 gene is composed of 8 exons spanning more than 20kb and produces a 1.4kb mRNA²⁰² encoding a 227 amino acid molecule with 4 hydrophobic domains and one N-glycosylation site. The CD9 gene belongs to a new cell surface protein family including the target of an antiproliferative antibody (TAPA1), a human tumor-associated antigen (CO-029), a B cell surface antigen (CD37), a melanoma-associated antigen (ME491), a prostate cancer metastasis suppressor (KAI1) and the Sm23 antigen of the trematode parasite *Schistosoma mansoni*. A murine monoclonal antibody (M31-15) specific to CD9 remarkably inhibited the motility of various cancer cell lines.²⁰³ Overexpression of the CD9 gene in human lung adenocarcinoma cell line MAC10 resulted in the suppression of cell motility associated

Table 5-3:
List of candidate genes involved in suppression of tumorigenicity.

Differential Fragment	Gene Name	Status	Chromosomal location
F1	Leukocyte homing receptor	H	1q23-25
F2	G protein α_1 2-like	I	12p12-p13
F5	Laminin β 1	H	7q31-32
F6	Calcium sensing receptor	H	?
F8g	CD9/MRP1	I	12p12.3-p13.3
F8f	FAT tumor suppressor	H	4q34-q35
F17	CENP-E	I*	4q24-q25
F19	Rab5b	H	12q13

H = sequence homology; I = identical sequence

* complete homology to half of the cloned fragment.

with considerable inhibition of cell growth. Mouse melanoma cells transfected with CD9 DNA demonstrated decreased ability to metastasize to the lung.²⁰⁴ It was also recently demonstrated that the level of CD9 expression was inversely related to the clinical stage of a given carcinoma of the breast and that low CD9 expression by tumors of the lung may be associated with poor prognosis.²⁰⁵ In light of these results, and the fact that it is located on del(12)(q13) and differentially expressed in our hybrids, we propose that CD9 represents a strong candidate as the tumor suppressor. It will be interesting to verify whether CD9 exercises its suppressive effect in different prostate cell lines and whether reduced expression of CD9 mRNA correlates with poor prognosis in prostate tumors.

Another gene (F19c) was isolated from the del(12)(q13) region which encodes the Ras-related protein Rab5b, located at band q13.²⁰⁶ This gene belongs to a family of small

monomeric GTPases thought to play a role in membrane trafficking. Rab5b was shown to be localized to the plasma membrane and early endosomes.

Sequence F8f was homologous to the *Drosophila* fat tumor suppressor gene in the coding region corresponding to one of the cadherin domains. This gene encodes a transmembrane protein containing 34 cadherin repeats,¹⁸⁹ and recessive (loss-of-function) mutations lead to hyperplastic overgrowth of the imaginal discs, suggesting its involvement in contact-dependent cell interactions.²⁰⁷ The human counterpart, named FAT, was recently cloned by Dunne et al,²⁰⁸ and localized to chromosome 4q34-q35. FAT mRNA was shown to be present in many epithelial and some endothelial and smooth muscle cells.

We have also cloned what might be an isoform of the voltage-dependent anion channel-2, VDAC-2 (F7a). The region of the gene subcloned by DDRT-PCR corresponded to a region of homology to VDAC1 and VDAC2. The third isoform was shown to be downregulated in suppressed cells containing chromosome 12. VDAC molecules are anion channels of the outer mitochondrial membrane and are thought to play a role in transport of metabolites including ATP across mitochondrial membranes. VDAC binds hexokinase and glycerol kinase to the mitochondrion and therefore may modulate respiration processes. Although only two VDAC genes were reported (VDAC1 at Xq13-q21 and VDAC2 on chromosome 21),²⁰⁹ the existence of other VDAC homologs has been proposed, some of which could be located in the plasma membrane.²¹⁰ It was recently demonstrated that the two human VDAC genes produce three proteins that differ

most significantly at their amino termini. This group could not substantiate previous results suggesting their presence in the plasma membrane. Based on this report, it is possible that the downregulated transcript that we have detected is an alternatively spliced isoform of VDAC2. Its downregulation during suppression of tumorigenesis could reflect its role in energy metabolism of tumor cells. Whether this is a cause or an effect of suppression has not yet been established.

Fragment F5 had homology to laminin B1, a gene localized to chromosome 7q31, a region of frequent deletions in prostate cancer.^{90,91} Laminin B1 is part of a heterotrimer of chains that form part of the extracellular matrix. A sequence in the B1 chain was linked to cell attachment, chemotaxis and binding to the laminin receptor. This sequence was found to reduce metastasis to the lung in mice injected with melanoma cells and to inhibit the invasiveness of the cells in vitro.²¹¹

A trend was observed in the roles displayed by several of the genes we have isolated, in that many are related to cell-cell or cell-matrix interactions: CD9 is a transmembrane protein involved in motility and metastasis suppression, Rab5b plays a role in trafficking at the plasma membrane, laminin beta 1 is part of the extracellular matrix and interacts with an integrin-like receptor, also involved in cell adhesion. The FAT tumor suppressor gene contains many cadherin domains, usually found in cell adhesion molecules such as E-cadherin. Cadherin molecules have often been linked to metastasis suppression as well. In fact, cell adhesion appears to be a key factor in prostate cancer progression. At an early stage in the search for PC tumor suppressor

genes, several genes, such as KAI1, E-cadherin, C-CAM1 and DCC have already been associated with tumorigenic and metastatic events occurring in the prostate. Because cell adhesion represents a complex series of inter-related events involving many different molecules, it is likely that many more genes involved in adhesion will be linked to tumor suppression. Therefore, we propose that the genes identified in this work represent possible candidates and that further analysis will provide insight as to their specific roles in prostate tumorigenesis.

Although we have identified candidate cDNAs involved in tumor suppression pathways, several of them are novel and will need to be further analyzed. Due to the anchored-primers used (T₁₁MN), most fragments are expected to be located in the 3'-UTR (at the polyA tail) of the corresponding mRNA. Since the 3'UTR of cloned genes are not always entered into sequence databases, it becomes necessary to isolate full-length cDNAs. This step is now greatly facilitated by the use of PCR techniques such as 3' and 5' RACE (Rapid Amplification of cDNA Ends). Once full length cDNAs are obtained, their sequence can be compared again to the gene databases to reveal their identity. Sequence motifs can also be identified to reveal possible functions of the gene product. The most promising candidates can be analyzed in different prostate cancer cell lines and other tumor cell lines to establish the degree of significance of the gene in cancer in general. Their expression can also be assessed in different stage tumor samples to investigate their possible uses as a prognostic markers. Candidate genes can also be introduced in tumor cells to establish their ability to suppress tumorigenicity.

6. CONCLUSIONS

The work presented in this thesis focused on the identification of chromosomes that can suppress the tumorigenic phenotype of a prostate cancer cell line, DU 145. The assessment of tumor suppression was achieved by subcutaneous injections of cells into athymic nude mice, the best system to determine tumorigenicity to date. We obtained evidence for the tumor suppressive potential of genes encoded on chromosome 8 in one microcell hybrid, DHM-6. The loss of two normal chromosomes 8 in the suppressed hybrid resulted in the re-emergence of the ability to form tumors. Although we have not identified the genes responsible for this suppression, many reports suggest that several tumor suppressor genes on the p arm of chromosome 8 are frequently deleted in prostate tumors. Other groups are now in the process of fine-mapping the regions of these genes in the aim to isolate them.

Evidence was also presented for the presence of a tumor suppressor in a specific region of chromosome 12, del(12)(q13), in three distinct microcell hybrids derived from DU 145 cells. In each of these hybrids, the loss of the introduced del(12)(q13) caused the prostate cells to regain their original tumor-forming ability.

Therefore, microcell fusion technology allowed the identification of two chromosomes, 8 and 12, that encode putative tumor suppressor genes inactivated in a prostate cancer cell line. The nature of the marker inserted into these chromosomes was crucial to confirm the results by allowing easy generation of segregant clones. The loss of normal chromosomes 8 and 12 in the segregant cells caused a reversion to a tumorigenic

phenotype, thus establishing that the changes in phenotype observed were caused by the introduced normal chromosomes.

Radiation hybrids of DU 145 cells were generated, which contained even smaller regions of the tagged del(12)(q13). After assessment of their effect on the tumorigenic phenotype in DU 145 cells, non-suppressive tagged portions of del(12)(q13) were transferred by microcell fusion into mouse cells to facilitate mapping. This strategy led to the localization of the tumor suppressor gene to smaller regions of chromosome 12. Since we initially started our search from the whole genome, and subsequently narrowed it down to a region of approximately 13.5Mb, we have increased the resolution of the gene location by over two hundred fold. The increase in availability of chromosome 12-specific PCR markers should allow even better resolution of the suppressor region than was achieved in the present work.

The last section of this thesis described the use of differential display methods to isolate either genomic or cDNA clones specific to suppressed DU145 hybrids. The technique of differential display reverse transcriptase PCR led to the identification of cDNAs which are specifically upregulated (or downregulated) during suppression of DU145 cells. A few of the cDNAs mapped to the del(12)(q13) region, demonstrating the specificity of the technique. Many novel cDNAs were isolated, which may also map to chromosomes 12. Other upregulated cDNAs represented known genes encoded by other chromosomes. The system established in this thesis provided optimal tools to identify not only the gene on chromosome 12 but also other genes, either oncogenes or tumor

suppressors involved in causing the suppression of tumorigenicity. We speculate that some of the genes we have isolated are involved in the suppressive pathways, presumably in downstream events initiated by the introduction of a chromosome 12 gene aberrant or absent in DU 145. The work presented in this thesis generated tools that will help future researchers to implicate new or known genes in the development of prostate cancer. Further characterization of the cDNAs isolated by differential display should provide insights into the suppressive events incurred by chromosomes 12 in prostate cancer and possibly in other types of cancers.

7. APPENDICES

7.1 APPENDIX I: OVERVIEW OF TUMOR SUPPRESSOR GENES

Even though the first TSG was cloned less than 10 years ago, an enormous amount of information pertaining to this type of gene exists, and could not possibly be covered here. Consequently, the following discussion will only attempt to briefly and selectively describe the functions of tumor suppressor genes which have had an impact in the still growing field of tumor suppression.

Tumor suppressor genes and the cell cycle

The two most extensively characterized tumor suppressor genes are without doubt the *p53* and Retinoblastoma (*Rb*) genes, whose inactivation lead to unrestrained cell proliferation and malignancy. Although their functions are distinct from one another, they are linked in the same cell cycle regulatory mechanism and each compensates for the loss of the other's tumor suppressor function.²¹² The *p53* gene is composed of 11 exons spanning about 20kb on the short arm of chromosome 17 (17p13.1).²¹³ The product of this gene is a 393-aminoacid nuclear phosphoprotein (53kd) with sequence-specific DNA-binding potential that allows it to function as a transcription factor.²¹⁴ Loss of normal *p53* function is one of the most common changes yet identified in human cancers.^{215,216} The most frequent alterations found are point mutations in conserved regions of the gene, clustered between exons 5 and 8. A second common mechanism of

loss of normal *p53* function is due to binding to mutated forms of *p53* and to viral oncoproteins.^{217, 218}

Studies have implicated *p53* as a control factor in response to DNA damage, resulting in two possible outcomes: arrest at the G₁/S phase of the cell cycle or induction of apoptosis. Mice with a deleted *p53* gene progress through fetal development normally, but spontaneous cancers occur after a few months. Cells from *p53*-null mice have defects in both the G₁ checkpoint activated by DNA damage and radiation-induced apoptosis of lymphoid cells. Consequently, these cells display a notable increase in genomic instability.^{219,220} Several genes are involved in these processes, especially the cyclins D and E and the cyclin-dependent kinases (CDKs) which allow passage through G₁ and S phases. The cyclin-dependent kinase inhibitors (CKIs) such as *p21*^{WAF1/Cip1}, *p16*^{INK4}, *p18*, *p19*, *p27*^{KIP-1}, and *p57*^{KIP2} are also implicated. In response to DNA damage, *p53* levels increase and cause G₁ arrest. It functions as a transcription factor to heighten the expression of specific genes such as the oncogene *MDM2*²²¹, the DNA repair gene *GADD45*²²² and the CKI *p21*^{WAF1/Cip1} gene.²²³ *p21* inhibits the ability of CDKs to interact with cyclins D and E and stimulates passage through the G₁ phase of the cell cycle.^{224,225,226} It now appears that *p53* can respond to DNA damage and cause G₁ arrest through *p21*^{WAF1/Cip1}²²⁷ and the growth arrest DNA damage-inducible *GADD45* gene. Both proteins interact with the proliferating cell nuclear antigen (PCNA), a molecule known to drive DNA replication and exert DNA repair functions.^{228,229} Moreover, the administration of wild-type *p21* using a recombinant adenoviral vector into a *p53*-

deficient mouse prostate cancer cell line significantly decreased tumor volumes in nude mice.²³⁰ This regulatory cascade of events involving activation of several negative regulators and inactivation of positive modulators of the cell cycle, results in prolonged G₁ arrest. This allows time for DNA damage repair before replication occurs.

As well as acting through a p53-PCNA-dependent pathway, p21^{WAF1/Cip1} has been shown to inhibit cell-cycle progression by binding and inhibiting CDKs. These two activities are functionally independent and reside in separate protein domains.²³¹ Induction of p21 by transcription factors such as MyoD or the E1A-associated p300 protein can cause cell cycle withdrawal during terminal differentiation.^{232,233} Moreover, mice lacking p21 develop normally but their fibroblasts cannot arrest in G₁ phase in response to DNA damage.²³⁴ Thus, it appears that CKIs are excellent candidates to act as tumor suppressor genes. In fact, the so-called multiple tumor suppressor gene (MTS1) located on chromosome 9p21 encodes the p16^{INK4} protein, which specifically binds CDK4, resulting in inactivation of the growth-promoting cyclin D/CDK4 complexes.²³⁵ The p16 gene has been found to be structurally abnormal in some tumor cells,^{236,237} and its introduction into lung cancer or glioma cell lines arrested tumor cell growth at G₁ and led to a pronounced inhibition of tumor cell growth both *in vitro* and *in vivo*.^{238,239} Merlo et al²⁴⁰ have demonstrated that transcriptional repression of the p16 gene can be achieved by promoter methylation rather than DNA mutation in some types of cancers. The MTS2 gene encodes another CDK inhibitor named p15^{INK4B} which binds CDK4 and CDK6, thus competing with their interactions with the D cyclins.²⁴¹

Other CKIs exist which have not yet been linked with tumor suppression but are certainly good candidates. *p19* and *p18*²⁴² are CDIs with homology to *p16*, while *p27*^{KIP-1} and *p57*^{KIP2} share homology with *p21*.^{243,244} The levels of p21, p15 and p27 proteins can be induced by the transforming growth factor TGF-beta.^{245,246,247} The latter causes an increased *p15* expression resulting in the displacement of *p27* from cyclin D-CDK complexes. *p27* is then available to bind to cyclin E/CDK2 complexes and prevent entry into S phase.^{248,249}

An additional consequence of the activation of CKIs is the inability of CDK molecules to phosphorylate and inactivate the RB protein.^{250,251} This allows RB to bind and sequester E2F transcription factors which are essential for progression to S phase.^{252,253} The *Rb* gene, named after the rare childhood eye tumor in which it was discovered, has served as an archetype for tumor suppressor genes. It spans 180 kb encoding a 928 amino acid product²⁵⁴ and its inactivation is involved in several human tumors.²⁵⁵ In many different cultured tumor cells, replacement of a normal *Rb* gene and expression of normal RB protein results in suppression of neoplastic properties. Moreover, germline mutation of the *Rb* gene leads to retinoblastomas or pituitary tumors, demonstrating its role in tissue-specific tumor predisposition. It has recently been demonstrated that RB is the critical target acted upon by cyclin D-dependent kinases in the G₁ phase of the cell cycle. In fact, *p16*^{INK4} can only suppress the growth of human cells that contain functional RB, establishing that RB is crucial for growth suppression by *p16*^{INK4}.^{256,257} It has been proposed that RB confers transcriptional regulation and tumor

suppression through an interaction with the TATA-binding protein-associated factor, TFIIID.²⁵⁸ RB can also specifically inhibit the activity of the RNA polymerase I transcription factor UBF (upstream binding factor) *in vitro*, thus repressing transcription of the ribosomal RNA genes.²⁵⁹

Tumor suppressor genes and transcriptional regulation

The association of a subset of Wilms' tumors with a chromosomal deletion of chromosome 11p13 led to the isolation of the Wilms' tumor suppressor gene (*WT1*) by positional cloning.^{261,260} The *WT1* protein has a proline-rich amino-terminus that mediates transcriptional repression and four Cys-Cys-His-His zinc fingers that bind DNA.^{261,262} Mutations in *WT1* have been detected in subsets of Wilms' tumor and in patients with the Denys-Drash Syndrome. The gene encodes four isoforms of a zinc finger protein,²⁶³ and is implicated in controlling normal urogenital development.²⁶⁴ Promoters of a large number of cellular growth- or development-related genes have been shown to be transcriptionally regulated by *WT1*.²⁶⁵ Candidate target genes for repression include insulin-like growth factor 2 (*IGF2*),²⁶⁶ platelet-derived growth factor chain-A (*PDGF-A*),^{267,268} insulin-like growth factor I receptor (*IGF-I-R*),²⁶⁹ *Pax-2*,²⁷⁰ the retinoic acid receptor-alpha 1 (*RAR-alpha 1*),²⁷¹ and the oncogenes *bcl-2* and *c-myc*.²⁷²

It is believed that dominant negative *WT1* alleles may play a role in tumorigenesis by associating with wild-type *WT1* proteins and decreasing their transcriptional activity, a situation reminiscent of *p53*.²⁷³ However, recent evidence suggest other roles for *WT1* in the cell. Larsson et al²⁷⁴ have shown that two *WT1* isoforms directly associate with

components in the spliceosomes and proposed that WT1 plays roles in posttranscriptional processing of RNA. WT1 has also recently been implicated in cell cycle regulation. Microinjection of the *WT1* cDNA blocked serum-induced cell cycle progression into S phase. The WT1-induced cell cycle block was mediated through inhibition of cyclin E/CDK2 or cyclin D1/CDK4 complexes.²⁷⁵ Luo et al²⁷⁶ have also suggested that tumor inhibition by WT1 may be achieved by interference with the *Ras*-mediated signaling pathway.

Another tumor suppressor gene plays a role in modulating the rate of transcriptional elongation. Mutations of von Hippel-Lindau gene (*VHL*) at locus 3p21 occur in sporadic and hereditary forms of renal carcinoma.^{277,278} Individuals with germ-line mutations are predisposed to multiple forms of cancers. In patients with von Hippel-Lindau disease, *VHL* is defective and can no longer negatively control the transcription elongation factor Elongin.^{279,280} The direct consequence is that RNA polymerase II can escape out of pause sites at much higher rates, thus speeding up transcription elongation of some genes.²⁸¹ The transfection of the wild-type *VHL* gene into two renal carcinoma cell lines that lacked normal expression of the gene resulted in dramatic growth suppression of these cells.²⁸²

Tumor suppressor genes and mismatch repair

The discovery of genome-wide microsatellite alterations was uncovered as the genetic defect in hereditary non-polyposis colorectal cancer (HNPCC). Subsequently, five different genes were cloned which are involved in the human equivalent of the

bacterial MutHLS DNA repair pathway. The *msh2* and G/T mismatch-binding protein (*GTBP*) genes, which both map to chromosome 2, encode proteins which target and bind to mismatched nucleotides.^{283,284,285,286} The *mlh1* gene product then excises the mismatched pair identified by MSH2.²⁸⁷ Taken together, these genes are frequently mutated in HNPCC (90%), and their inactivation is responsible for the replication error phenotype.^{288,289,290} Two other genes called *pms-1* and *pms-2* (for postmeiotic segregation) encode proteins which can also bind to mismatched DNA and with each other, presumably cooperating in the same repair pathway.^{284,291} Thus, this type of tumor suppressor gene helps to proofread DNA to detect and restore any base pair errors that occur during DNA replication. Lesions of these genes lead to genetic instability and the accumulation of mutations which can affect cancer-related genes.²⁹²

Tumor suppressor genes and cell adhesion

At the periphery of the cell, more tumor suppressor molecules act to modulate cell adhesion properties to prevent destabilization of junctions, invasion and metastasis. For instance, the cell adhesion molecule called E-cadherin acts to suppress metastasis of cancer cells.^{293,294} The E-cadherin gene has been shown to be mutated in gastric and ovarian carcinomas.^{295,296} E-cadherins are transmembrane proteins present in adherens junction complexes and play a key role in maintaining the epithelial phenotype. The cytoplasmic domain of E-cadherin interacts with beta catenin or gamma catenin (plakoglobin).^{297,298} These molecules, in turn, bind to α -catenin (vinculin homologue)²⁹⁹

which makes contact with components of the cytoskeleton such as F-actin.^{300,301} E-cadherin's cell adhesion properties are dependent on its interaction with the network of catenins.³⁰² The *APC* gene located on chromosome 5q21 is a frequent germline mutation in the familial syndrome of colon cancer adenomatous polyposis coli and is also altered in sporadic cases of this cancer.^{303,304,305} Re-introduction of this gene in colon cancer cell lines suppressed growth and tumorigenicity.³⁰⁶ Both β -catenin and plakoglobin were shown to directly interact with the tumor suppressor gene product APC in direct competition with E-cadherin.^{307,308} They also bind the *c-erbB-2* oncogene product³⁰⁹ and are substrates for tyrosine phosphorylation following epidermal growth factor (EGF) stimulation of cells.^{310,311} These results suggest that catenins must be central regulators of cell adhesion and perhaps tumor suppression. Interestingly, the human plakoglobin gene is localized on chromosome 17q21 in the vicinity of the breast cancer gene (*BRCA1*), a region which is frequently deleted in sporadic breast and ovarian cancer. Aberle et al³¹² have shown loss of heterozygosity for plakoglobin in breast and ovarian tumors and have determined a low-frequency polymorphism in the gene, suggesting that plakoglobin might represent a putative tumor suppressor gene. A phosphotyrosine substrate of pp60src, called p120, could also be involved in the modulation of cadherin function and hence in the tumor cell adhesion machinery. It is structurally similar to β -catenin and plakoglobin and although it was shown to bind E-cadherin, it does not bind to alpha-catenin or the *APC* gene product.^{313,314,315}

Hypermethylation of the promoter region is another mechanism responsible for the inactivation of the E-cadherin gene in carcinomas. Yoshiura et al³¹⁶ have demonstrated that the treatment of E-cadherin-inactivated cells with a demethylating agent resulted in reexpression of the gene leading to reversion to an epithelial cell morphology.

A new putative tumor suppressor, the neurofibromatosis type 2 (*NF2*), maps to chromosome 22q and encodes a protein called merlin/schwannomin, a member of the band 4.1 family of proteins. It has homology to other proteins that play key roles in regulating membrane physical properties, such as ezrin, radixin and moesin. Similar to the E-cadherin/catenins/APC network, these proteins are involved in maintaining the focal contact points between the plasma membrane and cytoskeleton and it has been postulated that they may act as tumor suppressor genes. There is evidence that *NF2* acts as a tumor suppressor because it displays an autosomal dominant mode of inheritance, it is inactivated in the germline of *NF2* patients, and mutations of *NF2* result in multiple tumors at a young age. Loss of heterozygosity of chromosome 22 occurs at high frequency in sporadic and *NF2*-associated meningiomas and schwannomas.³¹⁷ Furthermore, transfection of the *NF2* gene into NIH 3T3 cells in culture resulted in morphological changes and decreased growth rate.³¹⁸

Other tumor suppressor genes

Ras mutations appear in varying frequencies in a large number of tumors, suggesting a major role in tumor development. The products of the three *ras* genes (H-ras, K-ras, N-ras) couple growth-signaling molecules at the plasma membrane to

intracellular targets. This ensures the transmission of growth-regulatory signals from the cytoplasm to the nucleus, leading to regulation of DNA transcription. Ras proteins bind guanine nucleotides with high affinity and have intrinsic guanosine triphosphatase (GTPase) activity. They cycle between two conformational states: an active, GTP-bound state and an inactive, guanosine diphosphate (GDP)-bound state. Oncogenic mutants of ras *p21* escape hydrolysis of GTP. They are trapped in an active conformation by being insensitive towards GTPase Activating Proteins (GAPs). Five ras-specific GAP proteins have been cloned that share homology in the catalytic domain. p100-GAP and p120-GAP are generated by alternative splicing of a single gene on chromosomes 5q13.3.³¹⁹ The Von Recklinghausen Neurofibromatosis Type 1 disease gene, NF1, has homology to the GAP gene and can also stimulate the GTPase activity of p21 specifically. It was identified by positional cloning and maps to chromosome 17.³²⁰ It encodes a Ras GTPase activating protein (rasGAP) named neurofibromin.³²¹ Most evidence implicating *NF1* as a tumor suppressor comes from analysis of *NF1*-deficient mice and from the analysis of *NF1* mutations in tumors and in NF1 patients.

A breast and ovarian cancer susceptibility gene, *Brcal*, has been isolated . The gene spans more than 100kb on chromosome 17, and encodes a zinc-finger protein of unknown function.⁸⁸ Germline mutations of the *BRCA1* gene accounts for a major proportion of familial breast and ovarian cancers.^{322,323,324} Mutant alleles of the gene predispose carriers to carcinomas of the breast, ovary, colon and prostate, suggesting a role in epithelial cells which are hormonally-regulated.³²⁵ Recently, Marquis et al have

analyzed the pattern of *Brcal* expression during development and found that it is expressed during proliferation and differentiation in multiple tissues, and in the mammary gland in response to ovarian hormones.³²⁶

This review of tumor suppressors, although not complete, aimed to illustrate that many such molecules are being isolated and studied and attempted to demonstrate the various functions of tumor suppressor genes.

7.2 APPENDIX II: LIST OF DU 145-DERIVED HYBRIDS

HYBRID	TUMORIG.	SELECTION	DESCRIPTION
DU145-N19	+	G418 400µg/mL	Human prostate cell line DU145 tagged with pSV2neo plasmid.(clone #19)
DBM9-4	-	Hygromycin B 400µg/mL	Microcell hybrid; contains tagged piece of Hsa12 originating from B78MC9.
DBM9-4R6	+	None	GCV revertant of DBM9-4; lost tagged piece of Hsa12.
DBM9-7	-	Hygromycin B 400µg/mL	Microcell hybrid; contains tagged piece of Hsa12 originating from B78MC9.
DBM9-7R2	+	None	GCV revertant of DBM9-7; 70% cells lost tagged Hsa12.
DBM9-7R2RS	+	None	Tumor-derived cells from DBM9-7R2; 100% cells have lost tagged Hsa12.
DBM9-10	-	Hygromycin B 400µg/mL	Microcell hybrid; contains tagged piece of Hsa12 originating from B78MC9.
DBM9-10R9	+	None	GCV revertant of DBM9-10
DU12RH-3	+	Hygromycin B 400µg/ml	Radiation hybrid; tagged piece of Hsa12 originates from DBM9-7.
DU12RH-4	+	Hygromycin B 400µg/mL	Radiation hybrid; tagged piece of Hsa12 originates from DBM9-7.
DU12RH-5	+	Hygromycin B 400µg/mL	Radiation hybrid; tagged piece of Hsa12 originates from DBM9-7.
DU12RH-6	early passage:- late passage:+	Hygromycin B 400µg/mL	Radiation hybrid; tagged piece of Hsa12 originates from DBM9-7.
DU12RH-6R2	-	None	GCV revertant of DU12RH-6. Still contains 2 small translocated pieces of Hsa12.
DU12RH-6R4	-	None	GCV revertant of DU12RH-6. Still contains 2 small translocated pieces of Hsa12.
DU12RH-19	+	Hygromycin B 400µg/mL	Radiation hybrid; tagged piece of Hsa12 originates from DBM9-7.
DU12RH-20	+	Hygromycin B 400µg/mL	Radiation hybrid; tagged piece of Hsa12 originates from DBM9-7.

7.3 APPENDIX III: LIST OF CHEMICALS

dATP [α - ³³ S] (3000Ci/mmol)	Amersham	Formaldehyde	BDH
dCTP [α - ³² P] (3000Ci/mmol)	Amersham	Formamide	Gibco
15ml conical tubes	Coming	G418	Gibco
5X First Strand buffer	Gibco	Glycerol	Gibco
Acetic acid, glacial	BDH	Glycogen	Boehringer
Acrylamide	Boehringer		Mannheim
	Mannheim	HEPES	Boehringer
Agarose	Gibco		Mannheim
<i>Alu</i> primers	BRI, MRC	Hygromycin B	Boehringer
Ammonium persulfate	BDH		Mannheim
Ampicillin	Boehringer	KaryoMAX colcemid	Gibco
	Mannheim	Long Ranger hydrolink gel solution	Johns
AmpliTaq			Scientific
DNA polymerase	Perkin	MapPairs	Research
	Elmer,		Genetics
	Applied	MOPS	USB
	Biosystems	NaCl	BDH
	BDH	NuSeive GTG agarose	FMC
Amyl alcohol	PDI		Bioproducts
Anti-avidin antibody	PDI	Original TA Cloning Kit	Invitrogen
Antifade solution	NRC	Ouabain	Sigma
Arbitrary decamers	Difco	Pall Biodyne membrane	Gibco
Bacto trypsin	Laboratories	Paraffin oil	Fisher
	Gibco		Scientific
Blotting pads	Gibco	PEG 1500	NBS
Boric acid	Sigma		Biologicals
Bovine serum albumin	BDH	Phenol	Toronto
Bromophenol blue	BDH		Research
Chloroform	BDH		Chemical
Chromosome <i>in situ</i>		Placental RNase Inhibitor	Gibco
Hybridization System	Oncor	Prime-It™ II Random	
Cloning cylinder	Belco Glass	Primer Labeling Kit	Stratagene
Concanavalin A type III	Sigma	Ready Safe liquid	
Coplin staining dish	Canlab	scintillation cocktail	Beckman
Cot1 DNA	Gibco	Sequenase Version 2.0 DNA	
Cytochalasin B	Sigma	Sequencing kit	Amersham
DEPC	Sigma	Sodium citrate	BDH
Dimethylsulfoxide	BDH	Sodium dodecyl sulfate	Sigma
DMEM powder	Gibco	SP6 and T7 primers	Invitrogen
DME/Ham's F-12 (1:1) medium	PDI	Spermine 4HCl	Sigma
DNA, salmon sperm	ICN	SuperScript™ Reverse	
DNAse I	Gibco	Transcriptase	Gibco
DNA standards		T ₁₁ MN primers	NRC
for fluorometer	Canberra	TCA	BDH
	Packard	TEMED	BDH
DNA lambda	Gibco	Tris	Gibco
dNTPs	Boehringer	Urea	BDH
	Mannheim	X-gal	Gibco
Dithiothreitol (DTT)	Gibco	Xylene cyanol	BDH
Eco RI	Gibco	Yeast tRNA	Boehringer
EDTA	BDH		Mannheim
Ethidium bromide	BDH	Zetabind membrane	Terochem
Fetal bovine serum	Gibco		Scientific
Film for autoradiography	Picker		
Film Polaroid type 669	Treck-Hall		
FITC-Avidin	PDI		

7.4 APPENDIX IV:CURRICULUM VITAE

NATHALIE BÉRUBÉ

Date of birth: May 21, 1968

Citizenship: Canadian

Languages: French, English

Business Address: University of Ottawa
Department of Biochemistry
Faculty of Medicine
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Home Address: 1056 Secord Avenue
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Canada, K1H 8C8

EMPLOYMENT DATA

1) Academic degrees

- B. Sc. Honours Biochemistry, University of Ottawa, Ottawa, 1991.
Dean's Honour List; with mention "Magna Cum Laude".
- Comprehensive examination. Transfer from the M.Sc. program to the Ph.D. program in Biochemistry, May 1993.
- Completion of the Ph.D. thesis: May 1996.

2) **Previous positions held:**

March 1996 - April 1996

Coordinator and supervising professor
University of Ottawa, Biochemistry Dept.
3rd year Molecular Biology Laboratory
3 week session: Northern, Southern and PCR analysis.

March 1995 - April 1995

Teacher's assistant
University of Ottawa, Biochemistry Dept.
3rd year Molecular Biology Laboratory.

March 1994 - April 1994

Teacher's assistant
University of Ottawa, Biochemistry Dept.
3rd year Molecular Biology Laboratory.

January 1991 - April 1991

Teacher's assistant
University of Ottawa, Biochemistry Dept.
2nd year Biochemistry Laboratory.

May 1990 - August 1990

Summer student
University of Ottawa, Biochemistry Dept., Supervisor: Dr. I. Altosaar.
Research project in plant molecular biology.

PUBLICATIONS

Referred papers:

Bérubé, N.G., Speevak, M.D., and Chevette, M. (1994). Suppression of tumorigenicity of human prostate cancer cells by introduction of human del(12)(q13). *Cancer Res.* 54:3077-3081.

Speevak, M.D., Bérubé, N.G., McGowan-Jordan, I.J., Bisson, C., Lupton, S.D. and Chevette, M. (1995). Construction and analysis of microcell hybrids containing dual selectable tagged human chromosomes. *Cytogenet. Cell Genet.* 69:63-65.

Abstracts:

Chevette, M., Gourdeau, H., and Bérubé, N.G. Analysis of tumor suppression in prostatic whole cell hybrids. Abstract from American Association for Cancer Research Special Conference: Negative Controls on Cell Growth and their Breakdown during the Pathogenesis of Cancer. October 20-24, 1991.

Speevak, M.D., Bérubé, N.G., Chevette, M. Analysis of Tumor Suppression in Mouse Melanoma Microcell Hybrids. American Association for Cancer Research, Genetics of Cancer Meeting, Hilton Head, South Carolina, November, 1992.

Speevak, M.D., Bérubé, N.G., McGowan-Jordan, I.J., Ekker, M., and Chevette, M. Fluorescence *in situ* hybridization of microcell hybrids and their derivatives. Great Lakes Chromosome Conference, Toronto, Ontario, May 25 - May 27, 1994.

Bérubé, N.G. and Chevette, M. Evidence for inactivation of more than one tumor suppressor gene in prostate cancer. Abstract from Canadian Federation of Biological Societies, 34th annual meeting, June 9-11, 1991.

Bérubé, N.G., Speevak, M.D. and Chevette, M. Analysis of tumor suppression in microcell hybrids derived from prostate cell line DU 145. American Association for Cancer Research, Genetics of Cancer meeting, Hilton Head, South Carolina, Nov. 4, 1992.

Bérubé, N.G., Amjadi, K. and Chevette, M. Mapping of a human chromosome 12 tumor suppressor gene implicated in prostate cancer. American Association for Cancer Research, Basic and Clinical Aspects of Prostate Cancer Special Conference, Palm Springs, CA, USA, December 1994.

Bérubé, N.G., Amjadi, K. and Chevette, M. Mapping a tumor suppressor gene on human chromosome 12. Canadian Society of Laboratory Technologists Congress 1995, Ottawa, Ontario, June 18-23, 1995.

Bérubé, N.G. and Chevette, M. Identification de gènes impliqués dans la suppression tumorale de cellules prostatiques. 64^e Congrès de l'Association canadienne-française pour l'avancement des sciences Inc., 1996, Montréal, Ontario, May 15, 1996.

AWARDS AND SCHOLARSHIPS

1. Cancer Research Society (CRS) (\$15 050) University of Ottawa, Biochemistry Dept., Supervisor: Dr. M. Chevette. July 1995 to June 1996.

2. University of Ottawa Excellence Scholarship
May 1995 to May 1996.

3. American Association for Cancer Research (AACR) Young Investigator's grant (\$1 100)
Travel grant for attendance at AACR conference "Basic and Clinical Aspects of Prostate Cancer", Palm Springs, CA, USA. December 8-12, 1994.

4. University of Ottawa Excellence Scholarship
Scholarship covering tuition fees.
September 1994 to September 1995.

5. Ontario Graduate School (OGS) Scholarship (\$12 000)
University of Ottawa, Biochemistry Dept.,
Supervisor: Dr. M. Chevette.
September 1993 to September 1994.

6. University of Ottawa Excellence Scholarship
Scholarship covering tuition fees.
September 1993 to September 1994.

7. Ontario Graduate School (OGS) Scholarship (\$12 000)
University of Ottawa, Biochemistry Dept.,
Supervisor: Dr. M. Chevette.
September 1992 to September 1993.

8. University of Ottawa Excellence Scholarship
Scholarship covering tuition fees.
September 1992 to September 1993.

9. Entrance scholarship - Graduate Studies in Biochemistry (1991)

10. Natural Science and Engineering Research Council (NSERC) Scholarship
Summer Scholarship (\$3 200)
University of Ottawa, Biochemistry Dept.,
Supervisor: Dr. I. Altosaar. May to August 1990.

11. Entrance scholarship - Undergraduate program in sciences (1987)

SEMINAR PRESENTATIONS

1. Invited speaker: Seminars of the Urology Department: McGill University
December 4th, 1995.

RESEARCH EXPERIENCE

Tissue culture techniques:

Microcell fusions/irradiation hybrid production with mouse and human cells.
Karyotyping, G-banding - human chromosomes. (Basic knowledge only)
Nude mice injections.
Transfections of mammalian cells.

Molecular biology techniques:

Cloning; plasmid analysis.
Northern and Southern analysis, reverse Northern.
Sequencing.

General PCR techniques:

Microsatellite mapping.
Alu-PCR Fluorescent *in situ* Hybridization (*Alu*-PCR FISH).
Representational difference analysis (RDA).
Differential Display Reverse Transcriptase PCR (DDRT-PCR).

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