ERK4, A NEW TUMOR SUPPRESSOR GENE CANDIDATE IMPLICATED IN PROSTATE CANCER PROGRESSION

by

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À mes parents,
Ruth et Léger

ABSTRACT

In Canada, prostate cancer is the most frequently diagnosed cancer in man and the third leading cause of cancer related death. In order to identify genes implicated in prostate cancer progression, we used microcell-mediated chromosome transfer to introduce chromosomes 10, 12, 17 and 18 into human PC-3-derived cells lines called PC-3M-Pro4GP1 and PC-3M-LN4GP2. The properties of the hybrids were determined by invasion assay, growth in soft agar and injection into nude mice (either subcutaneously or orthotopically). After the transfer of chromosome 18 in our cell lines, we have identified two sets of hybrids that show opposite phenotypes. Genotyping studies indicated that one locus on chromosome 18 (*D18S51*) contains an additional allele in the less tumorigenic hybrids. This allele is absent in the highly tumorigenic hybrids as well as in tumors derived from the injection of less tumorigenic cells into nude mice. Preliminary microarray data analysis showed that two genes located in the *D18S51* region (*ERK4* and *BCL2*) were differentially expressed in the less tumorigenic hybrids compared to the tumorigenic hybrids. We examined publicly available microarray data sets and found that *ERK4* was down regulated in prostate, adrenal and lung cancers. We have also examined the possibility that the metastasis suppressor gene *MASPIN* (also located in the *D18S51* region) could be responsible for the less tumorigenic phenotype seen in our hybrids. Real-time PCR studies showed that *ERK4* and *MASPIN* were up regulated in the less tumorigenic hybrids and that *BCL2* was down regulated in these same cells. However, Western immunoblot experiments did not show any difference in the MASPIN or the BCL2 protein levels. Thus, *MASPIN* or a BCL2 repressor are most likely not responsible for the less tumorigenic phenotype of our hybrids. Interestingly, re-introduction of *ERK4* in our tumorigenic hybrids completely abolished their ability to form colonies in soft agar and to invade through Matrigel. However, there is no difference in tumor growth rate upon injection of ERK4-over-expressing cells compared to mock-transfected controls. We also found a non-synonymous polymorphism in the coding sequence of the *ERK4* gene. This polymorphism is present at the same frequency in Asian, European

and Ashkenazi Jewish populations but less frequently in the African (Yoruba) population. This is the first report of a non-synonymous polymorphism in the coding sequence of the *ERK4* gene. However, based on a limited number of prostate cancer cases, the presence of this poylmorphism is not associated with the disease. Taken together, these results demonstrate that *ERK4* is a good candidate for a tumor suppressor gene implicated in prostate cancer progression.

RÉSUMÉ

Au Canada, le cancer de la prostate est le cancer le plus diagnostiqué chez l'homme et est la troisième cause de mortalité tumorale. Afin d'identifier les gènes impliqués dans la progression du cancer de la prostate, nous avons utilisé la technique de transfert de chromosome par microcellules pour introduire les chromosomes 10, 12 17 et 18 dans deux lignées cellulaires humaines dérivées de PC-3 (PC-3M-Pro4GP1 et PC-3M-LN4GP2). Les propriétés des différents hybrides ont été déterminées par invasion, croissance en agar mou et par injection dans des souris nues (sous-cutanée ou orthotopique). Suite au transfert du chromosome 18 dans nos lignées cellulaires, nous avons identifié deux groupes d'hybrides qui démontraient des phénotypes opposés. Des études de génotypage ont montré qu'un locus sur le chromosome 18 (*D18S51*) contient un allèle supplémentaire dans les hybrides moins tumorigènes. Cet allèle est absent dans les hybrides très tumorigènes ainsi que dans les tumeurs dérivées de l'injection des hybrides moins tumorigènes dans des souris nues. Des données préliminaires de micropuces ont démontré que deux gènes situés dans la région *D18S51* (*ERK4* et *BCL2*) étaient différentiellement exprimés dans les hybrides moins tumorigènes comparativement aux hybrides très tumorigènes. Nous avons examiné les bases de données publiques de résultats de micropuces et avons observé que l'expression de *ERK4* est diminuée dans le cancer de la prostate, des glandes surrénales ainsi que dans le cancer du poumon. Nous avons également examiné la possibilité que le gène de suppression métastatique *MASPIN* (également situé dans la région *D18S51*) soit responsable du phénotype moins tumorigène de nos hybrides. Nos expériences de PCR en temps réel ont démontré que l'expression de *ERK4* et de *MASPIN* est augmentée dans les hybrides moins tumorigènes et que celle de *BCL2* est diminuée. Par contre, des expériences d'immunobuvardage ont révélé qu'il n'y pas de différence dans l'expression de la protéine MASPIN ou BCL2 dans nos hybrides tumorigènes et moins tumorigènes. Donc, le gène *MASPIN* ou un répresseur de *BCL2* ne sont probablement pas responsable du phénotype moins tumorigène de nos hybrides. De façon intéressante, la ré-

introduction du gène *ERK4* dans les hybrides tumorigènes a complètement aboli leur capacité à former des colonies en agar mou et à digérer le Matrigel. Par contre, il n'y a pas de difference dans la croissance tumorale lorsque les cellules surexperimant ERK4 et les cellules contrôles sont injectées dans des souris nues. Cependant, nous avons identifié un polymorphisme dans la séquence codante du gène *ERK4*. La fréquence de ce polymorphisme est la même chez les populations asiatique et européenne mais beaucoup moins fréquente chez les populations africaines. Nous sommes les premiers à rapporter la présence d'un polymorphisme dans la séquence codante du gène *ERK4*. Toutefois, des résultats préliminaires obtenus à partir d'un nombre restreint de patients atteints du cancer de la prostate semblent indiquer que ce polymorphisme ne serait pas associé à cette maladie. Cependant, l'ensemble de nos résultats démontre que le gène *ERK4* est un nouveau gène de suppression tumorale potentiellement impliqué dans la progression du cancer de la prostate.

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CONTRIBUTION OF CO-AUTHORS

-In Chapter 2 and 3, we introduce the B78MC hybrid panel that consists of a mouse/human hybrid panel that contains one or more human tagged chromosomes. Dr. Marsha Speevak generated this B78MC hybrid panel.

-In Chapter 3, we perform PCR to assess which human chromosome is present in each B78MC hybrid. Ms. Geneviève Lavoie performed these PCRs. This allowed us to choose which B78MC hybrids were going to be used to transfer each selected chromosome into our prostate cancer cell lines. She also performed the Fluorescence *In Situ* Hybridization experiments to confirm which chromosome was transferred into our prostate cancer cell lines.

-In Chapter 3, we assess the *in vitro* and *in vivo* properties of our hybrids. Mr. Jean-Sébastien Ripeau performed some soft agar assay experiments and helped Dr. Valerii Zvieriev to inject our hybrids subcutaneously and orthotopically in nude mice. Mrs. Audrey Gagnon performed the chromosome transfers, invasion assays, most of the soft agar assays, the nude mice monitoring and tumor processing. She also performed the microsatellite mapping of our chromosome 18 hybrids.

-In Chapter 4, we compared the gene expression profiles of our tumorigenic and less tumorigenic hybrids by microarray. Mr. Jean-Sébastien Ripeau performed the hybridization, the scanning and the data quantification of the microarray experiments. Mrs. Audrey Gagnon did the data normalization with the help of Mr. Peter Wilkinson. The data analysis, the selection of candidate genes, real-time PCR experiments and Western immunoblots were done by Mrs. Audrey Gagnon. Mr. François Gougeon amplified the coding sequence of the *ERK4* gene to get it sequenced at the McGill University and Genome Quebec Innovation Center by Mr. David Roquis and Mr. Sébastien Brunet. Mr. Gougeon, under the supervision of Mrs. Audrey Gagnon, also treated some of our cell

lines and hybrids with the proteasome inhibitor MG132 and extracted proteins from them. All the other proteasome inhibition experiments and Western immunoblots were done by Mrs. Audrey Gagnon. Ms. Justine Rousseau generated the polyclonal anti-human ERK4 antibody that we used in Chapter 4 for the detection of the ERK4 protein by Western immunoblot. She also constructed the *ERK4*-wt vector that Mrs. Audrey Gagnon then cloned into pcDNA4 His/Max B. Mr. Jean-Sébastien Ripeau injected our *ERK4*-over-expressing cells into nude mice and Mrs. Audrey Gagnon did the monitoring and tumor processing. Mrs. Audrey Gagnon did the screening of the *ERK4* V38M polymorphism in human cell lines and prostate cancer patients.

-Mrs Audrey Gagnon did the polymorphism screening in the three populations described in Chapter 5.

CLAIM TO ORIGINALITY

- In Chapter 3, we show that chromosome 18 suppresses the tumorigenicity of human prostate cancer cells both *in vitro* and *in vivo*. We also show that the *D18S51* region might contain a tumor suppressor gene implicated in prostate cancer progression.

- Chapter 4 shows that *MASPIN* and *ERK4* mRNA are down regulated in human prostate cancer cells and that *BCL2* is up regulated in these same cells. We show that the overexpression of *ERK4* into human prostate cancer cells completely abolishes their ability to form colonies in soft agar and to invade through Matrigel. We show that mice injected with ERK4 over-expressing cells still form tumors at the same rate as the ones injected with non-transfected cells.

- In Chapter 5, we show that *ERK4* contains a non synonymous polymorphism located in its coding sequence and that it is present at a lower frequency in the African population compared to European and Asian.

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aa	amino acid
AI	allelic imbalance
AIPC	androgen-independent prostate cancer
AR	androgen receptor
ARE	androgen responsive element
BMP	bone morphogenic proteins
bp	base pair
BPH	benign prostate Hyperplasia
$CaCl_2$	calcium chloride
CDK	cyclin-dependent kinase
CGH	comparative genomic hybridization
CHO	Chinese hamster ovary cells
ConA	concanavalin A
dATP	desoxyadenosine triphosphate
DBD	DNA binding domain
dCTP	desoxycytosine triphosphate
DEPC	diethyl polycarbonate
dGTP	desoxyguanidine triphosphate
DHT	dihydroxytestosterone
DMEM	Dulbecco's modified eagle medium
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
dTTP	desoxythymidine triphosphate
EC	embryonic carcinoma cells
ECM	extracellular matrix
EDTA	ethylenediamine-tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular-regulated kinase
ES	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
FISH	fluorescence *in situ* hybridization
GM-CSF	granulocyte macrophage colony stimulating factor
G418	geneticin

HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
IGF	insulin growth factor
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic
LBD	ligand binding domain
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
ml	milliliter
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
mM	millimolar
mm	millimeter
MMCT	microcell-mediated chromosome transfer
MSG	metastasis suppressor gene
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$NaHPO_4$	sodium phosphate
Na_2HPO_4	sodium phosphate dibasic
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_3VO_4	sodium orthovanadate
PBS	phosphate balanced solution
PCa	prostate cancer
PCNA	proliferation of cellular nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PHA	phytohemagglutinin A
PIN	prostatic intraneoplasia
PI3K	phosphatidylinositol kinase
PIP3	phosphatidylinositol 3,4,5-triphosphate
PSA	prostatic-specific antigen
PSMA	prostate-specific membrane antigen
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
TBS	tris buffer saline
TBST	tris buffer saline tween 20
TE	tris-EDTA
TF antigen	Thomsen-Friedenreich antigen
TGF beta	transforming growth factor beta
TK	thymidine kinase

TM4SF	transmembrane 4 surperfamily
TNM	tumor, node, metastasis staging
TSG	tumor suppressor gene
TURP	transurethral prostatectomy
ul	microliter
ug	microgram
WSC	1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-o-toluene-sulfonate
YAC	yeast artificial chromosome

PREFACE

In accordance with the "Guidelines concerning thesis preparation" of the Faculty of Graduate Studies and Research, three manuscripts (one published in Genes Chromosomes and Cancer, one to be submitted to Cancer Research and one to be submitted to Human Mutation) have been incorporated in this thesis. The Division of Experimental Medicine, Department of Medicine, has approved this thesis preparation format.

This thesis is written in a form that includes manuscripts submitted (or to be submitted) for publication, according to the instructions to authors of thesis that can be found on the McGill University Graduate and Post-doctoral Studies Faculty web site. Sections of Chapter 1 (Introduction and Literature review) are part of a book chapter written by the author of this thesis. Thus, permission from the editor can be found in Appendix A. Each manuscript presented in this thesis (Chapters 3, 4 and 5) possesses its own Abstract, Introduction, Material and Methods, Results, Discussion and Conclusion. Permission from publisher and proof for manuscript 1 can be found in Appendix B and C. For manuscripts 2 and 3, permissions from co-authors are in Appendix D and E, respectively. Prefaces, used to connect the chapters, are present at the beginning of each chapter. An Abstract (p.III), an Introduction and Literature review (Chapter 1) and a General discussion and conclusions (Chapter 6) are included in order to present an overall view of the subject matter contained in this thesis. The references for the entire thesis are found at the end. Contributions of each author to this thesis and manuscripts are clearly indicated on page XVIII.

CHAPTER 1

INTRODUCTION, LITERATURE REVIEW AND OBJECTIVES

1.1 Overview of prostate cancer (PCa)

1.1.1 The prostate gland

The prostate is a male gland located under the bladder of about the size and the shape of a chestnut. Its main function is to produce seminal fluid that is part of the semen. The prostate is made up of epithelial glands and a fibro muscular stroma. The glandular epithelium (which gives rise to prostate adenocarcinoma) is composed of three types of cells: basal cells, luminal secretory cells and neuroendocrine cells. Luminal cells secrete the different components of the prostatic fluid, express the androgen receptor (AR) and secrete prostatic specific antigen (PSA) in an androgen-dependent manner. The stroma is composed of fibroblasts, smooth muscle cells, dendritic cells, nerves and infiltrating mast cells and lymphocytes. Stromal cells are androgen-responsive and produce growth factors, which act in a paracrine fashion on epithelial cells. This stromal-epithelial crosstalk is an important regulator of growth, development and hormonal responses of the prostate [1].

1.1.2 Prostate malfunctions

Many problems are associated with the prostate, as men get older. Benign prostatic hyperplasia (BPH) is the most common benign tumor in men older than 60 years old [2]. Benign growth of the prostate gland is accompanied by a significant increase in the proliferation rate of epithelial cells in the hyperplasic acini [3]. This then leads to the constriction of the prostatic part of the urethra, resulting in problems to urinate. BPH also increases the risk of bladder and kidney infections. Therapies for BPH are mainly medical in nature, including

alpha-adrenergic blockade or 5-alpha-reductase inhibition. When medical therapy fails then surgery is contemplated, including microwave treatment to decrease prostate volume [4] and transurethral ablation of the prostate (transurethral prostatectomy: TURP). Although BPH was originally thought to lead to prostate cancer, it is now established that it is not the case.

1.1.3 Incidence

Prostate cancer is the second leading cause of cancer death in the male population in North America with an estimated 230,110 new cases and 29,900 deaths in USA in 2005 [5]. These incidences are similar to the Canadian ones, since for the same year, it is predicted that 20,500 Canadian will have the disease while 4,300 men will die from it [6]. In mature prostate, the maintenance of prostate-specific cellular functions requires continuous stimulation by androgens. Despite improved early diagnosis of PCa, approximately one-third of patients are still diagnosed at a clinically advanced stage [5]. Therefore, metastatic disease presents a continuing therapeutic challenge and is the most common cause of cancer-related death [7]. Moreover, progression to an androgen-independent state remains the primary cause of mortality in these patients [8].

1.1.4 Risk factors

There are well-established risk factors for PCa, including: ethnic origin (American black males), ageing, family history and patients in the highest quartile of IGF-I (insulin growth factor I) blood concentrations [9]. Diet is also a

well-studied risk factor for PCa: while dietary vitamin E, carotenoids and selenium protect against the disease, a diet rich in fat, red meat and dairy products exerts a promotional effect [10]. Familial history is also important in PCa since susceptibility loci have been found on chromosomes 1, 3, 8, 17, 20 and X. For example, a locus on chromosome 3p24 is associated with a lod score of 3.08 in families from Finland [11]. However, some putative risk factors have been excluded: cigarette smoking, alcohol, vasectomy and physical activity [10].

Environment also plays an important role in the aetiology of PCa. Indeed, the incidence of PCa has risen by 5 to 118% in Asian countries. Thus, with gradual westernisation, many Asian countries are losing their environmental protective factors such as low fat diet and are acquiring high-risk ones like obesity and a diet with a high content of red meats [12].

1.1.5 Diagnosis

PCa is usually diagnosed upon palpation by putting a finger in the rectum and looking at the volume and the texture of the prostate. It can also be diagnosed by dosage of PSA in the patient's serum. PSA has been identified in 1979 and is a very useful marker for tumorigenicity as its concentration follows the clinical stage of the disease [13]. When the PSA concentration in the serum is high (more than 10 ng/ml), the clinician can confirm the PCa diagnosis. The screening for early-stage disease is logical but its utility is limited by the inability of conventional diagnostic and histologic parameters to predict accurately the true extent and prognosis of a substantial proportion of clinically localized cancers.

This limitation is due to the subjectivity of current grading and staging systems ([14] and section 1.1.6). There is thus a critical need for new markers that will distinguish accurately those histologic lesions and disseminated cells that have a high probability of causing clinically important metastatic disease from those who will remain indolent.

1.1.6 Histological and clinical staging

PCa is hard to stage because of its cellular heterogeneity. In humans, heterogeneity is a common phenomenon that includes different histological grades among different tumor foci and different genotypes among phenotypically similar foci in one primary tumor [15].

The Gleason score system is still the most frequently used grading system for PCa [16]. Unusually, the overall grade is not based on the highest grade within the tumor but on an intermediate between the most predominant pattern of cancer and the second most prevalent one. These patterns are graded from 1 (most differentiated) to 5 (least differentiated) and the two grades are added. If a tumor has only one histological pattern, the primary and secondary scores are the same. The combined Gleason scores (called the Gleason sum) range from 2 (for tumors uniformly of grade 1) to 10 (for undifferentiated tumor of grade 5). However, the Gleason score system has difficulties with inter-observer reproducibility. Other problems include grading of cribriform patterns, grading of small foci of cancer at biopsy, borderline histology between grades and how to account for a tertiary pattern [17]. Nonetheless, the Gleason score is a powerful

prognostic indicator. It correlates with all important pathological variables seen in the radical prostatectomy sample, with the prognosis after surgery and with the outcome after radiotherapy [18, 19, 20]. For predicting the outcome, the following combinations are very helpful: score 2-4 (well differentiated), 5-6 (moderately differentiated), 7 (moderately to poorly differentiated) and 8-10 (poorly differentiated). The Gleason score grade also has an influence on the treatment given to patients [17]. Clinicians use this grading system to predict the probability of tumor extension outside of the prostate [21]. However, it has not been shown if the tumor grade changes or gets worse over time.

The TNM tumor staging is a standardized system for classifying the extent of the disease in order to compare therapeutic intervention and to estimate the outcome. The "T" (T1 to T4) describes the tumor topography and is characterized by the size of the tumor at its primary site and/or the involvement of local structures. The "N" (N1 to N3) evaluates the presence and extent of regional lymph nodes involvement while the "M" (M1 and M2) indicates the spread of the tumor by documenting evidence of distant metastasis [22].

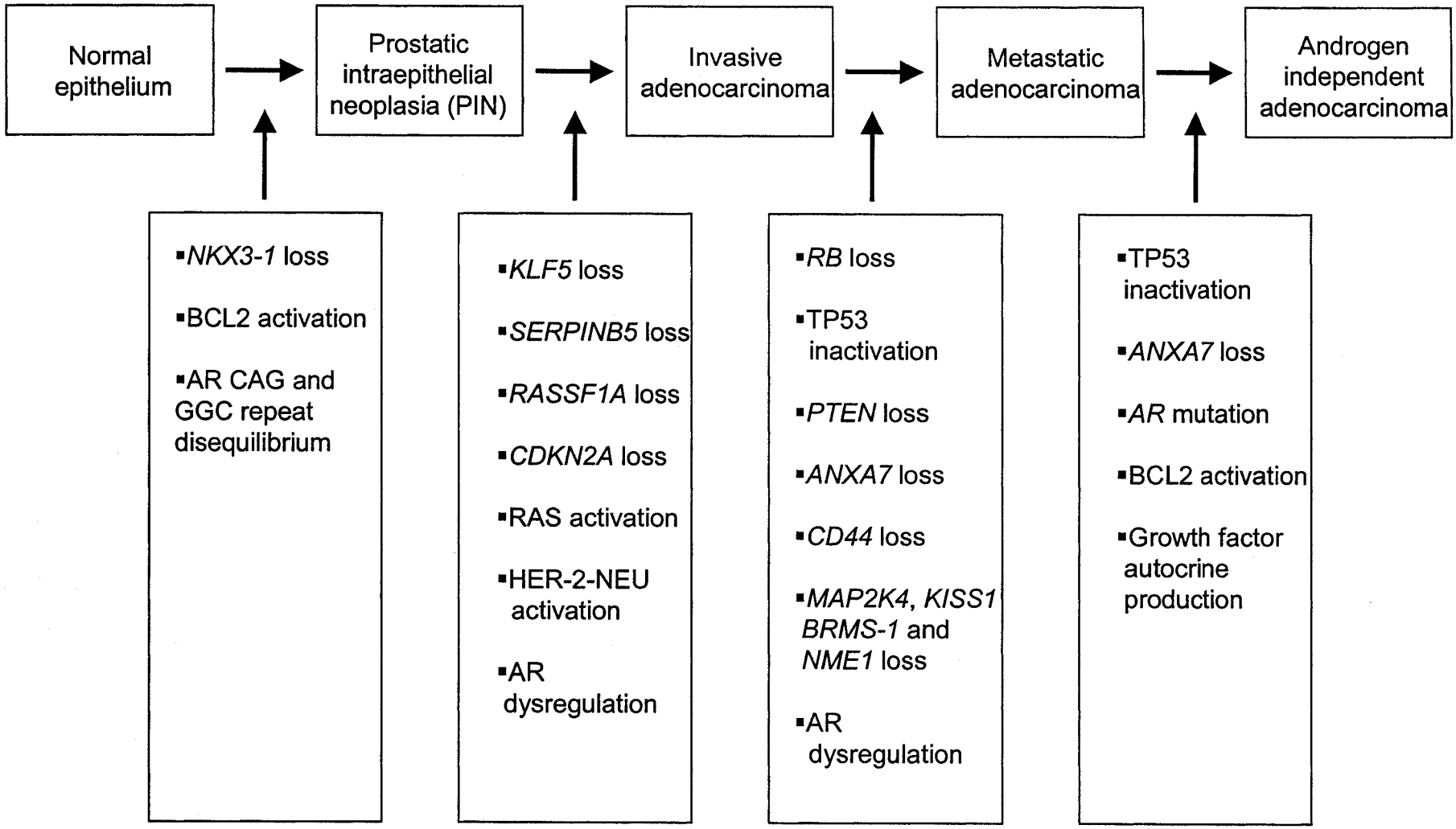
After modifications of TNM staging system in 1992 and 1997, clinicians found that this staging system could still predict progression-free survival after radical prostatectomy [23]. However, some controversies and suggestions for additional modifications were proposed [24, 25]. Moreover, by conducting a study with 12 PCa cases to assess the reproducibility of TNM staging system among 20 physicians with special expertise, Campbell et al. found that the overall staging

reproducibility is less than 80% of consensus [26]. Furthermore, in this study, they showed that the levels of PSA seem to be even more indicative of the extent of PCa than the current clinical staging. Thus, whether the clinical TNM staging system reveals the true biological significance of PCa is still questionable [26].

1.1.7 Clinico-pathology

PCa is a multiple stage disease (Figure 1) that often originates from prostatic intraepithelial neoplasia (PIN), which is characterized by architecturally benign prostatic acini lined by cells that seem to be malignant [17]. PIN can lead to localized PCa where cancer cells are confined into the prostate capsule and still androgen-dependent. Unfortunately, PCa often become more aggressive (advanced PCa) as it metastasizes to adjacent and remote tissues to form new foci of cancer cells in regional lymph nodes, bones and sometimes lungs and liver. At this stage, hormonal therapy to reduce the level of dihydrotestosterone (DHT), the active metabolite of testosterone, is used to slow down the progression of the disease. However, it is mostly genetic changes that are associated with the progression of the disease and it is likely the accumulation of these changes, rather than their specific order that transform a normal cell into a tumorigenic one (Figure 1).

Figure 1. Prostate cancer stages. During PCa evolution, different gene alterations are associated with the diverse stages, from PIN to androgen-independent adenocarcinoma.


Normal epithelium
Prostatic intraepithelial neoplasia (PIN)
Invasive adenocarcinoma
Metastatic adenocarcinoma
Androgen independent adenocarcinoma
▪*NKX3-1* loss
▪BCL2 activation
▪AR CAG and GGC repeat disequilibrium
▪*KLF5* loss
▪*SERPINB5* loss
▪*RASSF1A* loss
▪*CDKN2A* loss
▪RAS activation
▪HER-2-NEU activation
▪AR dysregulation
▪*RB* loss
▪TP53 inactivation
▪*PTEN* loss
▪*ANXA7* loss
▪*CD44* loss
▪*MAP2K4*, *KISS1* *BRMS-1* and *NME1* loss
▪AR dysregulation
▪TP53 inactivation
▪*ANXA7* loss
▪*AR* mutation
▪BCL2 activation
▪Growth factor autocrine production

Although the number of men presenting with metastatic PCa has decreased significantly over the last several years, the death rate for those men is essentially unchanged. To alter the currently inevitable progression of hormone refractory PCa to death, new targets and new therapies are needed [27]. In PCa tissue, the reciprocal loss of cellular differentiation, which occurs during the progression of the disease, is largely concomitant with a loss of androgen responsiveness by prostatic cells [28]. Thus metastatic spread is almost always accompanied by eventual insensitivity to androgens and results in patient death.

1.1.8 Androgen independence

In normal conditions, androgens regulate the development and the homeostasis of the prostate. They act on prostatic epithelial cells by binding to an intracellular protein, the AR that binds both testosterone and DHT. The nuclear AR is composed of an activating domain, a DNA-binding domain (DBD) and a ligand-binding domain (LBD). The LBD binds androgens, anti-androgens and selective AR modulators [29]. AR dimerizes upon stimulation and binds to androgen-responsive elements (ARE) in the promoter region of target genes [30]. One way that AR regulates the proliferation of prostate cells is by stimulating cyclin-dependent kinases (CDK) [31].

When androgen-ablation therapy is given to a patient, PCa cells can circumvent it by increasing their sensitivity to very low amounts of androgens, by up regulating AR expression, or by amplifying the AR gene. PCa cells can increase the local production of androgens to compensate for the overall decline,

increase the amount of AR in the nucleus, the specificity of the AR can be changed and thus be activated by other ligands than androgens (like epidermal growth factor (EGF)), or it can bypass the androgen necessity [30].

1.1.9 Metastasis

1.1.9.1 The metastatic cascade

Metastasis is defined as the formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion. In the past, extravasation and survival at the metastatic site were thought to be inefficient processes as only 0.1% of injected cells in an animal model went into secondary organs to form a tumor [32]. The main factors that could explain why these processes are inefficient was the low survival rate of cells shed in the circulation, and the low percentage of them that can escape from blood vessels [33]. While disseminated cells are likely to be present in numerous organs, only certain environments appear to allow their survival and subsequent growth [34]. However, a recent study by PS Steeg in 2003 showed that extravasation and survival at the metastatic site were relatively efficient processes [35].

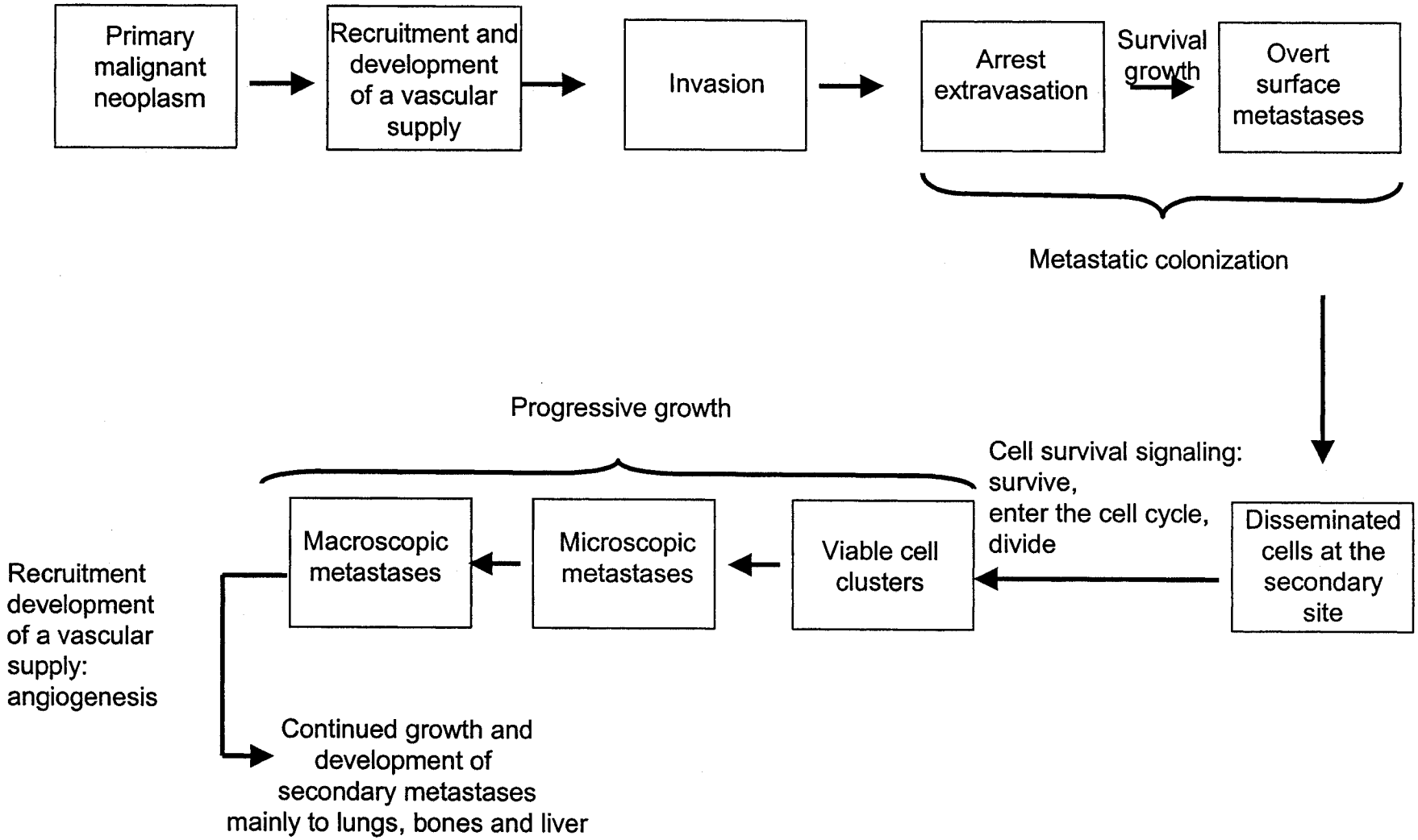
The formation of a primary tumor requires a cadre of molecular and cellular alterations that enable a cell to circumvent normal growth control mechanisms as well as to manipulate its local environment [36]. One of these changes is the development of blood supply that allows tumor cells to be nourished [37]. The development of clinically significant metastases requires that a cancer cell

complete a series of well-defined steps, generally referred to as the metastatic cascade (Figure 2).

Acquisition of invasive abilities involves loss of adhesion molecules (E-cadherin/CDH1), initiation of motility and extracellular-matrix (ECM) proteolysis, which culminates in the shedding of cells into the blood circulation either directly or via the lymphatics [38]. Beyond this step, survival of cells in the blood stream, their arrest at a distant organ, and initial extravasation were found to be efficient processes [35]. At the secondary site, cells arrest by binding to specific molecules like β-galactoside-binding lectin, galectin 3 (LGALS3), or TF (Thomsen-Friedenreich) antigen [39] in particular organs or tissues [7]. They can thus form a metastatic focus in this organ.

The next phase of the metastatic colonization is subdivided into the survival of cells after extravasation, and their persistence of growth. However, survival and persistence of growth were shown to occur inefficiently and the rate of invasion and metastatic colonization predicted the overall metastatic ability [35].

Figure 2. The metastatic cascade. Primary prostate cancer needs to undergo different steps to develop into macroscopic metastases.


Primary malignant neoplasm
Recruitment and development of a vascular supply
Invasion
Arrest extravasation
Survival growth
Overt surface metastases
Metastatic colonization
Progressive growth
Cell survival signaling: survive, enter the cell cycle, divide
Disseminated cells at the secondary site
Viable cell clusters
Microscopic metastases
Macroscopic metastases
Recruitment development of a vascular supply: angiogenesis
Continued growth and development of secondary metastases mainly to lungs, bones and liver

1.1.9.2 Bone metastases

To explain the homing of cancer cells to specific organs, Ewing proposed in 1928 the haemodynamic theory. This states that the development of metastases in a given organ is dependent on the blood volume flowing into that organ [40]. Unfortunately, this cannot be applied to bones since they do not have a high blood flow in the red marrow. It is now accepted that PCa cells possess inherent capacities, which not only direct them to bones, but also enable them to survive, proliferate and colonize bones [41]. Indeed, PCa cells loss their E-cadherin expression for the cadherin 11 (or OB cadherin) which targets them to the bones [42].

Bones have several unique features in their morphological structure and cellularity. They have a hard calcified matrix, which shows relatively low cellularity and metabolic activity. This matrix stores a variety of osteoblast-derived growth factors which may serve as essential nutrients for cancer cells which localize in bones [41]. These growth factors are constantly released into the bone marrow cavity. Thus, bones provide a fertile microenvironment which facilitates colonisation by metastatic cancer cells [41]. Bones are a target organ for both breast and PCa metastases, which validates the "seed and soil" theory proposed by Paget more than a century ago [43]. This theory states that PCa cells (the "seeds") need to encounter a fertile microenvironment (the "soil") to grow and develop metastases.

There are two patterns by which cancer cells can affect bones: osteolytic (breast cancer cells) and osteoblastic (PCa cells). PCa cells themselves produce factors like endothelin 1 (ET-1) [44] which stimulate osteoblastic bone formation. Moreover, some factors like bone morphonogenic proteins (BMPs), transforming growth factor-β1 (TGFβ1) and TGFβ2, insulin-like growth factor 1 (IGF1) and IGF2, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) [45] regulate osteoblast function by activating signalling pathways involved in the regulation of osteoblast proliferation and differentiation.

Primary PCa grows at a relatively slow rate. When spread to bones, its growth is accelerated, suggesting that the bone microenvironment provides proliferation-stimulating factors for metastatic PCa cells.

1.1.10 Treatments

When detected early enough, choices have to be made in order to treat organ-confined PCa. Here is an extensive list of possible treatments: watchful waiting; early, deferred, intermittent, or sequential androgen suppression; external or conformal external beam radiotherapy; iodine or palladium seed brachytherapy; high dose rate brachytherapy; cryotherapy; and/or radical retropubic, perineal or laparoscopic prostatectomy [46].

It is encouraging that a new generation of treatments is emerging to help patients with advanced PCa. Those are; combination chemotherapy; biphosphonates; calcitriol; antibodies against PSMA; bortezomib; thalidomide;

immunotherapy; *BCL2* antisense; inhibition and/or blockade of growth factor receptor or growth factor receptor pathways [27]. However, it is still too early to say if any of these treatments will really improve the survival time of patients with advanced PCa.

1.2 Genetic alterations in PCa

In order to become malignant, normal cells have to activate oncogenes and inactivate tumor suppressor genes (TSGs). For example, proto-oncogenes participate in normal cell proliferation, encoding a variety of proteins that function as growth factors, growth factor receptors, regulators of replication or transcription, modifiers of protein function, or in other signalling pathways. Alterations of these critical processes can cause aberrant growth and contribute to neoplastic transformation. Oncogenes can be activated by insertional mutagenesis, amplification, point mutation, and translocation [47]. As they function in a dominant manner, the presence of an altered and activated oncogene is sufficient to cause tumors despite the presence of one normal allele, [47].

On the other hand, TSGs are defined as genes that suppress tumor growth and function in a recessive manner. If one copy of the gene is already missing because of an inherited defect, then the loss or inactivation of the sole remaining allele results in complete absence of that gene's product, which can lead to cancer. Indeed, the inactivation of both alleles of a gene, termed the "two-hit" hypothesis by Knudson in 1971, explained the epidemiology of retinoblastoma

[48]. Later on, biallelic inactivation of the *RB1* gene was confirmed to be causative [49].

TSGs can be inactivated by different mechanisms, which allow a cell to progress through the cell cycle, to become resistant to apoptosis, and to escape the normal controls of the cell and immune system. There are three mechanisms by which a cell can inactivate a TSG: point mutation, deletion, often seen as loss of heterozygosity (LOH), and promoter methylation.

The metastatic spreading of cancer cells to other tissues requires the inactivation of another set of genes called metastasis suppressor genes (MSGs). MSGs are defined as genes whose encoded proteins suppress the formation of spontaneous macroscopic metastases, without affecting the growth of the primary tumor [7]. Using mainly MMCT, metastasis-suppressor activities have been reported on chromosomes 1, 6, 7, 8, 10, 11, 12, 16, and 17 [50, 51]. So far, thirteen genes have been shown to meet the MSG criteria: *KAI1*, *KISS1*, *MAP2K4*, *CD44*, *BRMS1*, *NME1*, *NME2*, *RHOGDI2*, *RKIP*, *SSECKs*, *VDUP1*, *CDH1*, and *TIMPs* [52]. However, only *KAI1*, *MAP2K4*, and *CD44* have been implicated in PCa and are described below.

1.3 Chromosomal alterations in prostate cancer

Genetic alterations are common features of cancer. Indeed, chromosomal rearrangements or loss of particular regions have been reported for almost all chromosomes. Moreover, MMCT (see section 1.4) experiments have shown that

TSGs lie on human chromosome 1, 3-13, 17-19, 22, and X [53]. However, these genetic modifications are not randomly distributed since specific chromosomal regions have been associated with certain types of cancers [54]. Indeed, some genetic alterations are specific to PCa, and it is thought that PCa arises as a consequence of at least five cumulative genetic changes [55]. Allelic losses of chromosomes 2q, 3p, 5q, 6q, 7q, 8p, 9p, 10p, 10q, 11p, 11q, 12p, 13q, 16q, 17p, 17q, 18q, and 21q have been shown to be associated with progression and/or initiation of PCa [56]. The most frequent alterations in advanced PCa are the loss of chromosomes 7q [57], 8p [58, 59, 60], 10q [61, 62, 63], 16q [64, 65, 66], 17q [67, 68], and 18q [69, 66] and the gain of chromosome arms 7q, 8q, and Xq [70]. Furthermore, the more sensitive Comparative Genomic Hybridization (CGH) technique has led to the identification of more genetic alterations in PCa, including losses of genetic material on chromosomes 2q, 5q, 6q, 9p, 13q, 15q, and 17p [71, 72]. The loss of these regions suggests that they contain one or more TSG. Moreover, introduction of human chromosomes 2 [73], 6 [74], 7 [75], 8 [76], 10 [77], 11 [78], 12 [79], 13 [80], 16 [81], 17 [82], 18 [83], 19 [84], and 20 [85] in human or rat Dunning PCa cell lines resulted in reduction or loss of their metastatic properties, without affecting their tumorigenicity.

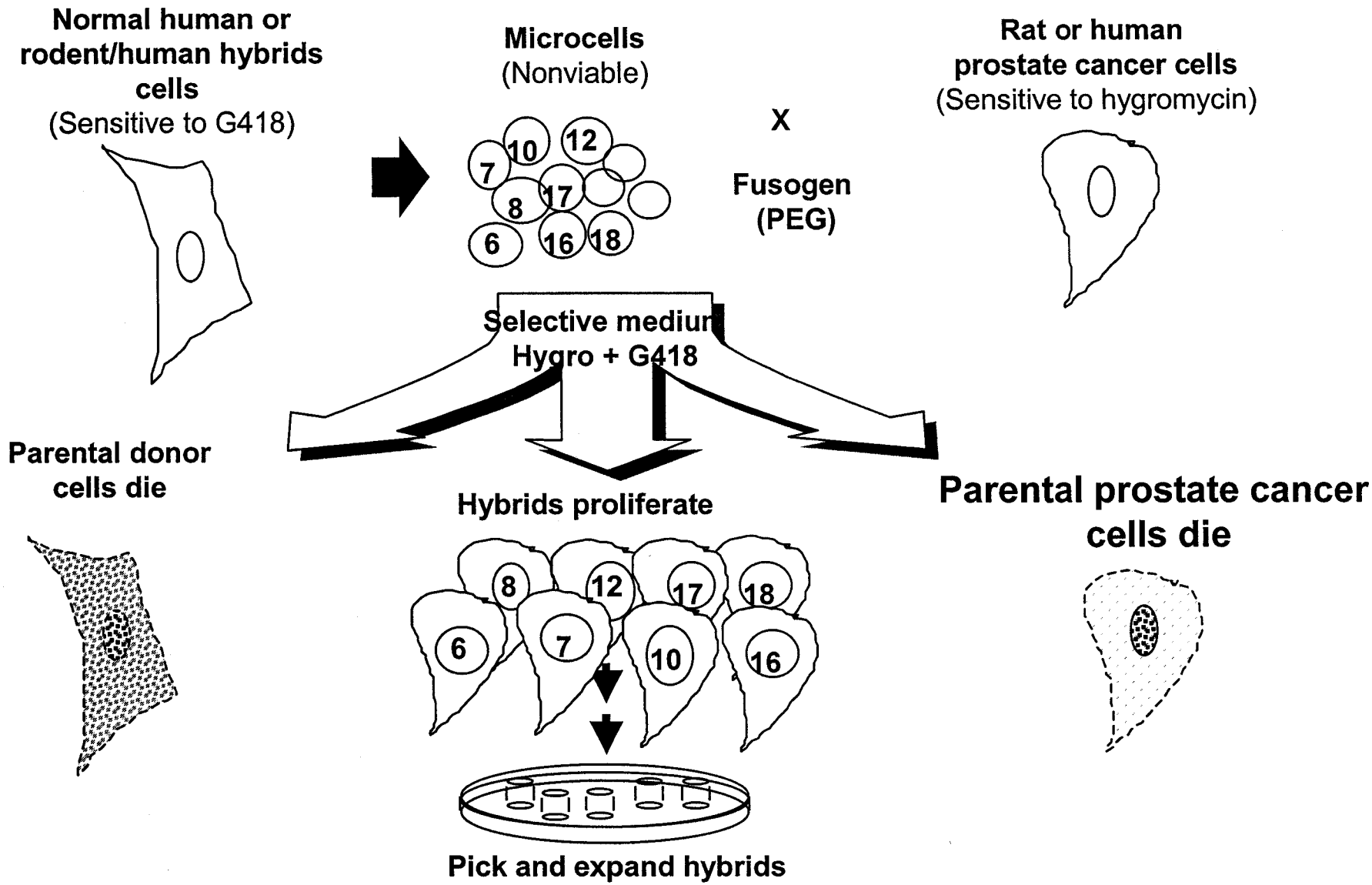
1.4 The use of microcell-mediated chromosome transfer to clone TSG/MSG

1.4.1 The technique

MMCT is a technique that has been in use for more than thirty years; it allows the transfer of a single chromosome (or chromosome fragments) from one cell to another. MMCT necessitates the generation of microcells, which are small

nuclei containing one or few chromosomes, surrounded by a plasma membrane. Although such microcells are not viable, they can be fused to other cells, and upon appropriate selection, the resulting microcell hybrids will have incorporated the chromosome(s) from the microcells into the genetic material of the recipient cells (Figure 3). MMCT can be carried out with somatic cells, embryonic carcinoma (EC) or embryonic stem (ES) cell recipient, to study *in vitro* or *in vivo* the effects of the transferred genetic material [53].

Figure 3. Scheme of the microcell-mediated chromosome transfer technique. Rodent/human donor cells are fused with recipient rat or human PCa cells. Each hybrid contains a tagged human chromosome, which allows selection for cells that has incorporated it.


Normal human or rodent/human hybrids cells
(Sensitive to G418)
Microcells
(Nonviable)
X
Fusogen
(PEG)
Rat or human prostate cancer cells
(Sensitive to hygromycin)
10
12
7
8
17
6
16
18
Selective medium
Hygro + G418
Parental donor cells die
Hybrids proliferate
Parental prostate cancer cells die
8
12
17
18
6
7
10
16
Pick and expand hybrids

The first successful cell fusion experiments were carried out in the 1950s, using Sendai or other viruses as fusing agents. The major goal at this time was to increase efficiency of fusion between two complete cells. The potential of this method was apparent as a major gathering of the leaders took place in 1968 to explore the study of intergenomic relationship and predict future uses for cell hybrids. Meanwhile, it had been known since the 1950s that cells became multinucleate following treatment with mitosis-inhibiting agents such as colcemid. These multinucleated cells can then be enucleated by cytochalasin B treatment followed by centrifugation. This process drags the condensed metaphase chromosomes out of the cells, along with a covering of nuclear and cell membrane, thus forming microcells. These two technologies (cell fusion and microcell formation) were then combined into a single process for fusing donor microcells containing single or small numbers of chromosomes with recipient cells to make fully functional hybrids. In 1977, Fournier and Ruddle at Yale University reported the fusion of mouse microcells with human HeLa or Chinese hamster ovary (CHO) cells. These hybrid cells contained small numbers of mouse chromosomes in a human or hamster background. Thus, the MMCT technology had arrived, at a time when less than 100 genes were assigned to specific human chromosomes.

In the early cell fusion experiments, it was noted that certain chromosomes tended to be preferentially lost, but this was unpredictable in individual experiments. Approaches were then developed for selection of specific chromosomes. In 1967, Weiss and Green used culture medium containing

hypoxanthine, aminopterin and thymidine to select for an endogenous human locus (the thymidine kinase (*TK*) gene), by maintaining the chromosome encoding the human *TK* gene in a mouse background [86]. Using other hybrids, the human *TK* gene was eventually mapped to human chromosome 17 [87]. New plasmid vectors and transfection technologies allowed integration of exogenous selectable markers into host chromosomes, thus allowing their selection in culture [88]. Since these transfected markers are inserted randomly into the host genome, the selection of any of these tagged chromosomes became possible. Presently, dual selectable markers (such as HyTK fusion protein) are used to select microcell hybrids in culture (in media containing hygromycin B), or revertant cells that will have lost the introduced chromosome (in media supplemented with ganciclovir).

1.4.2 Usage

MMCT experiments have shown that TSGs lie on almost all human chromosomes (see section 1.3). Indeed each normal human chromosome has been transferred into cancer cell lines, and the resulting hybrids experienced either suppression of *in vitro* growth, or diminished tumorigenicity *in vivo* [53]. Such experiments indicate that these human chromosomes encode one of more tumor/metastasis suppressor genes implicated in PCa progression.

1.4.3 TSG/MSG identified using MMCT

In PCa, MMCT has lead to the cloning of the ***KAI1*** (*CD82*) TSG/MSG. This gene was indeed identified when introduction of human chromosome 11 into Dunning

AT6.1 rat PCa cells completely abolished their tumorigenic properties both *in vitro* and *in vivo* [89]. This MSG also decreases PCa cell invasiveness and motility both *in vitro* and *in vivo* [90]. The KAI1 protein is a member of the tetraspanin superfamily (also known as the transmembrane 4 superfamily (TM4SF)) which is characterized by four highly conserved transmembrane domains and is involved in regulation of cell proliferation and motility [91]. *KAI1* is also implicated in breast carcinoma, since its expression is inversely correlated with the metastatic potential of some human breast cancer cells lines. The nearly ubiquitous expression of this gene suggests that it could function as a metastasis suppressor gene in other tumor types. Accordingly, exogenous expression of KAI1 in highly metastatic B16-BL6 murine melanoma cells also alters cell-cell interactions [92]. The exact mechanisms by which KAI1 is able to suppress metastasis are still not known. However, it could be through the interactions between KAI1 and its partners, such as E-cadherin, β1 integrin, and epidermal growth factor receptor (EGFR). These membrane proteins have indeed already been implicated in metastasis progression. [93].

Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (*MAP2K4*) gene is another MSG that has been identified following transfer of chromosome 17 into Dunning rat PCa cells [94] and is described below.

Using MMCT, Ichikawa et al. transferred a copy of human chromosomes 7, 8, 9, 10, 11, 12, and 17 into the highly metastatic Dunning R-3327 rat PCa cells.

They found that hybrids containing human chromosomes 7, 8, 10, 11, 12, or 17 showed decreased ability to metastasize to the lung without any loss of tumorigenicity [94]. Further molecular and cytogenetic analyses of microcell hybrids demonstrated that putative metastasis suppressor genes were located on human chromosomes 7q21-22, 7q31.2-32, 8p21-12, 10q11-22, 11p13-11.2, 12p11-q13, 12q24-ter, and 17pter-q23. The identification of regions on chromosomes 11p and 17p is not surprising since they encode respectively the *KAI1* (at 11p11.2), and *MAP2K4* (at 17p12) suppressor genes. This proves that MMCT is useful to identify tumor and metastasis suppressor genes implicated in PCa initiation and/or progression. Although other suppressor genes have been identified using other methods, such tumorigenic or metastasis suppression in hybrids likely indicates that more suppressors exist.

1.5 Chromosomes and genes implicated in prostate cancer

As mentioned in Figure 1 and section 1.2, many genetic alterations are seen in PCa cells. Indeed, allelic losses of 2q, 3p, 5q, 6q, 7q, 8p, 9p, 10p, 10q, 11p, 11q, 12p, 13q, 16q, 17p, 17q, 18q, and 21q are associated with PCa progression and/or initiation [56]. An extensive list of putative TSGs/MSGs classified by chromosome can be found with the corresponding reference in Table 1. Here, we review the chromosomes that are the more often affected during PCa progression, and the different oncogenes, TSGs and MSGs they encode.

Table 1. Tumor suppressor and metastasis suppressor genes implicated or not yet tested in prostate cancer progression

Chromo-some	Gene name	TSG or MSG	In prostate cancer	Reference
1p31	*CLCA2*	TSG	To be tested (TSG in breast cancer)	[95]
1p36	*RUNX3*	TSG	To be tested (TSG in gastric cancer)	[96]
1q21.2	*TXNIP*	MSG	To be tested (MSG in melanoma)	[97]
1q32	*KISS1*	MSG	To be tested (MSG in melanoma and breast cancer)	[93]
2p11.2	*RKIP*	MSG	Decreased expression in metastasis, decreases metastasis in a murine model	[98]
2q14	*BIN1*	TSG	Inactivated in advanced PCa, suppress growth *in vitro*	[99]
3p21.3	*RASSF1A & C*	TSGs	Inhibit LNCaP growth, Methylated in PCa cell lines (PC-3, DU 145, LNCaP)	[100] [101]
3q25.32	*TIG1*	TSG	Decreases PC-3 invasiveness, decreases tumors *in vivo*	[102]
6q22.32-q24.1	*CRSP3*	MSG	To be tested (MSG in melanoma)	[97]
6q24-25.2	*GRAVIN*	MSG	Decreased in PC-3, LNCaP, DU 145, decreases colonies in soft agar and metastasis *in vivo*	[103]
6q24.1	*DRG-1*	MSG	Inhibits lungs metastases	[104]

7q31.1	*CAV1*	TSG	Methylated in tissues	[105]
7q31	*TES*	TSG	Decreased expression in tissues	[106]
7q31.1-q31.3	*ST7*	TSG	Suppresses tumor formation from PC-3 cells *in vivo*	[107]
8p11.2	*LSM1*	TSG	Decreases invasion through Matrigel and metastases in mice	[108]
8p21	*NKX3-1*	TSG	Expression loss is associated with advanced PCa	[109]
8p22	*LZTS1*	TSG	Inhibits colony formation of LNCaP cells	[110]
8p22	*DLC1*	TSG	Methylated in tissues	[111]
9p21	*CDKN2A*	TSG	Decreased in primary PCa, lost in PC-3 cells	[112]
10p14-p15	*KLF6*	TSG	Mutated in PCa tissues, decreases cell proliferation	[113]
10q21	*ANXA 7*	TSG	Decreased in metastases and localized PCa, decreases colony formation and cell growth	[114]
10q23	*PTEN*	TSG	Decreases PC-3 cells growth, inhibits metastases and decreases primary tumor size in mice	[115]
10q24-q25	*MXI1*	TSG	Decreases cell proliferation and colony formation	[116]
11p11.2	*KAI1*	MSG	Decreases metastases in mice, lost in 100% lymph node metastases	[117] [93]
11p13	*CD44*	MSG	Methylated in tissues, lost in almost all metastases	[93]
11q13-q13.2	*BRMS1*	MSG	To be tested (MSG in breast cancer)	[93]
11q23.2	*TSLC1*	TSG	Methylated in PPC-1, suppresses PPC-1 tumors in mice	[118]
11q23-q24	*BCSC-1*	TSG	Suppresses colony formation, suppresses tumors *in vivo*	[119]

12p11.23	*PTX1*	TSG	Decreases PC-3 cells growth	[120]
12p13.1-p12	*CDKN1B*	TSG	Lost in advanced PCa, has to be inactivated along with PTEN	[121]
12p12.3	*RhoGDI2*	MSG	To be tested (MSG in bladder cancer)	[122]
12q14.1	*WIF1*	TSG	Decreased in primary PCa	[123]
12q21.1	*RTVP-1*	TSG	Decreased expression by methylation	[124]
12q24.1	*NME2*	MSG	To be tested (MSG in breast, colon cancer and melanoma)	[93]
13q21	*KLF5*	TSG	Suppressed DU 145 growth *in vitro*	[125]
16q22.1	*DERPC*	TSG	Decreased in prostate tumors, inhibits cell growth	[126]
16q22.1	*CDH1*	TSG	Inhibits invasion of DU 145 cells	[127]
16q23.1	*WWOX, WFDC1, MAF, FOXF1, MVD*	Puta-tive TSGs	Decreased in PCa cells	[128]
17p11.2	*MAP2K4*	MSG	Lost in metastases	[129]
17p13.1	*P53*	TSG	Inhibits cell growth, suppresses tumor growth and metastases	[130]
17q12-q21.1	*LCRG1*	TSG	To be tested (TSG in laryngeal cancer)	[131]
17q21	*BECN1*	TSG	To be tested (TSG in breast cancer)	[132]
17q21.3	*NME1*	MSG	Suppresses DU 145 colony formation and invasion	[133] [134]
18q21.3	*SERPINB5*	MSG	Decreases tumorigenesis, metastasis, down-regulates migration and invasion of PCa cells	[135]

19q13.2	*CEACAM1*	TSG	Inhibits angiogenesis, suppresses tumors *in vivo*	[136]
19q13.4	*PEG3*	TSG	Decreases tumors *in vivo*	[137]

Chromosome 3

In 1997, Dahiya R. et al. found LOH on two regions of chromosome 3: 3p24-26 and 3p22-12. However, the deletions at 3p24-26 and 3p22-12 were not related to the stage or grade of the tumor [138]. Since then, the TSG ***RASSF1A*** (Ras association domain family 1A) has been implicated in PCa and its location is close to the deleted regions reported by Dahiya et al. It has two promoters that produce *RASSF1A* and *RASSF1C* transcripts [101]. The *RASSF1A* gene is a member of the *RAS* family of proto-oncogenes that play a fundamental role in signal transduction pathways involved in cell proliferation and survival. The protein has a Ras-binding domain that binds RAS in a GTP-dependent manner and its overexpression induces apoptosis. The *RASSF1A* product may function in signal transduction pathways involving Ras-like proteins and the pro-apoptotic effect of the protein may require heterodimerization with NORE1 [139]. The *RASSF1A* gene is inactivated in the PCa cell lines LNCaP, PC-3 and DU 145 by methylation of its promoter. Interestingly, its re-introduction suppresses the growth of LNCaP cells *in vitro* [101].

Chromosome 6

Deletion of the long arm of chromosome 6 (6q) frequently occurs in many neoplasms including carcinoma of the prostate, suggesting the location of TSGs at 6q. Indeed, the 6q24 region can suppress the tumorigenicity of ovarian cancer cells when introduced by MMCT [140]. The loss of 6q13-q23 is the second most frequently deleted chromosome region in PCa after 10q [141]. The well-known oncogene ***P21/CDKN1A*** is located on this chromosome. It is implicated in the

regulation of cell growth by modulating TP53-induced apoptosis [142]. It is thought to have a paracrine effect by releasing mitogenic and antiapoptotic factors. The P21 protein induces G_1-phase arrest by inhibiting the activity of CDK, and interacting with the proliferation of cell nuclear antigen (PCNA), thereby directly preventing DNA synthesis. P21 expression is associated with the metastatic potential and androgen-independent PCa (AIPC) phenotype [142].

Chromosome 7

The 7q31chromosome region contains many putative TSGs such as *TES*, *CAV2*, *CAV1*, *MET*, *CAPZA2*, *ST7*, and *WNT2*. Using real-time RT-PCR, Chene L. et al. demonstrated that *CAV1*, *CAV2*, *MET*, and *TES* mRNA expression was lower in prostate tumors than in normal prostate tissues [106]. The transfer of human chromosome 7 into the PCa cell line PC-3 led to a two-fold increase in tumor latency in mice [143]. Moreover, a 1.5 megabase region at 7q31.1 (between microsatellite markers *D7S486* and *D7S655*) was shown to be essential for suppression of the malignant phenotype of PC-3 cells [143].

The oncogene ***EZH2*** is located on human chromosome 7q and encodes a transcription repressor, which is activated in advanced PCa. This suggests that repression by this protein could enhance the capability of a tumor to progress towards metastasis, possibly by down-regulating a TSG. A report showed that *EZH2* mRNA is increased in prostate tumor metastases, compared with confined tumors [144]. The up-regulation of *EZH2* in PCa cells leads to transcriptional repression and increased cell proliferation. A number of EZH2-

repressed genes are TSGs such as the transcription factor ZNFNF1A1, or proteins that inactivate cell signalling such as the RHO GTPase-activating protein 1 [145].

Chromosome 8

Several regions on the short arm of chromosome 8 (8p) play different roles in prostate tumors. Indeed, Oba et al have shown that gene(s) located at 8p22-p21.3 are implicated in tumor differentiation, while 8p21.1-p21.2 deletions could influence progression of prostate tumors [146]. There is also a statistical correlation between advanced tumor grade and frequency of 8p deletions [147]. By transferring yeast artificial chromosomes (YACs) spanning the frequently deleted region 8p21-p22 into rat PCa cells, Cabeza-Arvelaiz et al. have identified a candidate suppressor gene known as *FEZ1* (*LZTS1*). Although the exact function of the FEZ1 protein is not known, it contains a leucine-zipper domain, which suggests that the *FEZ1* gene encodes a putative DNA transcription regulator. This gene inhibits stable colony-forming efficiencies when transfected into LNCaP human PCa cells [110].

The 8p21-p12 region also encodes a MSG able to suppress the metastatic ability of rat PCa cells. When transferred into rat AT6.3 PCa cells, a 60kb region of chromosome 8p inhibits metastatic spreading to the lung, following injections in the flank of nude mice. Interestingly, this 60kb is located in the frequently 8p21-p12 deleted region in human PCa cells, a further indication that this region harbors a MSG for PCa [76]. Due to its location at 8p21, the androgen-regulated

homeobox *NKX3-1* gene had become a TSG candidate for PCa [148]. It encodes a transcription factor homeodomain highly expressed specifically in the prostate. *NKX3.1* undergoes LOH associated with tissue differentiation, and loss of androgen responsiveness during PCa progression. Furthermore, its expression is lost in the two androgen-independent cell lines PC-3 and DU 145, suggesting that its expression might be androgen-regulated. *NKX3.1* could play a role in the opposing processes of androgen-driven differentiation of prostatic tissue, and loss of differentiation during the progression of the disease [148]. Moreover, mice with a single *NKX3.1* allele (+/-) develop prostatic hyperplasia and PIN. The hyperplasic lesions of these mice retain *Nkx3.1* protein expression from the remaining wild-type allele, suggesting that haploinsufficiency contributes to tumorigenic progression [149]. Indeed, when only one allele is lost, the function of the gene is abrogated and it contributes to tumor progression [150].

Chromosome 9

The TSG ***CDNK2A*** (*P16*) is located on chromosome 9p21 and encodes a CDK inhibitor. It acts as a negative regulator of cell cycle progression through the G1/S phase. Various mechanisms have been described to explain its inactivation: homozygous deletion, point mutation, and aberrant methylation. All these mechanisms lead to an increased progression through the cell cycle. In patients with either primary or metastatic PCa, no mutations were detected in the *CDKN2* gene. LOH at this locus is found in about 20% of the primary tumors and in 46% of the metastatic cancers. The commonly used PCa cell line PC-3

has a dense methylation pattern of the *CDKN2* promoter, while no normal prostate tissues contain aberrant methylation [151].

Chromosome 10

Introduction of human chromosome 10p into human PCa resulted in suppression of their malignant phenotype, suggesting the presence of prostate TSG on 10p [152]. Moreover, a 1.2Mb region located at 10p15.1 and flanked by markers *D10S1172* and *D10S226* has been shown to contain a TSG for PCa [153]. This TSG could well be the *KLF6* gene, since LOH at this locus is often seen in PCa [113]. Human chromosome 10 also encodes two other important TSG for PCa progression: *PTEN* located at 10q23.3 and *ANXA7* at 10q21.1-q21.2. The ***ANNEXIN 7*** (*ANXA7*) gene is located at 10q21, which has been shown to contain a TSG for PCa [154]. The protein has calcium binding properties and GTPase activity. The expression of *ANXA7* is lower in metastatic and local recurrence of hormone refractory PCa. Its expression inversely correlates with the stage of the disease. ANXA7 protein expression is decreased in recurrent PCa [155]. Moreover, 35% of the primary tumor examined by Srivastava et al. showed LOH near the *ANXA7* gene [114]. It has been reported that human tumor cell proliferation, and colony formation were markedly reduced when the gene was introduced in LNCaP and DU 145 cells [114].

The TSG ***PTEN*** (Phosphatase and tensin homologue deleted on chromosome ten)/**MMAC1** (mutated in multiple advanced cancers 1) on chromosome 10q23 encodes a protein tyrosine phosphatase which has PIP3 (phosphatidylinositol

3,4,5-triphosphate) (a product of the PI3 (phosphatidylinositol) kinase pathway) for substrate [156]. PTEN acts as a negative regulator for the PI3 kinase/Akt signaling pathway, which control cell cycle progression and apoptosis [157]. It is mutated in its catalytic domain at high frequency (60%) in primary PCa [158, 159], although *PTEN* loss is a late event in PCa progression [160].

Chromosome 11

Glutathione-S-transferase (***GSTP1***) is located on chromosome 11, and encodes for a family of isoenzymes that provide protection to cells by catalyzing intranuclear detoxification of electrophilic metabolites of carcinogens and reactive oxygen species. Their epigenetic silencing is the most common (>90%) genetic alteration so far reported in PCa [161]. The *GSTP1* promoter is heavily methylated in PCa cell lines and tissues. Furthermore, a significant association between *GSTP1* promoter hypermethylation both with a Gleason score of 7 or more and the presence of Gleason grade 4 and/or grade 5 was found in patients [162].

The MSG ***CD44*** is a transmembrane protein that mediates cell adhesion by binding to specific ECM components [93]. Its expression inversely correlates with the grade and metastatic stage of prostate tumors [163]. It is thought that CD44-mediated metastasis suppression may occur through contact inhibition of cancer cell growth within micrometastatic foci at the secondary site [93].

Chromosome 12

Jaeger et al. (2004) identified a 4.2Mb discontinuous region at 12q24.3 that suppresses the metastatic properties of rat PCa cells by more than 90%. Interestingly, a number of potentially important genes, such as vitamin D receptor, multiple integrin subunits, and WNT inhibitory factor 1 are not retained in the non-metastatic hybrids. This suggests the activity of a novel MSG or a previously-uncharacterized function of a known gene [79]. Furthermore, we have previously shown that introduction of a portion of chromosome 12 (12pter-12q13) into the human PCa cell line DU 145 suppressed their tumorigenic properties [164]. Taken together, these results argue for the presence of at least two tumor suppressor genes on this chromosome. To our knowledge, no TSG implicated in PCa has yet been mapped to these regions.

Chromosome 13

Dong JT., et al studied the LOH of different regions of chromosome 13 in PCa specimens. They showed that the frequencies of LOH at 13q14, 13q21, and 13q33 were 62%, 57% and 34%, respectively [165]. Two TSGs located in these regions were implicated in PCa: *KLF5* and *RB1*. **Kruppel-like factor 5 and 6** (*KLF5* and *KLF6*) are TSGs implicated in PCa. They are ubiquitously expressed zinc finger transcription factors of unknown function that are located on chromosome 13 and 10, respectively. *KLF6* can up-regulate the cell cycle regulatory protein CDKN1A (described previously) in a TP53-independent manner, and also reduces cell proliferation, whereas *KFL5* activates the transcription of genes implicated in cell proliferation and differentiation. LOH and

hemizygous deletion are the main mechanisms by which *KFL5* is inactivated. LOH of *KLF6* is detected in 77% of primary PCa. Frequent genomic deletion and loss of expression, as well as cell growth suppression indicate that *KLF5* is a reasonable candidate for the TSG at 13q21 [125], a region that is frequently deleted in PCa [113].

The long arm of chromosome 13 (13q) is frequently deleted in PCa [71]. The TSG ***RB1*** is a cell cycle regulator, which is located in this commonly lost region. In normal cells, RB1 sequesters the E2F transcription factor, thus preventing the transcriptional activation of genes implicated in the S-phase progression. In the late G1 phase, RB is phosphorylated by the CDK2/cyclin E complex, which inhibits RB binding to E2F. The S-phase can then occur. When the *RB* gene is mutated in a cancer cell, it cannot be dephophorylated by a phosphatase at the end of the cell cycle. Thus, the RB protein is always free and the E2F transcription factor is constitutively active. This allows the cell to escape the G1/S-phase control point. *RB* was originally implicated in retinoblastoma [166] but since then, its inactivation has been associated with many other tumors [167]. *RB* gene expression is decreased or absent in a significant proportion of sporadic PCa [168].

Chromosome 16

A PCa susceptibility locus has been mapped to the long arm of chromosome 16, at 16q23.2 [169]. Indeed, this region exhibits a high frequency of allelic imbalance (AI) in tumors from 51 men diagnosed with PCa using the same linked markers.

This suggests the presence of a candidate TSG implicated in the development of both familial and possibly non-familial forms of PCa [169]. Numerous studies have suggested the presence of one or more TSG on 16q22.1. A new gene has been mapped to this region: ***DERPC*** (decreased expression in renal and prostate cancer). This gene encodes a basic, proline- and glycine-rich nuclear protein. The protein is ubiquitously expressed and its expression is decreased in renal (67%), and prostate carcinomas (33%). Exogenous *DERPC* over expression in LNCaP cells causes alterations of nuclear morphology, and has an inhibitory effect on PCa cell growth [126].

1.6.7 Chromosome 17

Loss of 17p is associated with higher Gleason score [170], a finding that is not surprising considering the numerous TSGs/MSGs located on this chromosome. Indeed, the oncogene *HER-2-NEU*, the TSGs *TP53*, and *BRCA1*, and the MSG *MKK4* are all located on this chromosome.

It has been suggested that the breast cancer susceptibility genes *BRCA1* at 17q21 and *BRCA2* at 13q12 might be involved in PCa [171, 172]. However, only few mutations of these genes were detected in PCa tissues. Indeed, Sinclair CS et al (2000) screened 22 high-risk PCa families for *BRCA1* and *BRCA2* mutations, but found only one previously identified *BRCA2* mutation, and two unreported *BRCA2* intron polymorphisms. Contrary to what is seen in breast cancer, no truncating mutations were found [173]. This suggests that *BRCA1* and *BRCA2* have a limited role in familial PCa susceptibility.

Located on chromosome 17, the oncogene ***HER-2-NEU*** is a receptor tyrosine kinase that belongs to the EGF family. The over expression of the receptor tyrosine kinase is associated with the progression to AIPC, and activates PSA transcription. Initially, HER-2-NEU confers survival to tumor cells by activating the AR pathway in an androgen-independent manner [174].

TP53 modifications represent the most common genetic change in human malignancies [175]. This TSG is located on chromosome 17p13, and encodes a 53kDa nuclear phosphoprotein which functions as a cell-cycle regulator that inhibits the progression of genetically damaged cells through the S-phase. Therefore, when *TP53* function is disrupted, cells with damaged DNA can divide and propagate the genetic defect to daughter cells. *TP53* mutation leads to genetic instability, a hallmark of malignant progression [176]. *TP53* is classically inactivated by point mutations [177]. The short-arm of chromosome 17 is commonly deleted in human tumors and the remaining *TP53* allele frequently harbours point mutations [178]. Another major function of TP53 appears to be in mediating the cellular response to DNA damage, and helping to maintain genomic stability [179]. TP53 is involved in the regulation of expression of genes induced during cell growth arrest triggered by DNA-damaging agents [179]. When a cell encounters agents that cause DNA damage, TP53 halts cell cycle progression by inducing transcription of mitotic inhibitors including CDKN1A. CDKN1A binds to and inhibits cyclin kinase complexes, preventing phosphorylation of specific substrates which in turn inhibits the passage from G1 to S phases [180]. During this time, the cell attempts to repair the damaged DNA. If

such damage is irreparable, TP53 triggers apoptosis. The *TP53* status is different in the three commonly used PCa cell lines DU 145, PC-3 and LNCaP. For example, in DU 145, two mutations were found in the *TP53* gene: a mutation at codon 274 (pro > leu), and a second mutation at codon 223 (val > phe) were present. The PC-3 cells are hemizygous for chromosome 17p, and the single copy of the *TP53* gene has a base pair deletion at codon 138 that generates a frame shift and a new in-frame stop codon at position 169 [181], thus completely inactivating the putative encoded protein .

The **mitogen-activated protein kinase kinase 4** (*MAP2K4*) gene at 17p11.2 can suppress rat PCa *in vivo* [182]. It is also a member of the MAP kinase family (mitogen-activated protein kinase). Its activation occurs in response to cell stress, leading to proliferation, apoptosis, and differentiation [93]. In normal prostatic tissue, there are high levels of MAP2K4 in the epithelial but not in the stromal compartment. In PCa, there is an inverse correlation between Gleason pattern and MAP2K4 expression. The protein is down regulated during PCa progression, arguing that *MAP2K4* has a role for deregulation of its signalling cascade in clinical disease. There is also LOH in 31% of PCa tissues [129].

Chromosome 18

Deletions of chromosome 18 have been associated with many cancers including colorectal [183], pancreatic [184], ovarian [185], head and neck squamous cell carcinoma [186], lung [187], and PCa [188, 189, 190, 191, 192]. Functional evidence for the presence of a TSG on human chromosome 18 was demonstrated when the

introduction of this chromosome into pancreatic cancer cells resulted in suppression of their tumorigenic phenotype [184].

Cytogenetic, allelotype and somatic cell hybrid studies have shown that 18q may carry a TSG that plays a role in the carcinogenesis of PCa. The introduction of 18q into the commonly used PCa cell line DU 145 and PC-3 completely abolished their tumorigenicity [189, 193]. Moreover, LOH on 18q was reported in 30-40% of PCa patients, indicating that it may play a role in PCa progression [66]. Two minimally lost regions have been described: one of them is between *D18S1119* and *D18S64*, and the other is located between *D18S848* and *D18S58*. However, no previously isolated TSG maps to these intervals [191].

The chromosomal band 18q21 harbours the tumor suppressor genes *DCC* (deleted in colorectal cancer), *SMAD4/DPC4* (deleted in pancreatic cancer 4), and *SMAD2/DPC2* (deleted in pancreatic cancer 2) [194]. *SMAD2* and *SMAD4* are components of the signalling pathways of serine-threonine kinase receptors for TGF-beta [190], but none of these genes is mutated in PCa specimen [195]. However, the loss of SMAD2 and SMAD4 protein expression is linked to increased Gleason score[196]. The long arm of chromosome 18 also encodes for the well-known oncogene *BCL2*, as well as the MSG *MASPIN/SERPINB5*, which is implicated in PCa progression.

The ***BCL-2*** oncogene is located on the long arm of chromosome 18, and is implicated in PCa progression. It encodes an anti-apoptotic protein that is

localized to the mitochondrial outer membrane. It prevents apoptosis by suppressing the release of the caspase-activating protein cytochrome c from mitochondria [197]. In normal prostate tissue, the expression of BCL-2 is confined to basal cells and undetectable in secretory cells. This correlates with the level of differentiation of these two kinds of cells. This protein was also found in BPH but not in PIN [198]. BCL2 and BCL-X levels were found to correlate with the development of PCa, which suggests that they are associated with a hormone-insensitive metastatic phenotype [199].

However, despite all the studied oncogenes (*RAS*, *CDKN1A*, *EZH2*, *HER-2-NEU*, *BCL2* for example), none of them has been linked conclusively to the initiation or early progression of PCa.

The MSG ***MASPIN*** (*SERPINB5*) is located on chromosome 18, and encodes a serine protease inhibitor. In human primary PCa, *MASPIN* expression is consistently down regulated at the critical transition from non-invasive, low-grade disease to highly invasive, high-grade PCa [200]. The reduced *MASPIN* expression in these patients correlates with a high Gleason grade [201]. Stable cell lines expressing MASPIN had decreased tumorigenic potential, as assessed by anchorage independent growth in soft agar assay compared to controls. *MASPIN* stable transfectants showed decreased metastatic potential and increased adhesion to fibronectin and laminin [135].

Chromosome 19

The TSG ***CEACAM1*** is located on chromosome 19, and encodes a cell-adhesion molecule of the immunoglobulin superfamily. The protein has four extracellular Ig-like domain and a cytoplasmic domain [136]. It has also been shown to mediate homophilic cell adhesion through its first Ig domain [202]. The anti-tumor effect of *CEACAM1* may be due to inhibition of tumor angiogenesis by increased secretion of antiangiogenic factors from the cells. Its expression in DU 145 cells induces the production of a factor that specifically blocks the growth of endothelial but not epithelial cells [136]. In patients, down regulation of *CEACAM1* is associated with loss of cell polarity. It coincides with the formation of the complex glandular architecture of Gleason grade 4 pattern [203].

1.6 Hypothesis

As described in this chapter, almost every human chromosome has been shown to encode TSGs and/or MSGs implicated in PCa progression. Thus, we hypothesize that by transferring the chromosomes that are the most frequently lost during PCa progression, we could identify chromosomal regions encoding TSGs/MSGs implicated in this disease. Such an approach also represents the first step in identifying the suppressor genes themselves.

As mentioned previously, we chose to use the MMCT technique, as it is a powerful tool to identify chromosomes/regions encoding TSGs/MSGs. The PC-3 cell line was isolated from a bone metastasis of a patient; bone metastases represent the deadly part of this disease. We used PC-3-derived cell lines PC-

3M-Pro4 and PC-3M-LN4 since they have, when orthotopically injected, different tumorigenic and metastatic properties (PC-3M-Pro4 grows rapidly in the prostate, whereas PC-3M-LN4 is also very aggressive and metastasizes to lymph nodes). These cell lines have been previously selected *in vivo* by several passages in the prostate of nude mice. Thus, they have been exposed to the prostate microenvironment and are most likely to mimic the disease than other models isolated from a brain metastasis (DU 145) or rat PCa cells (Dunning).

1.7 Goals of this study

In order to further characterize the suppressor genes implicated in PCa progression, the aims of this study were to:

1. Transfer human chromosomes that are implicated in PCa progression into two PC-3-derived cell lines (PC-3M-Pro4GP1 and PC-3M-LN4GP2).

2. Identify chromosome(s) that suppress (es) the tumorigenic properties of these PCa cells both *in vitro* and *in vivo.*

3. Characterize *in vitro* and *in vivo* the hybrids containing an exogenous copy of human chromosome 18.

4. Identify the region on chromosome 18 that is responsible for the les-tumorigenic phenotype seen in our hybrids.

5. Characterize candidate genes present in this region

CHAPTER 2

MATERIAL AND METHODS

2.0 Preface

The material and methods are described in five major sections corresponding to the order of the experiments in Chapters 3, 4, and 5.

(a) Microcell-mediated chromosome transfer into prostate cancer cells.

(b) Characterization of microcell hybrids *in vitro*.

(c) Nude mice injections of microcell hybrids.

(d) Chromosome 18 mapping and identification of the region containing the tumor suppressor gene.

(e) Characterization of candidate genes

2.1 Microcell-mediated chromosome transfer into prostate cancer cells

2.1.1 Cell lines and culture

2.1.1.1 PCa cell lines

PC-3M-LN4 and PC-3M-Pro4, obtained from I.J. Fidler (Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, 77030), were derived from orthotopic injections of PC-3M cells into the prostate of nude mice [204]. PC-3M-LN4 is a highly metastatic (lymph node) human PCa cell line, while PC-3M-Pro4 grows very aggressively in the prostate. PC-3M was derived from the PC-3 cell line obtained from a bone metastasis of a patient with PCa [205]. PC-3M-LN4 and PC-3M-Pro4 were kept in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON, Canada). The geneticin-resistant PC-3M-LN4GP2 and PC-3M-Pro4GP1 cell lines were generated upon Lipofectamine 2000 (LF2000, Invitrogen) transfection of the pQBI25 plasmid (Q-BIOgene, Carlsbad, CA, USA). These cells were kept in RPMI 1640 with 10% FBS and 400 ug/ml of geneticin (Invitrogen). B78MC hybrids are microcell hybrids from our mouse/human hybrid panel [206], and contain tagged human chromosomes in the B78 mouse melanoma background. B78MC hybrids were kept in DMEM (Dulbecco's modified Eagle medium; Invitrogen) supplemented with 10% FBS and 400 ug/ml of hygromycin B (Roche Diagnostics, Laval, QC, Canada). The Pro4B- and LN4B-microcell hybrids were grown in RPMI 1640 with 10% FBS, 400 ug/ml of geneticin and 400 ug/ml of hygromycin B.

2.1.1.2 Generation of cell lines expressing green fluorescent protein

Prior to transfection, the cells were plated at 40% confluency. The next day, 5 ug of pQBI25 plasmid DNA suspended in 250 ul of Opti-MEM (Invitrogen), was mixed with 5 ul of LF2000 (1mg/ml) diluted in 250 ul of Opti-MEM, and incubated 20 mins at room temperature. Meanwhile, the cells were washed with 2 ml of 1X PBS (0.137 M NaCl; 2.7 mM KCl; 10.14 mM Na_2HPO_4; 1.76 mM K_2HPO_4; pH 7.4). Then, serum-free media (RPMI 1640) and the DNA-LF2000 mix were added to the flasks. The cells were then placed at 37°C with 5% CO_2 for 5 hours. The DNA-LF2000 complex was removed and replaced with complete media (RPMI 1640 supplemented with 10% FBS). On the next day, the cells were diluted 1:10 and incubated for 24 hours after which selective media was added (RPMI 1640 supplemented with 10% FBS and 400 ug/ml of geneticin). Clones were picked 10 days after transfection, and were called PC-3M-Pro4GP1 to 3, and PC-3M-LN4GP1 to 5.

2.1.2 Microcell-mediated chromosome transfer

The microcell-mediated chromosome transfers were done essentially as described by Fournier in 1981 [207] (Table 2). Briefly, plastic bullets, made from the bottom of 150 mm Petri dishes, were sterilized overnight in 95% ethanol. Bullets were then dried in a laminar flow hood for 1 hour (bullets were inverted after 30 mins). A sterile 15 mg/ml solution of concanavalin A (Sigma, Oakville, ON, Canada), and a sterile 75 mg/ml WSC (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-o-toluene-sulfonate, Sigma) solution were prepared in 0.9% NaCl. The bullets were crosslinked with concanavalin A and WSC for 1-2 hours.

Table 2. Microcell-mediated chromosome transfer schedule

Day 1	Day 2	Day 3	Day 4	Day 5
Sterilize bullets	Crosslink bullets	Prepare microcells	Trypsinize fused cells	Add selection
	Micronucleate donor cells	Perform fusion		

The mixture was removed, and the bullets were washed and stored in sterile PBS overnight at 4°C. The next day, donor cells (1 x 10^6 B78MC hybrids in 1.2 ml of PBS) were spread on dried bullets. The bullets were flooded with 40 ml of complete media (DMEM with 10% FBS and 400 ug/ml of hygromycin B), and incubated at 37°C for 2 hours. B78MC hybrids containing different tagged chromosomes (10, 10p, 12, 18, translocated 3 and 17) were micronucleated in presence of colcemid (0.06 μg/ml) (Sigma, Oakville, ON, Canada) for 24 hours. Micronucleated cells were harvested in enucleation media (500 ml of serum-free media with 10 ug/ml of cytochalasin B (Sigma)) at 27,000g for 30 mins at 28 to 34°C. The microcells were then resuspended in 15ml of serum-free media, and filtered through 8 um and 5 um polycarbonate filters (Fisher Scientific) to eliminate intact cells. The microcells were centrifugated and resuspended in 1 ml of serum-free media. Meanwhile, recipient cells (PC-3M-Pro4GP1 and PC-3M-LN4GP2) were plated in 25 cm^2 flasks (Fisher Scientific, Nepean, ON, Canada) until 70-80% confluency on the day of microcell fusion. These recipient cells were rinsed with serum-free media, and then 1ml of microcells suspended in serum-free media and 1ml of phytohemagglutinin (PHA; 200 ug/ml) were added to each flask and incubated 15 mins at 37°C. PHA was removed and cell fusion was performed in presence of 50% (w/w) polyethylene glycol 1600 (Fisher Scientific) for 60 sec. The cells were rinsed three times with serum-free RPMI 1640 medium, and complete medium (RPMI 1640 with 10% FBS) was added to the flask and incubated overnight. The resulting microcell hybrids

(Pro4B... and LN4B...) were selected in RPMI 1640 medium containing 10% FBS + 800 μg/ml hygromycin B + 800 μg/ml geneticin. Individual hybrids (clones) were picked after 21 days, expanded and characterized.

2.2 *In vitro* characterization of microcell hybrids

2.2.1 Confirmation of chromosome transfer

2.2.1.1 *Alu*-PCR FISH

B78MC hybrids could contain between one and few human chromosomes. Since mouse chromosomes do not contain *Alu* repetitive elements in contrast to human chromosomes, a rapid screening of these hybrids was performed using the *Alu* PCR FISH technique [208, 209]. Briefly, human inter-*Alu* DNA sequences are amplified using the following four *Alu* primers:

451 (5'-GTGAGCCGAGATCGCGCCACTGCACT-3'),

450 (5'-AAAGTGCTGGGATTACAGG-3'),

153 (5'-GGGATTACAGGCGTGAGCCAC-3') and

154 (5'-TGCACTCCAGCCTGGGCAACA-3') (Sigma).

The amplified products were labelled with biotin, and used as probe in Fluorescence *In situ* hybridization (FISH) on normal human chromosome spreads [210]. Under these conditions human chromosomes present in the B78MC hybrids could be identified, based on their size and on the position of their centromere as mouse chromosomes are often acrocentric and will not hybridize to the Alu probe.

2.2.2 Microsatellite mapping

To determine which specific human chromosome(s), or chromosome fragment(s) was/were transferred in each B78MC hybrids; genomic mapping using microsatellite markers was performed.

2.2.2.1 Genomic DNA extraction

High quality genomic DNA was obtained as follows: 70-80% confluent cells were rinsed twice with 0.9% NaCl, scraped off the plate, and centrifugated at 16,250g. The cell pellets were mixed to 300ul of fresh TSM (140 uM NaCl, 10 uM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$) + 0.5% NP40 (nonidet P 40, Sigma) and incubated on ice for 3 mins. The cells were then harvested 10 sec by centrifugation at 16,250g, and the pellets were resuspended in nuclei dropping buffer (0.075 M NaCl, 0.024 M EDTA, pH 8.0) containing 0.2 mg/ml of proteinase K (Roche Diagnostics) and 0.5% sodium dodecyl sulfate (SDS), and incubated overnight at 37°C. 5 ml of water and 5 ml of 6M NaCl were added and the samples were mixed for 2 hours. The cells debris were removed by centrifugation at 755g for 20 mins, and two volumes of 100% ethanol were added to the supernatant. The precipitated DNA was then spooled and resuspended overnight in TE (10mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and re-precipitated by adding ½ volume of 7.5 M NH_4acetate

and two volumes of 100% ethanol. The DNA was then spooled and resuspended in TE^{-4} (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0).

2.2.2.2 Microsatellite mapping

PCR reactions were carried out in 1X PCR buffer, 75 uM of dNTPs without dCTP, 0.66 uM dCTP, 0.01 umol of Alpha ^{32}P-dCTP, 0.05U of Taq DNA polymerase, 1.6 uM of each primer, and 100 ng of genomic DNA. The following PCR program was used: 94°C for 2 mins, followed by 35 cycles of 94°C for 30 secs, annealing temperature (from 53 to 65°C according to each primer pair) for 30 secs, and 72°C for 30 secs, and a final extension at 72°C for 10 mins. After PCR amplification, 10 ul of sequenase stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) were added. The stopped PCR reactions were denatured at 95°C for 5 mins, and electrophoresed through a 4% acrylamide gel. The gel was dried for 2h at 80°C, and autoradiographed overnight in order to reveal the amplified microsatellites.

2.2.3 Invasion assay

We used a Boyden chamber assay [211] to assess invasive potential. Briefly, 25,000 cells were added to the chamber coated with Matrigel (Fisher Scientific), and RPMI 1640 medium supplemented with 5% FBS was added to the bottom chamber to serve as chemoattractant. The chambers were incubated at 37°C for 22 hours, and filters were stained with a Diff-Quick

staining kit (Dade Behring, Newark, DE). Invasive cells from five random fields were counted under a contrast phase microscope at 200X magnification. All invasion experiments were performed twice in quadruplicate (n=8), and the results were expressed as percentage of the invasion obtained with the parental cell line.

2.2.4 Soft agar assay

Soft agar assays were performed according to Rizzino, 1987 [212]. Briefly, a 1 ml base layer of 0.5% agar is poured into each of 6 wells. Then, the cells are trypsinized, counted and resuspended to a concentration of 3,000 cells per ml. The top layer of agar (2 ml of media containing 0.5% agar) is mixed with one ml of cells (3000 cells), and put on the top of the base layer. The plates are incubated for 10 days in a 37°C incubator with 5% CO_2, stained with crystal violet, scanned with a PowerLook 2100XL scanner (UMAX, Dallas, TX), and counted on the computer screen. In some cases, qualitative assessment of growth was performed; where colony formation of hybrids was compared to those of parental cell lines which were arbitrary assigned the symbol +++ (with a varying scale of 0 to ++++). Each soft-agar assay was performed three times on six wells per cell line (n=18) and the p values were calculated using a two-tailed Student's t-test (see Chapter 4 for details).

2.3 *In vivo* characterization of microcell hybrids

2.3.1. Nude mice injections

All mouse injections were done according to Canadian Council for Animal Care guidelines, after approval by the McGill University Animal Care Committee. For the nude mouse injections (*nu/nu;* six to eight weeks of age; Charles River Laboratory, St-Constant, QC, Canada), the cells were trypsinized, counted, and resuspended in 1X HBSS (Hank's balanced salt solution, 0.4 mM KH_2PO_4, 0.6 mM $MgSO_4$, 5.4 mM KCl, 1.3 mM $CaCl_2$,; Invitrogen) at 1 million cells in 200 ul for subcutaneous injections, and one million cells in 16.6 ul for orthotopic injections. Mice were monitored, and weighed three times per week, and were sacrificed when the subcutaneous tumor reached 1000mm^3, or when the animals became moribund for orthotopic injections. Five mice were injected for each cell line tested (see chapter 4 for details).

2.3.2 Tumor processing

Tumors were excised, and digested twice for 15 mins at 37°C with type I collagenase (Invitrogen). The cells were seeded into flasks, and fed with complete media supplemented with antibiotics. Two days later, complete media containing selective agents and antibiotics was added (see chapter 4 for details).

2.4 Chromosome 18 mapping and identification of the region encoding the tumor suppressor gene

2.4.1 Genotyping of human chromosome 18

We characterized our chromosome 18 hybrids by using 27 microsatellite markers (see Table 5 in chapter 4 and section 2.2.2.2 for details). Briefly, 100 ng of genomic DNA from our hybrids was amplified in presence of ^{32}P-dCTP in triplicate with microsatellite markers and electrophoresed through a 4% acrylamide gel. The gel was dried, and autoradiographed to X-Ray film overnight.

2.4.2 Microarray experiment

2.4.2.1 RNA extraction

Briefly, the cells were scraped off the tissue culture plate, centrifugated, and mixed with TSM + NP40 as described previously (section 2.2.2.1). After a 3 mins lysis on ice, the cells were harvested, and the supernatant was mixed with TSE + S (10mM Tris-HCl, pH 7.4, 150mM NaCl, 5mM EDTA, pH 7.4, 0.2% SDS). After, two phenol-chloroform-isoamyl alcohol (25:24:1) (Fisher Scientific), and one chloroform-isoamyl alcohol (24:1) extractions were done. The RNA was then precipitated with 1/10 volume of 3 M sodium acetate (NaOAc), 2 volumes of 100% ethanol, and 10 ug of glycogen (Roche Diagnostics) at -80°C overnight. The samples were centrifugated for 15 mins at 4°C and the pellets were rinsed twice with 70% ethanol, air-dried,

and resuspended in DEPC-treated water (Sigma). Each sample was electrophoresed through agarose gel to assess its quality.

2.4.2.2 Microarray experiments

We used a 19k3 cDNA microarray (Microarray Center at University Health Network, Ontario Cancer Institute, Toronto, ON, Canada) containing more than 19 200 human genes or ESTs spotted in duplicate. Three separate experiments were done comparing the expression profile of different sets of hybrid cells, as well as a subcutaneous tumor derived from our less tumorigenic hybrids: LN4B156-2 vs LN4B156-6 in duplicate by swapping the dyes and TLN156-6-3 vs LN4B156-6.

The reverse transcriptase labelling reactions were prepared in the following mix: 1X First strand buffer (Invitrogen), 150 pmol of AncT primer (Sigma), 5 mM of dNTP-dTTP (Amersham Pharmacia, Baie d'Urfée, QC, Canada), 1.5 mM of dTTP (Amersham Pharmacia), 1.5 mM of AA-dUTP (Sigma), 0.1 M of DTT (Invitrogen), 2 ng of *Arabidopsis* control RNA, and 10 ug of total RNA (see section 2.4.2.1). The reactions were incubated at 65°C for 5 mins, then at 42°C for 5 mins. SuperScriptII reverse transcriptase (0.03U; Invitrogen) was added, and the reactions were incubated at 42°C for 2 to 3 hours. The reactions were incubated at 95°C for 5 mins. 2 mM of NaOH was added, and the reactions were heated at 65°C for 15 mins. Then, 2 mM of HCl and 1 mM of Tris-HCl, pH7.5 were added to neutralize the reactions. The labelled

cDNA were purified using the Amicon Microcon PCR purification columns (Fisher Scientific) following manufacturer's instructions. To the purified samples, 1.8 mM of $NaHCO_3$ pH 9.0, and each dye (Alexa 555 and Alexa 647, Molecular Probes, Burlington, ON, Canada) were added. The reactions were incubated in the dark for 1hour. They were purified using microcon column as described previously, and were combined together. Then, 1/10 volume of 3M NaOAc, 1 volume of 95% isopropoanol (Fisher Scientific), and 10 ug of glycogen were added. The samples were precipitated at -20°C for 30 mins, centrifugated at 16,250g for 10 mins, the pellets were washed with 70% ethanol, and resuspended in 5 ul of Sigma water.

A hybridization solution was prepared (20:1:1, Dig Easy Hyb (Roche Diagnostics): sonicated calf thymus DNA (10 mg/ml) (Sigma): yeast tRNA (10 mg/ml) (Invitrogen)), and incubated at 65°C for 2 mins. The combined Alexa 555/Alexa 647 reactions were added to the hybridization solution, and incubated at 65°C for 3 mins. The reaction was then loaded onto the microarray, and incubated in a moist chamber (VWR, Mississauga, ONT, Canada) (filled with Dig Easy Hyb) overnight at 37°C. Washing solutions were prepared as following: 1X SSC + 0.1% SDS (preheated to 50°C), and 1X SSC at room temperature. Three staining dishes (Diamed, Mississauga, ONT, Canada) were filled with preheated 1X SSC + 0.1% SDS, and placed in a 50°C water bath. Two staining dishes were filled with room temperature 1X SSC. The first 1X SSC container was used to split the two slides by

dipping and agitating them. The slides were washed three times 15 mins each in 1X SSC + 0.1% SDS at 50°C. The slides were rinsed in 1X SSC, then in 0.1X SSC, spin-dried at 45g for 5 mins, and stored at 4°C in the dark. The microarrays were scanned using SCANarray 5000 (Perkin Elmer, Woodbridge, ON, Canada), and the fluorescent signals of the dyes were quantified using QuantArray version 3 (Perkin Elmer). The data analysis was done using the R software version 2.1.1 (www.r-project.org). The data were normalized within each slide using the loess sub-grid method. They were further normalized between the slides with the quantile method. Then, the data were submitted to a linear model (SAM, significant analysis of microarrays) to get the significant differentially expressed genes.

2.5 Characterization of candidate genes located around the *D18S51* region

2.5.1 Real-time PCR

The expression of the *ERK4* and *MASPIN* genes were assessed by real-time quantitative PCR. The amplification was adjusted to the *GAPDH* expression level, and gene-specific Quantitect primers (Qiagen, Mississauga, ON, Canada) were used for PCR amplification. A PCR mix was prepared for each sample tested: 1X of 2X DyNAmo SYBR Green qPCR kit (New England Biolabs, Pickering, ON, Canada), 1X Quantitect primer mix (Qiagen), 50 ng of Reverse Transcriptase reaction in a final volume of 25 ul. The PCR efficiency of each primer set was determined by sequential dilutions of the

purified PCR amplicon (1:4 to 1:256). PCR reactions were electrophoresed through an agarose gel, and a dissociation curve was added to the PCR program to make sure that the right amplicon was amplified; each reaction was repeated 8 times. The following PCR program was run on an MXI4000 (Stratagene, LaJolla, CA, USA) thermal cycler: stage 1: 50°C for 2 mins, stage 2: 95°C for 15 mins, stage 3: 40 cycles of 95°C for 15 secs, 60°C for 15 secs, and 72°C for 32 secs and stage 4: 95°C for 15 secs, 50°C for 15 secs, and 95°C for 15 secs. The mean Ct (cycle threshold) and standard deviation were calculated using Microsoft Excel 2000. The delta Ct (Ct_{TARGET} – Ct_{GAPDH}) and its standard deviation were calculated using the same program. The delta-delta Ct method was use to calculate the fold changes as recommended by the manufacturer, and a Student t-test was used to determine the significance of the findings.

2.5.2 Western immunoblot

Cells were grown to 90% confluency and washed twice with cold PBS. Then, the cells were scraped off in Brij97 lysis buffer (1% Brij 97, 25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM pyrophosphate, 100 units/ml aprotinin, 10 µg/ml leupeptin, 25 µM *p*-nitrophenyl *p'*-guanidino-benzoate, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide) (all reagents are from Sigma), and incubated on ice for 5 mins. The lysed cells were then centrifugated at 13,000g for 10 mins at 4°C. The supernatants were

combined and the proteins were quantified with the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA), according to the manufacturer's protocol. Forty micrograms of proteins were boiled 5 mins in 2X Laemmli buffer (62.5 mM Trsi-HCl pH 6.8, 40% v/v glycerol, 5% SDS, 0.005% bromophenol blue containing 10% of β-mercaptoethanol (Sigma)), and electrophoresed through a 10% acrylamide SDS-PAGE gel. The proteins were then transferred (1 hour) onto a nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA, USA), and blocked for 1h in TBST (25 mM Tris-HCl pH 8.0, 125 mM NaCl, 0.1% tween 20) + 5% non-fat dry milk (Bio-Rad Laboratories). The membranes were incubated overnight with the appropriate primary antibody in TBST + 5% non-fat dry milk. For the BCL2 and MASPIN proteins, monoclonal anti-human antibodies were used (Chemicon, Temecula, CA, USA) at a 1:5000 dilution. For the ERK4 protein, a polyclonal antibody was obtained from Dr. Sylvain Meloche's laboratory (IRIC, Université de Montréal, Montreal, QC, Canada), and used at a dilution of 1:2000 (a peptide located in the C-terminal portion of ERK4 (amino acids 361 to 497) was used to immunize rabbits and generate a polyclonal antibody). After 16 hours, the membranes were washed 3 times for 15 mins in TBST, and incubated 1h at room temperature with the secondary antibody conjugated to horseradish peroxydase (donkey anti-rabbit for ERK4 (Bio/Can Scientific, Etobicoke, ON, Canada) and goat anti-mouse for MASPIN and BCL2 (Fisher Scientific)) in TBST + 5% non-fat dry milk. The bound

antibodies were detected using the ECL detection kit (GE Healthcare, Baie d'Urfe, QC, Canada).

2.5.3 Proteasome inhibition

Since the ERK4 protein was undetectable in our cell lines, they were treated for 12 hours with 25 uM of the proteasome inhibitor MG132 (Cedarlane, Hornby, ON, Canada). As a control, cells were also incubated in PBS for 12 hours. Proteins were extracted and Western immunoblotted using the polyclonal anti-ERK4 antibody (see section 2.5.2 for the procedure).

2.5.4 Sequencing

The entire coding sequence of the *ERK4* gene was amplified using the Phusion high-fidelity DNA polymerase (New England Biolabs), according to manufacturer's instructions. Briefly, a PCR reaction containing 1X Phusion HF buffer, 200 uM of each dNTP, 0.5 uM of each primer, 0.02 U/ul of Phusion DNA polymerase, 100 ng of genomic DNA or 50 ng of cDNA was prepared in a final volume of 20 ul. The following PCR program was ran: 98°C for 30 secs, followed by 40 cycles of 98°C for 10 secs, annealing temperature for 30 secs and 72°C for 30 secs with a final extension of 72°C for 10 mins. The primers used for amplification of the *ERK4* mRNA or genomic DNA were:

mapk464 (5'-TGGGCAGCTCCAGATCACTG-3') and

mapk802c (5'-ACGATGTACGCCACGCTGAA-3')

(for mRNA or genomic DNA);

mapk672 (5'-CACGCGCTCCGAGAGATCAA-3') and

mapk1520c (5'-GTTAGCGGCCATCAGCACGA-3') (for mRNA);

mapk1487 (5'-GAGATCGACGACATCGTGCT-3') and

mapk1841c (5'-AGTAGTGGTGCGGCTTGTTG-3') (for mRNA).

The annealing temperatures varied from 60 to 69°C. The PCR reaction products were electrophoresed through a 1.5% agarose gel, the amplified product was extracted from the gel using the Qiagen gel extraction kit, and sent to the McGill University and Genome Quebec Innovation Centre (Montreal) for sequencing. The primers used for sequencing were the same as the ones used to perform the PCR amplification. Briefly, the PCR products were purified and resuspended in 2 ul to be amplified with the Big Dye Terminator (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The following PCR conditions were used for amplification: 96°C for 1 min, 25 cycles of 96°C for 30 secs, 50°C for 10 secs, 60°C for 4 mins. The PCR products were precipitated, centrifugated, washed, dried, and the pellets were resuspended in formamide. The fluorescence was then read on an ABI 3730xl DNA analyser (Applied Biosystems).

2.5.5 *ERK4* vector construction and transfection

We obtained the *ERK4* expression vector from Dr. Sylvain Meloche. The vector pcDNA3ERK4-wt encodes the normal sequence of the *ERK4* gene. The full-length ERK4 insert was introduced into the pcDNA4 His/Max B vector (Stratagene). This introduces a His tag at the N-terminal of the protein. The construct was then transfected into PC-3M-LN4GP2 cells (see section 2.1.1.2 for transfection procedure). Clones were selected in presence of zeomycin (800 ug/ml). ERK4 protein over expression was confirmed by Western immunoblot (see section 2.5.2 for the Western blot protocol), using the polyclonal antibody obtained from Dr. Sylvain Meloche's laboratory (see section 2.5.2 for details).

2.5.6 Characterization of *ERK4* transfectants

Transfected cells over expressing or not the ERK4 protein were assessed by invasion and soft agar assays as described previously (sections 2.2.3 and 2.2.4).

CHAPTER 3

Published manuscript:

Chromosome 18 suppresses the tumorigenic properties of human prostate cancer cells

Gagnon A., Ripeau J-S, Zvieriev V., and Chevrette M.
Genes Chromosomes and Cancer, 2006,
Mar; 45(3): 220-230.

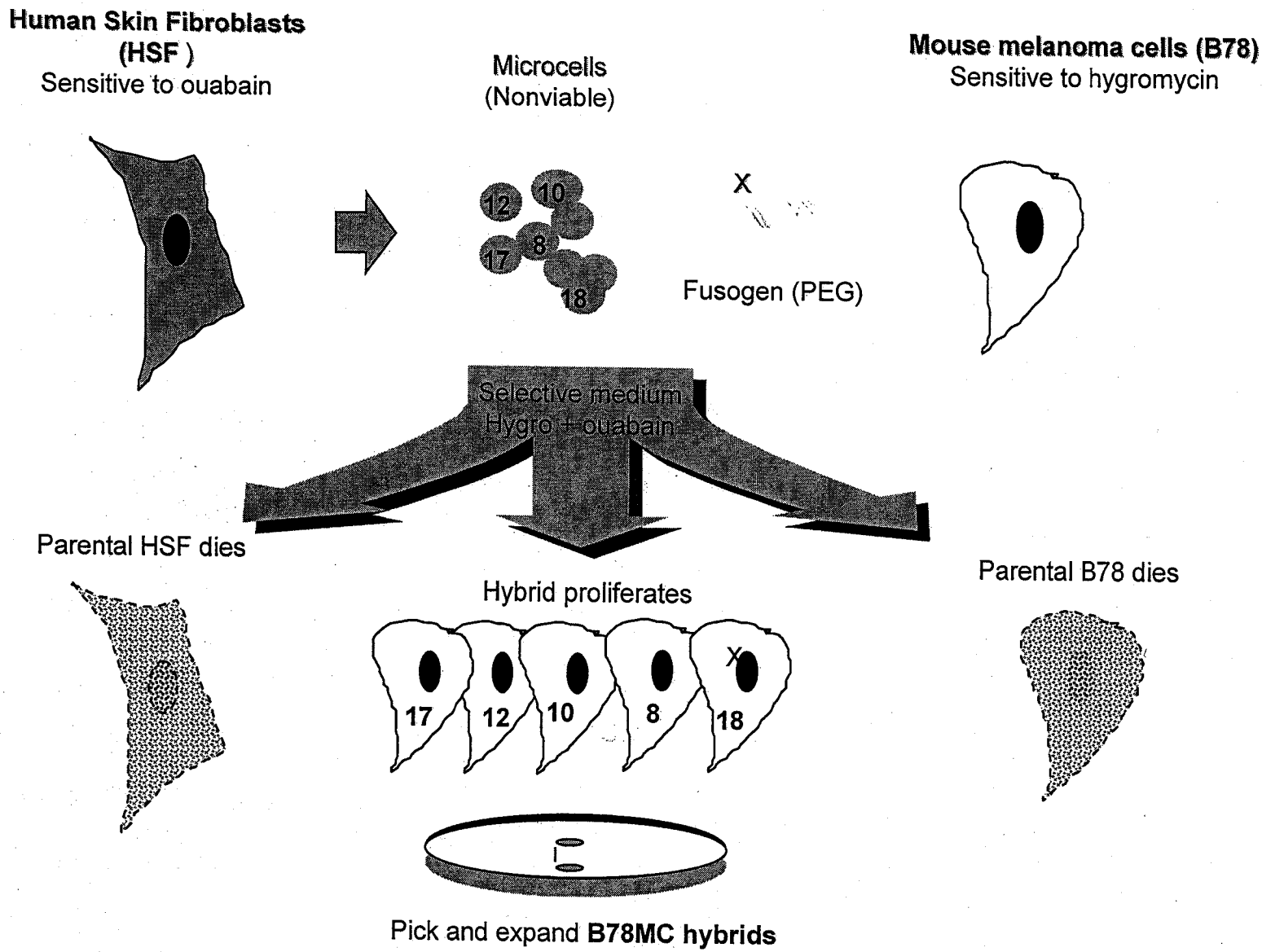
3.0 Preface

This published manuscript summarizes the results obtained by transferring human chromosome 18 into the PC-3-derived prostate cancer cell line PC-3M-Pro4GP1 and PC-3M-LN4GP2. It highlights the different properties of our chromosome 18 hybrids and the process by which we narrowed down the region encoding a new TSG to about 10Mb on the long arm of chromosome 18. The proof of this published manuscript can be found in Appendix C and the permission from publishers in Appendix B. We also included a preface summarizing the construction of the B78MC hybrid panel as well as the transfer of chromosomes 8, 10, 12, and t(17:3, with unknown breakpoints).

3.0.1 A mouse/human hybrid panel

In the 1990's, Dr. Chevrette's laboratory used normal human foreskin fibroblasts (HSF), along with the mouse melanoma cell line B78, to generate a mouse/human hybrid panel [206]. Briefly, the tgCMV-HyTk plasmid encoding the hygromycin phosphotransferase/thymidine kinase fusion gene [213] was introduced into the HSF cell population. By doing so, at least one human chromosome per cell was tagged, and could then be transferred into another cell. This allows positive selection of the tagged chromosome with hygromycin B, and its negative selection using gangiclovir. Microcell-mediated chromosome transfer (see section 2.1.2) was used to transfer the tagged chromosomes from HSF to the B78 mouse melanoma cell line. The resulting hybrids (over 200 were isolated) were named B78MC hybrids (where MC stands for microcell) (Figure 4).

Figure 4. Scheme of the construction of the B78MC hybrid panel. Microcell-mediated chromosome transfer was used to introduce normal human chromosomes from HSF into the mouse melanoma cell line B78. The resulting hybrids, selected in presence of hygromycin B and ouabain (mouse cells are naturally more resistant to ouabain than their human counterparts) were mouse/human hybrids containing one or more normal human tagged chromosomes.


Human Skin Fibroblasts
(HSF)
Sensitive to ouabain
Microcells
(Nonviable)
Mouse melanoma cells (B78)
Sensitive to hygromycin
12
10
17
8
18
X
Fusogen (PEG)
Selective medium
Hygro + ouabain
Parental HSF dies
Parental B78 dies
Hybrid proliferates
17
12
10
8
18
X
Pick and expand B78MC hybrids

3.0.2 Characterization of the B78MC hybrids by PCR

A PCR based assay was used to identify which human chromosomes were present in each B78MC hybrid. Briefly, PCR reactions were done using human based microsatellite markers from Invitrogen. Three different markers per chromosome were used (Table 3). The PCR program was as follow: 94°C 2 mins, 94°C 45 secs, annealing temperature between 53°C and 62°C 45 secs, 72°C 1 min, 35 cycles and 7 mins at 72°C. The reactions were electrophoresed through an agarose gel (Figure 5). To ensure that no contaminant was amplified, a normal human cell line (HSF) was used as positive control and the mouse melanoma cell line B78 as a negative control (data not shown).

3.0.3 Microcell-mediated chromosome transfer

As mentioned in Chapter 1, some genetic alterations are specific for PCa [70]. Therefore, using our mouse/human hybrid panel, we have transferred some of the most important chromosomes for PCa progression (chromosomes 10, 12, 17, and 18) into two PCa cell lines. The following B78MC hybrids, and the human chromosomes (chr) they contained were used. B78MC75 (chr 10), B78MC77 (chr 10), B78MC9 (chr 12), B78MC57 (t(17:3) with unknown breakpoints) and B78MC156 (chr 18). The presence of these human chromosomes in different hybrids was also confirmed using *Alu*-PCR-FISH (data not shown).

Table 3. Microsatellite makers used to characterize B78MC hybrids

Chromosome (estimated size based on NCBI database)	Polymorphic microsatellite marker	Position on the chr (Mb) (based on NCBI database)	PCR product length (bp)	PCR-annealing temperature
1 (254Mb)	*D1S468*	p arm (3Mb)	171-223	53
	D1S2809	p arm (11Mb)	123-147	53
	D1S423	p arm (23Mb)	162-168	53
6 (171Mb)	*D6S254*	p arm (70Mb)	250-264	57
	D6S278	p arm (108Mb)	125-139	55
	D6S305	p arm (162Mb)	204-230	55
7 (158Mb)	*D7S639*	p arm (67Mb)	261	53
	D7S527	p arm (93Mb)	273-297	53
	D7S666	p arm (99Mb)	155-169	57
	D7S498	q arm (142Mb)	137-153	53
8 (146Mb)	*D8S1824*	p arm (3Mb)	226-248	53
	D8S511	p arm (13Mb)	135	55
	D8S1731	p arm (14Mb)	217-241	57
	D8S1821	p arm (38Mb)	144-168	57
10 (135Mb)	*D10S196*	q arm (47Mb)	99-109	53
	D10S1692	q arm (104Mb)	182-211	57
	D10S169	q arm (128Mb)	99-117	53
12 (132Mb)	*D12S368*	q arm (51Mb)	200-216	62
	D12S325	q arm (53Mb)	209-229	55
	D12S90	q arm (57Mb)	166-182	55
13 (114Mb)	*D13S279*	q arm (70Mb)	241-257	53
	D13S1230	q arm (88Mb)	217-275	53
	D13S261	q arm (110Mb)	169	53
16 (88Mb)	*D16S415*	q arm (44Mb)	208-234	55
	D16S400	q arm (54Mb)	192-202	53
	D16S504	q arm (70Mb)	267	55
17 (80Mb)	*D17S1850*	q arm (29Mb)	232-274	55
	D17S1853	q arm (54Mb)	130-156	55
	D17S1154	q arm (60Mb)	354	57
18 (76Mb)	*D18S1148*	q arm (56Mb)	132-154	53
	D18S1147	q arm (57Mb)	204-232	57
	D18S554	q arm (72Mb)	167	53
	D18S1097	q arm (73Mb)	177-215	54
Y (57Mb)	*DYS390*	q arm (15Mb)	212	57
	DYS58S2	q arm (22Mb)	267	54

Figure 5. Confirmation of chromosomes present in B78MC hybrids. The *D17S1154* microsatellite marker was PCR amplified from different B78MC hybrid DNA. The presence of the 264 bp amplicon indicates that these hybrids contain this chromosome 17 region (60 Mb).

264bp
B78MC19
B78MC57
B78MC59
B78MC61
B78MC67
B78MC69
B78MC123
B78MC126
B78MC140

We have chosen two very aggressive PC-3-derived cell lines, called PC-3M-Pro4 and PC-3M-LN4 [204], as recipients for our microcell-mediated chromosome transfers. For ease of detection, we have transfected both cell lines with the pQBI25 plasmid (Q-BIOgene, Carlsbad, CA, USA) encoding the green fluorescent protein gene (GFP), and allowing for the selection of cells with geneticin (G418). The resulting cell lines were called PC-3M-Pro4GP1 to 3, and PC-3M-LN4GP1 to 5 (see section 2.1.1.2 for details).

We fused the different B78MC hybrids with both PC-3M-Pro4GP1 and PC-3M-LN4GP2, and obtained Pro4BX- (Pro4B75-, Pro4B77-, Pro4B9-, Pro4B57-, Pro4B156-), and LN4BX- hybrids (LN4B77-, LN4B9-, LN4B57-, LN4B156-). Analysis of the different hybrids showed that transfer of these different human chromosomes into PCa cells affects a variety of pathways implicated in different aspects of tumorigenicity (Table 4). We have choosen to concentrate our efforts on the human chromosome 18.

The results obtained by transferring human chromosome 18 into our two PC-3-derived cell lines are detailed in the following published manuscript entitled "Chromosome 18 suppresses the tumorigenic properties of human prostate cancer cells".

Chromosome 18 suppresses tumorigenic properties of human prostate cancer cells

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Footnotes:

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Running title:

Chromosome 18 suppresses human prostate cancer tumorigenicity.

Key Words:

Prostate cancer, chromosome transfer, chromosome 18, suppression of tumorigenic/metastatic properties.

[3]The abbreviations used are: PCa, prostate cancer; FBS, fetal bovine serum; FISH, Fluorescence *in situ* hybridization; TSG, tumor suppressor gene; chr, chromosome.

3.1 Abstract

Although prostate cancer is still the most diagnosed cancer in men, most genes implicated in its progression still remain to be identified. Chromosome abnormalities have been detected in human prostate tumors, and many of them are associated with prostate cancer progression. Indeed, alterations (including deletions or amplifications) of more than fifteen human chromosomes have been reported in prostate cancer. We hypothesized that transferring normal human chromosomes into human prostate cancer cells will interfere with their tumorigenic and/or metastatic properties. We have used microcell-mediated chromosome transfer to introduce human chromosomes 10, 12, 17, and 18 into highly tumorigenic (PC-3M-Pro4) and highly metastatic (PC-3M-LN4) PC-3-derived cell lines. We have tested the *in vitro* and *in vivo* properties of these hybrids. Introducing chromosome 18 into the PC-3M-LN4 prostate cancer cell line greatly reduced its tumorigenic phenotype. We observed retarded growth in soft agar, decreased invasiveness through Matrigel, and delayed tumor growth into nude mice, both subcutaneously and orthotopically. This phenotype is associated with a marker present in the 18q21 region. Combined with the loss of human chromosome 18 regions often seen in patients with advanced prostate cancer, our results show that chromosome 18 encodes one or more tumor suppressor genes whose inactivation contributes to prostate cancer progression.

3.2 Introduction

With an estimation of 232,090 new cases and 30,350 deaths in the USA in 2005, prostate cancer (PCa) is still the most diagnosed cancer in men, and the second leading cause of their cancer deaths [5]. However, despite these alarming figures, there is still no treatment that can cure the most advanced stages of the disease, when the cancer has become androgen-independent. Furthermore, as it progresses, PCa metastasizes to bones and lymph nodes, which further complicates its treatment [214]. Thus, there is an urgent need for new therapies, but these are likely to require a better understanding of the mechanisms and genes implicated in the progression of this disease.

Genetic alterations are a common feature of cancer. Indeed, in different tumor types, chromosomal rearrangements or loss of particular regions have been reported for almost all chromosomes. However, these modifications are not randomly distributed, but specific regions have been associated with certain types of cancer [54]. Unfortunately, in PCa, such analysis is complicated by the heterogeneity of tumor samples. Cytogenetic analysis of human prostate tumors revealed that alterations of many chromosomes are associated with PCa [56]. Indeed, numerous studies have shown deletions of chromosome arms 7q [215, 57], 8p [60, 58, 59], 10q [63, 61, 62], 16q [216, 66, 64], 17q [68, 67], and 18q [66, 217, 69] in advanced PCa. The sensitive comparative genomic hybridization method has led to the identification of more genetic alterations in PCa, including losses of genetic material on human chromosome arms 2q,

5q, 6q, 9p, 13q, 15q, and 17p [72, 71]. The loss of these regions could indicate that they encode tumor suppressor genes. Functional evidence to that effect has been gathered from the transfer of normal human chromosomes in PCa cell lines.

Almost every human chromosome has been transferred into either rat or human PCa cells for assessment of their tumor-suppressive properties. Thus, human chromosome arm 8p [76] and chromosome 12 [51] suppressed the tumorigenicity of rat PCa cells. Similarly, introduction of human chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17, 19, and 20 in rat Dunning cell lines resulted in reduction or loss of their metastatic properties, sometimes without affecting their tumorigenicity [81, 84, 218, 85, 80].

The analysis of rat/human hybrids containing human chromosome 11 has led to the identification of a new metastasis suppressor gene called *CD82 antigen* that encodes the KAI1 protein [89]. Reduced expression of KAI1 is associated with progression of PCa [219] and epithelial ovarian carcinoma [220], as well as with bladder [221], breast [222], gastric [223], and colorectal cancer [224]. Reduced expression of KAI1 was also detected in metastatic forms of oesophageal squamous cell [225] and hepatocellular carcinoma [226]. Introduction and expression of KAI1 into melanoma cells was accompanied by a reduction of their invasive and metastatic properties [92], further establishing the metastasis-suppressive activity of the KAI1 protein.

Other genes have also been shown to suppress metastasic properties without affecting primary tumor growth. These genes are *CD44* (located at 11p13), *MAP2K4* (17p11.2), *NME1* (17q21.3), *NME2* (12q24.1), *KISS1* (1q32), and *BRMS1* (11q13.1-2). However, only three of them (*CD82*, *CD44*, and *MAP2K4*) act as metastasis-suppressor genes in PCa [93].

Chromosome transfer has also been performed in human PCa cell lines, where introduction of chromosomes 2 [73], 8p [227], 10p [152], 12 [164], and 19 [84, 228] showed tumor-suppressing activity.

Cytogenetic, allelotyping, and somatic-cell hybrid studies have shown that the long arm of human chromosome 18 (18q) likely carries one or more tumor or metastasis suppressor gene(s) that play a role in PCa progression. Indeed, loss of heterozygosity on 18q was reported in 30-40% of PCa patients [66]. However, introduction of human chromosome arm 18q into the DU 145 PCa cell line abolished its tumorigenicity [189], whereas transfer of an intact chromosome 18 into the PC-3 PCa cell line interferes with both the tumorigenic and metastatic properties of these cells [193].

Tumor suppressor genes have been identified on the long arm of chromosome 18. Indeed, cytogenetic band 18q21 encodes the tumor suppressor genes *DCC* (deleted in colorectal cancer), *SMAD4/DPC4* (deleted in pancreatic cancer 4), and *SMAD2/DPC2* (deleted in pancreatic

cancer 2). However, their implication in PCa is still controversial[229]. Indeed, the SMAD2 and SMAD4 proteins seem to be lost as the Gleason score increases[230]. Moreover, and although studies in PCa patients identified loss of two chromosome 18 minimal regions (between markers *D18S1119* and *D18S64* (52940 and 55575 kbp), and between *D18S848* and *D18S58* (40053 and 70193 kbp)), no tumor-suppressor gene has yet been mapped to these intervals [231].

To identify tumor- or metastasis-suppressor genes, we have used microcell-mediated chromosome transfer to introduce different tagged normal human chromosomes (10, 12, 17, and 18) into the tumorigenic PC-3M-Pro4 and metastatic PC-3M-LN4 PCa cell lines. Here we show that introduction of chromosome 18, and not the others, into human PCa cells is sufficient to affect both their *in vitro* and *in vivo* tumorigenic properties. Indeed, such hybrids experienced decreased invasiveness, lost their anchorage-independent growth capacity, and harboured a delayed tumor growth upon both subcutaneous and orthotopic injections in nude mice.

3.3 Materials and methods

3.3.1 Cell Lines

PC-3M-Pro4 and PC-3M-LN4 human PCa cell lines [232] were obtained from I.J. Fidler (Department of Cancer Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX). PC-3M-Pro4 cells are highly

tumorigenic, whereas PC-3M-LN4 cells are highly metastatic to lymph nodes when injected orthotopically into the prostates of nude mice. Both cell lines were derived upon orthotopic injections of PC-3M into the prostates of nude mice. PC-3M was derived from the PC-3 cell line obtained from a bone metastasis of a patient with PCa [205]. PC-3M-LN4 and PC-3M-Pro4 were kept in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON, Canada). Geneticin-resistant PC-3M-Pro4GP1 and PC-3M-LN4GP2 cell lines were generated upon Lipofectamine 2000 (Invitrogen) transfection of the pQBI25 plasmid (Q-BIOgene, Carlsbad, CA). This plasmid encodes the green fluorescent protein gene and contains the neomycin phosphotransferase gene that confers resistance to geneticin. These cells were kept in RPMI 1640 with 10% FBS and 400 μg/ml of geneticin (Invitrogen). B78MC9, B78MC57, B78MC75, B78MC77, and B78MC156 are microcell hybrids from our mouse/human hybrid panel [233], and contain a tagged human chromosome (12, a translocated chromosome 3 and 17 with unknown breakpoints, 10p, 10, and 18, respectively) in the B78 mouse melanoma background. B78MC hybrids were kept in DMEM (Dulbecco's modified Eagle medium; Invitrogen) supplemented with 10% FBS and 400 μg/ml of hygromycin B (Roche Diagnostics, Laval QC, Canada). PC-3M-Pro4- and PC-3M-LN4-derived microcell hybrids (such as Pro4B156- and LN4B77- hybrids; see below) were grown in RPMI 1640 with 10% FBS, 400 μg/ml of geneticin, and 400 μg/ml of hygromycin B.

3.3.2 Microcell-mediated chromosome transfer

Microcell fusion was done as described [207]. B78MC hybrids containing different tagged chromosomes (10, 10p, 12, translocated 3 and 17, 18) were micronucleated in the presence of colcemid (0.06 μg/ml) (Sigma, Oakville, ON, Canada) for 24 hours. The resulting microcells were fused to either PC-3M-Pro4GP1 or PC-3M-LN4GP2 cells in the presence of 50% (w/w) polyethylene glycol 1600 (Fisher Scientific, Nepean, ON, Canada) for 60 sec. The cells were rinsed three times with serum-free RPMI 1640, and complete medium (RPMI 1640 with 10% FBS) was added to the flask and incubated overnight. The resulting microcell hybrids (Pro4B... and LN4B..., Table 4) were selected in RPMI 1640 medium containing 10% FBS + 800 μg/ml hygromycin B + 800 μg/ml geneticin.

3.3.3 Invasion assay

We used a Boyden chamber assay [211] to assess invasive potential. Briefly, 25,000 cells were added to the chamber coated with Matrigel (Fisher Scientific), and RPMI 1640 medium supplemented with 5% FBS was added to the bottom chamber to serve as chemoattractant. The chambers were incubated at 37°C for 22 hours, and filters were stained with a Diff-Quick staining kit (Dade Behring, Newark, DE). Filters were then put onto microscope slides (Fisher Scientific), and invasive cells from five random fields were counted under a contrast phase microscope at 200X magnification. All invasion experiments were performed twice in

quadruplicate (n=8), and the results were expressed as percentage of the invasion obtained with the parental cell line. The p values were calculated using a two-tailed Student's t-test.

3.3.4 Soft-agar assay

The soft-agar assays were performed as described previously [234]. Briefly, 1 ml of 0.5% agar (Fisher Scientific) diluted in appropriate media was put into 6-well plates to form the basal layer. Then 3,000 cells were mixed with 0.5% agar and put on top of the basal layer. Plates were incubated at 37°C for 21 days. Growing cell foci were stained with 200 µl of a 0.03% crystal violet (Sigma, Oakville, ON, Canada) solution in 100% methanol (Fisher Scientific). Qualitative assessment of growth was performed; where colonies formation of hybrids was compared to those of parental cell lines, which were arbitrary assigned the symbol +++. For quantitative evaluation, plates were scanned on a PowerLook 2100XL scanner (UMAX, Dallas, TX). The number of colonies was counted on the computer screen, and converted to a percentage of colonies obtained with the parental cell line. Each soft-agar assay was performed three times on six wells per cell line (n=18) and a Student's t-test was used to assess the significance of the findings.

3.3.5 Nude mouse injections

All mouse injections were done according to Canadian Council for Animal Care guidelines, after approval by the McGill University Animal Care

Committee. Male nude mice (*nu/nu;* six to eight weeks of age; Charles River Laboratory, St-Constant, QC, Canada) were injected with PCa cells. One million cells, in 200 µl of Hanks balanced saline solution (HBSS; Invitrogen) were injected subcutaneously in the upper backs of nude mice. Mice were sacrificed when tumors reached a size of 1000 mm^3. For orthotopic injections, one million cells were resuspended in 16.6 µl of HBSS and injected with a push button-controlled dispensing device (Hamilton Syringe Company, Reno, NY) into the dorsal lobe of the prostate. The mice were sacrificed when they became moribund. A Kaplan-meier graph was used to determine if there was a statistically significant difference between the surival of the mice injected with our hybrids versus the ones injected with parental cells.

3.3.6 Tumors in culture

Tumors were excised and digested two times for 15 min at 37°C with 350 U/ml of type I collagenase (Invitrogen), and cell suspensions were seeded into culture flasks containing RPMI 1640 supplemented with 10% FBS and 1% penicillin (5000 IU/ml) / streptomycin (5000 µg/ml) (Invitrogen). Two days later, complete selective medium (RPMI 1640 with 10% FBS + 400 µg/ml geneticin + 1% penicillin (5000 IU/ml) / streptomycin (5000 µg/ml) was added.

3.3.7 Genotyping of hybrids

To identify B78MC hybrids that contained the selected tagged human chromosomes, we performed PCR by using polymorphic microsatellite markers. We used *D10S196*, *D10S1692*, and *D10S169* for chromosome 10; *D12S43*, *D12S90*, *D12S325*, and *D12S368* for chromosome 12; *D17S1850*, *D17S1853*, and *D17S1154* for chromosome 17; as well as *D18S66*, *D18S1148*, and *D18S554* for chromosome 18. Subsequent analysis by fluorescence *in situ* hybridization (FISH) of B78MC57 revealed that this hybrid contains a translocation between chromosomes 3 and 17; however the breakpoints were not determined. We thus selected five hybrids that contained human chromosomes 10, 10p, 12, a translocated 3;17, and 18 (B78MC77, B78MC75, B78MC9, B78MC57, and B78MC156, respectively). The presence of the desired chromosome was also confirmed by Alu-PCR-FISH as described previously [164]. To characterize LN4B156- hybrids further, we used 27 human chromosome 18 microsatellite markers (Table 5). The polymorphic markers (heterozygosity > 0.8) used were: *D18S59*, *D18S63*, *D18S976*, *D18S42*, *D18S843*, *D18S542*, *D18S44*, *D18S877*, *D18S847*, *D18S1133*, *D18S67*, *D18S1157*, *D18S535*, *D18S65*, *D18S1145*, *D18S46*, *D18S487*, *D18S1109*, *D18S39*, *D18S1155*, *D18S51*, *D18S1270*, *D18S382*, *D18S541*, *D18S58*, *D18S50*, and *D18S1141* (www.invitrogen.com). Markers *D18S847*, *D18S39*, *D18S58*, *D18S50*, and *D18S1141* were non-informative as they could not distinguish the exogenous chromosome 18 from the

endogenous, whereas markers *D18S63*, *D18S843*, *D18S1157*, *D18S1109*, and *D18S382* were not transferred.

3.4 Results

3.4.1 Introduction of human chromosome 18 into PC-3M-LN4GP2 cells interferes with their *in vitro* tumorigenic properties

From our mouse/human microcell hybrid panel [206], we have identified B78MC-hybrids (Table 4) containing tagged human chromosomes that have been implicated in PCa progression (chromosomes 10, 12, 17, and 18). These tagged chromosomes were transferred into highly tumorigenic PC-3M-Pro4GP1 and highly metastatic PC-3M-LN4GP2 cell lines to generate the Pro4B- and LN4B-derived hybrids (Table 4). We have compared the anchorage dependence and invasiveness of our hybrids to their parental human PCa cells by using growth in soft agar and a Boyden chamber assay. Although transfer of the translocated chromosome (3;17, with unknown breakpoints) into PC-3M-LN4GP2 cells could significantly decrease its tumorigenic potential (see tumor formation of LN4B57-5 in Table 4), this translocated chromosome did not significantly reduce the *in vitro* tumorigenic properties of the two parental cell lines (see invasion and soft agar assays for Pro4B57- and LN4B57- hybrids; Table 4). Similarly, the *in vitro* and *in vivo* properties of the PC-3M-Pro4GP1 cells were not affected when chromosome 8 was transferred into them (data not shown), confirming that

chromosome transfer per se is not sufficient to inhibit the malignant properties of these cells.

We then quantitatively measured the ability of all our LN4B156 hybrids (LN4B156-1, 2, 3, 6, 13, 14, 16, and 20) to grow in an anchorage-independent manner. Four hybrids (LN4B156-6, 13, 16, and 20) formed statistically fewer colonies than the parental cells ($P<0.001$; Table 4). Although clonal variation is often seen in soft-agar assays, these highly significant results contrast with the anchorage-independent growth obtained with another geneticin-resistant PC-3M-LN4 clone (PC-3M-LN4GP1) that formed 74% as many colonies as did PC-3M-LN4GP2. Thus, introduction of human chromosome 18 into PC-3M-LN4GP2 cells suppresses the ability of some LN4B156 hybrids to invade through Matrigel, and to form anchorage-independent colonies. Although LN4B156-14 cells were not able to invade through Matrigel, they formed four times more colonies in soft agar than did the parental PC-3M-LN4GP2 cells ($P<0.00001$; Table 4). Such important clonal variation is similar to what was observed in chromosome 18 hybrids generated by Padalecki et al. (2003) and could indicate that the integrity of the transferred chromosome 18 was not the same in all hybrids (some chromosomes for example could harbour deleted portions), a characteristic often seen in microcell hybrids[193]. Moreover, the properties of the LN4B156-14 hybrid could argue that invasiveness and anchorage-independent growth are under the control by different genes on chromosome 18.

TABLE 4. Phenotypic Changes Associated with Human Chromosome Transfer in PC-3M-Pro4GP1 and PC-3M-LN4GP2 Prostate Cancer Cell Lines

Mouse/human parental hybrids (transferred chromosomes)	Properties of hybrids			
	Invasion assays (Parental = 100%)	Soft agar assays (Parental = +++ or 100%)	Tumor formation * (subcutaneous)	Tumor formation ** (orthotopic)
B78MC75 (chr 10p)	n/a	Pro4B75-2 ++ Pro4B75-3 ++ 1/2 Pro4B75-11 +++ Pro4B75-12 + Pro4B75-14 +	Pro4B75-12: 3/10 (p = 0.002)	Pro4B75-12: 2/5 (p = 0.14)
B78MC77 (chr 10)	n/a	Pro4B77-2: + 1/2 Pro4B77-10: + 1/2 Pro4B77-15: +		
B78MC9 (chr 12)	Pro4B9-4: 88% (p = 0.49) Pro4B9-8: 217% (p = 0.02) Pro4B9-17: 568% (p=0.0007) Pro4B9-41: 107% (p = 0.75)	Pro4B9-11: +++ Pro4B9-17: ++ Pro4B9-18: ++ Pro4B9-21: ++ 1/2 Pro4B9-22: +++	n/a	n/a
B78MC57 (chr t(3;17))	n/a	Pro4B57-1: ++ 1/2 Pro4B57-3: ++ Pro4B57-6: +++ Pro4B57-7: ++ 1/2 Pro4B57-8: + 1/2	n/a	n/a
B78MC156 (chr 18)	n/a	Pro4B156-10: + 1/2 Pro4B156-12: ++	Pro4B156-10: 3/5 (p = 0.07)	n/a

Mouse/human parental hybrids (transferred chromosomes)	Properties of hybrids			
	Invasion assays (Parental = +++ or 100%)	Soft agar assays (Parental = 100%)	Tumor formation * (subcutaneous)	Tumor formation ** (orthotopic)
B78MC77 (chr 10)	LN4B77-9: 35% (p=0007) LN4B77-50:: 73% (p = 0.17) LN4B77-60: 63% (p = 0.09) LN4B77-80: 22% (p=0.00007)	LN4B77-2: + 1/2 LN4B77-9: ++ LN4B77-37: + 1/2 LN4B77-44: + LN4B77-45: ++	LN4B77-44: 2/5 (p = 0.54)	n/a
B78MC9 (chr 12)	LN4B9-34: 2% (p=0.000006) LN4B9-35: 10% (p=0.00016)	LN4B9-31: ++++ LN4B9-33: +++ LN4B9-34: +1/2 LN4B9-35: ++ LN4B9-36: +++	n/a	n/a
B78MC57 (chr t(3;17))	LN4B57-4: 250% (p=0.007) LN4B57-5: 142% (p=0.15) LN4B57-6: 265% (p=0.003) LN4B57-7: 281% 9p=0.0011)	LN4B57-4: ++ LN4B57-5: +1/2 LN4B57-6: ++ LN4B57-7: ++	LN4B57-5: 2/10 (p = 0.04)	n/a
B78MC156 (chr 18)	LN4B156-1: 58% (p=0.98) LN4B156-2: 75% (p=0.16) LN4B156-6: 42% (p=0.0002) LN4B156-14: 19% (p=0.0000009)	LN4B156-1: ++++, 289% (p=0.005) LN4B156-2: +++, 91% (p=0.45) LN4B156-3: ++++, 227% (p=0.0003) LN4B156-14: ++++, 396% (p=0.00000001) LN4B156-6: +, 4% (p=0.00001) LN4B156-13:+, 23% (p=0.00002) LN4B156-16: ++, 58% (p=0.0002) LN4B156-20: ++, 58% (p=0.0006)	LN4B156-2: 5/5 (p=0.08) LN4B156-6: 2/10 (p=0.05)	 LN4B156-6: 2/3 (p=0.03)

* In subcutaneous injections, PC-3M-Pro4GP1 formed tumors in 9/10 mice and PC-3M-LN4GP2 formed tumors in 2/5 mice.

** In orthotopic injections into the prostate, PC-3M-Pro4GP1 formed tumors in 3/3 mice and PC-3M-LN4GP2 formed tumors in 9/13 mice.

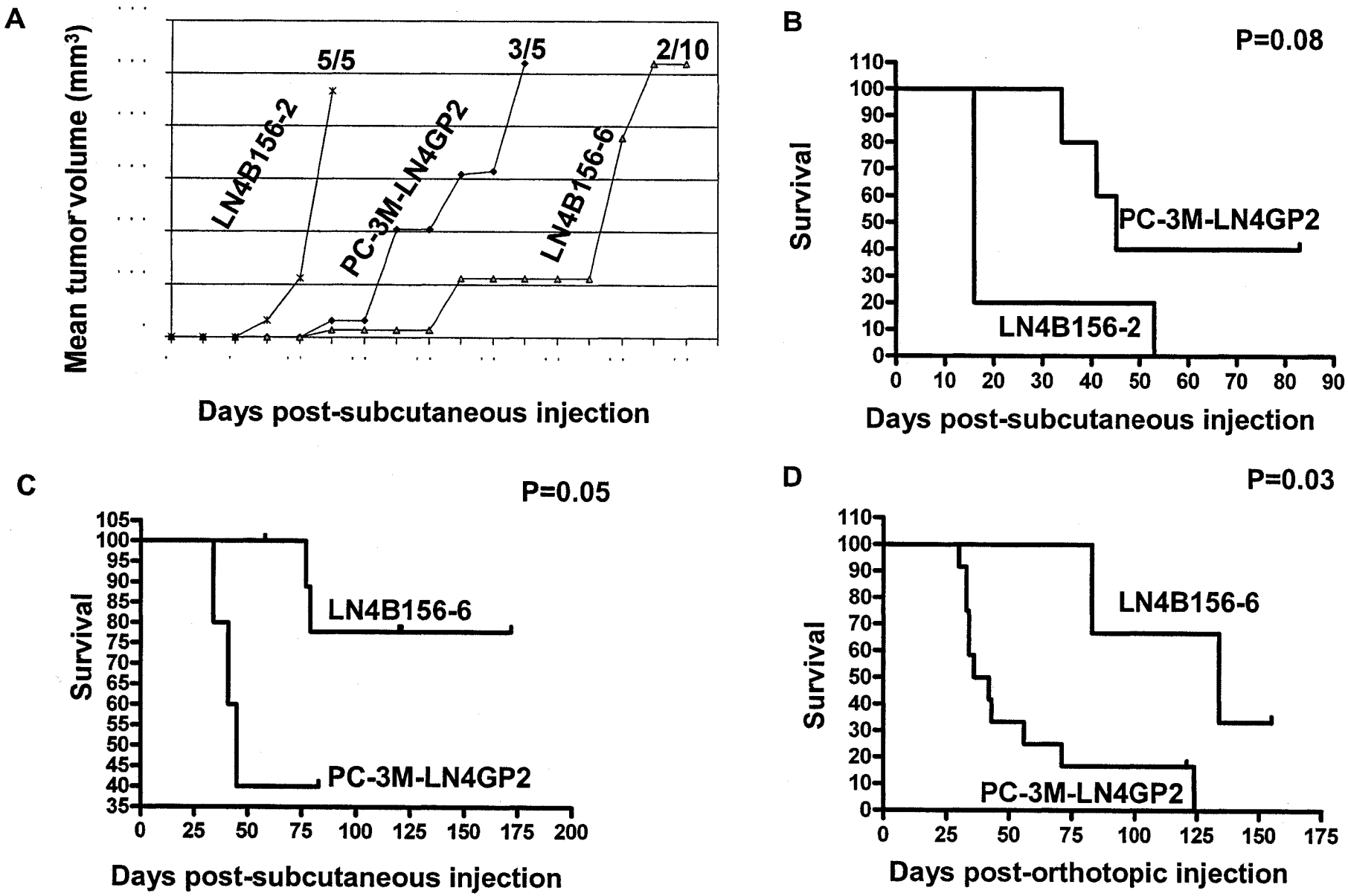
3.4.2 Introduction of human chromosome 18 into PC-3M-LN4GP2 cells interferes with their *in vivo* tumorigenic properties

To test the *in vivo* tumorigenic properties of our hybrids, one million cells from the selected hybrids, and from parental PC-3M-Pro4GP1 and PC-3M-LN4GP2 cells were injected subcutaneously into nude mice. We tested LN4B77-44 and Pro4B75-12 hybrids, containing, respectively, an introduced chromosome 10 or a 10p fragment. A statistically significant difference in tumor formation was obtained with the Pro4B75-12 hybrid (P=0.002; Table 4). Because they displayed opposite phenotypes in invasion and in soft-agar assays, we also injected LN4B156-2 and LN4B156-6 hybrids. Indeed, LN4B156-2 hybrid cells were 75% as invasive as was the parental cell line and formed as many colonies (91%) in soft agar (Table 4). In contrast, LN4B156-6 cells were 42% as invasive as PC-3M-LN4GP2 cells, and formed 25 times fewer colonies (3.8%; Table 4). The few tumors (2 out of 10) obtained upon injection of LN4B156-6 cells grew much slower than those obtained by either PC-3M-LN4GP2 or LN4B156-2 cells (Fig. 6A). Indeed, mice injected with PC-3M-LN4GP2 or LN4B156-2 cells became moribund much earlier (on average, 31 and 50 days earlier, respectively) than did mice injected with LN4B156-6 cells (Fig. 6C, P=0.05).

Furthermore, there was no statistically significant difference in tumor growth between parental cells and the LN4B156-2 (Fig. 6B; P=0.08), or LN4B77-44 hybrid containing an extra human chromosome 10 (P=0.54, Table 4). This

difference in tumor formation was also observed when LN4B156-6 cells were injected directly into the dorsal lobe of the prostate of nude mice. Indeed, there was a significant delay (Fig. 6D; P=0.03) in orthotopic tumor growth between LN4B156-6 and PC-3M-LN4GP2 cells.

Figure 6. Subcutaneous and orthotopic injections of prostate cancer cells into nude mice. A. Graphic representation of subcutaneous tumor growth following injections of 1 x 10^6 PC-3M-LN4GP2, LN4B156-2, and LN4B156-6 cells. Tumor growth was measured three times a week. B and C. Kaplan-Meier curves comparing survival rate (until mice became moribund) of mice upon subcutaneous injection of PC-3M-LN4GP2 cells and LN4B156-2 cells (B) and LN4B156-6 cells (C). D. Kaplan-Meier curve comparing survival rate of mice upon orthotopic prostatic injections of 1 x 10^6 PC-3M-LN4GP2 and LN4B156-6 cells. Mice were sacrificed as they became moribund.


A
Mean tumor volume (mm³)
5/5
3/5
2/10
LN4B156-2
PC-3M-LN4GP2
LN4B156-6
Days post-subcutaneous injection
B
P=0.08
Survival
PC-3M-LN4GP2
LN4B156-2
Days post-subcutaneous injection
C
P=0.05
Survival
LN4B156-6
PC-3M-LN4GP2
Days post-subcutaneous injection
D
P=0.03
Survival
LN4B156-6
PC-3M-LN4GP2
Days post-orthotopic injection

Taken together, these experiments demonstrate that the chromosome 18 introduced into LN4B156-6 cells was responsible for the suppression of their tumorigenic phenotype. However, once orthotopic tumor growth was established, both hybrid and parental cells formed regional lymph node metastases. Thus, even if LN4B156-6 cells showed delayed tumor growth, they still escaped the primary tumor mass to form metastases in retroperitoneal lymph nodes.

After sacrificing the mice, we excised and cultured the tumors from both subcutaneous and orthotopic groups. All cultured tumor cells were still expressing the green fluorescent protein gene (data not shown) and still contained polymorphic markers from the introduced chromosome 18 (TLN156-hybrids; Table 5), confirming that tumors were derived from the injected hybrid cells.

3.4.3 A region of human chromosome 18 is associated with the reduced tumorigenicity of LN4B156-6 cells

FISH with a chromosome 18-specific painting probe did not reveal any major differences in the chromosome 18 introduced in LN4B156 hybrids (data not shown). We complemented this analysis by genotyping the hybrids using twenty-seven polymorphic markers (Figure 7 and Table 5). To identify which allele was present in the parental cell lines, we tested each marker with DNA from the B78MC156 mouse/human hybrid and from the PC-3M-LN4GP2

human prostate cancer cell line. Five markers (*D18S487*, *D18S39*, *D18S58*, *D18S50*, and *D18S1141*; Table 5) could not distinguish the alleles present in B78MC156 from those of PC-3M-LN4GP2 cells, whereas five markers (*D18S63*, *D18S843*, *D18S1157*, *D18S1109*, and *D18S382*; Table 5) were not transferred into PC-3M-LN4GP2 hybrids.

We then tested all twenty-seven polymorphic markers on LN4B156 hybrids to determine which regions of chromosome 18 had been transferred. As seen in Table 5 (see also *D18S1133* in Figure 7), most markers had been transferred into both LN4B156-2 and LN4B156-6 hybrids. However, an additional allele of *D18S51* was present in the LN4B156-6 hybrid (allele 1A; Figure 7 and Table 5). This additional allele was also present in LN4B156-13 (Figure 7), another hybrid that forms fewer colonies in soft agar (Table 4). Interestingly, *D18S51* allele 1A was lost in one of the two tumors that eventually developed from subcutaneous injections (TLN156-6-3; Figure 7) and in one of the two tumors occurring after orthotopic injections of LN4B156-6 cells (OLN156-6-5; Figure 7). Allele 1A was also seen faintly in B78MC156 DNA, which could indicate that a subpopulation of these cells contained either only allele 1A, or both 1 and 1A alleles. Indeed, FISH experiments with a human chromosome 18-specific painting probe showed that a small portion of chromosome 18 was present in 23% of the B78MC156 cell spreads examined. This result could explain why the allele 1A band was hardly detectable in B78MC156. Moreover, direct sequencing

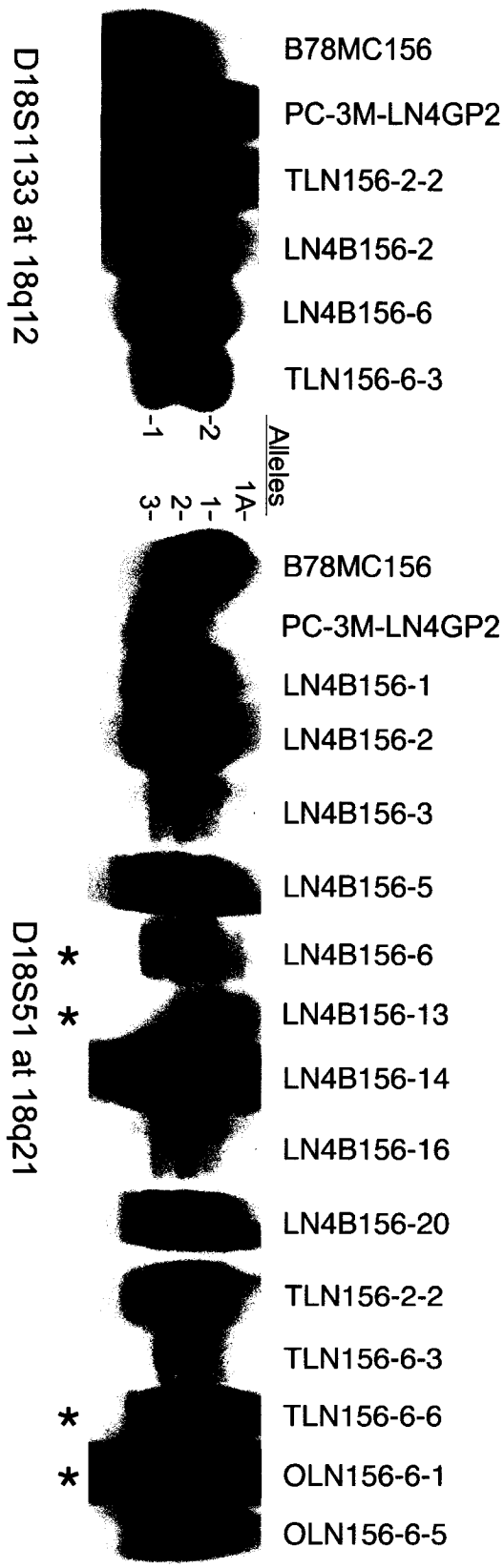
of the *D18S51* amplification products obtained from B78MC156 DNA revealed that this cell line contains two *D18S51* alleles.

TABLE 5. Genotyping of Chromosome 18 in Parental Cells and LN4B156- Hybrids and Tumors

Marker	Cytoband	Position (kbp)	B78	B78MC156	PC3MLN4GP2	LN4B156-2	TLN156-2-2	LN4B156-6	TLN156-6-3 & -6-6
D18S59	18p11.3	634	0	1	2	1,2	1,2	1,2	1,2
D18S63	18p11.3	3426	0	1	2,3	2,3	2,3	2,3	2,3
D18S976	18p11.2	5238	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S42	18p11.2	5891	0	1	2	1,2	1,2	1,2	1,2
D18S843	18p11.1	8603	0	1	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4
D18S542	18p11.1	11550	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S44	18q11.1	19875	0	1	2	1,2	1,2	1,2	1,2
D18S877	18q11.1	26613	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S847	18q11.2	27591	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S1133	18q12	31986	0	1	2	1,2	1,2	1,2	1,2
D18S67	18q12	35855	0	1	2	1,2	1,2	1,2	1,2
D18S1157	18q12	37751	0	1	2,3,4,5	2,3,4,5	2,3,4,5	2,3,4,5	2,3,4,5
D18S535	18q12	38036	0	1	2	1,2	1,2	1,2	1,2
D18S65	18q12	40425	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S1145	18q12	40435	0	1	2	1,2	1,2	1,2	1,2
D18S46	18q21	48376	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S487	18q21	51673	0	1	1,2	1,2	1,2	1,2	1,2
D18S1109	18q21	57430	0	1	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4
D18S39	18q21	54001	0	1	1, 2	1,2	1,2	1,2	1,2
D18S1155	18q21	56848	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S51	18q21	60734	0	1, 1A	2,3	1,2,3	1,2,3	1,1A,2,3	1,2,3
D18S1270	18q21	61178	0	1	2,3	1,2,3	1,2,3	1,2,3	1,2,3
D18S382	18q22	63354	0	1	2,3	2,3	2,3	2,3	2,3
D18S541	18q22	69959	0	1	2	1,2	1,2	1,2	1,2
D18S58	18q23	72125	0	1	1,2	1,2	1,2	1,2	1,2
D18S50	18q23	75252	0	1	1,2	1,2	1,2	1,2	1,2
D18S1141	18q23	76658	0	1	1	1	1	1	1

For each marker, the allele present in the mouse/human hybrid B78MC156 (donor cells) is identified by "1", polymorphic alleles derived from the prostate cancer cell line PC-3M-LN4GP2 are represented by "2" to "5" ; "0" indicates the absence of this marker.

Figure 7. Genotype of *D18S1133* and *D18S51* markers in prostate cancer cells and hybrids. The left panel shows a representative autoradiogram of PCR analysis of microsatellite polymorphic marker *D18S1133* for DNA extracted from cell cultures of mouse/human hybrid B78MC156 (allele 1), human prostate cancer cell line PC-3M-LN4GP2 (allele 2), microcell hybrids LN4B156-2 and LN4B156-6, and from tumor derived from nude mice injection of these two microcell hybrids (indicated by TLN156-2-2 and TLN156-6-3). The right panel shows a representative autoradiogram of PCR analysis of microsatellite polymorphic marker *D18S51* for DNA extracted from cell cultures of mouse/human hybrid B78MC156 (alleles 1 and 2), human prostate cancer cell line PC-3M-LN4GP2 (alleles 2 and 3), microcell hybrids LN4B156-1, -2, -3, -5, -6, -13, -14, -16, and -20, and from tumor-derived from nude-mouse injection (both subcutaneous (TLN) and orthotopic (OLN)) of LN4B156-2 and LN4B156-6 microcell hybrids (TLN156-2-2, TLN156-6-3, TLN156-6-6, OLN156-6-1, and OLN156-6-5). For each marker, PCR amplification was performed three times.


D18S1133 at 18q12
B78MC156
PC-3M-LN4GP2
TLN156-2-2
LN4B156-2
LN4B156-6
TLN156-6-3
-1
-2
Alleles
1A-
1-
2-
3-
B78MC156
PC-3M-LN4GP2
LN4B156-1
LN4B156-2
LN4B156-3
LN4B156-5
LN4B156-6
LN4B156-13
LN4B156-14
LN4B156-16
LN4B156-20
TLN156-2-2
TLN156-6-3
TLN156-6-6
OLN156-6-1
OLN156-6-5
D18S51 at 18q21
*
*
*
*

3.5 Discussion

Deletions of chromosome 18 regions have been associated with many cancers, including colorectal [183], pancreatic [184], ovarian [235], head and neck squamous cell carcinoma [186], lung [187], and prostate carcinoma [192, 231, 236, 229, 188]. Functional evidence for the presence of tumor suppressor gene(s) on human chromosome 18 was demonstrated when introduction of this chromosome into pancreatic cancer cells resulted in suppression of their tumorigenic phenotype [184]. Similar results were obtained in PCa cell lines DU 145 and PC-3 [236, 193]. As compared to DU 145 cells, PC-3 and its derivatives likely represent a more appropriate model for study of prostate cancer. Indeed, PC-3 cells were derived from bone metastasis, a common feature seen in patients at an advanced stage of the disease. In contrast, the DU 145 cell line was derived from a brain metastasis, which rarely occurs in patients. Although PC-3 cells form metastases upon injection into the left cardiac ventricle [193], the use of a more aggressive PC-3-derived cell line (PC-3M-LN4) allows the study of metastatic cancer progression as it appears in human patients. Indeed, PC-3M-LN4 cells form lymph-node metastases upon orthotopic injection into the prostate of nude mice [204]. Thus, injecting cancer and hybrid cells orthotopically more appropriately mimics the conditions present in PCa patients and further confirms that chromosome 18 is implicated in PCa progression.

Introduction of human chromosome 18 into the metastatic PC-3M-LN4GP2 cell line considerably affects its tumorigenic properties. Both LN4B156-6 and

LN4B156-14 hybrids have a reduced capacity to invade through Matrigel, whereas LN4B156-6, LN4B156-13, LN4B156-16, and LN4B156-20 hybrids have a reduced ability to grow in an anchorage-independent manner, a feature also observed by Padalecki et al. (2003) in their PC-3-derived hybrids[193]. Furthermore, the LN4B156-6 hybrid also shows significantly delayed tumor growth upon subcutaneous (P= 0.05) and orthotopic (P=0.03) injections into nude mice. However, in the prostate environment, LN4B156-6 cells did form proportionally more tumors (two mice out of four; 50%) than when injected subcutaneously (two mice out of ten; 20%). Such results could indicate that the natural prostatic environment is a more appropriate site than is under the derma (subcutaneous) for the establishment and growth of tumorigenic cells. Moreover, once established in the prostate, all tumors were also able to metastasize to lymph nodes. Upon orthotopic injection, all mice became moribund due to uremia and had to be sacrificed. As seen in Figure 6, almost all mice orthotopically injected with parental PC-3M-LN4GP2 cells became moribund in less than 75 days, whereas it took up to 134 days after LN4B156-6 orthotopic injections.

Our results thus confirm and complement those obtained by Padalecki et al. (2003) and further suggest the presence of one or more chromosome 18 tumor/metastasis suppressor genes implicated in PCa progression[193]. Such suppressor genes are likely to be inactivated during the time it took the tumors to form in the animal. According to this hypothesis, it is worth noting that LN4B156-6-tumor-derived cells (TLN156-6-3) displayed a significant

increase in their capability to form colonies in soft agar (409%; P= $1x10^{-7}$) when compared to PC-3M-LN4GP2 (data not shown). As reported for nonpapillary renal cell carcinoma, such inactivation could result from chromosomal rearrangement [237]. In renal cell carcinoma-derived microcell hybrids, such rearrangements often not only occur in the introduced chromosome, but also affect endogenous homologous chromosomes and other polysomic chromosomes [238].

The characterization of LN4B156-hybrids (Table 4 and Figure 6) revealed that some hybrids had reduced *in vitro* and *in vivo* tumorigenic properties (see, for example, LN4B156-6, LN4B156-13), whereas others (like LN4B156-1 and LN4B156-2) were at least as tumorigenic as were the parental PC-3M-LN4GP2 cells. Such differences could be due to the fact that hybrids like LN4B156-2 contain an introduced chromosome 18 in which the suppressor gene is already inactivated or deleted. Although FISH analysis did not show any chromosomal differences between LN4B156-2 and LN4B156-6 hybrid cells (data not shown), genotype analysis of these hybrids pointed to the 18q21 region. Indeed, when compared to tumorigenic hybrids, many hybrids having a reduced tumorigenic phenotype (either *in vitro* or *in vivo*) carried an extra *D18S51* allele (allele 1A). The fact that this allele is often lost in cancer cells derived from LN4B156-6 subcutaneous or orthotopic injections further validates that this region is important in PCa.

The chromosome band 18q21 region encodes few identified tumor suppressor genes. Among them, the *DCC* gene has been associated with colorectal cancer progression, whereas the *SMAD2* and *SMAD4* genes are inactivated in pancreatic cancer. However, because these genes are rarely mutated in PCa [239, 195, 192, 229], the involvement of these three genes in this disease is probably limited. Accordingly, a preliminary analysis using the 19k7 microarray containing 19,200 human genes (www.microarrays.ca/products/types.html) did not reveal any variation in the expression level of the *SMAD4* gene when PC-3M-LN4GP2 cells were compared to LN4B156-6 cells (data not shown). This further suggests that *SMAD4* is not implicated in the decreased tumorigenic phenotype observed in the LN4B156-6 hybrid and that a not yet identified suppressor gene could be present in the 18q21 region. Moreover, these three genes are located more than 10 megabases (Mb) away (*DCC* is located at 49 Mb, *SMAD2* at 43.5 Mb, and *SMAD4* at 47 Mb) from the *D18S51* locus (59 Mb) (www.ncbi.nlm.nih.gov/mapview/maps.cgi?taxid= 9606&chr=18). Thus, it is very unlikely that these genes are implicated in the less tumorigenic phenotype of our LN4B156-6 hybrid.

Whatever its inactivation mechanism, the chromosome 18-encoded suppressor gene is likely to be implicated (directly or indirectly) in cell-cell adhesion or cell-matrix interaction, because some of our LN4B156 hybrids had a decreased ability to grow in an anchorage-independent manner and to invade through Matrigel. Inactivation of the suppressor gene is unlikely to

happen over the time required for performing the invasion assay (22 hours) or even the soft-agar assay (21 days). Such suppressive *in vitro* properties are similar to what has been reported for the *CD82* metastatic suppressor gene [93] in both prostate and breast cancer cells. However, the presence of the suppressor gene in the LN4B156-6 hybrid was unable to inhibit lymph node metastasis formation once tumors had developed after orthotopic injections in the prostate. Presently, we cannot rule out the probability that this suppressor gene could also affect metastatic development, because the suppressor gene is likely to be inactivated by the time the cells form tumors in the mice. Such inactivation could also interfere with its putative metastasis-suppressing properties.

3.6 Acknowledgments

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CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE GENES

4.0 PREFACE

This chapter is written in a manuscript format with its own Introduction, Material and methods, Results and Discussion sections. It will be submitted to Cancer Research in early 2006. Contribution of co-authors can be found at page XVIII and permission from co-authors in Appendix D.

ERK4, a new tumor suppressor gene candidate implicated in prostate cancer progression

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For contribution of co-authors, see p.XVIII.

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Running title:

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Prostate cancer, chromosome 18, tumor suppressor, mitogen-activated protein kinase

Footnotes:

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[4]The abbreviations used are: PCa, prostate cancer; FBS, fetal bovine serum; FISH, Fluorescence *in situ* hybridization; TSG, tumor suppressor gene; chr, chromosome; MAPK, mitogen-activated protein kinase; ERK, extracellular-regulated kinases.

4.1 Abstract

Prostate cancer is the second leading cause of cancer death in the USA. However, the mechanisms underlying this important disease are not well understood. Thus, there is a need for identifying new tumor suppressor genes implicated in prostate cancer progression. We have assessed the expression of the *MASPIN*, *BCL2* and *ERK4* genes in tumorigenic and less tumorigenic hybrids containing an extra copy of normal human chromosome 18. Here we show that mRNA levels of the *MASPIN* and *ERK4* genes are down regulated in highly tumorigenic cells compared to less tumorigenic hybrids, and that the level of *BCL2* is up regulated in these same cells. However, there is no difference in the MASPIN or BCL2 protein level between tumorigenic and less tumorigenic cells, suggesting that MASPIN or a BCL2 repressor are not implicated in the less tumorigenic phenotype of our chromosome 18 hybrids. Introduction of the wild-type *ERK4* gene into human prostate cancer cells completely abolished their ability to invade through Matrigel and their anchorage-independent growth potential. However, subcutaneous injections of the *ERK4*-over-expressing cells into nude mice did not result in any reduced growth rate or delay. That could argue that *in vivo*, ERK4 needs a partner to perform its tumor suppressing function. Alternatively, it could also be necessary to inject these cells orthotopically to fully assess the effect of ERK4 on these cells. Nevertheless, *ERK4* is a good candidate for a new tumor suppressor gene for prostate cancer since it is down regulated in human prostate cancer cells and suppresses colony formation and invasiveness *in vitro*.

4.2 Introduction

Prostate cancer (PCa) is still the second leading cause of cancer death among American males. Indeed, 232,090 new cases and 30,350 deaths are expected in 2005 [5]. Despite these alarming statistics, there is still no efficient treatment for advanced stages of the disease. Moreover, the molecular mechanisms of this disease are not well understood. Thus, there is a need for identifying molecular markers and genes such as tumor suppressor genes implicated in PCa progression.

There are specific chromosomes that are lost during PCa progression. Indeed, cytogenetic analyses showed the alteration of specific chromosomes in PCa [56]. Indeed, numerous studies showed deletions of chromosome arms 7q [215, 57], 8p [60, 58, 59], 10q [63, 61, 62], 16q [216, 66, 64], 17q [68, 67], and 18q [66, 240, 69] in advanced PCa. The loss of these chromosomes indicates that they encode one or more tumor suppressor gene(s) (TSG) implicated in PCa progression.

Almost all human chromosomes have been tested for their tumor suppressive properties by transferring them into either rat of human PCa cells. Indeed, introduction of chromosomes 2 [73], 8p [227], 10p [152], 12 [164], 18 [241], and 19 [84, 228] showed a tumor suppressing activity when transferred into human PCa cells. The loss of chromosome arm 18q is found in 30-40% of PCa tumors [66]. The transfer of this chromosome into the PCa cell lines DU 145 and PC-3 completely abolished their tumorigenic potential [189, 193, 242].

However, even if studies in PCa patients identified two minimal regions of chromosomal loss, no TSG was identified in these regions [231].

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that play a role in transmitting extracellular signals inside the cell [243]. Indeed, these proteins are key components of intracellular signalling pathways implicated in a variety of cellular processes such as proliferation, differentiation, and survival [243]. The extracellular-regulated kinases (ERK) family of threonine/tyrosine kinases is composed of eight members named *ERK1* to *ERK8*. The classical members of the family (*ERK1* and *ERK2*) are ubiquitously expressed kinases that, once phosphorylated, translocate to the nucleus, and activate transcription factors involved in important cellular processes such as survival, proliferation, and differentiation [243]. The ERK1 and ERK2 proteins are up regulated in PCa specimens compared to normal prostate tissues [243]. Two non-classical members of the *ERK* family (*ERK3* and *ERK4*) share 50% homology with *ERK1* and *ERK2* in their kinase domain. They also contain a long C-terminal tail that is not present in the ERK1 and ERK2 proteins. Moreover, their phosphoacceptor site (SEG) is different from the Thr-X-Tyr site commonly found in the activation loop of ERK1 and ERK2 [244]. However, the exact cellular function of ERK3 and ERK4 is still unknown.

As reported previously [245], we have transferred a copy of normal human chromosome 18 into the PC-3-derived cell line PC-3M-LN4GP2. Subsequent

in vitro and *in vivo* characterization of these hybrids demonstrated that the chromosome cytoband 18q21 suppresses the tumorigenicity of these human PCa cells. Genotyping studies showed that the *D18S51* locus was linked to the suppressed phenotype of the chromosome 18 hybrids. In the present study, we characterized three candidate genes located close to the *D18S51* locus (*MASPIN*, *BCL2*, and *ERK4*) to determine if they are candidates for new tumor suppressor genes implicated in PCa progression.

4.3 Material and methods

4.3.1 PCa cell line.

The generation of the geneticin-resistant PC-3M-LN4GP2 cell line was described previously [246]. Briefly, the PC-3M-LN4 cell line was obtained from Dr. I.J. Fidler (M.D. Anderson Cancer Centre, Houston, TX) and was stably transfected with the green fluorescent protein (GFP)-encoding plasmid pQBI25 (Q-BIOgene, Carlsbad, CA). The cells were then kept in RPMI 1640 media supplemented with 10% FBS and 400ug/ml of geneticin (Invitrogen, Burlington, ON, Canada).

4.3.2 ERK4 vectors construction and stable transfection.

The ER*K4*-wt sequence was cloned into the pcDNA4 His/Max B vector (Stratagene, LaJolla, CA), introducing a His tag at the N-terminal region of the gene. The V38M polymorphism in the *ERK4* gene was introduced by PCR amplification to generate the ERK4V38M expression vector. Integrity of the sequence and of the open reading frame of these constructs was

confirmed by sequencing. The *ERK4*-wt expression plasmid was stably transfected into PC-3M-LN4GP2 cells. Briefly, the cells were plated at 50% confluency, and transfected with 5 ug of plasmid DNA using the Lipofectamine^{TM} 2000 method (Invitrogen). Clones were picked 10 days after transfection, and were named LN4GPerk4wt-1 to 10. Over-expression of the ERK4 transcript was confirmed by real-time PCR and the presence of the protein (wild-type) was confirmed by Western immunoblot using the polyclonal antibody described in Chapter 2 (section 2.5.2).

4.3.3 Real-time PCR

The expression of the *ERK4* mRNA was assessed by real-time quantitative PCR. The amplification was adjusted to *GAPDH* expression level and gene-specific Quantitect primers (Qiagen, Mississauga, ON, Canada) were used for PCR amplification. Briefly, a PCR mix was prepared for each sample tested: 1X of 2X DyNAmo SYBR Green qPCR kit (New England Biolabs, Pickering, ON, Canada), 1X Quantitect primer mix (Qiagen), 50 ng of RT reaction in a total volume of 25 ul. The PCR efficiency of the primer sets was determined by sequential dilutions of the purified PCR amplicons (1:4 to 1:256). PCR reactions were electrophoresed through an agarose gel, and a dissociation curve was added to the PCR program to insure that the right amplicon was amplified; each reaction was repeated 8 times. The following PCR program was ran on an thermal cycler MX4000 from Stratagene: stage 1: 50°C for 2 mins, stage 2: 95°C for 15 mins, stage 3: 40 cycles of 95°C for

15 secs, 60°C for 15 secs and 72°C for 32 secs and stage 4: 95°C for 15 secs, 50°C for 15 secs and 95°C for 15 secs. The mean Ct (cycle threshold) and standard deviation were calculated using Microsoft Excel 2000 (Microsoft, Mississauga, ON, Canada). The delta Ct (Ct *ERK4* – Ct *GAPDH*) and its standard deviation were calculated using the same program. The delta-delta Ct method was use to calculate the fold changes as recommended by the manufacturer, and a Student t-test was used to determine significance of the findings.

4.3.4 Western immunoblot

Cells were seeded to 90% confluency, washed twice with ice cold PBS, scraped off in Brij97 lysis buffer (1% Brij 97, 25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM pyrophosphate, 100 units/ml aprotinin, 10 µg/ml leupeptin, 25 µM *p*-nitrophenyl *p'*-guanidino-benzoate, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide; all reagents are from Sigma, Oakville, ON, Canada), and incubated on ice for 10 mins. Extracts were centrifugated at 13,000g for 10 mins at 4°C. Supernatants of matched samples were combined, and proteins were quantified with the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL), according to the manufacturer's protocol. 40 ug of proteins were boiled 5 mins in 2X Laemmli buffer (62.5mM Tris-HCl pH 6.8, 40% v/v glycerol, 5% SDS, 0.005% bromophenol blue containing 10% of B-mercaptoethanol (Sigma)), and electrophoresed through a 10%

acrylamide SDS-PAGE gel. The proteins were then transferred (1 hour) onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and blocked for 1h in TBST (25 mM Tris-HCl pH 8.0, 125 mM NaCl, 0.1% tween 20) + 5% non-fat dry milk (Bio-Rad Laboratories). The membranes were incubated overnight with the appropriate primary antibody in TBST + 5% non-fat dry milk. For the MASPIN and the BCL2 proteins, a monoclonal anti-human antibody was used (Chemicon, Temecula, CA) at a 1:5000 dilution. For the ERK4 protein, a polyclonal antibody obtained from Dr. Sylvain Meloche's laboratory (IRIC, Universite de Montreal, Montreal) was used at a dilution of 1:2000 (see section 2.5.2 for details). After 16 hours, the membranes were washed 3 times for 15 mins in TBST, and incubated 1h at room temperature with the secondary antibody conjugated to horseradish peroxydase (donkey anti-rabbit for *ERK4* (Bio/Can Scientific, Etobicoke, ON, Canada) and goat anti-mouse for MASPIN and BCL2 (Fisher Scientific, Nepean, ON, Canada)) in TBST + 5% non-fat dry milk. The bound antibodies were detected using the ECL detection kit (GE Healthcare, Baie d'Urfé, QC, Canada), and each experiment was performed three times.

4.3.5 Sequencing.

The entire coding sequence of the *ERK4* gene was amplified using the Phusion high-fidelity DNA polymerase (New England Biolabs), according to manufacturer's instructions. Briefly, a PCR reaction containing 1X Phusion HF buffer, 200 uM of each dNTP, 0.5 uM of each primer, 0.0 2U/ul of Phusion DNA polymerase, 100 ng of genomic DNA or 50 ng of cDNA was

prepared in a final volume of 20 ul. The following program was ran: 98°C for 30 secs, followed by 40 cycles of 98°C for 10 secs, annealing temperature for 30 secs and 72°C for 30 secs with a final extension of 72°C for 10 mins. The primers used for amplification of the *ERK4* nucleic acids were:

mapk464 (5'-TGGGCAGCTCCAGATCACTG-3') and

mapk802c (5'-ACGATGTACGCCACGCTGAA-3')

(for mRNA or genomic DNA);

mapk672 (5'-CACGCGCTCCGAGAGATCAA-3') and

mapk1520c (5'-GTTAGCGGCCATCAGCACGA-3') (for mRNA);

mapk1487 (5'-GAGATCGACGACATCGTGCT-3') and

mapk1841c (5'-AGTAGTGGTGCGGCTTGTTG-3') (for mRNA);

annealing temperatures varied from 60 to 69°C. The sequencing on the coding sequence of the *ERK4* gene was done using primers specific for *ERK4* mRNA. Primers mapk464 and mapk802c were used to screen the V38M polymorphism in our PCa cases and controls as they can amplify genomic DNA. The PCR reactions were electrophoresed through a 1.5% agarose gel, the PCR products were extracted from the gel using the gel extraction kit (Qiagen), and sent to the McGill University and Genome Quebec Innovation Centre (Montreal) for sequencing. Primers used for sequencing were the same as for PCR amplification. Briefly, the PCR products were purified and resuspended in 2 ul to be amplified with the Big Dye Terminator (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The following conditions were used for

amplification: 96°C for 1 min, 25 cycles of 96°C for 30 secs, 50°C for 10 secs, 60°C for 4 mins. The reactions were precipitated, centrifugated, air-dried, and the pellets were resuspended in formamide. The fluorescence was then read on ABI 3730xl DNA analyser (Applied Biosystems).

4.3.6 Soft agar assays

The soft-agar assays were performed as described previously [212]. Briefly, 1 ml of 0.5% agar noble (Fisher Scientific) diluted in appropriate media was put into 6-well plates to form the basal layer. Then 1,000 cells were mixed with 0.5% agar and put on top of the basal layer. Plates were incubated at 37°C for 10 days. Growing cell foci were stained with 200 μl of a 0.03% crystal violet (Sigma) solution in 100% methanol (Fisher Scientific). The number of colonies was counted, and converted to a percentage of colonies obtained from the parental cell line (number of colonies formed by PC-3M-LN4GP2 cell line = 100%). Each soft-agar assay was performed in six wells per cell line and a Student's t-test was used to calculate p values.

4.3.7 Invasion Assays.

We used a Boyden chamber assay [211] to assess invasive potential. Briefly, 25,000 cells were added to the chamber coated with Matrigel (Fisher Scientific), and RPMI 1640 medium supplemented with 5% FBS was added to the bottom chamber to serve as chemoattractant. The chambers were incubated at 37°C for 22 hours, and filters were stained with a Diff-Quick

staining kit (Dade Behring, Newark, DE). Invasive cells from five random fields were counted under a contrast phase microscope at 200X magnification. All invasion experiments were performed in quadruplicate, and the results were expressed as percentage of the invasion obtained with the parental cell line (parental = 100%). The significance of the findings was calculated using a two-tailed Student's t-test.

4.3.8 Nude mice injections.

All *in vivo* tumorigenicity assays were done according to Canadian Council for Animal Care guidelines, after approval by the McGill University Animal Care Committee. Male nude mice (balb/c, *nu/nu;* six to eight weeks of age; Charles River Laboratory, St-Constant, QC, Canada) were injected with PCa cells. One million cells, in 200 μl of Hanks balanced saline solution (HBSS; Invitrogen) were injected subcutaneously in the upper back of nude mice. Mice were sacrificed when tumors reached 1000 mm^3.

4.4 Results

4.4.1 Selecting candidate genes

After confirming that the *D18S51* locus was linked to the less tumorigenic phenotype of our hybrids, we chose to analyze three candidate genes located around that locus. Based on preliminary microarray data, we decided to characterize the only two genes on chromosome 18 (*ERK4* and *BCL2*) that were differentially expressed between a tumorigenic (LN4B156-2) and a

less tumorigenic hybrid (LN4B156-6). *BCL2* is an oncogene in PCa but the loss of a *BCL2* repressor could be responsible for its up regulation in the highly tumotigenic hybrids. Thus, this repressor could be a candidate TSG for PCa. Furthermore, and mainly due to its function of a metastasis suppressor gene implicated in PCa progression [135], we also analyzed the *MASPIN* gene.

4.4.2 Real-time PCR.

We used real-time quantitative PCR to determine if the *ERK4*, *BCL2* and *MASPIN* genes were differentially expressed in our tumorigenic and less tumorigenic hybrids. When compared to the less tumorigenic hybrid LN4B156-6, *MASPIN* is significantly down regulated in the parental cell line PC-3M-LN4GP2 (-1.57 fold, p=0.03), in the tumorigenic hybrid LN4B156-2 (-1.86 fold, p=0.02) and in tumors derived from subcutaneous injections of these cells (TLN156-6-3 -3.10 fold, p=0.05). Interestingly, *MASPIN* was reported to be down regulated in PCa [247, 248] as well as in lung cancer [249] and in lymphoma [250]. As expected for an oncogene, *BCL2* is over-expressed in the parental cell line PC-3M-LN4GP2 (12.68 fold, p=0.138), in the LN4B156-2 hybrid (3.88 fold, p=0.01), as well as in the tumors (2.22 fold, p=0.01, data not shown), which is consistent with three previous reports mentioning that *BCL2* is up-regulated in PCa [251, 247, 252]. *ERK4* is down regulated in the parental cells (-6.295 folds, p=0.226), in the LN4B156-2 hybrid (-20.645 folds, p=0.03), and in the tumors (-1.775, p=0.005, data not shown) when compared to the less tumorigenic LN4B156-6 hybrid. This

gene is down-regulated in two other studies comparing PCa cells and normal prostate cells [247, 253], as well as in adrenal cancer [254], lung cancer [249] and small cell lung cancer[249].

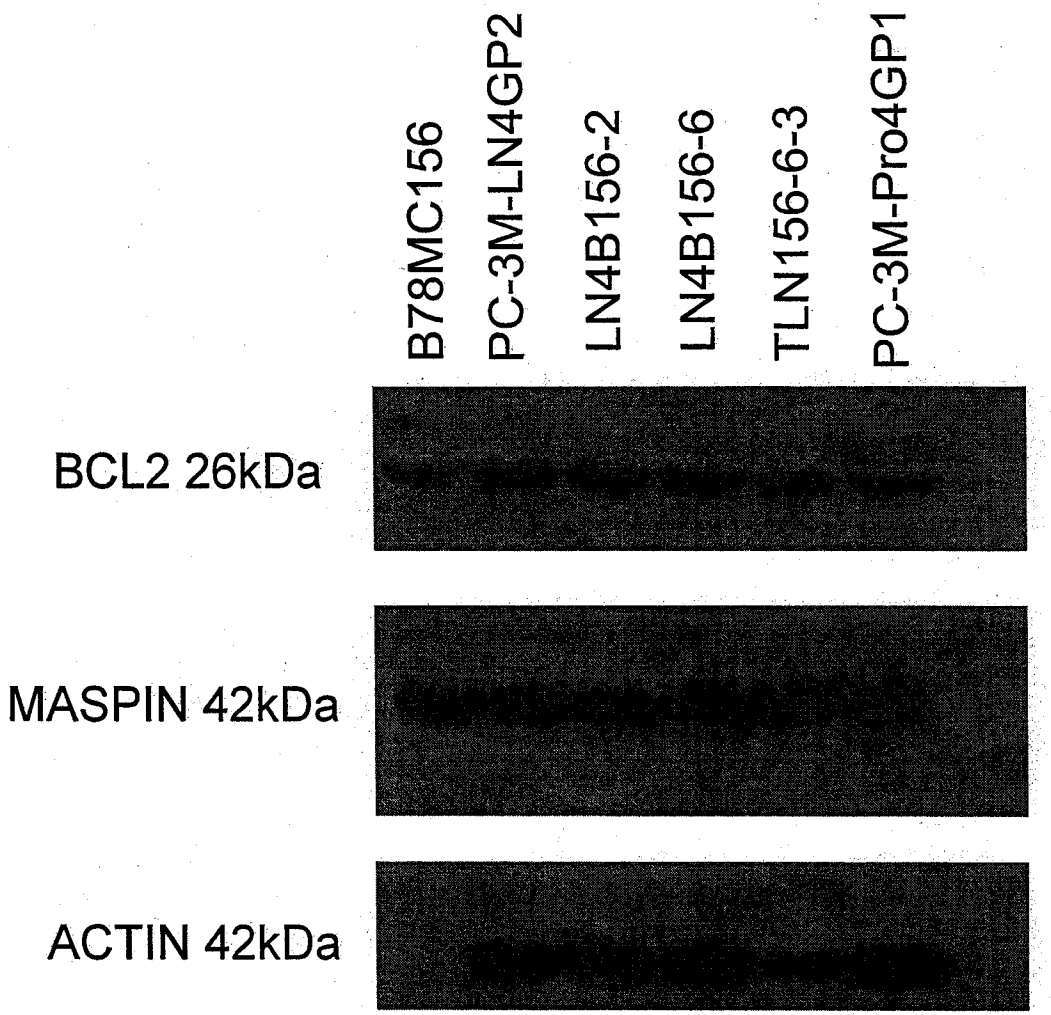
4.4.3 Western immunoblot

To verify that these differences in mRNA expression also affect the protein level of these genes, we performed Western immunoblot. Antibodies for each protein were blotted on a membrane containing 40 ug of total proteins from the parental B78MC156 and PC-3M-LN4GP2 cells, from LN4B156-2, and LN4B156-6 hybrids, from the derived TLN156-6-3 tumor cells and from PC-3M-Pro4GP1, another prostate cancer parental cell line of some of our hybrids. This cell line was included since the transfer of chromosome 18 in these cells did not have any effect on their tumorigenicity (see Chapter 3). This will allow us to see if the protein expression varies among tumorigenic and less tumorigenic cells.

As shown in figure 8, there was no difference in the protein expression levels of BCL2 or MASPIN between our less tumorigenic and our highly tumorigenic hybrids. Moreover, quantification of these blots by phosphorimager did not show any significant difference between our hybrids (n=3, data not shown). Even if BCL2 or MASPIN protein levels do not vary between tumorigenic and less tumorigenic cells, this doesn't rule out the possibility that a *BCL2* repressor or MASPIN could be responsible for the

less tumorigenic phenotype of our hybrids since these genes could still be mutated in our hybrids, rendering the produced protein non-functional.

Figure 8. MASPIN and BCL2 Western immunoblot. Top and middle panel. 40ug of proteins were loaded on a 10% SDS-PAGE. BCL2 or MASPIN were detected using monoclonal anti-human BCL2 or anti-MASPIN antibody. Lanes 1 and 2. Parental mouse/human hybrid B78MC156 and human PC-3M-LN4GP2 cells. Lane 3. Highly tumorigenic hybrid LN4B156-2. Lane 4. Less tumorigenic hybrid LN4B156-6. Lane 5. Tumor-derived cell line TLN156-6-3. Lane 6. Human prostate cancer cell line PC-3M-Pro4GP1 in which the transfer of chromosome 18 had no effect on their tumorigenic properties. Bottom panel. ACTIN, used as a loading control, was detected on the same membrane.


B78MC156
PC-3M-LN4GP2
LN4B156-2
LN4B156-6
TLN156-6-3
PC-3M-Pro4GP1
BCL2 26kDa
MASPIN 42kDa
ACTIN 42kDa

Since endogenous ERK4 protein was not detectable in our cell lines (PC-3M-LN4GP2, PC-3M-Pro4GP1, LN4B156-2, LN4B156-6, TLN156-2-2 and TLN156-6-3) by Western immunoblot, we treated the cells with the proteasome inhibitor MG132. It has been shown that treating cells with this inhibitor greatly increases the protein level of ERK3, a very unstable member of the *ERK* family that is highly degraded by the proteasome complex [255]. Considering the homology between ERK3 and ERK4, it was worthy to determine if such treatment will also affect the stability of ERK4. However, even in these stabilizing conditions, the ERK4 protein was not detectable. Thus, these experiments assessing mRNA and protein levels of our three candidate genes suggest that they could all still be responsible for the less tumorigenic phenotype seen in our hybrids. Since *ERK4* has no known function, we have decided to pursue its characterization.

4.4.4 ERK4 over-expression

The wild-type *ERK4* gene was transfected (along with an empty vector (mock)) into PC-3M-LN4GP2 cells to assess its effect of the tumorigenic properties of these cells. Over-expression of the *ERK4* mRNA was confirmed by real-time PCR. Indeed, the LN4GPerk4wt-1, LN4GPerk4wt-3 and LN4GPerk4wt-4 transfected cells are expressing 290 to 650 fold more ERK4 mRNA than the non-transfected PC-3M-LN4GP2, or the mock-transfected LN4GPmock-1 (data not shown). The over-expression of the ERK4 protein in stable transfectants was confirmed by Western immunoblot (Figure 9). Since endogenous ERK4 protein is not detectable in our non-transfected cells, we

assessed the detection threshold level of our polyclonal ERK4 antibody. To do so, we serially diluted a protein extract from an ERK4 over expressing cell line 1 to 1/10,000 and performed a Western blot (Figure 10). The ERK4 protein was detectable in the 1:100 lane (Figure 10, top panel), while ACTIN was detectable in the 1:1000 lane (Figure 10, bottom panel). This indicates that transfected cells express at least 100 fold more ERK4 protein than the parental cells. Cells over-expressing wild-type ERK4 protein have significantly reduced ability to form colonies in soft agar as compared to the non-transfected parental cell line (Table 6, see LN4GPerk4wt-1, wt-3, wt-4 and wt-10 vs PC-3M-LN4GP2, $p<0.05$). The cells transfected with the empty vector (mock) have a slight decrease in their anchorage-independent ability but still form 10 times more colonies than the ERK4-over-expressing cells ($p= 0.0003$ to $p=0.000005$). Moreover, over-expression of ERK4 also affects the invasive properties of these cells (Table 7, compare LN4GPerk4wt-1, 3 and 10 versus LN4GPmock-6 and PC-3M-LN4GP2). Indeed, the LN4GPerk4wt-1, 3, and 10 cells are respectively 28% ($p=0.002$), 23% ($p=0.0015$), and 38% ($p=0.008$) as invasive as the parental PC-3M-LN4GP2 cells. There is no significant difference between the invasive properties of the non-tranfected PC-3M-LN4GP2 cells and the mock-transfected LN4GPmock-6 cells. Taking together, these results show that over-expression of ERK4 protein greatly reduces the *in vitro* invasive and anchorage-independent properties of human prostate cancer cells.

Figure 9. ERK4 is over-expressed in transfected cells. The top panel shows the expression of ERK4 in transfected (LN4GPerk4wt-1, LN4GPerk4wt-3, LN4GPerk4wt-4 and LN4GPerk4wt-10) and mock-transfected cells (LN4GPmock-6). The bottom panel illustrates the expression of ACTIN as a loading control. In the right panel, the positive control is an extract of fibroblasts transiently transfected with ERK4 and the negative control is the non-transfected PC-3M-LN4GP2 cell line.

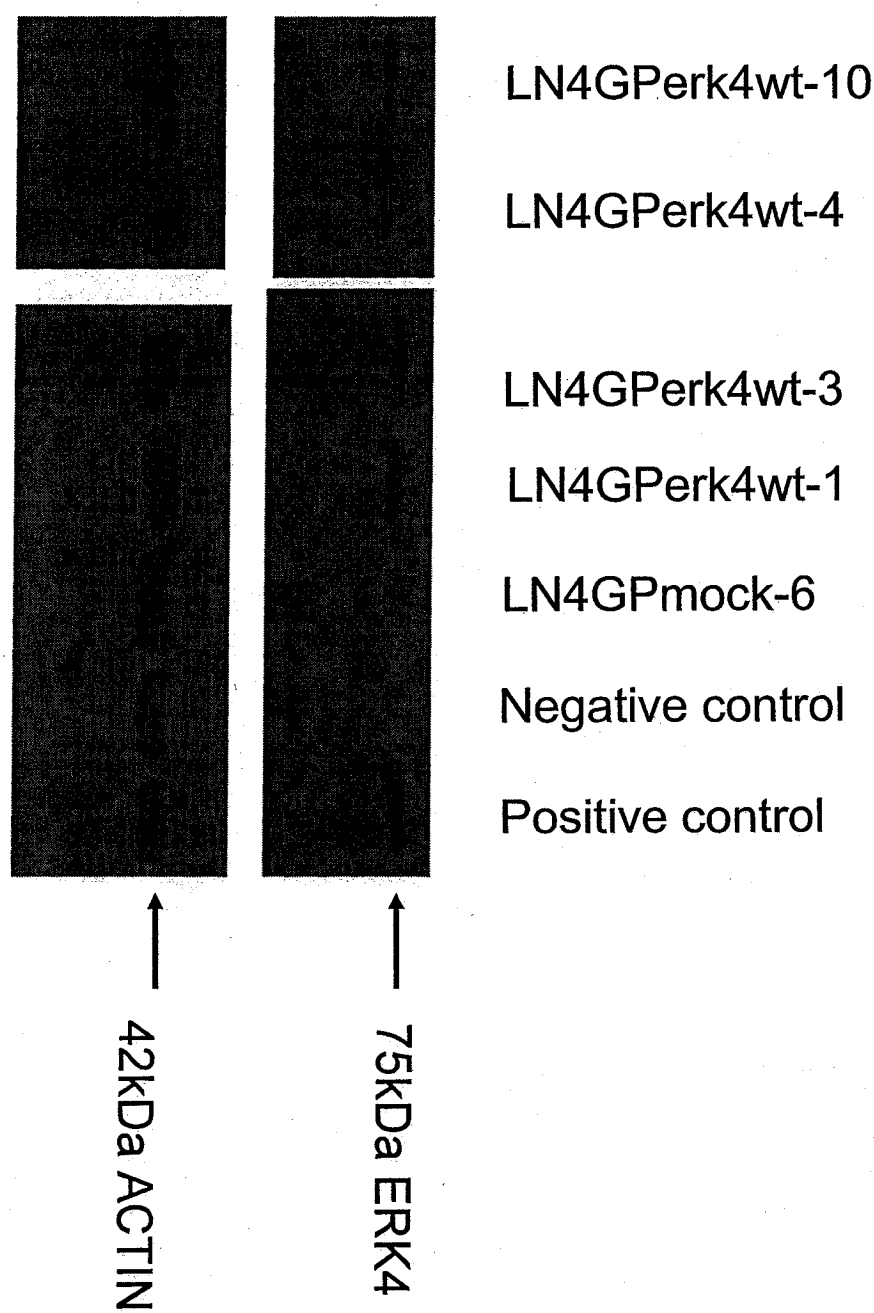

LN4GPerk4wt-10
LN4GPerk4wt-4
LN4GPerk4wt-3
LN4GPerk4wt-1
LN4GPmock-6
Negative control
Positive control
42kDa ACTIN
75kDa ERK4

Figure 10. Quantification of ERK4 over-expression. Top panel. Serial dilution from 1 to 1:10,000 of an ERK4 over expressing protein extract ran of acrylamide gel and detected with ERK4 polyclonal antibody. Bottom panel. ACTIN detection on the same membrane to control for loading.

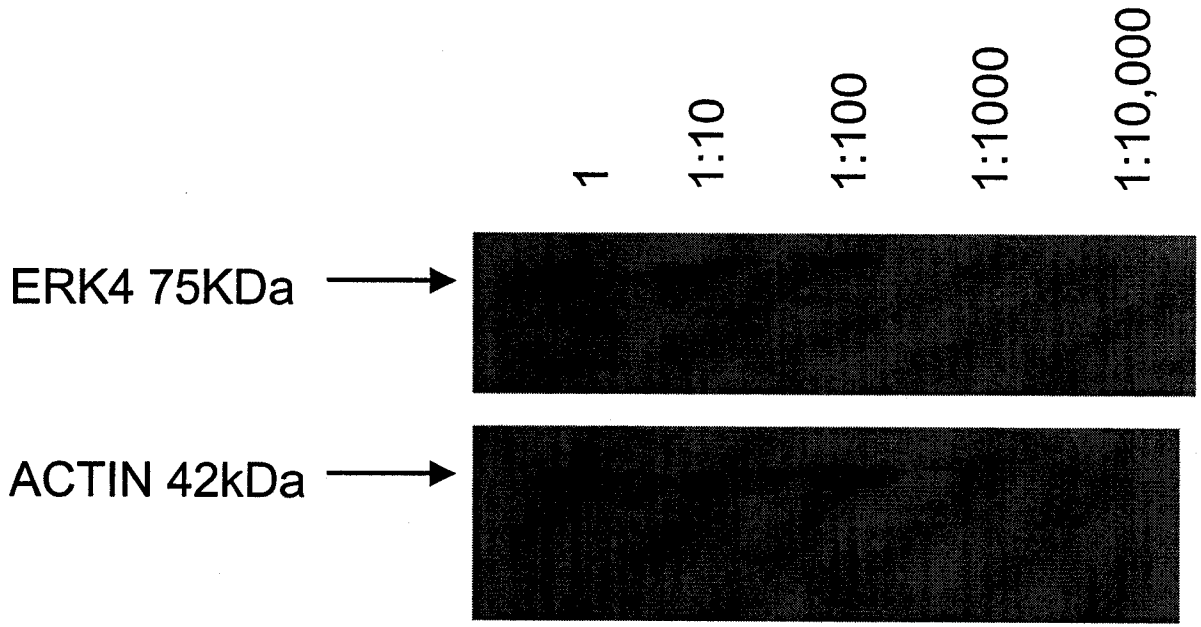

1
1:10
1:100
1:1000
1:10,000
ERK4 75KDa
ACTIN 42kDa

Table 6. Anchorage independent growth of prostate cancer cells over-expressing ERK4, as determined by soft agar assays

Cell line	Colony formation and significance *
LN4GPerk4wt-1	2.74%, p=0.000004
LN4GPerk4wt-3	6.16 % p=0.000038
LN4GPerk4wt-4	17% p=0.00005
LN4GPerk4wt-10	16.58% p=0.000005
LN4GPmock-6	66.7% p=0.003

* The number of colonies obtained with PC-3M-LN4GP2 (varying from 511 to 626) was established at 100%. The experiments were repeated 6 times. The p value was generated by a two-tailed Student's t-test.

Table 7. Invasive properties of prostate cancer cells over-expressing ERK4, as determined by Boyden chamber assays

Cell line	Invasiveness *	P values compared to parental
PC-3M-LN4GP2	100%	-
LN4GPmock-6	93%	0.794
LN4GPerk4wt-1	28%	0.002
LN4GPerk4wt-3	23%	0.0015
LN4GPerk4wt-10	38%	0.008

* The number of cells migrating through Matrigel obtained with PC-3M-LN4GP2 (varying from 75 to 115) was established at 100%. The experiments were repeated 4 times. The p values were caculated by a two-tailed Student's t-test.

4.4.5 Nude mouse injections

Cells over-expressing the ERK4 protein (LN4GPerk4wt-1, and LN4GPerk4wt-3), non-transfected parental PC-3M-LN4GP2, and mock transfected LN4GPmock-2 cells were injected subcutaneously in nude mice. The tumors formed by the ERK4 over-expressing cells grow at the same rate as the tumors formed by the parental cells, or the mock transfected cells (data not shown), and are still over-expressing the ERK4 protein at the same level. These results contrast with the ones obtained when chromosome 18 was transferred into the same PC-3M-LN4GP2 cells, where tumor formation was significantly delayed (see Chapter 3). Such differences argue that *in vivo* suppression of tumorigenicity requires the presence of an additional suppressor gene on human chromosome 18.

4.4.6 *ERK4* sequencing

We have sequenced the full coding sequence of the *ERK4* mRNA (bp 414 to 2153) in three commonly used PCa cell lines, and found a polymorphism at bp 603, located in the kinase domain of the ERK4 protein (called V38M since it substitutes a valine at position 38 for a methionine). This polymorphism is present in all our hybrids derived from PC-3M-Pro4 and PC-3M-LN4, and in two of the commonly used PCa cell lines (LNCaP and PC-3). All PC-3-derived cell lines (PC-3, PC-3M-Pro4 and PC-3M-LN4) are homozygous for the V38M polymorphism, while LNCaP cells are heterozygous. A preliminary screening was done on 111 Ashkenazi Jewish controls and 70 consecutive Ashkenazi

Jewish PCa patients since they are a genetically closed population that would allow us to find any link between PCa and the presence of this polymorphism. However, no significant difference was found in the frequency of the polymorphism between the controls and the cases (p=0.27 (Chi/Fisher exact test) and Table 8). Indeed, the allelic frequency is 41% in the controls and 39% of the Pca cases. However, there was a difference (p=0.06) in the Gleason score of the homozygous (-/-) and heterozygous (+/-) V38M patients versus the wild type allele (+/+). Indeed, Gleason score was higher in patients with a homozygous or heterozygous polymorphism, as compared to the patients with no polymorphism. However, the analysis of additional cases is needed to statistically establish if this V38M polymorphism is implicated in the more aggressive form of the disease. It is interesting to note that polymorphisms in other genes, such as the length of the CAG repeat in the androgen receptor have been linked to higher risk of developing an aggressive PCa [256].

Table 8. V38M polymorphism screening

Position 38	Jewish controls	Jewish cases
Normal +/+	35/111	29/70
Heterozygous +/-	60/111	28/70
V38M homozygous -/-	16/111	13/70
Total	111	70

4.5 Discussion

We have suppressed the tumorigenicity of human PCa cells by transferring into them a normal copy of human chromosome 18. We have then narrowed down the region responsible for this phenotype to a 10 Mb region around the *D18S51* marker [257].

In this study, we selected three candidate genes located around the *D18S51* locus, and determined their mRNA and protein level in our tumorigenic and less tumorigenic hybrids. mRNA levels of both *MASPIN* and *ERK4* were significantly down regulated in tumorigenic cells as compared to less tumorigenic cells. The mRNA level of *BCL2* was up regulated in tumorigenic cells when compared to less tumorigenic cells. However, the protein levels of MASPIN and BCL2 were not different between tumorigenic and less tumorigenic cells. This suggests that MASPIN or a BCL2 repressor might not be responsible for the less tumorigenic phenotype of our hybrids. However, these two genes could be mutated, which would reder the produced protein non-functional. Thus, sequencing these two genes in our hybrids would be required before we can rule them out as candidate TSGs in our cell system. Moreover, *MASPIN* has been shown to suppress tumorigenicity when introduced into TRAMP C2N PCa cells [135], LNCaP or DU 145 cells [258]. However, we have not found any independent evidence through a literature search that *MASPIN* suppresses the tumorigenicity of PC-3-derived cells.

The ERK4 protein levels were undetectable by regular Western immunoblot, or upon proteasome inhibitor treatment. This shows that our cells express very low levels, if any, of ERK4 protein. Cell treatment with the proteasome inhibitor MG132 failed to allow the detection of these proteins. This is interesting since the other non-classical ERK protein ERK3 was shown to be very unstable with a half-life of 30 minutes, accordingly that protein could be stabilized by proteasome inhibition [255]. ERK3 is rapidly degraded by the proteasome, but this might not be the case for ERK4. Further studies are needed to assess ERK4 protein stability..

Here we report that *ERK4* is down regulated in tumorigenic cells and, that its overexpression in human PCa cells completely abolishes their anchorage-independent growth potential. Furthermore, the ERK4 overexpressing cells show reduced invasiveness through Matrigel. However, when injected subcutaneously into nude mice, the ERK4 overexpressing cells form as many tumors as the parental cells, or the mock-transfected cells. This could suggest that, *in vivo*, ERK4 needs a partner to perform its tumor suppressing function. Such partner would be located around the *D18S51* region and was directly brought into the LN4B156-6 cells by the transfer of chromosome 18, or was induced by a gene located in this region. This partner would act synergically with *ERK4 in vivo* to suppress the tumorigenictiy of human PCa cells.
Alternatively, it is also possible that *ERK4* is not implicated in *in vivo* tumorigenicity and that it has only an effect *in vitro*.. In such case, ERK4 will

not be the TSG revealed by the transfer of the chromosome 18 in our PCa cells, but just a gene located in this region, which happens to inhibit *in vitro* growth properties of these cells. Thus, *in vitro*, *ERK4* could be sufficient to suppress the invasiveness and the anchorage-independent growth of PCa cells. However, *in vivo*, there could be a need for adaptation to the mouse microenvironment in order to suppress tumor formation, and this can be achieved only in presence of ERK4 and its partner(s). There are about 50 genes around the *D18S51* locus, and many of them could be putative *ERK4* partners. For example, there is a serpin and a cadherin cluster that could also be TSG implicated in PCa progression.

The *ERK4* gene contains a SNP called V38M that is present in PCa patients at the same frequency than in the populations tested. Thus, the V38M SNP does not seem to be linked to PCa. However, with the limited number of patients and controls tested, we had only an 80% power of detecting a 2.55 odd ratio (with 95% confidence intervals). This means that even if there was a significant difference in the allelic frequency of V38M between controls and PCa cases, we wouldn't have been able to detect it. Thus, more patients and more controls are needed to establish if there is any link between PCa and the V38M polymorphism. When the allelic frequency of the 471delAAAG SNP of the *RNASEL* gene was tested in the same Ashkenazi Jewish controls and cases, Kotar et al. [259] found a non-significant odd ratio of 0.47 (p=0.61, confidence intervals 0.02). They also argue that a bigger sample size is

needed to fully analyse the significance of the allelic frequency of polymorphisms linked to PCa in this population.

In 2000, Padalecki S. et al. reported two minimal regions of chromosomal loss located between markers *D18S1119* and *D18S64* (52 and 55Mb), and between *D18S848* and *D18S58* (40 and 70Mb) [231] but so far, no TSG has been mapped to these intervals. Interestingly, the *ERK4* gene is located at 47Mb (in the second minimal lost region), and could thus be the first of many TSGs implicated in PCa progression that maps in these intervals.

4.6 Conclusion

Even if the final proof is still lacking, our *in vitro* results suggest that *ERK4* could be implicated in PCa progression. We are currently generating an *ERK4*-V38M construct to assess the influence of this polymorphism on *ERK4* function. Furthermore, we are generating different truncated *ERK4* constructs that will be stably transfected in our PC-3-derived cells to assess the function of the C-terminal tail and the kinase domain of *ERK4*. These studies should help us understand the function of the C-terminal tail of *ERK4*.

4.7 Acknowledgements

The authors would like to thank Francois Gougeon for ERK4 sequencing PCRs and Jean-Sébastien Ripeau for nude mice injections.

CHAPTER 5

Manuscript 3:

Report of the first SNP located in the coding sequence of the ERK4 gene

Gagnon A., and Chevrette M.

To be submitted to Human Mutation early 2006.

5.0 Preface

This manuscript is a study reporting a comparison of the frequency of the V38M polymorphism described in Chapter 4 in three different human populations (Asian, European and African). Since it is not related to prostate cancer, we have decided to put it in a different chapter. The permission from the co-author can be found in Appendix E.

Report of the first SNP located in the coding sequence of the *ERK4* gene

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Short Title: A new SNP in the coding sequence of the *ERK4* gene

Permission from co-author can be found in Appendix E.

5.1 Abstract

Mitogen-activated protein kinases (MAPK) are implicated in a variety of cellular processes such as differentiation, survival, and proliferation. In this study, we have identified a polymorphism located in the coding sequence of the *ERK4* kinase gene. This polymorphism creates an *Nco*I site. We have screened DNA samples from individuals from Asia, Africa (Yoruba), and Europe for the presence of this SNP. Interestingly, we found the SNP at the same frequency in Asian and European individuals, but at a much lower frequency in the individuals from Africa. The SNP homozygosity frequency was very low in this group (4/90). To this date, the MAPK4 protein encoded by the *ERK4* gene has no known cellular function.

5.2 Introduction

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that play a role in transmitting extra cellular signals inside the cell [260]. These proteins are key components of intracellular signalling pathways implicated in a variety of cellular processes such as proliferation, differentiation, and survival [261]. The extracellular-regulated kinases (ERK) family of threonine/tyrosine kinases is composed of eight members named *ERK1* to *ERK8*. The classical members of the family (ERK1 and ERK2, official name MAPK3 and MAPK1) are ubiquitously expressed kinases that, once phosphorylated, translocate to the nucleus, and activate transcription factors involved in proliferation and differentiation [243]. The ERK1 and ERK2 proteins are upregulated in PCa specimens, compared to the normal

prostate tissue [262]. Some members of the ERK family are also activated in colorectal cancer [263]. Two non-classical members of the *ERK* family (ERK3 and ERK4, official name MAPK6 and MAPK4) share 50% homology with *ERK1* and *ERK2* in their kinase domain. They also contain a long C-terminal tail that is not present in ERK1 and ERK2 proteins. Moreover, their phosphoacceptor site (SEG) is different from the Thr-X-Tyr site commonly found in the activation loop of ERK1 and ERK2 [244]. However, the exact cellular function of ERK3 and ERK4 is still unknown.

5.3 Methods

5.3.1 r.603G>A SNP screening

We have compared the DNA sequence of controls to the NCBI sequence NM_002747. We have searched the NCBI SNP database and found the same SNP only in one brain tumor. To establish the frequency of the r.603G>A SNP in different populations worldwide, we used DNA from 93 Asian, 92 European, and 90 African individuals. The r.603G>A SNP introduces an *Nco*I restriction site, which facilitates its detection. Briefly, we PCR amplified the sequence spanning the SNP and digested the products with *Nco*I. The primer set mapk464 (5'-TGGGCAGCTCCAGATCACTG-3') and mapk802c (5'-ACGATGTACGCCACGCTGAA-3') was used to amplify the sequence surrounding this SNP. Briefly, a PCR reaction containing 1X Phusion HF buffer (New England Biolabs, Pickering, ON, Canada), 200 uM of each dNTP (Invitrogen, Burlington, ON, Canada), 0.5 uM of each primer (Sigma, Oakville, ON, Canada), 0.02 U/ul of Phusion DNA polymerase (New

England Biolabs), 200 ng of genomic DNA was prepared in a final volume of 20 ul. The following program was ran: 98°C for 30 secs, followed by 40 cycles of 98°C for 10 secs, 65°C for 30 secs and 72°C for 30 secs with a final extension of 72°C for 10 mins. The reactions were prepared in a final volume of 50 ul using the same reagent concentrations as above. Then, 10ul of the PCR reaction was electrophoresed through an agarose gel to verify the quality of amplification. 10 ul of the PCR reaction was digested with 10U of *Nco*I in a total volume of 40 ul. The digestions were electrophoresed through an agarose gel to verify the presence of the SNP (homozygous normal G/G= 338 bp; heterozygous G/A = 100 bp, 238 bp; and 338 bp; homozygous V38M A/A = 100 bp and 238 bp). An undigested PCR reaction was also electrophoresed as a control.

5.4 Results

5.4.1 *ERK4* sequencing

We have sequenced the coding region of the *ERK4* mRNA (bp 414 to 2153) in three commonly used PCa cell lines and found the SNP NM_002747:r.603G>A, located in the kinase domain of the ERK4 protein (the SNP results in Val38Met since it changes valine 38 for a methionine). The commonly used PCa cell lines LNCaP is heterozygous whereas PC-3 is homozygous for r.603G>A. A screen of different populations from Asia, Africa (Yoruba), and Europe was performed to assess the frequency of this SNP. As shown in Table 9, there is a significant difference in the frequency

of the SNP between the Asian and the European, compared to the African Yoruba population ($p<0.00005$, two-sided Chi/Fisher exact test). Indeed, the allelic frequency is 15% in the African population compared to 33% and 41% among Asian and European individuals, respectively. Also, careful analysis of the NCBI publicly available SNP database, failed to reveal any other polymorphism or SNP in the whole coding region of the *ERK4* gene. Thus, the Val38Met polymorphism is the first SNP reported in the coding sequence of the *ERK4* gene.

Table 9. r.603G>A SNP screening

SNP status	Asian	African	European
Normal GTG/GTG	43/93	67/90	34/92
Heterozygous GTG/ATG	38/93	19/90	40/92
V38M homozygous ATG/ATG	12/93	4/90	18/92
Total	93	90	92

5.5 Discussion

In this study, we showed that the *ERK4* gene contains a SNP called r.603G>A that is present at the same frequency in two of the three populations tested. Few SNPs have been reported for the members of the ERK family. Indeed, this is the first report of a SNP located in the coding sequence of the *ERK4* gene. All the 213 reported SNPs for this gene are located in untranslated regions or in introns. For the *ERK1* gene, only 16 SNPs were reported, and two synonymous SNPs are located in its coding sequence (both at amino acid 336). In the *ERK2* gene, 307 SNPs were reported, and only 3 synonymous ones are located in its coding sequence. In the *ERK3* gene, 99 SNPs were identified, and this is the only *ERK* gene in which non-synonymous SNPs were reported. Indeed, two nonsynonymous SNPs p.Lys254Glu and p.Cys256Arg were identified. All other *ERK* genes have SNPs that do not change the encoded amino acids. Based on other *ERK* genes, the kinase domain of ERK4 is thought to be responsible for its cellular function. The SNP identified in this study is located at amino acid 38 (p.Val38Met), which is located in the kinase domain of ERK4. Since it changes an amino acid, it might also have an influence on the function or cellular localization of the ERK4 protein. Further characterization of ERK4 function is needed to assess the putative influence of this SNP on the function of this protein.

CHAPTER 6

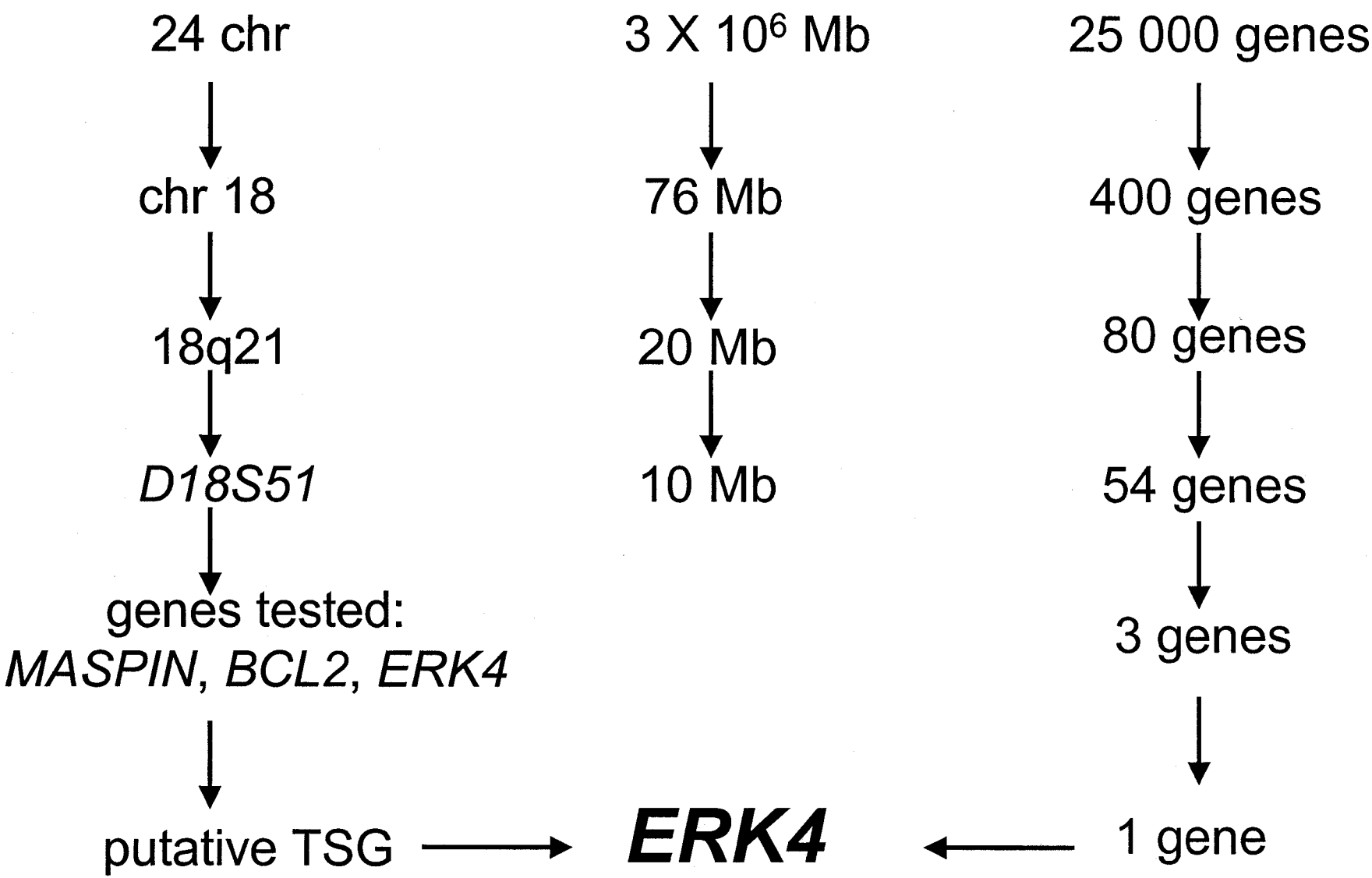
GENERAL DISCUSSION

AND CONCLUSION

6.0 Thesis summary

In this thesis, we have used the microcell-mediated chromosome transfer technique to assess which human chromosome(s) can affect the tumorigenic properties of human prostate cancer cells *in vitro* and *in vivo*. To do so, we transferred chromosomes that are the most frequently lost during PCa progression into two very aggressive human PCa cell lines called PC-3M-Pro4GP1 and PC-3M-LN4GP2. Indeed, we showed that the transfer of different chromosomes can have an effect on either *in vitro* and/or *in vivo* characteristics of human PCa cell lines. This thesis mainly concentrates on the transfer of chromosome 18 into PC-3M-LN4GP2 cells. This transfer demonstrated that chromosome 18 contains a tumor suppressor gene implicated in PCa progression. We narrowed down the region encoding this TSG to the *D18S51* region using polymorphic microsatellite markers and PCR mapping. We characterized three candidate genes (*BCL2*, *MASPIN* and *ERK4*), and showed that *ERK4* could be responsible for the less tumorigenic phenotype seen in our chromosome 18 hybrids (Figure 11).

Figure 11. Summary of the thesis showing that we started this thesis with 24 chromosomes and about 25,000 genes and finished by identifying a good candidate for a new tumor suppressor gene implicated in prostate cancer progression.


24 chr
chr 18
18q21
D18S51
genes tested:
MASPIN, BCL2, ERK4
putative TSG
3 X 10^6 Mb
76 Mb
20 Mb
10 Mb
ERK4
25 000 genes
400 genes
80 genes
54 genes
3 genes
1 gene

In this thesis:

1) In Chapter 3, we show that the transfer of chromosome 10 reduces the ability of the PC-3M-LN4 human prostate cancer cells to invade through Matrigel. However, chromosome 10 hybrids (LN4B77-) did not show any reduced growth ability in nude mice. We also show that chromosome 12 can decrease cell invasiveness in PC-3M-LN4, but did not have any effect on their anchorage-independent growth in soft agar. The transfer of a translocated chromosome t(17:3, with unknown breakpoints) had an effect on cell colony formation potential, as well as on *in vivo* tumorigenic properties but increased their invasiveness.

2) In Chapter 3, we show that the transfer on chromosome 18 into PC-3M-LN4GP2 cells decreases their ability to invade through Matrigel. It also abolishes their colony formation potential. The cells also have a delayed and greatly reduced tumor growth in nude mice, both subcutaneously and orthotopically. This suggests that chromosome 18 encodes a tumor suppressor gene implicated in prostate cancer progression.

3) In Chapter 3, we use polymorphic microsatellite markers to narrow down the region that encodes the tumor suppressor gene to the vicinity of the *D18S51* locus.

4) In Chapter 4, we characterize three candidate genes (*BCL2*, *MASPIN* and *ERK4*) that could be implicated in the less tumorigenic phenotype of our chromosome 18-derived LN4B156-hybrids. We use real-time PCR and Western immunoblot to examine the possibility that a *BCL2* repressor or *MASPIN* could be responsible for this phenotype.

5) In Chapter 4, we show that prostate cancer cells express very little amount (if any) of ERK4 protein. The treatment of these cells with a proteasome inhibitor did not allow us to detect the ERK4 protein by Western immunoblot.

6) In Chapter 4, we show that *ERK4* mRNA expression is significantly reduced in tumorigenic compared to less tumorigenic cells. We also show that cells over-expressing ERK4 have a decreased invasive potential. The *ERK4*-transfected cells also form significantly fewer colonies in soft agar compared to the non-transfected or mock-transfected cells. However, we show that ERK4 overexpressing cells do not have reduced or delayed tumor formation when injected subcutaneously in nude mice.

7) In Chapter 5, we describe a new SNP, r.603G>A, located in the coding region of the *ERK4* gene which results in p.Val38Met. This is the first SNP reported in the coding sequence of this gene.

6.1 General discussion

In this thesis, we transfer chromosomes 10, 12, t(17:3, with unknown breakpoints), and 18 into two human prostate cancer cells called PC-3M-LN4GP2 and PC-3M-Pro4GP1. As shown in Chapter 3, transfer of chromosomes 10, 12, and t(17:3, with unknown breakpoints) affected differently the *in vitro* and *in vivo* characteristics of these two prostate cancer cell lines. Indeed, the two cell lines chosen for these transfers have been isolated differently *in vivo* [204]. The PC-3M-Pro4GP1 cells are very aggressive cells in the prostate since they were selected in the prostate of nude mice. However, the PC-3M-LN4GP2 cells are also aggressive but form lymph nodes metastases upon injection in nude mice. Thus, their expression profile is very different since they have different phenotypes *in vitro* and different tumorigenic and metastatic abilities *in vivo*.

When we transferred chromosome 10, we observed reduced invasiveness and decreased colony formation in soft agar in both cell lines. The transfer of the t(17:3, with unknown breakpoints) into these two cell lines increased their invasiveness properties, but decreased their colony formation potential in

soft agar, and their tumor growth *in vivo*. Again, this shows that human chromosomes contain genes implicated in different processes *in vitro* and *in vivo*, and that their transfer in cell lines with different properties can have opposite effects. The transfer of chromosome 12 into these same cell lines had an opposite effect on their invasiveness. Indeed, LN4B9- cells are less invasive that parental PC-3M-LN4GP2 cells, whereas Pro4B9- cells are more invasive than parental PC-3M-Pro4GP1. The colony formation potential of both parental cell lines remained unchanged upon transfer of chromosome 12. This shows that chromosome 12 contains genes that could be implicated in invasiveness properties, but not in anchorage-independent growth. This contrasts with the previous results obtained from this laboratory [164] where it was shown that transfer of human chromosome del(12)(q13) into DU 145 prostate cancer cells completely abolished their tumor growth ability in nude mice. The DU 145 cell line was isolated from a brain metastasis from a 69 years old prostate cancer patient [264]. *In vitro* and *in vivo*, DU 145 cells have different properties than the PC-3-derived cell lines we have used. Thus, the endogenous properties of the cellular model used may explain that transfer of chromosome 12 into our PC-3-derived cell lines had a different effect than the one expected. Indeed, genetic alterations of the recipient cells such as lost TSGs/MSGs, chromosomal aberrations, methylated genes vary among cell lines and may account for the differences in the phenotype observed after the transfer of a specific chromosome.

We have transferred a copy of human chromosome 18 into PC-3M-LN4GP2 cells. This transfer resulted in two sets of hybrids: one set that were highly invasive, formed colonies in soft agar, and formed tumors in nude mice (subcutaneously), and a second set that were less invasive, formed less colonies in soft agar, and formed fewer and latent tumors in nude mice (subcutaneously and orthotopically). However, the transfer of chromosome 18 in PC-3M-Pro4GP1 cells did not have any significant effect on their *in vitro* or *in vivo* properties, suggesting that the choice of the recipient cell line has an impact and that the MMCT technique itselft does not affect these properties. Since we observed major differences in the phenotype of our two sets of LN4B156- hybrids, we suggested that these differences were due to the loss of a TSG in our highly tumorigenic hybrids. Indeed, all hybrids were generated upon transfer of chromosome 18 but the highly tumorigenic hybrids have lost (deleted) a TSG encoding region that is still present in the less tumorigenic hybrids. Using PCR mapping, we narrowed down the region encoding the tumor suppressor to the *D18S51* locus. Indeed, one allele (allele 1A) was linked to the less tumorigenic phenotype of our hybrids since it was present in all less tumorigenic hybrids, deleted in all tumorigenic hybrids and lost upon tumorigenesis. This argues that a TSG lies around that *D18S51* locus.

We then analysed three candidate genes located around the *D18S51* locus (*BCL2*, *MASPIN* and *ERK4*) to determine if any are/were responsible for the

less tumorigenic phenotype of our LN4B156- hybrids. As shown in Chapter 4, it is unlikely that a *BCL2* repressor or *MASPIN* are responsible for our less tumorigenic phenotype, based on Western immunoblot assays. However, we cannot rule them out yet since these two genes might contain a mutation that renders the protein inactive, even if it's still produced. Thus, sequencing these two genes is needed before stating that they are not implicated in the less tumorigenic phenotype seen in our hybrids.

We have shown that the mRNA expression level of *ERK4* was significantly reduced in tumorigenic cells, compared to less tumorigenic cells. Moreover, *ERK4* overexpressing cells show significantly decreased invasiveness, and their colony formation in soft agar was completely abolished. However, *ERK4* alone was not sufficient to inhibit tumor growth *in vivo*, as the mice injected with ERK4 over expressing cells formed as many tumors as the ones injected with mock transfected or parental cells. This could argue that ERK4 needs one or more partners to perform its tumor suppressor function in the mouse environment. Moreover, based on the reduced invasiveness results obtained with *ERK4* overexpressing cells (Chapter 4), we could hypothesize that *ERK4* has a metastatic suppressor effect, rather than a tumor suppressor effect. Thus, orthotopic injection of these ERK4 overexpressing cells in the prostate of nude mice is needed to assess if ERK4 can suppress the formation of metastases to lymph nodes, lungs or bones. It is also possible that the inhibitory effects of ERK4 are seen only *in*

vitro and that ERK4 is not implicated in the less tumorigenic phenotype seen in our hybrids.

To assess the TSG/MSG potential of *ERK4*, we used tranfection (over expression). In this system, *ERK4* mRNA was overexpressed by more than 250 fold while the level of ERK4 protein increased at least by 100 fold. This most likely does not reflect physiological level since the ERK4 protein is not detectable in non-transfected cells. Thus, it is still possible that the reduced colony formation potential and invasiveness are due to the overexpression itself. Then, the *in vitro* effects of *ERK4* would have been over estimated. To avoid such unphysiological level, *ERK4* could be cloned under the control of a weaker promoter like its own promoter (once characterized). In such instance, the level of ERK4 expression should mimic the level seen in our chromosome 18 hybrids, thus representing a better estimate of what happens in the cell physiologically.

We have sequenced the coding sequence of the *ERK4* gene in the commonly used prostate cancer cell lines LNCaP, DU 145, and PC-3 and found that LNCaP was heterozygous, PC-3 homozygous for a SNP located at bp603 of the *ERK4* mRNA (r.603G>A) while DU 145 is normal at this SNP. This SNP is nonsynonymous since it changes a valine for a methionine (p.Val38Met). We have screened 111 Ashkenazi Jewish controls, and 70 Ashkenazi Jewish PCa cases for the presence of this SNP (Chapter 4). We

found it at the same frequency in controls and PCa cases, suggesting that it is not linked to PCa risk. These sample sizes provided a 80% power to detect significant odds ratios of approximately 2.55 or greater. Thus, if there were an obvious effect of this SNP with PCa, we would have detected it. However, very large sample sizes of more than 2500 cases, and 2500 controls would be needed to exclude an effect of 1.5 fold or less. Thus, a study with larger sample sizes is needed to know if the presence of this V38M SNP is linked to PCa establishment or its progression.

Our study was done on DNA extracted from patient's blood. Thus, we could have found a higher allelic frequency if we had used microdissected prostate tumors, and compared it to surrounding normal prostate tissue and blood from the same patient. Indeed, if this SNP is linked to PCa in some patients, the allelic frequency would be a lot higher in prostate tumors and surrounding tissues compared to the same patient's blood. This SNP could be the first hit of a two hit phenomenon [265]: a certain proportion of the population harbours this SNP on one allele, and just needs to modify the second allele directly in prostate cells to render these cells malignant. That could explain why we did not find any link between this SNP in the blood of Ashkenazi Jewish cases and controls.

We also screened an Asian, a European, and an African (Yoruba) population to assess the frequency of this SNP. We found the same frequency in the

Asian and European populations, as we found in the Ashkenazi Jewish group. Interestingly, the frequency of V38M homozygosity was very low (4.4%) in the African population compared to 12.9%, 19.6% and 14.4% respectively, in the three populations tested (Asian, European, and Ashkenazi Jewish). The allelic frequency was also lower in the African individuals (15%) compared to 33%, 41% and 40% for the three other populations examined. This variation in allelic frequency is probably due to the fact that the Yoruba population has a very low genetic diversity and that their genetic pool is closed. Thus, this polymorphism is probably recent since only the populations with a high genetic pool (Asian, Ashkenazi Jewish and European) have a high allelic frequency.

More impirtantly, this thesis sets the bases for many other studies. Indeed, we have transferred chromosome 10, 12, t(17:3, with unknown breakpoints) and 18 into PCa cells and characterized hybrids from each of these transfers. We have shown that other chromosomes than 18 encode TSG implicated in PCa progression and these could be further studied. We have narrowed down the region of chromosome 18 that encodes a TSG for PCa to a region of about 10 Mb. Many candiate genes in this region could be screened for their potential role in PCa. Moreover, we suggest three candidate genes that could be implicated in PCa progression: *ERK4*, *MASPIN* and a *BCL2* repressor. Even though the protein levels of BCL2 and MASPIN do not vary, they could still be responsible for the less tumorigenic

phenotype seen in our LN4B156-hybrids and should thus be sequenced and further analysed. We also provide some evidence that *ERK4* suppresses PCa cells invasiveness and colony formation *in vitro* and its MSG potential should be assessed *in vivo* by orthotopic injection in nude mice.

6.2 Future studies

The exact cellular function of the ERK4 protein is not known, and the role of its different domains is not well understood. Based on its homology to other members of the ERK family, it is possible that the kinase domain of ERK4 is responsible for its function. Interestingly, ERK3 and ERK4 are the only two members of the *ERK* family to have long C-terminal tails [244]. Thus, we are generating C-terminal tail truncated, and kinase domain mutated constructs that will be transfected in PC-3M-LN4GP2 cells. Then, these stable transfectants will be assessed for invasiveness and anchorage-independence to verify if these ERK4 constructs are still functional and still have a tumor suppressor activity. These experiments will allow us to understand the importance of the C-terminal tail and the kinase domain of ERK4 for its tumor suppressor function. We are also collecting prostate cancer tumors, normal tissue surrounding these tumors, as well as blood from the same patient to assess the frequency of the p.Val38Met SNP. That should tell us if this SNP is linked in any way to PCa. Lastly, nude mice orthotopic injection of ERK4 overexpressing cells will reveal if *ERK4* is a MSG implicated in PCa *in vivo*.

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APPENDIX A:

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APPENDIX B:

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Chromosome 18 suppresses the tumorigenic properties of human prostate cancer cells (Chapter 3)

APPENDIX C:

Proof of published Manuscript 1:
Chromosome 18 suppresses the tumorigenic properties of human prostate cancer cells
(Chapter 3)

GENES, CHROMOSOMES & CANCER 45:000–000 (2005)

RESEARCH ARTICLE

Chromosome 18 Suppresses Tumorigenic Properties of Human Prostate Cancer Cells

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AQ1 Although prostate cancer is still the most diagnosed cancer in men, most genes implicated in its progression are yet to be identified. Chromosome abnormalities have been detected in human prostate tumors, many of them associated with prostate cancer progression. Indeed, alterations (including deletions or amplifications) of more than 15 human chromosomes have been reported in prostate cancer. We hypothesized that transferring normal human chromosomes into human prostate cancer cells would interfere with their tumorigenic and/or metastatic properties. We used microcell-mediated chromosome transfer to introduce human chromosomes 10, 12, 17, and 18 into highly tumorigenic (PC-3M-Pro4) and highly metastatic (PC-3M-LN4) PC-3-derived cell lines. We tested the in vitro and in vivo properties of these hybrids. Introducing chromosome 18 into the PC-3M-LN4 prostate cancer cell line greatly reduced its tumorigenic phenotype. We observed retarded growth in soft agar, decreased invasiveness through Matrigel, and delayed tumor growth into nude mice, both subcutaneously and orthotopically. This phenotype is associated with a marker in the 18q21 region. Combined with the loss of human chromosome 18 regions often seen in patients with advanced prostate cancer, our results show that chromosome 18 encodes one or more tumor-suppressor genes whose inactivation contributes to prostate cancer progression. © 2005 Wiley-Liss, Inc.

INTRODUCTION

With an estimated 232,090 new cases and 30,350 deaths in the United States in 2005, prostate cancer (PCa) is still the most diagnosed cancer in men and the second leading cause of their cancer deaths (Jemal et al., 2005). However, despite these alarming figures, there is still no treatment that can cure the most advanced stages of the disease, when the cancer has become androgen independent. Furthermore, as it progresses, PCa metastasizes to bones and lymph nodes, which further complicates its treatment (Hanks et al., 1989). Thus, there is an urgent need for new therapies, but these are likely to require a better understanding of the mechanisms and genes implicated in the progression of this disease.

Genetic alterations are a common feature of cancer. Indeed, in different tumor types, chromosomal rearrangements or loss of particular regions have been reported for almost all chromosomes. However, these modifications are not randomly distributed; specific regions have been associated with certain types of cancer (Mitelman et al., 1997). Unfortunately, in PCa, such analysis is complicated by the heterogeneity of tumor samples. Cytogenetic analysis of human prostate tumors revealed that alterations of many chromosomes are associated with PCa (Kawana et al., 2002). Indeed, numerous studies have shown deletions of chromosome arms 7q (Zenkluzen et al., 1994; Takahashi et al., 1995), 8p (Bova et al., 1993; Macoska et al., 1995; Suzuki et al., 1995), 10q (Gray et al., 1995; Ittmann, 1996; Komiya et al., 1996), 16q (Bergerheim et al., 1991; Kunimi et al., 1991; Suzuki et al., 1996), 17q (Gao et al., 1995; Williams et al., 1996), and 18q (Kunimi et al., 1991; Gao et al., 1993; Latil et al., 1994) in advanced PCa. The sensitive comparative genomic hybridization method has led to the identification of additional genetic alterations in PCa, including loss of genetic material on human chromosome arms 2q, 5q, 6q, 9p, 13q, 15q, and 17p (Visakorpi et al., 1995; Cher et al., 1996). The loss of these regions could indicate that they encode tumor-suppressor genes. Functional evidence to that effect has been gathered from the transfer of normal human chromosomes in PCa cell lines.

Almost every human chromosome has been transferred into either rat or human PCa cells for assessment of their tumor-suppressive properties. Thus, human chromosome arm 8p (Nihei et al., 2002) and chromosome 12 (Luu et al., 1998) sup-

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pressed the tumorigenicity of rat PCa cells. Similarly, the introduction of human chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17, 19, and 20 in rat Dunning cell lines resulted in reduction or loss of their metastatic properties, sometimes without affecting their tumorigenicity (Mashimo et al., 1998; Gao et al., 1999; Ichikawa et al., 2000; Goodarzi et al., 2001; Hosoki et al., 2002).

Analysis of rat/human hybrids containing human chromosome 11 has led to the identification of a new metastasis-suppressor gene called *CD82* that encodes the KAI1 protein (Dong et al., 1995). Reduced expression of KAI1 is associated with progression of PCa (Lijovic et al., 2002) and epithelial ovarian carcinoma (Liu et al., 2000), as well as with bladder (Su et al., 2004), breast (Yang et al., 2000), gastric (Hinoda et al., 1998), and colorectal (Lombardi et al., 1999) cancer. Reduced expression of KAI1 was also detected in metastatic forms of esophageal squamous cells (Uchida et al., 1999) and hepatocellular carcinoma (Guo et al., 1998). The introduction and expression of KAI1 into melanoma cells was accompanied by a reduction of their invasive and metastatic properties (Takaoka et al., 1998), further establishing the metastasis-suppressive activity of the KAI1 protein.

Other genes have also been shown to suppress metastatic properties without affecting primary tumor growth. These genes are *CD44* (11p13), *MAP2K4* (17p11.2), *NME1* (17q21.3), *NME2* (12q24.1), *KISS1* (1q32), and *BRMS1* (11q13.1–2). However, only three of them (*CD82*, *CD44*, and *MAP2K4*) act as metastasis-suppressor genes in PCa (Kauffman et al., 2003).

Chromosome transfer has also been performed in human PCa cell lines, where introduction of chromosomes 2 (Mashimoto et al., 2000), 8p (Xu et al., 2003), 10p (Murakami et al., 2000), 12 (Bérubé et al., 1994), and 19 (Gao et al., 1999; Astbury et al., 2001) showed tumor-suppressing activity.

Cytogenetic, allelotyping, and somatic-cell hybrid studies have shown that the long arm of human chromosome 18 (18q) likely carries one or more tumor- or metastasis-suppressor genes that play a role in PCa progression. Indeed, loss of heterozygosity on 18q was reported in 30%–40% of PCa patients (Kumini et al., 1991). However, introduction of human chromosome arm 18q into the DU 145 PCa cell line abolished its tumorigenicity (Padalecki et al., 2001), whereas transfer of an intact chromosome 18 into the PC-3 PCa cell line interfered with both the tumorigenic and metastatic properties of these cells (Padalecki et al., 2003).

Tumor-suppressor genes have been identified on the long arm of chromosome 18. Indeed, cytogenetic band 18q21 encodes the tumor-suppressor genes *DCC* (deleted in colorectal cancer), *SMAD4/DPC4* (deleted in pancreatic cancer 4), and *SMAD2/DPC2* (deleted in pancreatic cancer 2). However, these genes are not implicated in PCa progression (Yin et al., 2001). Moreover, and although studies in PCa patients identified loss of two chromosome 18 minimal regions (between markers *D18S1119* and *D18S64*, kbp 52940 and 55575, and between *D18S848* and *D18S58*, kbp 40053 and 70193), no tumor-suppressor gene has yet been mapped to these intervals (Padalecki et al., 2000).

To identify tumor- or metastasis-suppressor genes, we used microcell-mediated chromosome transfer to introduce different tagged normal human chromosomes (10, 12, 17, and 18) into the tumorigenic PC-3M-Pro4 and metastatic PC-3M-LN4 PCa cell lines. We showed that only the introduction of chromosome 18, not the others, into human PCa cells was sufficient to affect both their in vitro and in vivo tumorigenic properties. Indeed, such hybrids experienced decreased invasiveness, lost their anchorage-independent growth capacity, and harbored a delayed tumor growth after both subcutaneous and orthotopic injections into nude mice.

MATERIALS AND METHODS

Cell Lines

PC-3M-Pro4 and PC-3M-LN4 human PCa cell lines (Pettaway et al., 1996) were obtained from I. J. Fidler (Department of Cancer Biology, the University of Texas M. D. Anderson Cancer Center, Houston, TX). PC-3M-Pro4 cells are highly tumorigenic, whereas PC-3M-LN4 cells are highly metastatic to lymph nodes when injected orthotopically into the prostates of nude mice. Both cell lines were derived upon orthotopic injections of PC-3M into the prostates of nude mice. PC-3M was derived from the PC-3 cell line obtained from a bone metastasis of a patient with PCa (Kaighn et al., 1979). PC-3M-LN4 and PC-3M-Pro4 were kept in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Burlington, Ontario, Canada). Geneticin-resistant PC-3M-Pro4GP1 and PC-3M-LN4GP2 cell lines were generated upon Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada) transfection of the pQBI25 plasmid (Q-BIOgene, Carlsbad, CA). This plasmid encodes the green fluorescent protein gene and contains the neomycin phosphotransferase gene, which confers resistance to geneticin. These cells were kept in RPMI 1640 with 10%

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FBS and 400 μg/ml of geneticin (Invitrogen, Burlington, Ontario, Canada). B78MC9, B78MC57, B78MC75, B78MC77, and B78MC156 are microcell hybrids from our mouse/human hybrid panel (Speevak et al., 1995) and contain a tagged human chromosome (chromosome 12, chromosome 3 translocated with chromosome 17 with unknown breakpoints, chromosome 10p, chromosome 10, and chromosome 18, respectively) in the B78 mouse melanoma background. B78MC hybrids were kept in DMEM (Dulbecco's modified Eagle medium; Invitrogen, Burlington, Ontario, Canada) supplemented with 10% FBS and hygromycin B at a concentration of 400 μg/ml (Roche Diagnostics, Laval Quebec, Canada). PC-3M-Pro4- and PC-3M-LN4-derived microcell hybrids (such as Pro4B156 and LN4B77 hybrids; see below) were grown in RPMI 1640 with 10% FBS, 400 μg/ml of geneticin, and 400 μg/ml of hygromycin B.

Microcell-Mediated Chromosome Transfer

Microcell fusion was done as described (Fournier, 1981). B78MC hybrids containing different tagged chromosomes (10, 10p, 12, translocated 3 and 17, and 18) were micronucleated in the presence of Colcemid (0.06 μg/ml; Sigma, Oakville, Ontario, Canada) for 24 hr. The resulting microcells were fused to either PC-3M-Pro4GP1 or PC-3M-LN4GP2 cells in the presence of 50% (w/w) polyethylene glycol 1600 (Fisher Scientific, Nepean, Ontario, Canada) for 60 sec. The cells were rinsed three times with serum-free RPMI 1640, and complete medium (RPMI 1640 with 10% FBS) was added to the flask and incubated overnight. The resulting microcell hybrids (Pro4B... and LN4B...; Table 1) were selected in RPMI 1640 medium containing 10% FBS + 800 μg/ml hygromycin B + 800 μg/ml geneticin.

Invasion Assay

We used a Boyden chamber assay (Albini et al., 1987) to assess invasive potential. Briefly, 25,000 cells were added to a chamber coated with Matrigel (Fisher Scientific, Nepean, Ontario, Canada), and RPMI 1640 medium supplemented with 5% FBS was added to the bottom chamber to serve as a chemoattractant. The chambers were incubated at 37°C for 22 hr, and filters were stained with a Diff-Quick staining kit (Dade Behring, Newark, DE). Filters were then put onto microscope slides (Fisher Scientific, Nepean, Ontario, Canada), and invasive cells from five random fields were counted under a contrast phase microscope at 200×. All invasion experiments were performed twice in quadruplicate ($n = 8$), and the results were expressed as the percentage of the invasion obtained with the parental cell line.

Soft-Agar Assay

The soft-agar assays were performed as described previously (Rizzino, 1987). Briefly, 1 ml of 0.5% agar (Fisher Scientific, Nepean, Ontario, Canada) diluted in appropriate media was poured into six-well plates to form the basal layer. Then 3,000 cells were mixed with 0.5% agar and poured on top of the basal layer. Plates were incubated at 37°C for 21 days. Growing cell foci were stained with 200 μl of a 0.03% crystal violet (Sigma, Oakville, Ontario, Canada) solution in 100% methanol (Fisher Scientific, Nepean, Ontario, Canada). Qualitative assessment of growth was performed, in which the colony formation of hybrids was compared to that of the parental cell lines, which were arbitrarily assigned the symbol +++. For quantitative evaluation, plates were scanned on a PowerLook 2100XL scanner (UMAX, Dallas, TX). The number of colonies was counted on the computer screen and converted to a percentage of colonies obtained with the parental cell line. Each soft-agar assay was performed three times on six wells per cell line ($n = 18$).

Nude Mouse Injections

All mouse injections were done according to Canadian Council for Animal Care guidelines, after approval by the McGill University Animal Care Committee. Male nude mice (*nu/nu;* 6–8 weeks of age; Charles River Laboratory, St-Constant, Quebec, Canada) were injected with PCa cells. One million cells in 200 μl of Hanks balanced saline solution (HBSS; Invitrogen, Burlington, Ontario, Canada) were injected subcutaneously in the upper backs of nude mice. Mice were sacrificed when tumors reached a size of 1,000 mm^3. For orthotopic injections, 1 million cells were resuspended in 16.6 μl of HBSS and injected with a push-button-controlled dispensing device (Hamilton Syringe Company, Reno, NY) into the dorsal lobe of the prostate. The mice were sacrificed when they became moribund.

Tumors in Culture

Tumors were excised and digested two times for 15 min at 37°C with 350 U/ml of type I collagenase (Invitrogen, Burlington, Ontario, Canada), and cell suspensions were seeded into culture flasks containing RPMI 1640 supplemented with 10% FBS and 1% penicillin (5,000 IU/ml)/streptomycin (5,000 μg/ml) (Invitrogen, Burlington, Ontario, Canada). Two days

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TABLE I. Phenotypic Changes Associated with Human Chromosome Transfer in PC-3M-Pro4GP1 and PC-3M-LN4GP2 Prostate Cancer Cell Lines

Mouse/human parental hybrid (transferred chromosome)	Properties of hybrids			
	Invasion assay (Parental = 100%)	Soft-agar assay (Parental = +++ or 100%)	Tumor formation[a] (subcutaneous)	Tumor formation[b] (orthotopic)
B78MC75 (chr 10p)	n/a	Pro4B75-2 ++ Pro4B75-3 ++ 1/2 Pro4B75-11 +++ Pro4B75-12 + Pro4B75-14 +	Pro4B75-12: 3/10 ($P = 0.002$)	Pro4B75-12: 2/5 ($P = 0.14$)
B78MC77 (chr 10)	n/a	Pro4B77-2: + 1/2 Pro4B77-10: + 1/2 Pro4B77-15: +		
B78MC9 (chr 12)	Pro4B9-4: 88% ($P = 0.49$) Pro4B9-8: 217% ($P = 0.02$) Pro4B9-17: 568% ($P = 0.0007$) Pro4B9-41: 107% ($P = 0.75$)	Pro4B9-11: +++ Pro4B9-17: ++ Pro4B9-18: ++ Pro4B9-21: ++ 1/2 Pro4B9-22: +++	n/a	n/a
B78MC57 [chr t(3;17)]	n/a	Pro4B57-1: ++ 1/2 Pro4B57-3: ++ Pro4B57-6: +++ Pro4B57-7: ++ 1/2 Pro4B57-8: + 1/2	n/a	n/a
B78MC156 (chr 18)	n/a	Pro4B156-10: + 1/2 Pro4B156-12: ++	Pro4B156-10: 3/5 ($P = 0.07$)	n/a
B78MC77 (chr 10)	LN4B77-9: 35% ($P = 0007$) LN4B77-50: 73% ($P = 0.17$) LN4B77-60: 63% ($P = 0.09$) LN4B77-80: 22% ($P = 0.00007$)	LN4B77-2: + 1/2 LN4B77-9: ++ LN4B77-37: + 1/2 LN4B77-44: + LN4B77-45: ++	LN4B77-44: 2/5 ($P = 0.54$)	n/a
B78MC9 (chr 12)	LN4B9-34: 2% ($P = 0.000006$) LN4B9-35: 10% ($P = 0.00016$)	LN4B9-31: ++++ LN4B9-33: +++ LN4B9-34: +1/2 LN4B9-35: ++ LN4B9-36: +++	n/a	n/a
B78MC57 (chr t(3;17))	LN4B57-4: 250% ($P = 0.007$) LN4B57-5: 142% ($P = 0.15$) LN4B57-6: 265% ($P = 0.003$) LN4B57-7: 281% 9P = 0.0011)	LN4B57-4: ++ LN4B57-5: +1/2 LN4B57-6: ++ LN4B57-7: ++	LN4B57-5: 2/10 ($P = 0.04$)	n/a
B78MC156 (chr 18)	LN4B156-1: 58% ($P = 0.98$) LN4B156-2: 75% ($P = 0.16$)	LN4B156-1: ++++, 289% ($P = 0.005$) LN4B156-2: +++, 91% ($P = 0.45$) LN4B156-3: ++++, 227% ($P = 0.0003$) LN4B156-14: ++++, 396% ($P = 0.00000001$)	LN4B156-2: 5/5 ($P = 0.08$)	
	LN4B156-6: 42% ($P = 0.0002$) LN4B156-14: 19% ($P = 0.0000009$)	LN4B156-6: +, 4% ($P = 0.00001$) LN4B156-13:+, 23% ($P = 0.00002$) LN4B156-16: ++, 58% ($P = 0.0002$) LN4B156-20: ++, 58% ($P = 0.0006$)	LN4B156-6: 2/10 ($P = 0.05$)	LN4B156-6: 2/3 ($P = 0.03$)

[a]In subcutaneous injections, PC-3M-Pro4GP1 formed tumors in 9 of 10 mice, and PC-3M-LN4GP2 formed tumors in 2 of 5 mice.
[b]In orthotopic injections into the prostate, PC-3M-Pro4GP1 formed tumors in 3 of 3 mice, and PC-3M-LN4GP2 formed tumors in 9 of 13 mice.

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TABLE 2. Genotyping of Chromosome 18 in Parental Cells and LN4B156 Hybrids and Tumors

Marker	Cytoband	Position (kbp)	B78	B78MC156	PC3MLN4GP2	LN4B156-2	TLN156-2-2	LN4B156-6	TLN156-6-3 and TLN156-6-6
D18S59	18p11.3	634	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S63	18p11.3	3426	0	1	2, 3	2, 3	2, 3	2, 3	2, 3
D18S976	18p11.2	5238	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S42	18p11.2	5891	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S843	18p11.1	8603	0	1	2, 3, 4	2, 3, 4	2, 3, 4	2, 3, 4	2, 3, 4
D18S542	18p11.1	11550	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S44	18q11.1	19875	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S877	18q11.1	26613	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S847	18q11.2	27591	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S1133	18q12	31986	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S67	18q12	35855	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S1157	18q12	37751	0	1	2, 3, 4, 5	2, 3, 4, 5	2, 3, 4, 5	2, 3, 4, 5	2, 3, 4, 5
D18S535	18q12	38036	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S65	18q12	40425	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S1145	18q12	40435	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S46	18q21	48376	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S487	18q21	51673	0	1	1, 2	1, 2	1, 2	1, 2	1, 2
D18S1109	18q21	57430	0	1	2, 3, 4	2, 3, 4	2, 3, 4	2, 3, 4	2, 3, 4
D18S39	18q21	54001	0	1	1, 2	1, 2	1, 2	1, 2	1, 2
D18S1155	18q21	56848	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S51	18q21	60734	0	1, 1A	2, 3	1, 2, 3	1, 2, 3	1, 1A, 2, 3	1, 2, 3
D18S1270	18q21	61178	0	1	2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
D18S382	18q22	63354	0	1	2, 3	2, 3	2, 3	2, 3	2, 3
D18S541	18q22	69959	0	1	2	1, 2	1, 2	1, 2	1, 2
D18S58	18q23	72125	0	1	1, 2	1, 2	1, 2	1, 2	1, 2
D18S50	18q23	75252	0	1	1, 2	1, 2	1, 2	1, 2	1, 2
D18S1141	18q23	76658	0	1	1	1	1	1	1

1 = For each marker, Allele in the mouse/human hybrid B78MC156 (donor cell); 2–5 = polymorphic alleles derived from the prostate cancer cell line PC-3M-LN4GP2; 0 = absence of this marker.

later, a complete selective medium [RPMI 1640 with 10% FBS + 400 μg/ml geneticin + 1% penicillin (5,000 IU/ml)/streptomycin (5,000 μg/ml)] was added.

Genotyping of Hybrids

To identify B78MC hybrids that contained the selected tagged human chromosomes, we performed PCR by using polymorphic microsatellite markers. We used *D10S196*, *D10S1692*, and *D10S169* for chromosome 10; *D12S43*, *D12S90*, *D12S325*, and *D12S368* for chromosome 12; *D17S1850*, *D17S1853*, and *D17S1154* for chromosome 17; as well as *D18S66*, *D18S1148*, and *D18S554* for chromosome 18. Subsequent analysis by fluorescent in situ hybridization (FISH) of B78MC57 revealed that this hybrid contains a translocation between chromosomes 3 and 17; however, the breakpoints were not determined. We thus selected five hybrids that contained human chromosomes 10, 10p, 12, translocated 3;17, and 18 (B78MC77, B78MC75, B78MC9, B78MC57, and B78MC156, respectively). The presence of the desired chromosome was also confirmed by Alu-PCR-FISH as described previously (Bérubé et al., 1994). To characterize LN4B156 hybrids further, we used 27 human chromosome 18 microsatellite markers (Table 2). The polymorphic markers (heterozygosity > 0.8) used were: *D18S59*, *D18S63*, *D18S976*, *D18S42*, *D18S843*, *D18S542*, *D18S44*, *D18S877*, *D18S847*, *D18S1133*, *D18S67*, *D18S1157*, *D18S535*, *D18S65*, *D18S1145*, *D18S46*, *D18S487*, *D18S1109*, *D18S39*, *D18S1155*, *D18S51*, *D18S1270*, *D18S382*, *D18S541*, *D18S58*, *D18S50*, and *D18S1141* (www.invitrogen.com). Markers *D18S847*, *D18S39*, *D18S58*, *D18S50*, and *D18S1141* were noninformative, whereas markers *D18S63*, *D18S843*, *D18S1157*, *D18S1109*, and *D18S382* were not transferred. T2

RESULTS

Introduction of Human Chromosome 18 into PC-3M-LN4GP2 Cells Interferes with Their In Vitro Tumorigenic Properties

From our mouse/human microcell hybrid panel (Speevak et al., 1995), we have identified B78MC hybrids (Table 1) containing tagged human chromosomes that have been implicated in PCa pro-

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gression (chromosomes 10, 12, 17, and 18). These tagged chromosomes were transferred into highly tumorigenic PC-3M-Pro4GP1 and highly metastatic PC-3M-LN4GP2 cell lines to generate the Pro4B- and LN4B-derived hybrids (Table 1). We compared the anchorage dependence and invasiveness of our hybrids to their parental human PCa cells by using growth in soft agar and a Boyden chamber assay.

Although transfer of the translocated chromosome (3;17) into PC-3M-LN4GP2 cells could significantly decrease its tumorigenic potential (see tumor formation of LN4B57-5 in Table 1), this translocated chromosome did not significantly reduce the in vitro tumorigenic properties of the two parental cell lines (see invasion and soft-agar assays for Pro4B57 and LN4B57 hybrids; Table 1). Similarly, the in vitro and in vivo properties of the PC-3M-Pro4GP1 cells were not affected when chromosome 8 was transferred into them (data not shown), confirming that chromosome transfer per se is not sufficient to inhibit the malignant properties of these cells.

In contrast, the introduction of human chromosome 10 or chromosome 18 into PC-3M-LN4GP2 cells was accompanied by reduction of in vitro tumorigenic properties of some hybrids. Indeed, compared to their respective parental cells, Pro4B77-15 and LN4B77-44 hybrids formed about three times fewer colonies in soft agar, whereas LN4B77-9 and LN4B77-80 were significantly less invasive than PC-3M-LN4GP2 ($P < 0.001$; Table 1). Similarly, LN4B156-6 and LN4B156-14 hybrids showed significantly decreased invasiveness compared to PC-3M-LN4GP2 cells ($P = 0.0002$ and $P < 0.0001$, respectively; Table 1). However, this decrease in invasiveness was not seen in other LN4B77 or LN4B156 hybrids such as LN4B77-50, LN4B77-60, LN4B156-1, and LN4B156-2 (Table 1). Moreover, we noticed that, when cultured, LN4B156-6 cells could not be diluted more than 1:4. In comparison, parental PC-3M-LN4GP2 cells could easily sustain a 1:15 dilution. Furthermore, LN4B156-6 cells could not survive in medium containing less than 10% FBS, whereas parental PC-3M-LN4GP2 cells and LN4B156-2 hybrid cells could easily grow in medium supplemented with only 1% FBS. This further suggested that the introduction of human chromosome 18 into PC-3M-LN4GP2 cells could interfere with their growth properties.

We then quantitatively measured the ability of eight LN4B156 hybrids (LN4B156-1, -2, -3, -6, -13, -14, -16, and -20) to grow in an anchorage-independent manner. Four hybrids (LN4B156-6, -13, -16, and -20) formed statistically fewer colonies than did the parental cells ($P < 0.001$; Table 1). Although clonal variation is often seen in soft-agar assays, these highly significant results contrast with the anchorage-independent growth obtained with another geneticin-resistant PC-3M-LN4 clone, PC-3M-LN4GP1, which formed 74% as many colonies as did PC-3M-LN4GP2. Thus, the introduction of human chromosome 18 into PC-3M-LN4GP2 cells suppresses the ability of some LN4B156 hybrids to invade through Matrigel and to form anchorage-independent colonies. Although LN4B156-14 cells were not able to invade through Matrigel, they formed four times as many colonies in soft agar as did the parental PC-3M-LN4GP2 cells ($P < 0.00001$; Table 1). Such important clonal variation is similar to what was observed in chromosome 18 hybrids generated by Padalecki et al. (2003) and could indicate that the integrity of the transferred chromosome 18 was not the same in all hybrids, a characteristic often seen in microcell hybrids. Moreover, the properties of the LN4B156-14 hybrid could argue that invasiveness and anchorage-independent growth are controlled by different genes on chromosome 18.

Introduction of Human Chromosome 18 into PC-3M-LN4GP2 Cells Interferes with Their In Vivo Tumorigenic Properties

To test the in vivo tumorigenic properties of our hybrids, 1 million cells from the selected hybrids and from parental PC-3M-Pro4GP1 and PC-3M-LN4GP2 cells were injected subcutaneously into nude mice. We tested LN4B77-44 and Pro4B75-12 hybrids that contained an introduced chromosome 10 or 10p fragment, respectively. A statistically significant difference in tumor formation was obtained with the Pro4B75-12 hybrid ($P = 0.002$; Table 1). Because they displayed opposite phenotypes in the invasion and soft-agar assays, we also injected LN4B156-2 and LN4B156-6 hybrids. Indeed, LN4B156-2 hybrid cells were 75% as invasive as the parental cell line and formed as many colonies (91%) in soft agar (Table 1). In contrast, LN4B156-6 cells were 42% as invasive as PC-3M-LN4GP2 cells and formed 25 times fewer colonies (3.8%; Table 1). The few tumors (2 of 10) obtained on injection of LN4B156-6 cells grew much more slowly than those obtained by either PC-3M-LN4GP2 or LN4B156-2 cells (Fig. 1A). Indeed, F1
mice injected with PC-3M-LN4GP2 or LN4B156-2 cells became moribund much earlier (an average of 31 and 50 days earlier, respectively) than did mice injected with LN4B156-6 cells (Fig. 1C, $P = 0.05$).

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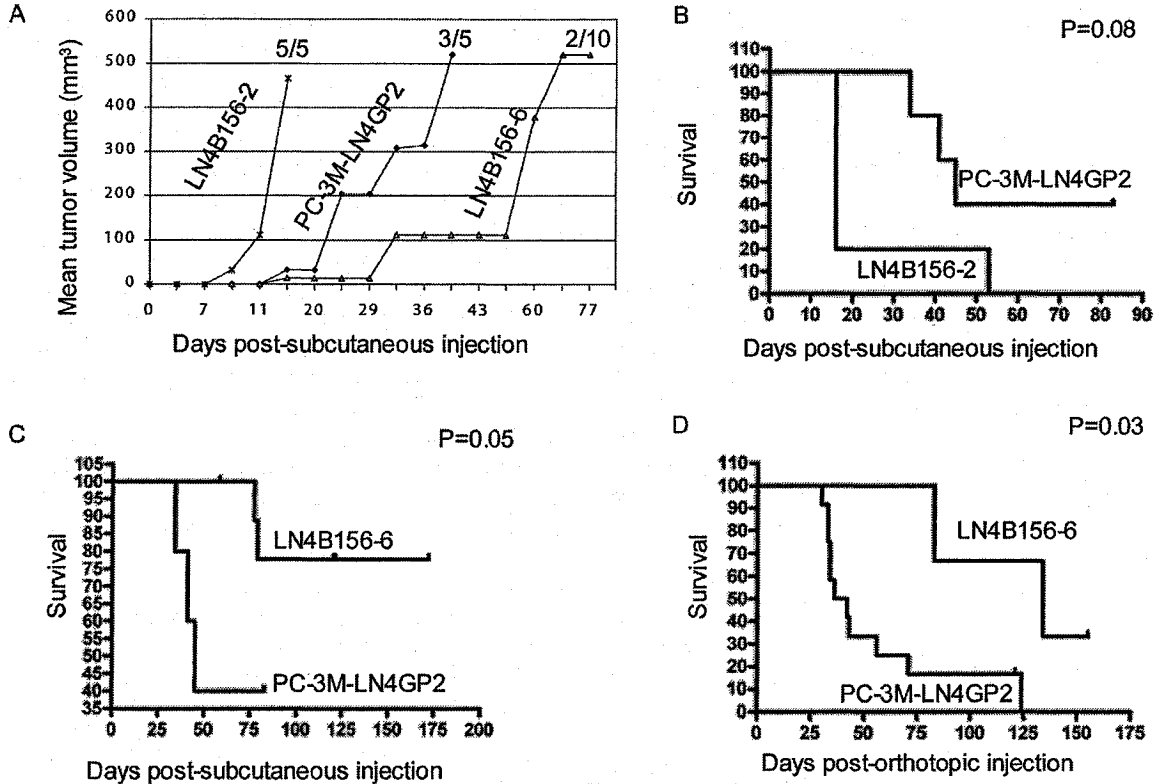

Figure 1. Subcutaneous and orthotopic injections of prostate cancer cells into nude mice. (A) Graphic representation of subcutaneous tumor growth following injections of 1×10^6 PC-3M-LN4GP2, LN4B156-2, and LN4B156-6 cells (tumor growth was measured three times a week); (B) Kaplan–Meier curves comparing survival rate (until mice became moribund) of mice upon subcutaneous injection of PC-3M-LN4GP2 cells and LN4B156-2 cells; (C) Kaplan–Meier curves comparing survival rate (until mice became moribund) of mice upon subcutaneous injection of LN4B156-6 cells; (D) Kaplan–Meier curve comparing survival rate of mice upon orthotopic prostatic injections of 1×10^6 PC-3M-LN4GP2 and LN4B156-6 cells. Mice were sacrificed as they became moribund.

Furthermore, there was no statistically significant difference in tumor growth between parental cells and the LN4B156-2 (Fig. 1B; $P = 0.08$) or LN4B77-44 hybrid containing an extra human chromosome 10 ($P = 0.54$, Table 1). This difference in tumor formation was also observed when LN4B156-6 cells were injected directly into the dorsal lobe of the prostate of nude mice. Indeed, there was a significant difference between LN4B156-6 and PC-3M-LN4GP2 cells in delay (Fig. 1D; $P = 0.03$) in orthotopic tumor growth.

Taken together, these experiments demonstrate that the chromosome 18 introduced into the LN4B156-6 cells was responsible for the suppression of their tumorigenic phenotype. However, once orthotopic tumor growth was established, both hybrid and parental cells formed regional lymph node metastases. Thus, even if LN4B156-6 cells showed delayed tumor growth, they still escaped the primary tumor mass to form metastases in retroperitoneal lymph nodes.

After sacrificing the mice, we excised and cultured the tumors from both subcutaneous and orthotopic groups. All cultured tumor cells were still expressing the green fluorescent protein gene (data not shown) and still contained polymorphic markers from the introduced chromosome 18 (TLN156 hybrids; Table 2), confirming that tumors were derived from the injected hybrid cells.

Region of Human Chromosome 18 Is Associated with Reduced Tumorigenicity of LN4B156-6 cells

FISH with a chromosome 18–specific painting probe did not reveal any major differences in the chromosome 18 introduced in LN4B156 hybrids (data not shown). We complemented this analysis by genotyping the hybrids using 27 polymorphic markers (Fig. 2 and Table 2). To identify which allele was present in the parental cell lines, we tested each marker with DNA from the B78MC156 mouse/human hybrid and from the PC-3M-LN4GP2

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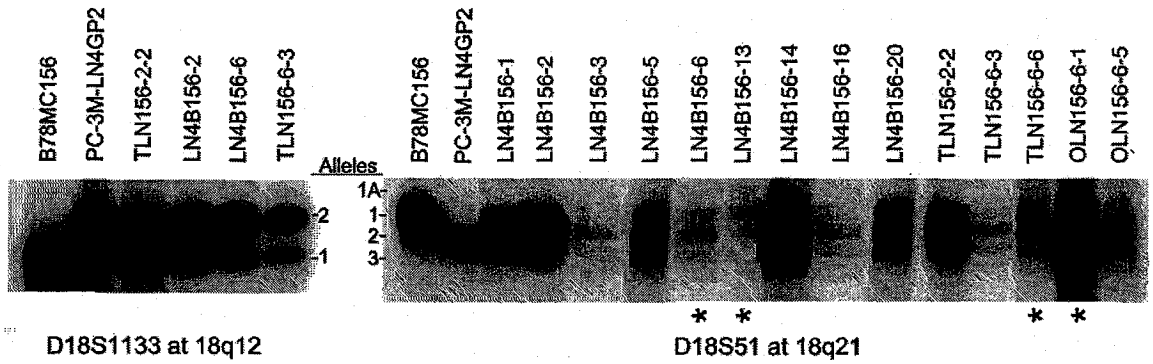

Figure 2. Genotype of *D18S1133* and *D18S51* markers in prostate cancer cells and hybrids. The left panel shows a representative autoradiogram of PCR analysis of microsatellite polymorphic marker *D18S1133* for DNA extracted from cell cultures of mouse/human hybrid B78MC156 (allele 1), human prostate cancer cell line PC-3M-LN4GP2 (allele 2), and microcell hybrids LN4B156-2 and LN4B156-6 and from tumor derived from nude-mouse injection of these two microcell hybrids (indicated by TLN156-2-2 and TLN156-6-3). The right panel shows a representative autoradiogram of PCR analysis of microsatellite polymorphic marker *D18S51* for DNA extracted from cell cultures of mouse/human hybrid B78MC156 (alleles 1 and 2), human prostate cancer cell line PC-3M-LN4GP2 (alleles 2 and 3), and microcell hybrids LN4B156-1, -2, -3, -5, -6, -13, -14, -16, and -20 and from tumor derived from nude-mouse injection [both subcutaneous (TLN) and orthotopic (OLN)] of LN4B156-2 and LN4B156-6 microcell hybrids (TLN156-2-2, TLN156-6-3, TLN156-6-6, OLN156-6-1, and OLN156-6-5). For each marker, PCR amplification was performed three times. An asterisk identifies hybrids and tumors that contain the 1A allele that is linked to the tumor-suppressor locus.

human prostate cancer cell line. Five markers (*D18S487*, *D18S39*, *D18S58*, *D18S50*, and *D18S1141*; Table 2) could not distinguish the alleles in B78MC156 from those in PC-3M-LN4GP2 cells, whereas five markers (*D18S63*, *D18S843*, *D18S1157*, *D18S1109*, and *D18S382*; Table 2) were not transferred into PC-3M-LN4GP2 hybrids.

We then tested all 27 polymorphic markers on LN4B156 hybrids to determine which regions of chromosome 18 had been transferred. As seen in Table 2 (see also *D18S1133* in Fig. 2), most markers had been transferred into both LN4B156-2 and LN4B156-6 hybrids. However, an additional allele of *D18S51* was present in the LN4B156-6 hybrid (allele 1A; Fig. 2 and Table 2). This additional allele was also present in LN4B156-13 (Fig. 2), another hybrid that formed fewer colonies in soft agar (Table 1). Interestingly, *D18S51* allele 1A was lost in one of the two tumors that eventually developed from subcutaneous injections (TLN156-6-3; Fig. 2) and in one of the two tumors occurring after orthotopic injections of LN4B156-6 cells (OLN156-6-5; Fig. 2). Allele 1A was also seen faintly in B78MC156 DNA, which could indicate that a subpopulation of these cells contained either allele 1A only or both 1 and 1A alleles. Indeed, FISH experiments with a human chromosome 18–specific painting probe showed that a small portion of chromosome 18 was present in 23% of the B78MC156 cell spreads examined. This result could explain why the allele 1A band was hardly detectable in B78MC156 cells. Moreover, direct sequencing of the *D18S51* amplification products obtained from B78MC156 DNA revealed that this cell line contains two *D18S51* alleles.

DISCUSSION

Deletions of chromosome 18 regions have been associated with many cancers, including colorectal (Salovaara et al., 2002), pancreatic (Lefter et al., 2002), ovarian (Lassus et al., 2001), head and neck squamous cell carcinoma (Blons et al., 2002), lung (Yanaihara et al., 2001), and prostate carcinoma (Latil et al., 1999; Padalecki et al., 2000; Padalecki et al, 2001; Yin et al., 2001; Chu et al., 2003). Functional evidence of tumor-suppressor genes on human chromosome 18 was demonstrated when introduction of this chromosome into pancreatic cancer cells resulted in suppression of their tumorigenic phenotype (Lefter et al., 2002). Similar results were obtained in PCa cell lines DU 145 and PC-3 (Padalecki et al., 2001, 2003). As compared to DU 145 cells, PC-3 and its derivatives likely represent a more appropriate model for study of prostate cancer. Indeed, PC-3 cells were derived from bone metastasis, a common feature seen in patients at an advanced stage of the disease. In contrast, the DU 145 cell line was derived from a brain metastasis, which rarely occurs in patients. Although PC-3 cells form metastases upon injection into the left cardiac ventricle (Padalecki et al., 2003), the use of a more aggressive PC-3-derived cell line (PC-3M-LN4) allows the study of metastatic cancer progression as it appears in human patients. Indeed, PC-3M-LN4 cells formed lymph node metastases upon orthotopic injection into the prostates of

nude mice (Pettaway et al., 1996). Thus, injecting cancer and hybrid cells orthotopically more appropriately mimics the conditions in PCa patients and further confirms that chromosome 18 is implicated in PCa progression.

The introduction of human chromosome 18 into the metastatic PC-3M-LN4GP2 cell line had a considerable effect on its tumorigenic properties. Both LN4B156-6 and LN4B156-14 hybrids had reduced capacity to invade through Matrigel, whereas LN4B156-6, LN4B156-13, LN4B156-16, and LN4B156-20 hybrids had reduced ability to grow in an anchorage-independent manner, a feature also observed by Padalecki et al. (2003) in their PC-3-derived hybrids. Furthermore, the LN4B156-6 hybrid also showed significantly delayed tumor growth upon subcutaneous ($P = 0.05$) and orthotopic ($P = 0.03$) injections into nude mice. However, in the prostate environment, LN4B156-6 cells did form proportionally more tumors (2 of 4 mice; 50%) than when injected subcutaneously (2 of 10 mice; 20%). Such results could indicate that the natural prostatic environment is a more appropriate site than under the derma (subcutaneous) for the establishment and growth of tumorigenic cells. Moreover, once established in the prostate, all tumors were also able to metastasize to lymph nodes. Upon orthotopic injection, all mice became moribund because of uremia and had to be sacrificed. As seen in Figure 1, almost all mice orthotopically injected with parental PC-3M-LN4GP2 cells became moribund in fewer than 75 days, whereas it took up to 134 days after LN4B156-6 orthotopic injections for mice to become moribund.

Thus, our results confirm and complement those of Padalecki et al. (2003) and, in addition, implicate one or more chromosome 18 tumor/metastasis-suppressor genes in PCa progression. Such suppressor genes are likely to be inactivated during the time it takes the tumors to form in an animal. Within the framework of this hypothesis, it is worth noting that LN4B156-6-tumor-derived cells (TLN156-6-3) displayed a significantly increased capability to form colonies in soft agar (409%; $P = 1 \times 10^{-7}$) compared to that of PC-3M-LN4GP2 (data not shown). As reported for nonpapillary renal cell carcinoma, such inactivation could result from chromosomal rearrangement (Yang et al., 2001). In renal cell carcinoma–derived microcell hybrids, such rearrangements often not only occur in the introduced chromosome but also affect endogenous homologous chromosomes and other polysomic chromosomes (Kost-Alimova et al., 2004).

The characterization of LN4B156 hybrids (Table 1 and Fig. 1) revealed that some hybrids had reduced tumorigenicity in vitro and in vivo (see, for example, LN4B156-6 and LN4B156-13), whereas others (like LN4B156-1 and LN4B156-2) were at least as tumorigenic as the parental PC-3M-LN4GP2 cells. Such differences could be a result of hybrids like LN4B156-2 having an introduced chromosome 18 whose suppressor gene is already inactivated or deleted. Although FISH analysis did not show any chromosomal differences between LN4B156-2 and LN4B156-6 hybrid cells (data not shown), genotype analysis of these hybrids pointed to the 18q21 region. Indeed, when compared to tumorigenic hybrids, many hybrids having a reduced tumorigenic phenotype (either in vitro or in vivo) carried an extra *D18S51* allele (allele 1A). That this allele is often lost in cancer cells derived from LN4B156-6 subcutaneous or orthotopic injections further validates the importance of this region in PCa.

Chromosome band 18q21 encodes few identified tumor-suppressor genes. Among those identified, the *DCC* gene has been associated with colorectal cancer progression, whereas the *SMAD2* and *SMAD4* genes are inactivated in pancreatic cancer. However, because these genes are rarely mutated in PCa (MacGrogan et al., 1997; Ueda et al., 1997; Latil et al., 1999; Yin et al., 2001), the involvement of these three genes in this disease is probably limited. Accordingly, a preliminary analysis using the 19k7 microarray, which contains 19,200 human genes (www.microarrays.ca/products/types.html), did not reveal any variation between PC-3M-LN4GP2 cells and LN4B156-6 cells in the expression of the *SMAD4* gene (data not shown). This further suggests that *SMAD4* is not implicated in the decreased tumorigenic phenotype observed in the LN4B156-6 hybrid and that a not-yet-identified suppressor gene could be in the 18q21 region. Moreover, these three genes are more than 10 megabases (Mb) (*DCC*, 49 Mb; *SMAD2*, 43.5 Mb; and *SMAD4*, 47 Mb away) from the *D18S51* locus, 59 Mb (www.ncbi.nlm.nih.gov/mapview/maps.cgi?taxid=9606&chr=18). Thus, it is very unlikely that these genes are implicated in the less tumorigenic phenotype of our LN4B156-6 hybrid.

Whatever its inactivation mechanism, the chromosome 18–encoded suppressor gene is likely to be implicated (directly or indirectly) in cell–cell adhesion or cell–matrix interaction because some of our LN4B156 hybrids had a decreased ability to grow in an anchorage-independent manner and to invade through Matrigel. Inactivation of the sup-

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pressor gene is unlikely to occur over 22 hr required to perform the invasion assay or even during the 21 days to perform the soft-agar assay. Such suppressive in vitro properties are similar to those reported for the *CD82* metastasis-suppressor gene (Kauffman et al., 2003) in both prostate and breast cancer cells. However, the suppressor gene in the LN4B156-6 hybrid was unable to inhibit lymph node metastasis formation once tumors had developed after orthotopic injections in the prostate. Nevertheless, at this point, we cannot rule out that this suppressor gene could also affect metastatic development because the suppressor gene is likely to be inactivated by the time the cells form tumors in mice. Such inactivation could also interfere with its putative metastasis-suppressing properties.

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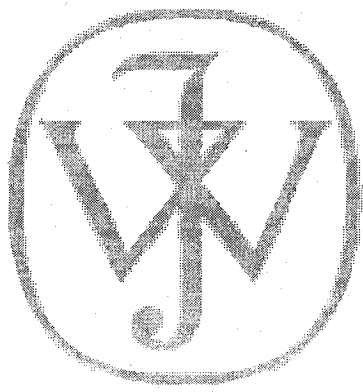
AQ1: Note that human genes are designated with capital letters in italics (including numbers) and that the symbols should only contain Latin letters and Arabic numerals (i.e., no Greek letters or Roman numerals) and that there are no dashes or punctuations within the gene designation. All genes should be italicized. Proteins are designated as genes but are not italicized.



Author Proof

APPENDIX D:

Permissions of Manuscript 2 co-authors:
ERK4, a new candidate tumor suppressor gene implicated in prostate cancer progression
(Chapter 4)

APPENDIX E:

Permission from Manuscript 3 co-author:

Report of the first SNP located in the coding sequence of the *ERK4* gene (Chapter 5)

APPENDIX F:

Radiation Safety Permits and certificate