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Novel Genes Involved in Tumor Progression

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Institut für Genetik

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Isolierung und Charakterisierung von neuen Genen, die in die Tumorprogression involviert sind

Zusammenfassung

Der Ausdruck Tumormetastasierung beschreibt die Streuung von Tumorzellen aus einem Primärtumor und die Bildung von Tochtertumoren an entfernten Stellen des Körpers. Trotz der immensen Bedeutung der Tumormetastasierung in der klinisch-onkologischen Praxis ist die molekulare Biologie dieses pathophysiologischen Vorgangs noch immer unverstanden. Hierfür gibt es mehrere plausible Gründe: Zum einen unterscheiden sich die möglichen Metastasierungswege verschiedener Tumortypen, zum anderen ist der Vorgang der Dissemination von Tumorzellen im Körper sehr komplex und erfordert vermutlich Änderungen in der Expression einer Vielzahl von Genen.

Ziel der vorliegenden Arbeit war es, einen möglichst umfassenden Überblick über die mit dem metastatischen Phänotyp einer Tumorzellinie verbundenen Änderungen in der Genexpression zu gewinnen. Als Modellsystem wurden dabei nicht-metastatische und metastatische Zellinien aus dem Ratten-Pankreas-Adenokarzinom Bsp73 gewählt. Durch molekulare Subtraktion der exprimierten mRNAs aus der nicht-metastatischen Tumorzellinie Bsp73-1AS von der mRNA aus der metastatischen Tumorzellinie Bsp73-ASML wurden solche Gene identifiziert, deren Expression in der metastatischen Tumorzelle hochreguliert ist. Die Analyse der subtraktiven Genbank ergab, daß sowohl eine Reihe bekannter Gene als auch eine große Zahl unbekannter Gene isoliert wurden. Durch Analyse der Expression dieser bislang unbekannten Gene in anderen metastatischen Rattentumoren konnten diejenigen Klone identifiziert werden, deren Expression nicht nur im Bsp73-Modellsystem, sondern auch in anderen Ratten-Tumorprogressionsmodellen mit dem metastatischen Phänotyp korreliert.

Unter den isolierten bekannten Genen befindet sich eine ganze Reihe bekannter Tumorprogressionsgene. Ihre Isolierung aus der metastasen-spezifischen, subtraktiven Genbank darf daher als Beweis für den Wert des gewählten experimentellen Ansatzes gelten. Neben diesen bekannten Genen wurde auch eine Vielzahl bekannter Gene identifiziert, deren Assoziation mit dem metastatischen Phänotyp bislang noch unbekannt war, deren funktionelle Beteiligung am Metastasierungsprozeß aufgrund der ihnen zugeschriebenen Eigenschaften jedoch naheliegt. Eines dieser Gene, Ezrin, wurde daher im Detail auf seine Funktion bei der Disseminierung von Bsp73-ASML - Zellen hin untersucht. Der Beweis für die funktionelle Beteiligung des Ezrin-Proteins am Tumor-Phänotyp von Bsp73-ASML - Zellen wurde durch Überexpression einer dominant-negativen Form des Ezrin-Proteins erbracht: ASML-Zellen, die dominant-negatives Ezrin überexprimieren, zeichnen sich durch vermindertes Tumorwachstum *in vivo* aus.

Abstract

The term tumor metastasis describes the formation and growth of a secondary tumor at a distant site from the primary tumor mass. Despite the immense importance of tumor metastasis in the context of a clinical setting, the molecular events that drives this pathophysiological process remains largely not understood. There are a number of plausible explanations for this fact. Firstly, the possible mechanisms by which tumor cells metastasize are different from one tumor cell type to another, and secondly, the dissemination of tumor cells in the body is extremely complex and probably requires changes in the expression status of many genes.

The primary goal of this work was to derive a comprehensive and near total overview of the alterations in gene expression that accompanies the metastatic phenotype. As a model system a metastatic and non-metastatic cell line originating from a rat pancreatic adenocarcinoma Bsp73 were taken. Through molecular subtraction, between expressed mRNA transcripts from the non-metastatic Bsp73-1AS line and the metastatic Bsp73-ASML cell line genes were isolated that were exclusively expressed in the metastasizing cell line. Sequence analysis of the isolated genes revealed that a large proportion were known genes, however an equally large proportion were novel. By analysing the expression of these novel genes in other metastatic rat cell lines, clones were identified whose expression was not restricted to the Bsp73-model system, but whose expression correlated with the metastatic phenotype in other tumor progression models. From the known genes isolated many were already known tumor progression genes. The isolation of such genes from a subtractive library indicates that such an experimental approach is highly valid. Alongside the known genes were genes whose association with a metastatic phenotype had not yet been described, however for some, their documented function would suggest a putative involvement in the metastatic process. The functional role played by one such gene, ezrin in the dissemination of Bsp73-ASML tumor cells was studied in detail. The proof that ezrin was indeed functionally involved in the tumor phenotype of Bsp73-ASML cells, was obtained through the overexpression of a dominant-negative mutant form of ezrin. ASML cells overexpressing dominant-negative ezrin demonstrated *in vivo* a dramatic reduction in tumor growth.

INDEX

		Page
1	**INTRODUCTION**	1
	PART ONE	1
1.1	The Genetic bases of Cancer Progression	1
	oncogenes vs Tumor Suppressor Genes	2
1.2	The Genetic bases of Colorectal Carcinogenesis	3
1.2.1	Hereditary Colorectal Carcinogenesis	3
1.3	The Genetic Bases of Breast Cancer Metastasis	5
1.3.1	Hereditary Breast Cancer	5
1.3.2	Sporadic Breast Cancer	6
	(a) proto-oncogen activation in Breast Cancer	6
	(b) Tumor suppressor Genes in	7
	The Oestrogen Receptor	8
1.4	Cancer: Mechanisms of Invasion and Metastasis	8
1.4.1	The Metastatic Cascade	9
1.4.2	Molecular Prossess Involved in Metastasis	10
1.4.3	Angiogenesis in Tumor Formation	11
1.4.4	Adhesion. Invasion and Motility of Tumor Cells	13
1.4.5	Intergrins in Tumor progression	13
1.4.6	Cadherins in Tumor Progression	15
1.4.7	Acquisition of Invasive Characteristics Mediated by the Secretion of proteases	16
	(a) Metalloproteinases	16
	(b) Urokinase Plasminogen activator	18
1.4.8	Motility Factors Involved in Tumor Cell Migration	20
1.4.9	Patterns of Metastatic Spread of Tumorigenic Cells	23
1.5	Genetic Aspects of Invasion and Metastasis	24
1.5.1	NM23 in Metastasis and Oncogenic Development	25
1.5.2	Metastasis-Related mts-1 Gene	26
	PART TWO	
1.6	Current Strategies and Methodologies that Allow Identification	
	of Differentially Expressed Genes	28
1.7	Project Outline and Specific Aims	29

2 MATERIALS & METHODS 31

2.1 **MATERIALS** 31

Oligos 31

Antibodies 31

2.2 **GENERAL METHODS** 32

Phenol/Chloroform extraction of nucleic acids 32

isolation/Purification of DNA from agarose gels 33

(a) Electrophoretic isolation of DNA 33

(b) Direct isolation from agarose gels

(c) DNAeasy kit(Biozyme) DNA isolation grom agarose gels 33

Precipitation of nucleic acids 33

Determination of nucleic acid concentration 34

Restriction endonuclease digest of DNA 34

DNA ligation 34

(a) Ligation of linkers/adaptors 34

(b) Ligation of PCR products 34

(c) Sub-cloning 34

Polymerase Chain Reaction (PCR) 34

Reverse transcription Polymerase Chain Reaction (RT-PCR) 35

Radioactive labelling of DNA/cDNA probes 35

Isolation of total RNA from tissue or human tumor material 35

Isolation of Poly $(A)^+$ RNA 36

First and second strand cDNA synthesis 36

(a) First strand synthesis using SuperScript™II for RT-PCR 36

(b) One-tube second strand synthesis 37

Mini-prep plasmid preparation from bacteria 37

(a) Standard method 37

(b) Wizard Mini-prep kit (promega) 38

Large scale plasmid preparation from bacteria 38

Preparation of competent bacteria (E.coli) 39

(a) Chemical competent E.coli (Calcium chloride method) 39

(b) Electrocompetent E.cloi 39

Transformation of bacteria 39

(a) Chemically 39

(b) Electroporation 39

Sequencing of double-stranded template DNA 40

(a) [a-^{33}P]-ddNTP method 40

(b) ™Vistra DNA sequencer 725 40
Generation of a subtracted library by SSH 41
Evaluation of subtraction efficiency 41
Cloning into TA vector 42
Reverse-northern high density blots and screening 42
Subtracted clone amplification and reverse northern screening 43
Coupled transcription/ translation in vitro (TNT) 43
Cell Culture and Transfections 44
Cell lines 44
Cell culture 44
Stable transfection of cells 45
(a) DOTAP liposomal transfection protocol 45
In vivo inoculation of eucaryotic cells 45
Human tumor samples 45
2.3 **ANALYTICAL METHODS** 46
Northern blot hybridisation 46
Southern blotting of ds cDNA and PCR products 46
Immunoblot analysis of proteins (Western blot) 47
(a) Separation of proteins by polyacrylamide gel electrophoresis (PAGE) 47
(b) Preparation of protein probes 47
(c) Coomassie blue staining of resolved proteins 47
(d) Electrophoretic transfer of resolved proteins onto membrane and subsequent antidoby detection 47
Immunoprecipitation of proteins 48

3 RESULTS

PART ONE

3.1 The Model System 49
3.2 Confirmatiom of the Metastatic Potential of the Bsp73 Cell Lines 50
3.3 Subtractive Suppression Hybridization (SSH) 51
3.4 Generation and Subtraction Efficiency Monitoring of the Bsp73-1AS/ASML Subtractive Library 54
3.4.1 Reverse Northern Screening of the Bsp73-1AS/ASML Subtracted Library 56
3.4.2 Confirmation of Differential Status by Northern Analysis 58
3.4.3 Sequence Identity of Isolated Differentially Expressed Clones 60
3.4.4 cDNA Subtraction of Bsp73 AS versus ASML Identifies Known Tumor Progression Genes 67

3.4.5	Functional Classification of Identified Genes	68
3.5	Identification of Subtracted Genes in other Cell Systems	70
3.5.1	Reverse Northern Screeing Identifies Common Genes in other Tumor Progression Cell Systems	71
3.5.2	Direct Northern Analysis Prooves Correlative Expression of Subtracted Clones	75
3.5.3	Identification of Subtracted Clones Expressed in Human Tumors	80
	PART TWO	
3.6	Functional Analysis of Rat Full Length Ezrin in the Bsp73 Model System	84
3.6.1	Full Length Cloning of Rat Ezrin from the ASML Cell Line and the Generation of a Dominant Negative Mutant Form of Ezrin	85
3.6.2	Generation of ASML Clones Expressing Dominant-Negative Ezrin	91
3.6.3	Dominant-negative ezrin reduces tumor formation capacity of ASML cells *in vivo*	92
4 DISCUSSION		94
4.1	Tumor Progression: A Change of Paradigms	95
4.2	Attempts to Identify Genes Involved in Tumor Progression	95
4.2.1	At the Genomic DNA level (DNA-mediated gene transfer)	95
4.2.2	At the Protein Level: Differential Antibody Screening	96
4.2.3	At the mRNA /cDNA Level: Subtractive cDNA Cloning	96
4.3	Technologies for Expression Profiling and Difference Analysis	97
4.4	Aim of the Work and Choice of the Model System	98
4.5	Proof of Concept: Subtractive cDNA Cloning as a Rapid Way to Identify Molecular Determinants of Metastasis	99
4.6	From Gene Expression to Protein Function	102
5 RESUME´		102
REFERENCES		104
ACKNOWLEDGMENTS		

Abbreviations

^{o}C	Degrees Centrigrade
APS	Ammonium-Persulfate
ATP	Adenosin-Triphosphate
bp	Base Pair
BSA	Bovine-Serum-Albumin
cDNA	copy DNA
cm	Centimeter (10^{-2} meter)
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DMEM	Dulbeccos-Modified-Eagles-Medium
DMSA	Dimercaptobernsteinsäure
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
ds cDNA	Double-strande copy DNA
DTT	Dithiothreitol
ECL	Enhanced Chemoluminiscence
ECM	Extracellular Martix
EDTA	Ethylendiamine-N,N-tetracetate
et al	and others (Lat. *et alii*)
f.c.	final contration
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
g/lt	Gramms per litre
h	hour
HEPES	N-2-Hydroxyethylpiperazine-N´-2-ethansulfonic acid
kb	Kilobase (1kb=1000bp)
kDa	Kilodalton (10^{3} daltons)
Kv	Kilovolts
M	Molar
mRNA	Messenger RNA
mA	Milliampere
mg	Milligram (10^{-3} gram)

min	Minute
ml	Milliliter (10^{-3} l)
µg	Microgram 10^{-6} gram)
µl	Microliter (10^{-6} l)
mM	Millimolar (10^{-3} molar)
µM	Micromolar (10^{-6} molar)
NAD^+	Nicotinamide adenosin dinucleotide (oxidized form)
ng	Nanogram (10^{-9} gram)
nm	nano meter (10^{-9} meter)
OD	Optical Density
O/N	Over Night
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
P/C/I	Phenol/Choloroform/Isopropanol
PCR	Polymerase Chain Reaction
pg	Picograme (10^{-12} gram)
ppt	Precipitate
RSV	Rous-Sarcom-Virus
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR
s.c	Subcutaneous
SDS	Sodium-Lauryl-Sulfate (sodium dodecyl sulfate)
sec	Second
SSC	Sodiumchloride-Sodiumcitrate
SSH	Surpression Subtractive Hybridization
TBE	Tris-Borate-EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	Tris-(hydroxymethyl)-aminomethane
U	Unit(s)
UV	Ultra-voilet light
vols	Volumes

1.INTRODUCTION

The general tendency of the global predisposition of cancer in most forms is on an increase as is our knowledge of the underlying genetic dysregulations of cancer cells. It is also apparent that the manifestation of a fully malignant tumor involves the progressive acquisition of mutations and epigenetic abnormalities in multiple genes (Liotta & Stetler-Stevenson, 1985). Due to the large number and enormous diversity of function of these genes, it is becoming clear that the two classical categories "oncogenes" and "tumor suppressor genes" are not adequate because they fail to indicate the specific biochemical functions of the individual genes or to consider the cellular contexts in which they function. As a consequence the need to dissect the specific molecular function of certain genes in the global frame work of total cellular function is of increasing importance. Over one hundred different oncogenic alleles generated from cellular proto-oncogenes by point mutation, gene amplification, or chromosomal translocation have been identified (Bishop, 1991; Rabbitts, 1994) and more than a dozen tumor suppressor genes have been identified and the existence of an ever greater number has been suggested through various studies (Weinberg, 1991; Knudson, 1993). For many of these genes a causual involvement in tumor progression has been demonstrated and although these studies have provided useful insights, they also typify the inadequacy concerning the orchestrated action of multiple genes in the instigation of tumor cell development and often the poor extrapolation of findings obtained from various rodent models into a clinical setting highlights the shortcomings of these models.

PART ONE

1.1 The Genetic bases of Cancer Progression

Although we know intricate details of the molecular genetic dysfunctions that underlie cancer, this has not translated into directed therapeutic strategies that result in substantial cures. This is analogous to sickle cell disease where the defect has been known for many years but still today there exists no effective therapy for the disorder.

Virtually every human cancer can occur in genetically predisposed individuals. The most striking form of genetic susceptibility involves mendelian dominant inheritance with high penetrance and appearance of cancer at earlier than usual age, as shown for colon cancer in persons with familial adenomatous polyposis (FAP). In this example, the heterozygous state of the germ-line mutation imparts a high risk for just one form of cancer, while in other examples, such as the Li-Fraumeni syndrome (LFS), it predisposes to several kinds of cancer, although never to all.

Colorectal and breast cancers account for a significant proportion of deaths due to malignant neoplasia in the industrialised world (Wingo et al., 1995). As a result these two forms of cancer

will be considered in detail concerning the dysregulation of known proto-oncogenes and tumor suppressor genes that have been functionally implicated in the manifestation of these two cancers types.

Oncogenes vs Tumor Suppressor Genes.

Over 100 oncogenes have to date been identified and the list is expanding. Proto-oncogenes are genes present in normal eucaryotic organisms whose exons are homologous to cancer-causing oncogenes in acutely transforming retroviruses or eucaryotic genes demonstrated to induce neoplastic transformation in transfection assays (reviewed by, Garrett & Sell, 1995). They participate in normal growth and proliferation, encoding a wide variety of proteins and fall primarily into one of five categories: (1) growth factors (e.g., *sis*, TGF-α); (2) growth factor receptors (e.g., *fms, c-erbB-1, c-erb-B2, c-met, kit, ret,* and *sea*); (3) molecules involved in signal transduction composed of cytoplasmic protein kinases (e.g., *src, raf, abl, fes, lck,* and *yes*); (4) GTP-binding oncogenes (e.g., H-, k-, and N-*ras, gip2,* and *gsp*), and (5) the transcriptional regulators, which makes up the largest group (e.g., *erbA-1, erbA-2, ets1/2, fos, jun, myb, rel, ski, c/L/N-myc, lyt-10, E2A, PBX-1, rhom-2,* and *Tgt-2*).
The alterations that converts a proto-oncogene can take several forms. For example a mutation (base substitution) as is in the case of *ras* , (reviewed by Barbacid, 1990), resulting in altered GTP-binding or GTPase activity, so that there is extended stimulation of effector molecules. Gene amplification is frequently observed in *N-myc* in neuroblastomas and *c-erb-2* in breast cancers (King et al., 1985). A third mechanism for conversion occurs when the oncogene is moved (or translocated) to a different region on the same chromosome, or to an entirely different chromosomal site. One of the most thoroughly studied translocations is the t(9;22) (q34;q11) or the Philadelphia chromosome, which occurs in 95 % of chronic myelocytic leukemia (CML; Weinberg, 1997). The cellular ABL gene on chromosome 9 translocates to chromosome 22, where it combines with the BCR gene in a head-to tail configuration, resulting in a hybrid BCR-c-ABL protein with tyrosine phosphokinase activity that can transform hematopoietic cells (McLaughin et al., 1985). As oncogenes tend to promote growth by increasing proliferation, or by allowing the cell to bypass growth inhibitory signals, there exits molecules that appear to have apposing actions these are the so called "tumor suppressor genes". Both oncogenes and tumor suppressor genes work in symmetry in that they function on opposite sides of the road, one promoting, the other stemming, cell proliferation. However there also existed a marked asymmetry as germline determinants of human cancer were found to be mutant alleles of tumor suppressor genes. The mutation mechanisms would inactivation tumor suppressor genes (TSG) resulting in loss of gene function. Usually, both alleles of TSGs are lost, inactivated or damaged, resulting in either loss or attenuation of the gene product which was necessary in suppressing the neoplastic phenotype. The two best characterised TSGs, are the retinoblastoma (RB1; Marshall, 1991) gene and p53 (Nigro et al., 1989; Hollstein, 1991). Other identified TSGs include Wilms tumor gene (WT1; Francke et al.,

1979), deleted in colorectal cancer (DCC; Fearon et al., 1990), adenomatosis polyposis coli gene (APC; Groden et al 1991), type 1 neurofibromatosis gene (NF1; Latif et al., 1993), multiple endocrine neoplasia gene (MEN; Larsson et al., 1988) and the BRCA1 and 2 breast cancer genes (Marshall, 1991). Some of these onocgenes and anti-oncogenes will be considered further in the pathogenetic mechanisms of two prevailing cancer types in our society

1.2 The Genetic Bases of Colorectal Carcinogenesis

In no other form of cancer are the genetic dysregulations that accompanies and predisposes to a major cancer of adults so well understood as that of colorectal cancer. It accounts for around 98 % of all malignant tumors of the large bowel and although most tumors are sporadic, several inherited colorectal cancer syndromes are recognised. The most common of these are the familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). The peak of incidence is in the seventh decade, with 20 % of cancers diagnosed before the age of 50 (HMSO. Mortality Statistics, 1992). The incidence of the disease in the UK is about 30,000 cases per year, resulting in 17,000 deaths per annum. Average 5-year survival is about 50 %, a figure that has improved only modestly over the past 40 years. The genetic features of FAP are discussed below.

1.2.1 Hereditary Colorectal Cancer

Following the path demarcated by germline mutations in identified FAP patients and somatic alterations in sporadic colorectal tumors, lead to the identification of the adenomatous polyposis coli gene (APC) which was subsequently shown to be the cause of FAP (Groden et al., 1991; Nishisho et al., 1991). Now there is good evidence that just two mutations are required to initiate the growth of a colorectal tumor, and in most cases, these mutations probably occur at the APC locus (5q21-q22) (Powell et al., 1992). This was deduced from studies in humans with documented FAP, whereby the rate limiting step in tumor initiation is a somatic mutation of the wild-type allele inherited from the unaffected parent (Levy et al., 1994; Luongo et al., 1994). This provided strong evidence for the "two-hit" hypothesis of Knudson, originally proposed to explain the familial and nonfamilial incidences of childhood tumors such as retinoblastoma and Wilms´ tumor (Knudson, 1993). APC is a 312 kDa protein comprising of 2850 amino acids and is coded for by a gene spanning several Mb (Groden et al., 1991; Kinzler et al., 1991). APC mutations, which almost always results in a truncated APC protein (Miyoshi et al., 1992) or take the form of allele loss (Solomon et al., 1987), are found in about 70 % of sporadic colorectal cancers (Powell et al., 1992; Miyaki et al., 1994) and <1 % of FAP patients. Additionally, APC mutations appear to be present in the earliest neoplastic lesions that can be examined (jen et al., 1994). Such lesions, called dysplastic aberrant crypt foci (ACF), are believed to be the precursors of adenomas. Are APC mutations always the first events in tumorigenesis, or do mutations of other genes suffice? Actually, such mutations do

not efficiently initiate the neoplastic phenotype. For example, p53 is altered in >80 % of colorectal carcinomas (Baker et al., 1990), yet patients with germline mutations of p53 do not develop colorectal cancer (Garber et al., 1991). Therefore, though it is apparent that p53 is involved in colorectal tumor progression, it is equally apparent that it cannot initiate the process in a fashion similar to APC.

Likewise, the *ras* protein is also commonly mutated in colonic tumors as they progress (Vogelstein et al., 1988; Shibata et al., 1993). However, *ras* mutations that occur in normal epithelial cells do not appear to lead to colorectal cancers (Jen et al., 1994). Furthermore, cells which exhibit *ras* mutations are amazingly common and form foci of hyperproliferating cells (Pretlow et al., 1993).

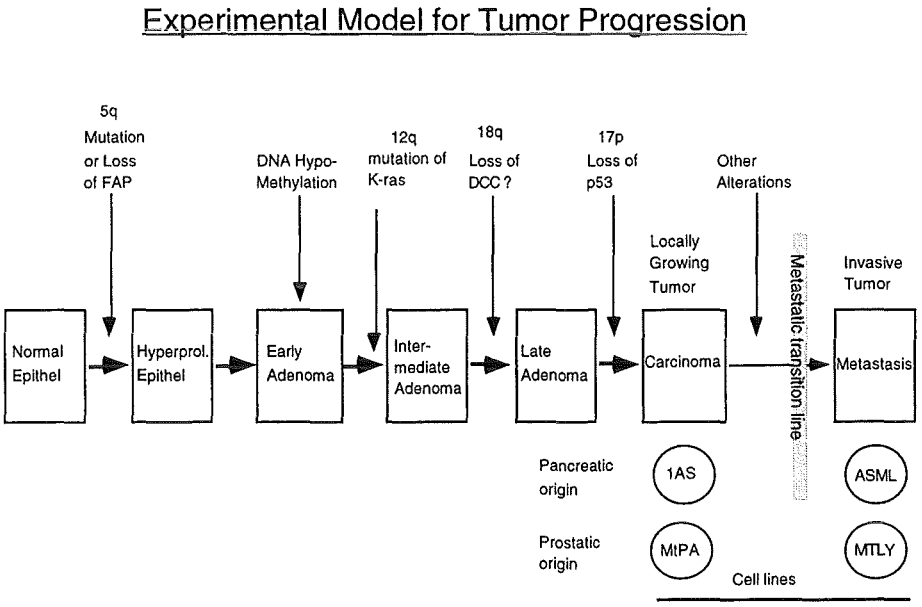


Fig 1. A genetic model for colorectal tumorigenesis.
Tumorigenesis is believed to proceed through a series of genetic alterations, the most common of which include 5q, 17p, and 18q are indicated together with the relative timing of the chromosome loss event. The three stages of adenomas in general represent tumors of increasing size, and dysplasia. In patients identified with FAP, the APC gene locus located on chromosome 5q is mutated and inherited in a dominant fashion. This mutation in turn may contribute to the hyperproliferative epithelium present in these patients. *Ras* gene mutations appears to occur in one cell of a pre-existing small adenoma and through clonal expansion produces a larger and more dysplastic tumor. Allelic deletions of chromosome 17p and 18q usually occurs at a later stage of tumorigenesis than is seen with 5q. However, the order of alterations is not invariant, and accumulation of these changes rather than their order with respect to one another, is of greatest significance. Taken from Fearon & Vogelstein, 1996.

Yet, these hyperplastic cells, unlike their dysplastic counterparts with mutant APC genes, have little or no potential to form tumors and may even regress through apoptosis (Shpitz et al., 1996). K-*ras* mutations occur in 50 % of colorectal tumors, mostly in adenomas larger than 1 cm, and often such adenomas have already altered APC function. In addition, a specific set of K-*ras* mutations have been identified in colorectal tumors (Burmer et al., 1990) suggesting that these may be specific to promoting tumorigenesis.

Point mutation of one allele of the p53 gene coupled with loss of the remaining wild-type allele appears to occur frequently in colorectal tumors, although such loss is relatively infrequent in adenomas at any stage (Baker et al., 1989; Delattre et al., 1989; Nigro et al., 1989). With the accumulating evidence from *in vitro* transformation systems pointing to a tumor suppressor function for the p53 protein, (Eliyahu et al., 1989; Finlay et al., 1989), a hypothesis arises whereby the wild-type p53 gene inhibits colorectal tumor growth, and removal of the wild-type p53 gene is the selective pressure underlying progression in these tumor types. The subsequent loss of the wild-type p53 allele is often associated with the progression from adenoma to carcinoma, and probably amplifies the growth advantage provided by the mutation.

Another common region of allelic loss is that of 18q, which is lost in more than 70 % of carcinomas (Delattre et al. 1989; Vogelstein et al., 1989). The candidate tumor suppressor gene in this region has been identified (Fearon et al., 1990) and is termed deleted in colorectal cancer (DCC). The gene exhibits high homology to the cell adhesion molecule N-CAM, and its expression is reduced or largely absent in the majority of colorectal carcinomas (usually before malignancy occurs). Characterised mutations in DCC usually produce truncated, non-functional proteins, however, no hereditary condition in which DCC is mutant is known. Recently, there has been doubt that allele loss of 18q may actually target a gene different from DCC (Thiagalingam et al., 1996).

So in light of the above facts, what emerges is a picture that suggest it is not simply the order of these alterations, but rather it is the accumulation of mutations, that determines the propensity for neoplasia (see figure 1) and that a specific subset of genes can initiate the process of tumorigenesis.

1.3 The Genetic Bases of Breast Cancer Metastasis

Breast carcinoma is the most common malignancy in women in North America and Western Europe and several environmental and epigenetic factors have been demonstrated to influence the development of breast cancer. These include exposure to therapeutic and contraceptive hormones, age of manarche and menopause, first pregnancy, and even breast feeding (Lipmman & Dickinson, 1988). In addition, the alteration of certain proto-oncogenes and tumor suppressor genes has been documented in the pathogenesis of beast cancer progression.

1.3.1 Hereditary Breast Cancer

The majority of breast cancers are due to the acquision of somatic mutations, with only 5 to 8 % of breast cancer patients having a strong family history, indicative of inheritance of mutations in an autosomal dominant fashion (Hall et al., 1990). Approximately, one half of genetically inherited breast cancer cases appear to be linked to a gene on 17q21 named BRCA1 (Miki et al., 1994), which encompassed over 100-kb composed of 24 exons encoding

a DNA binding zinc-finger protein. A second breast cancer susceptibility gene, designated BRCA2 and which is thought to account for much of the remaining genetically linked cases of breast cancer, has been localised to chromosome 13q12-13 (Wooster et al., 1995). Unlike BRCA1, the BRCA2 locus does not appear to be linked to ovarian cancer development (Hosking et al., 1995). Hogervorst et al. (1995) screened for mutations in exon 11 which encodes 61 % of BRCA1, using a protein truncation test but found no hard evidence that BRCA1 is important in sporadic breast cancers. Additionally, loss of heterozygosity (LOH) of BRCA1 and BRCA2 in sporadic cancers using PCR-based fluorescent DNA technology revealed little prognostic value (Beckmann et al., 1996).

1.3.2 Sporadic Breast Cancer

(a) Proto-oncogene Activation in Breast Cancer

Studies on *c-myc* in breast cancer have generally supported an important role for this proto-oncogene in the molecular pathogenesis of breast cancer (Bonilla et al., 1988). Tumorigenicity of the breast cancer cell line SW613-S has been reported to directly correlate with the degree of *c-myc* amplification (Lavialle et al., 1988), and overexpression of *c-myc* in primary breast tumors has been associated with increase frequency of lymph node involvement (Guerin et al., 1988; Berns et al., 1992). Although these studies suggest that overexpression and amplification of *c-myc* in breast cancer are an adverse finding, evidence confirming *c-myc* as an independent prognostic indicator has not been demonstrated.

A second proto-oncogene that has been widely evaluated as a prognostic index in breast cancer is *c-erbB-2*. This oncogene was first identified through transfection studies of a rat neuroblastoma and encodes a 185 kDa transmembrane glycoprotein with extensive homology to the epidermal growth factor receptor (EGFR; Coussens et al., 1985). Amplification of the gene is found in 20-30 % in invasive carcinomas (Varley et al., 1987; Borresen et al., 1990), and a correlation has been demonstrated between amplification and aggressive features and poor short term prognosis, although not by all (Clark & McGuire, 1991). Additionally, many investigators have found *c-erdB-2* overexpression to be an independent predictor of poorer disease free interval and survival (Lovekin et al., 1991; Winstanley, et al., 1991).

Two other proto-oncogenes regarded as potentially important in the molecular pathogenesis of human breast cancer are *int-1* and *int-2*, which are located adjacent to each other on chromosome 11q (Lidereau et al., 1988; Tsuda et al., 1990) These genes belong to the family of fibroblast growth factor related genes and evidence has been presented that amplified expression of *int-2*/FGF3 is seen in 10-20 % of breast cancers. An association was also found with the oestrogen receptor (Fanti et al., 1990) although others found that amplification was concomitant with poor prognosis (Borg et al., 1991; Henry, et al., 1993). More recently, another oncogene CCND1 was located at 11q13 and encodes cyclin D1 (Xiong et al., 1993). This, when complexed with its associated cyclin-dependent kinase, controls cell cycle progression in G1 by phosphorylating the retinoblastoma (RB) protein (Sherr, 1994). Over-

expression of cyclin D1 has been found in breast cancer cell lines in both the presence and absence of amplification and dysregulation of cyclin D1 could hold promise as a factor in the manifestation of breast cancer (Buckley et al., 1993). Gillet et al. (1996) could later show, using immunohistochemical detection of D1 and the oestrogen receptor, that cyclin D1 was a very good indicator of likely response to endocrine therapy.

(b) Tumor Suppressor Genes in Breast Cancer

Studies to identify specific tumor suppressor genes associated with breast cancer has not advanced to the degree achieved in colon cancer. The retinoblastoma gene (RB1) is the classical example of a tumor suppressor gene, and has been located on 13q14 (Friend et al., 1986) and encodes for a 105 kDa protein. This gene product in its under-phosphorylated state restricts cell cycle progression in G1, by direct interaction with transcriptional factor-2 (E2F; Chellappan et al., 1991). Alterations to chromosome 13q have been found at several loci in breast carcinomas (Lundberg et al., 1987) as have structural alterations in the RB gene (T´Ang et al., 1988). Of significance was that alterations were found either in advanced cases (Varley et al., 1989) or in aneuploid, high S phase cancers (Borg et al., 1992), suggesting that alterations to RB are not an initiating event in breast cancer but is an event occurring in an unstable genome.

Evidence for the involvement of p53 in the pathogenesis of breast cancer arises from several lines of evidence. Kindred's with the Li-Fraumeni syndrome (Malkin et al., 1990), in which a germ-line mutation occurs in one of the p53 alleles, are characterised by young onset sarcoma associated with breast cancer, primary brain tumor or leukaemia in a first degree relative under the age of 45 years. Interesting is the fact that only about half the Li-Fraumeni families have p53 mutations (Santibanez-Koref et al., 1991), and germ-line mutations are rarely found in cases of early onset breast cancer and those with strong family history (Sidransky et al., 1992; Warren et al., 1992). There is however an association between the presence of mutations and aggressive features within breast carcinomas e.g., lack of oestrogen receptor (Anderson et al., 1993b), and a high S-phase index (Merlo et al., 1993). Allred and colleagues examined tissue from 700 breast cancers of auxiliary lymph node-negative patients and found a significant association between overexpression of p53 and early death in node-negative breast cancer cases (Allred et al., 1993). Besides being of value for the prediction of prognosis, p53 can aid in the selection of therapy. Bergh et al. (1995) found adjuvant tamoxifen therapy to be of less value in p53 mutation lymph node positive cases. Moreover, response to chemotherapy and radiotherapy can be affected by altered p53 function, due to its role in regulating DNA damage response (Lowe et al., 1993).

In addition to the dysregulation of known oncogenes and tumor suppressor genes that have been documented in the manifestation of breast cancer, other molecules such as the oestrogen receptor (ER), epidermal growth factor receptor (EGFR; Klijn et al., 1992; Nicholson et al.,

1994) and certain growth factors (TGF-β; Dalal et al., 1993; Walker et al., 1994) have all been implicated in breast cancer.

(c) The Oestrogen Receptor

The evaluation of the oestrogen receptor (ER) status of a breast cancer has been used for over 20 years to aid the determination of likely response to hormonal therapy. The receptor can be detected in 60-70 % of breast cancers, of whom a half (i.e. a third overall) will respond to hormonal manipulation (Barnes & Millis, 1995). While the main stream of research has focused on the role of (ER) in the clinical management of breast cancer, some very intriguing observations have arisen. For example, (i) why are a proportion of carcinomas ER negative? (ii) why do some ER positive cancers fail to respond to endocrine therapy? and (iii) why do a small number of ER negative tumors respond to endocrine therapy? A number of alterations in ER have been identified that may contribute to explaining some of the above questions. For example, ER gene methylation of CpG islands in ER negative breast cancers leads to transcriptional block of the gene (Lapidus et al., 1996) and therefore lack of ER protein. Fuqua et al. (1992) identified in ER positive tumors a truncated ER lacking exon 7, which was unable to induce transcription and which functioned in a dominant negative manner with respect to normal ER function. These observations might offer insight into ER positive tumors that fail to react to hormonal manipulation.

1.4 Cancer: Mechanisms of Invasion and Metastasis

Metastasis, which is the spread of tumors throughout the body, is a pathophysiological process of profound clinical significance. The relentless and often intractable nature of metastatic spread can have a servere psychological impact on cancer patients (Hersh, 1985) and the overt manifestation and initial presentation of cancer usually occurs at a late stage in the disease process when the capacity for invasion has already been unleashed. By the time of diagnosis, a high proportion of patients have occult or clinically detectable metastasis and this often leads to treatment failure despite successful control of the primary tumor. From a clinical perspective, how metastasis develops, while of scientific interest, is not a critical issue. Most patients (50-75 %), depending on which tumors are included, have either regional or distant metastasis (Liotta & Stettler-Stevenson, 1985). Patients who do not have evidence of metastasis on presentation, generally undergo what is, presumptively, curative surgery. Although indications of how widespread a malignancy is at the time of presentation is directly relevant to the clinician's choice of strategy, knowledge of how the metastases developed offers little insight about control.

The difficult challenge remains the treatment of metastases and one might say that cancer is a disease that pushes out the limits of conventional methodology and thought. What is clear is that the propensity for metastasis formation varies significantly within different tumor entities and that a large multitude of widely varying gene products, whose expression status changes

on a temporal and spatial basis, forms the bases of a series of remarkably complex interactions between the cancer cell and its microenvironment. However, despite this apparent complexity, the process of metastasis from a solid primary tumor can be segregated to some degree into a number of discrete stages some of which, under an appropriate experimental setting and be separately investigated.

1.4.1 The Metastatic Cascade

It is an old truism that metastasis accounts for the majority of cancer deaths in industrialised countries. In these societies, the most frequent cancers are derived from epithelia of the gastrointestinal and urogenitary tract, as well as of mammary ducts and bronchi. Yet despite improved methods of diagnostics, mortality has not substantially decreased essentially because patients continue to succumb to metastatic disease. This clearly points to dissemination of tumor cells at an early stage of tumor growth, and implies that the acquisition of at least some characteristics of metastatic behaviour can occur prior to attainment of unrestrained growth observed in fully developed tumors. In this context, it is important to consider that tumorigenesis and metastasis development are not necessarily the result of the same genetic changes (Fidler & Radinsky, 1990; Liotta et al., 1991). In fact, defined mutations in proto-oncogenes often confer tumorigenic characteristics to cells while their metastatic potential is not activated (Price et al., 1989). This is not surprising in light of the fact that the characteristics of these two processes can, at least in part, be separated. Development of unrestricted growth is focused on the acquisition of growth factor independence and loss of contact inhibition while development of metastasis is focused on enhanced cell motility, expression of certain proteases and alterations in the expression of cell and matrix adhesion molecules (Hart et al., 1989).

Neoplastic transformation is now recognised to occur because of the progressive accumulation of mutations in genomic DNA within clones of cells. These mutations may enhance expression of genes that either increase cellular growth, mobility and angiogenesis, or suppress expression of genes that depress cellular replication or promote cell differentiation (Fearon et al., 1990). To equate cancer with uncontrolled growth, however, is a gross oversimplification. Cancer may be succinctly defined as the loss of growth and spatial control (Schwartz, 1993).

In the classic descriptions the initial carcinogenic events give rise to benign (non-invasive and non-metastatic) tumors that then "progress" to more malignant phenotypes. This view was first described by Foulds, 1953, and was based on histological examination of tumor specimens. He reasoned that if the same lymph-node metastasis contained a group of well, poorly and undifferentiated cells, then the latter populations had to derive from the well-differentiated cells. Some 24 years later, Nowell, (1976) proposed that the carcinogenic event induces a "genetic liability" that "permits stepwise selection of variant sublines and underlies tumor progression". Progression means selection of variants with increasing genomic and phenotypic abnormalities that in turn lead to tumor heterogeneity. The implication has been

that "genomic instability" (a term whose precise meaning is difficult to define) produces heterogeneity and progressively selects for the more malignant and diverse cells whose inherent heterogeneity enhances their malignant potential. Now, it is generally accepted that tumors arise from clonal expansion of a single or few transformed cells (Fialkow, 1976; Innaccone et al., 1987) and that this monotonous cell population eventually gives rise to multiple subpopulations. By the time a tumor is clinically detectable, it most likely consists of diverse subpopulations that differ in characteristics, such as response to growth factors (Poste & Greig 1982). For example, the response of most epithelial cells to transforming growth factor-β (TGF-β) is decreased proliferation and/or a more differentiated phenotype. Escape from the growth inhibitory effects of TGF-β is a hallmark of many transformed cells (Huggett et al., 1991; Steigerwalt et al., 1992). Additionally, tumor heterogeneity facilitates tumor survival because it minimises the likelihood that a single insult will eradicate all subpopulations (Heppner, 1989).

Although the precise mechanisms that regulate phenotypic diversity are unknown, it has been proposed that interactions of tumor cell populations maintain phenotypic diversity at an "equilibrium" level (Poste et al., 1981). Thus the concept that evolves is one of a highly integrated "tumor ecosystem" in which a heterogeneity "set-point" maintains a certain degree of heterogeneity among tumor cell populations in each patient. The tumor heterogeneity is so dynamic, that at the moment of definition (whether a tumor is invasive or metastatic), the tumor contains an undefined number of cells capable of expressing invasive and metastatic phenotypes.

1.4.2 Molecular Processes Involved in Metastasis

As noted before, the metastatic cascade encompasses a sequence of events that requires the production of different groups of molecules for each step. These molecules may be synthesised either by the metastatic cell or by host cells collaborating with the metastasising cells. Most generally (see figure 2) the process involves, after the initial transforming event, either unicellular or multicellular neoplastic cells grow progressively (if a tumor mass is to exceed 2 mm in diameter, extensive vascularisation must occur). Several angiogenic factors play key roles in establishing a neocapillary network from the surrounding host tissue.

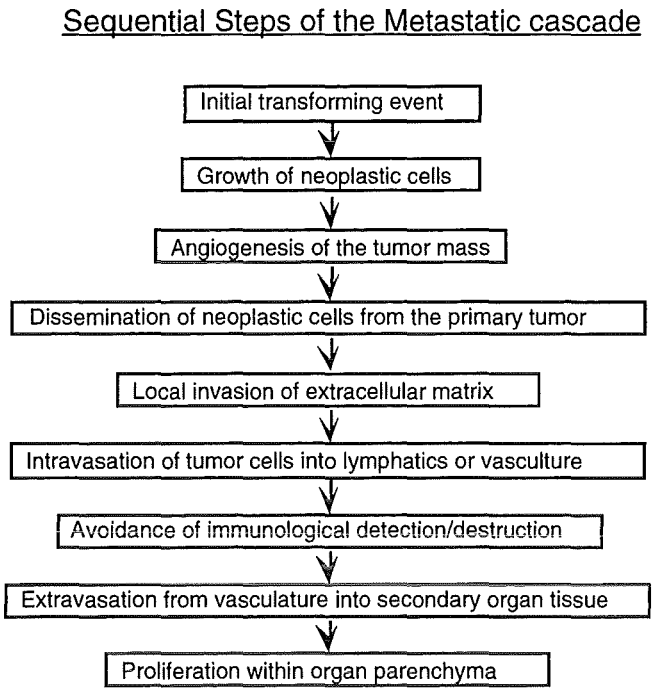


Fig 2. The individual steps that make up the metastatic cascade.
After an initial transforming event there are a total of eight separate steps recognized that must be successfully completed such that a secondary neoplasms results. Failure at any stage will prohibit metastasis and further more, metastatic cells that display low efficiency to carry out one particular step are not compensated by their high efficiency to carry out other downstream steps in the cascade. Details are provided in the text.

Once vascularisation occurs, local invasion of the host stroma by some tumor cells could occur by several mechanisms that are not mutually exclusive: thin walled venules, like lymphatic channels, offer very little resistance to penetration by tumor cells and provide the most common pathways for tumor cell entry into the circulation. Detachment and embolization of small tumor cell aggregates occur next; those that survive the circulation must then arrest in the capillary beds of organs. Extravasation then occurs, probably by the same mechanisms that influence initial invasion. Finally, proliferation within the organ parenchyma completes the metastatic process. To produce detectable lesions, these metastases must develop their own vascular network, evade the host immune system, and respond to organ-specific factors that influence their growth. Once they do so, the cells can invade host stroma, penetrate blood vessels, and enter the circulation to produce secondary metastases, the so-called metastasis of metastases (Fidler, 1995). Most of these stages that orchestrate a network of co-operative interactions will be considered separately and in the context in which they take place.

1.4.3 Angiogenesis in Tumor Formation

Although tumors 1-2 mm in diameter can receive all nutrients by diffusion, further growth is strictly dependent on the development of an adequate blood supply through angiogenesis (Folkman, 1987, 1989). Angiogenesis describes the process of new blood vessel formation

from parent micro vessels. Under physiological circumstances, angiogenesis is observed during embryonic development and growth (O´Rehilly, & Müller, 1992), and almost never occurs in adulthood except under strictly controlled conditions and cyclically in the ovarian follicle, corpus luteum and post-menstrual endometrium (Christenson et al., 1996; Modlich et al., 1996; Risau, 1995). Pathological situations accompanied by this process are wound healing and inflammation (Kovacs & DiPietro, 1994), ischemia (Harada et al., 1994), diabetic retinopathy (Frater-Schroder et al., 1987) and of course tumor growth (Folkman, 1995).

Most tumors in humans persist for months to years without neovascularization until a subset of tumor cells acquires an angiogenic phenotype. The switch is a deliberate change in the regulation of angiogenesis and many angiogenic signals have been identified that activate endothelial cells. Both stromal cells and tumor cells produce an enormous and complex assortment of angiogenic factors such as VEGF and TGF-β, both of which allow the tumor to recruit macrophages through their potent chemotractant properties. Macrophages themselves induce angiogenesis by secreting TNF-α (Leibovich et al., 1987). Additionally tumors secret proteases and heparanases which mobilize and activate angiogenic molecules stored in the ECM, such as bFGF, aFGF, SF/HGF and VEGF. Substances such as prostaglandins PEG1 and PEG2 (From et al., 1983) and degradation products of hyaluronic acid (West et al., 1985) are also capable of stimulating angiogenesis. PD-ECGF (platelet derived endothelial cell growth factor) induces angiogenesis but not by direct action on endothelial cells but via its thymidine phosphorylase (dThdPase) activity (Finnis et al., 1993). PD-ECGF is produced mainly by tumor infiltrating macrophages (Takahashi et al., 1996) whereas, dThdPase activity is up-regulated in tumor cells (Haraguchi et al., 1994).

Up-regulation of angiogenic factors, however is not sufficient in itself for a tumor cell to become angiogenic, and it is becoming evident that there are different classes of endogenous inhibitors of endothelial growth and motility that work in concert with inducer molecules to control angiogenesis. Reducing the concentration of inhibitor or increasing that of inducer results in an angiogenic switch. The tumor suppressor protein p53 depresses VEGF-A production and supports the secretion of thrombospondin-1 (TSP-1), a multi functional 450kDa glycoprotein which is a major constituent of platelet alpha granules. It functions as a angiogenesis inhibitor that modulates endothelial cell adhesiveness, motility and proteolytic activity, by sequestering angiogenic inducers. (Dameron et al., 1994; Van Meir et al., 1994). Thus, p53 mutations induce angiogenesis, which is a pivotal component of tumor progression.

In 1995 Folkman and co-workers published the first findings of an endogenous peptide that displayed antiangiogenic properties. Angiostatin (as it is now known) is a 38 kDa internal fragment of plasminogen and contains the first four "kringle domains" of plasminogen. It has a half life of 2.5 days in the blood and accumulates in the circulation in the presence of certain growing primary mouse tumors but disappears upon excision of the tumor mass. (O´Reilly, et al 1995). Later it was demonstrated in certain animal tumor models to suppress primary tumor growth and consequently metastasis (Holmgren, 1996) by inducing a state of "dormancy". Its

mode of action remains unclear but it is imagined that it acts as a decoy molecule and occupies the catalytic site of enzymes that proteolytically cleave plasminogen, thus preventing matrix remodelling required for angiogenesis.
The same group in 1997 described another member, endostatin, a 20 kDa carboxy-terminal fragment of collagen XVIII, that elicited even more potent antiangiogenic effects *in vivo* (O´Reilly, 1997). To date other peptide fragments have been shown to possess intrinsic antiangiogenic properties including a 6 kDa fragment of platelet factor 4 (PF4), and peptides from laminin, epidermal growth factor and TSP-1 (reviewed in Hanahan & Folkman, 1996).
Based on these findings, the principal target for antiangiogenic therapy is represented by proliferating endothelial cells. However it is important to distinguish between antiangiogenesis and vascular targeting. The former interferes with a wide range of biological processes involved in growth, migration and differentiation of blood vessels. The latter provides rapid destruction and cell death of the vessels, infarcting large areas of the tumor (reviewed in Fan et al., 1995).

1.4.4 Adhesion, Invasion and Motility of Tumor Cells

The molecular characterisation of diminished adhesive properties, invasiveness and sensitisation to motility factors of primary tumor cells has led to the identification of checkpoints that constitute intervention targets. As a consequence all three points will be considered in more detail.
A simplistic tale of sequences of events leading to dissemination of tumor cells would begin with the escape of cells from their local environment. For this to occur, homotypic and heterotypic adhesion bonds of tumor cells in their immediate surrounding must be actively modified. Cell adhesion molecules (CAMS) represents a very large group of cell surface structures that are involved in cell-cell and cell-matrix interactions. They can be divided structurally into four superfamilies, namely the cadherins, intergrins, immunoglobulins and other adhesion molecules.

1.4.5 Integrins in Tumor Progression

Integrins are a growing class of heterodimeric receptors composed of a common β subunit noncovalently associated with a variety of a subunits that confer ligand specificity, although there is redundancy at both the level of receptor and ligand. In total 16 α and 8 β subunits have been identified, which can result in over 20 different receptor types (reviewed by Hynes, 1992), and alternative splicing adds further complexity. These integral plasma membrane cell surface receptors are responsible for cell-ECM and cell-cell interactions, but above this they are also implicators or complex signal transduction events, including modulation of intercellular pH, activation of specific tyrosine kinases and alterations in inositol lipid metabolism (reviewed by Hynes, 1992; Schwartz, 1992). More recent evidence demonstrates that the integrins provide key survival signals for endothelial and epithelial cells

(Meredith et al., 1993; Frisch & Francis, 1994). Detachment from the ECM results in anoikis, however this can be reverted by transformation of the cells with v-Src or treatment with hepatocyte growth factor/scatter factor (HGF/SF) (Frisch & Francis, 1994). These observations support the notion that resistance to apoptosis correlates with acquisition of an anchorage independent state and / or increased motility.

Exhaustive studies have implicated the role of β_1 subclass of intergrin in tumorigenesis. Through transfection of Chinese hamster ovary (CHO) cells with cDNA coding for the $\alpha_3\beta_1$ fibronectin receptor inhibits their ability to grow in soft agar, to establish tumors in nude mice and to migrate (Giancolti & Rouslahti, 1990). In counter distinction, clonal sublines of erythroleukemia cells (Symington, 1990), or CHO cells expressing decreased levels of fibronectin receptor (Schreiner et al., 1991), are characterised by increased tumorigenicity. In human melanomas, up regulation of the expression of certain integrins, including $\alpha_2\beta_1$, $\alpha_3\beta_1$ and, $\alpha_6\beta_1$ has been shown to correlate with metastatic potential (Danen et al., 1993), or progression of the disease (Natali et al., 1993). For several integrin receptors whose functions can be competed by synthetic Arg-Gly-Asp (RGD) peptides, the addition of such peptides inhibits both *in vitro* and *in vivo* melanoma cell invasion (Gehlsen et al, 1988; Yamada, 1991). Experiments with the human melanoma cell line A375M have revealed that stimulation of the $\alpha_V\beta_3$ vitronectin receptor resulted in increased expression of the 72 kDa type IV collagenase (gelatinase A) (Seftor et al., 1992) and that this integrin is elevated in melanoma cells (Gehlsen et al., 1992). More recently, a highly metastatic melanoma cell line C8161 with little $\alpha_V\beta_3$ surface expression was found to increase expression of type IV collagenase in response to stimulation of $\alpha_5\beta_1$ but not $\alpha_V\beta_1$ (Seftor, 1993). Changes in integrin profiles also have been documented for both breast cancinoma and prostate carcinoma which show a down-regulation of $\alpha_5\beta_1$ (Zutter et al., 1993) and β_4 subunit (Knox et al., 1994), respectively. Consequently, it would appear that alterations in adhesive properties by changing of integrin expression profiles of tumorigenic cells in their immediate vercinity, could reflect a higher propensity for invasion as they are more motile.

In normal cells focal adhesion kinase (FAK) is the major tyrosine phosphorylated protein present in cells upon integrin activation (Kornberg et al., 1992; Guan & Shalloway, 1992). Phosphorylation of FAK creates a high affinity binding site for the SH2 domain of the Src family kinases and the recruitment of Src and / or Fyn to the FAK complex. The formation of this bipartite kinase complex is important in the tyrosine phosphorylation of other downstream substrates in intergrin signalling (Jewell et al., 1995). Successful phosphorylation of FAK and activation of MAP kinases requires the existence of an organised actin cytoskeleton (Guan, 1991; Chen, 1994). The small GTP binding protein *rho* regulates ECM cytoskeletal organisation (Ridley & Hall, 1992), and inactivation of *rho* by ADP-ribosylation, also inactivates FAK activity (Kumagai et al., 1993), indicating that *rho* is in the pathway of FAK activation by external stimuli (Ridley & Hall, 1994). These perturbations in *rho* activation could

be envisaged to be involved in malignant transformation due to uncoupling of the integrin-*rho* pathway (McGlade et al., 1993).

1.4.6 Cadherins in Tumor Progression

These constitute a family of cell surface molecules that generally mediate cell-cell and cell-matrix calcium dependent homophilic adhesions, although E-cadherin can also interact with an intergrin ($\alpha_E\beta_7$) expressed on a subset of lymphocytes (Cepek et al., 1990). Thus, cadherins may function outside of the narrow range of interactions previously assigned to them. Structurally, cadherins are transmembrane gylcoproteins with an extracellular region composed of four repeat domains, a transmembrane portion and a long cytoplasmic domain. The cytoplasmic domain of functional cadherins require association with accessory cytoplasmic proteins (Ozawa et al., 1989), namely the catenins, of which exists four types being α, β, γ and more recently, $p120^{cas}$ (Reynolds et al., 1994; Shibamoto et al., 1995), and this association is required for full adhesion activity (Takeichi, 1991; Kawanishi et al., 1995). Although there are numerous different cadherins, E (epithelial)-cadherin, P (placental)-cadherin and N (neural)-cadherin are the best characterised (reviewed by Gumbiner, 1988; Grunwald, 1993). E-cadherin, also known as uvomorulin, L-Cam, cell-CAM120/180 or Arc-1 is the best characterised in the context of neoplasia and metastasis.

The initial observation that a monoclonal antibody directed against E-cadherin altered the morphology of Madin-Darby Canine Kidney (MDCK) epithelial cells so as to acquire an invasive phenotype, was the first evidence that this molecule could be important in tumor invasion (Vestweber & Kemler, 1985; Behrens et al., 1985; Behrens et al., 1989) Concomitant with this acquisition of invasive capacity was a loss of E-cadherin expression seen in a variety of epithelial carcinoma cell lines (reviewed by Behrens, 1993). Although in some tumor cells E-cadherin may be normally expressed, the dysfunction or mutation of β-catenin may result in a cell-cell disengagement with consequent invasiveness (Kawanishi et al., 1995). There are however few *in vitro* studies showing the relationship between E-cadherin, catenins and tumor cell motility and invasiveness. Manipulations of E-cadherin (Vleminckx et al., 1991; Navarro et al., 1991; Doki et al., 1993), α catenin (Shimoyama et al., 1992), β-catenin (Kawanishi et al., 1995) and γ catenin by means of antibodies, gene deletion or mutation, genetic engineering, microinjection and antisense provides evidence that abnormalities of these proteins facilitate tumor cell dissociation and increase motility and invasiveness. Indeed, studies on human cancers ranging from liver tumors (Slagle et al., 1993), ovarian tumors (Veatch et al., 1994), invasive breast carcinomas (Glukhova et al., 1995), pancreatic cancer (Pignatelli et al., 1994) and renal cancers (Katagiri et al., 1995) confirm the relationship between these molecules and metastasis. Most recently, Guilford et al. 1998, could show that germ-line mutations in the gene coding for E-cadherin, leading to a truncated gene product, probably contributes to the molecular basis for familial gastric cancer. The adenomatous polyposis coli (APC) tumor suppressor gene codes for a protein that normally associates with catenins and

E-cadherin (Rubinfeld et al., 1993; Su et al., 1993). Mutations in APC occur in the germline of patients with familial adenomatous polyposis (FAP) and as a somatic event in more than two thirds of sporadic colorectal carcinomas. Despite being an early event in colorectal carcinogenesis, APC abnormalities may lead disruption of normal cell-to-cell adhesion, thereby contributing to the malignant phenotype.

1.4.7 Acquisition of Invasive Characteristics Mediated by the Secretion of Proteases

Invasion and metastasis largely determine the clinical course of tumors. As a rule, cell proliferation in most normal sedentary tissues is under strict control; cells respect specific boundaries and do not spread to distant sites. Malignant tumors also grow locally but they invade tissues other than their tissue of origin. Nevertheless, most tumor cells do not metastasise but remain within the primary tumor, held in place by their inability to overcome cell-cell interactions or interactions between cell surface receptors and basement membrane structures (Bresalier, 1994).

It should be noted that invasion is not metastasis but represents a crucial prerequisite for metastasis to occur. Rather invasion is the active translocation of neoplastic cells across tissue boundaries and through host cellular and extracellular matrix barriers (Duffy, 1992; Tryggvason et al., 1987). Invasion is not due to growth pressure but involves additional genetic deregulation over and above those molecular events that cause uncontrolled proliferation. That is, it is the tendency of malignant cells to cross tissue boundaries which distinguishes proliferative disorders and carcinomas *in situ* from true malignancy.

If one assumes that the malignant tumor cell is inappropriately expressing a pre-existing normal cell program of physiological invasion, then the fundamental difference between normal and malignant cells is regulation. The difference must lie in the proteins that start, stop, or maintain the invasion program at times and places that are totally inappropriate for non-malignant cells (Nicolson, 1991). However proteolysis of tissue barriers is not restricted to malignant processes and numerous examples of physiological invasion exist including, amongst others, smooth muscle cell migration from the media to the intima (Bendeck et al., 1994), nerve growth cone extension and homing (Wu & Goldberg, 1993), and trophoblast implantation (Lola & Graham, 1990).

(a) Metalloproteinases

For invasion to take place, subsequent release of a large cohort of proteolytic enzymes of differing substrate specificity must occur in a direct and controlled manner. Degradation of basement membrane and interstitial stroma followed by tumor cell traversal must be completed on three separate occasions during the transition from *in situ* to invasive carcinoma (Duffy, 1992): escape of tumor cells from the primary site and both intravasation to and extravasation from the vasculature. As the ECM is a highly complex and specialised, semielastic, continuous

structure composed of mainly various collagens, glycoproteins and proteoglycans (Timpl, 1989), by logical inference, metastatic cells require sufficient degradative enzymatic capacity to break down these proteinaceous structural barriers (Barsky et al., 1983). Implicated repeatedly, are the matrix metalloproteinases (MMPs) of which currently 16 members have been identified and have been associated with the malignant phenotype for several decades (DeClerk et al., 1997; Stack et al., 1998). However, from both rodent model systems and histopathologic assessment of patient material, correlations between protease levels and metastatic competence are not universal (Duffy, 1987. and 1990). Identified degrading enzymes include MMPs, plasmin, heparanases, urokinase plasminogen activator (uPA), cathepsins and tumor associated trypsinogen (TAT) 2, but only MMPs and the uPA/PAI system will be considered.

MMPs are a family of secreted or transmembrane proteins in a latent form (proenzymes) that require extracellular proteolytic cleavage for activation. They share a catalytic domain with the HEXGH motif responsible for complexing zinc required for catalytic function (Windsor et al., 1994) in addition to a PRCGVPD sequence in the pro-domain, which maintains latency of the zymogen (reviewed by Birkedal et al., 1993; Powell et al., 1996). There are three main subgroups of MMPs, identified by their substrate preferences: (1) the collagenases degrade fibrillar collagens, (2) the gelatinases are particularly potent in degrading denatured and basement membrane collagens (gelatin), and (3) the stromelysins degrade proteoglycans and glycoproteins. Activation of proforms of MMPs or serine proteases can occur through proteolytic cleavage by other pre-activated MMPs. For example, plasmin activates most MMPs by making one cleavage in the prodomain which initiates the autoactivation of the MMP. (Eeckhout & Vaes, 1997; Santibanez & Martinez, 1993). The MMP matrilysin can act on urokinase to liberate the NH_2-terminal fragment containing the receptor binding domain (Marcotte et al., 1992), which could effect subsequent cell surface activation of urokinase. These activations although documented in *in vitro* have not been suitably demonstrated *in vivo* (He et al., 1989; Marcotte et al., 1992).

Once MMPs are activated they are susceptible to inhibition by the general serum proteinase inhibitor α2-macroglobulin and by a family of naturally occurring specific tissue inhibitors of metalloproteinases (TIMPs; Woesner, 1991). There are currently four members of the TIMP family, each capable of inhibiting MMPs by forming tight stoichiometric, noncovalent complexes with either proenzyme or the activated enzyme (DeClerck & Imren, 1994), or by controlling their autocatalytic activation (DeClerck et al., 1991; Howard et al., 1991). The first member of the MMP family to be cloned was transin, the rat homologue of stromelysin-1, which was identified as an oncogene and growth factor inducible gene (Matrisian et al., 1985). Later it became clear that many oncogenes can regulate MMP levels, and to date all but one MMP family member has been shown to contain an AP-1 transcription element in their promotor (Gaire et al. 1994) which has been implemented in many cases to be involved in mediating the response (Gutmann & Wasylyk, 1990).

Evidence that MMPs play a functional role in metastasis came originally from experiments with recombinant or genetically manipulated levels of TIMP-1. Schultz et al, 1988, first showed that an intraperitoneal injection of recombinant TIMP-1 reduced lung colonisation of intravenously injected B16F10 melanoma cells. A reduction in TIMP-1 levels by antisense RNA in mouse fibroblasts resulted in formation of metastatic tumors in nude mice (Khokha et al., 1989), and targeted disruption of the TIMP-1 gene resulted in an increase in *in vitro* invasion (Alexander et al., 1992). Conversely, overexpression of TIMP-2 in c-Ha-Ras transfected rat embryo cells inhibited tumor growth (DeClerck et al., 1992). Transfection of TIMP-2 in the highly metastatic melanoma cell line M24met, that expresses both MMP2 (gelatinase A) and MMP9 (gelatinase B), results in reduced growth potential *in vivo* and in *in vitro* collagen gels, but their metastatic ability remains unaffected (Montgomery et al., 1994). Other transfection studies have demonstrated that forced expression of gelatinase A in a bladder cancer cell line increased the area of lung metastasis (Kawamata et al., 1996), and a transmembrane domain MMP, MT1-MMP (also known as MMP14) when overexpressed enhanced the survival of mouse lung carcinoma cells in the lungs of intravenously injected mice (Tsunezuka et al., 1996).
However the most definitive evidence for a role for MMPs in tumor cell establishment comes from studies in which levels of a specific MMP were manipulated. Stromelysin-3, originally isolated from the surrounding malignant breast carcinomas (Basset et al., 1990), when overexpressed in human breast carcinoma cells or ablated by anti sense RNA in mouse fibroblasts, demonstrated altered tumorigenicity in nude mice. However, growth of established tumors, their invasive capacity or their metastatic competence was completely unchanged (Noel et al., 1996). Collagenase expression in the skin of transgenic mice resulted in earlier onset and increased numbers of papillomas arising after chemical initiation and promotion (D´Armiento et al., 1995) and expression of stromelysin-1 in mammary glands of transgenic mice resulted in the development of aggressive malignant mammary tumors (Sympson et al., 1995). Most recently, mice in which the gene for gelatinase A (MMP-2) had been inactivated through targeted disruption, show a dramatic reduction of tumor progression and angiogenesis when B16-BL6 or Lewis lung carcinoma cells were implanted intradermally (Itoh et al., 1998). How MMPs and their inhibitors contribute functionally to metastasis is very difficult to address experimentally, and many initial conclusions have been derived by inference rather than direct experimentation. Nevertheless, in light of the above facts, MMPs expression can promote tumor take and that certain MMPs may favour cancer cell survival in a tissue environment initially not permissive for tumor growth. Yet ultimately it is the subtle balance of MMPs verses their inhibitors that will dictate the outcome.

(b) Urokinase Plasminogen activator

The plasminogen activator (PA)/plasmin system has been significantly characterised at the biochemical level in the context of tissue remodelling and particularly in malignancy (for reviews see Danø et al., 1985; Kwaan, 1992). There exists two known mammalian PAs,

tissue type PA (tPA) and urokinase type PA (uPA), both of which are serine proteases with narrow specificity cleaving the ubiquitous zymogen plasminogen and thereby generating the broad acting serine protease plasmin. This in turn can degrade fibrin, fibronectin and laminin and is also able to contribute to matrix degradation by activating some latent MMPs. Like MMPs, the PA/plasmin system is tightly controlled by specific inhibitors, including the PA inhibitors, PAI-1 and -2 and α2 antiplasmin, all of which belong to the serpin superfamily.

Studies with *in vitro* assays provide substantial evidence for an important role of the u-PA system in cell migration and invasion at the level of individual cells. The situation in tumors is more complex as non-malignant cells also may be migratory and invasive during processes of cancer cell-directed tissue remodelling (Dvorak et al., 1995). Using the model of dissemination of human tumors in nude mice, Ossowski et al. (1991) reported that antibodies against human uPA prevented local invasion of cancer cells, while lung metastasis was not inhibited. Quax et al. (1991) using the same approach, reported a correlation between cancer cell u-PA expression and lung metastasis with a series of human melanoma cell lines. Transfection of rat PC3 prostate carcinoma cells with uPA cDNA enhanced their metastatic potential *in vivo* when inoculated into ventricles of rats (Achbarou et al., 1994). Gene targeting of u-PA demonstrated that u-PA$^{-/-}$ mice exhibited a drastically reduced progression to malignant melanomas when challenged (Shapiro et al., 1996).

Early studies established that the level of u-PA in malignant tumors is significantly higher than in the corresponding normal tissue or in benign tumors of the same tissue type (reviewed by Duffy, 1996). Indeed, u-PA was the first proteinase shown to be a prognostic marker in human malignancies (Duffy et al., 1988). He showed that patients with breast tumors containing high levels of u-PA enzyme activity had a significantly shorter disease-free interval than patients with tumors with low levels of activity. Later, high u-PA antigen levels were found to correlate with a shortened overall survival (Duffy et al., 1990). These results have been substantiated by many groups (Jänicke et al., 1990; Spyratos et al., 1993; Fernø et al., 1996). u-PA antigen levels appear to be among the strongest prognostic factors so far described for breast cancer. Using both univariate and multivariate analysis, u-PA is at least as strong a marker as nodal status and better than other prognostic indices such as tumor size, oestrogen receptor status and cathepsin D levels (Duffy et al., 1990; Schmitt et al., 1990; Jänicke et al 1993).

Apart from breast cancer, u-PA has been shown to be of prognostic value in other malignancies, including cancers of the lung (Oka et al., 1991), stomach (Nekarda et al., 1994), colorectum (Mulcahy et al., 1994), cervix (Kobayashi et al., 1994), ovary (Kuhn et al., 1994) and brain (Bindahl et al., 1994). As with u-PA, its molecular inhibitor PAI-1 was demonstrated to be also a prognostic marker in breast cancers (Jänicke et al., 1993). In node-negative women, PAI-1 was almost as strong a prognostic marker as u-PA. Combining u-PA and PAI-1, patients with tumors containing low levels of both molecules had a very small risk of relapse (93 % disease-free survival at 3 years) in contrast to those patients with high amounts of both (55 % disease-free survival at 3 years). The cellular receptor for u-PA, u-PAR also holds

prognostic value in certain cancers, but constitutes an independent marker (Ganesh et al., 1994), and again, high levels are indicative of poor outcome. Interestingly, u-PAR on the surface of cells is mainly localised in caveolae (Stahl & Mueller, 1995), flask-shaped micro-invaginations of the plasma membrane that clusters cell surface receptors and signal transduction molecules in a characteristic lipid environment (Anderson, 1993a; Lisanti et al., 1994). The function of caveolae is currently not well understood, but the structural integrity of caveolae appears to be required for efficient plasmin generation on the cell surface. Evidence has been presented that uPA has other substrates, for example, hepatocyte growth factor/scatter factor (HGF/SF) and macrophage-stimulating protein (MSP) are secreted from cells as inactive single-chain pro-forms. They become active as growth factors after conversion to 2-chain forms by limited proteolysis (Donate et al., 1994). uPA is able to catalyse this conversion, at least in the case of HGF/SF (Naldini et al., 1992; Mars et al., 1993). This rises the possibility of tumorigenic cells being capable of activating their own HGF/SF and thereby increase their propensity to migrate. Additionally, HGF/SF-MET signalling has been shown to significantly increase the protein levels of both uPA and its receptor uPAR in certain cell types (Jeffers et al., 1996), through the induction of the ETS1 transcription factor (Fafeur et al., 1997). These data couples HGF/SF-MET signalling to the activation of proteases that mediate dissolution of the ECM, a critical step in the metastatic cascade (Vande-Woude et al., 1997).

The overall picture that is painted concerning *in vitro* data generally implicates u-PA as being able to enhance cell migration and invasion. However a growing body of evidence suggests that the function of the system does not rely on the unbridled enzyme activity of u-PA. Rather, the dynamic state of the system at the cell surface allows temporal and spatial re-arrangements of its components during migration and invasion. Results obtained from animal model systems, collectively and almost unanimously, point to the enzyme activity of u-PAR-bound u-PA at the surface of the cancer cells being causally involved in the metastatic process. In humans, the correlation between u-PA and u-PAR concentrations and poor prognosis is in agreement with the basic notion of u-PAR-bound u-PA at the surface of the cancer cells being necessary for cancer-cell invasion and metastasis..

1.4.8 Motility Factors Involved in Tumor Cell Migration

Active locomotion by invading tumor cells is a prerequisite step in the establishment of secondary tumors. The repeated observation of non-random patterns of metastatic dissemination suggested the existence of specific parameters governing cell migration (Nicolson, 1988a; Raz & Ben-Ze'Ev, 1987), and recent studies have now identified a group of secreted cytokines that have been described as cell motility factors termed autocrine motility factors (AMFs).

Secretion of AMF is largely confined to transformed or immortalized cells thus far and in addition to regulating the induction of both random and directional cell migration in self-producing cells, it

has been demonstrated they also act in a paracrine fashion (Watanabe et al., 1991). A tumor specific autocrine factor of less than 30 kDa was characterised from the Dunning R-3327 rat prostatic adenocarcinoma AT-2.1 subline which was shown to induce motility through the cyclic adenosine monophosphate (cAMP) pathway (Evens et al., 1991). Another AMF was purified to homogeneity from the A2058 human melanoma cell line (Liotta et al., 1986) This 125 kDa protein induces migration of the producer cells via a pertussis toxin-sensitive G protein and was termed autotaxin (Stracke et al., 1992). Stimulation of producer cells with AMF at concentrations as low as 15 pg/ml causes them to consistently clear an area two to three fold larger than controls when plated on a gold particle-coated substrate (Silletti et al., 1991). This nondirectional motility induction (chemokinesis) was described previously in addition to the chemotatic effect observed in a modified Boyden chamber assay (Liotta et al, 1986). It is however not known at present which of these activities, chemokinesis or chemotaxis is exploited *in vivo* during cancer cell dissemination. The receptor for AMF has been identified as a 78 kDa cell surface glycoprotein designated gp78 (Watanabe et al., 1991) and nucleotide sequence analysis revealed significant homology to the tumor suppressor gene p53 implying a common ancestral gene. It is now thought that the motility response of AMF on target cells functions through rearrangement of focal adhesion plaque proteins into thin filamentous structures orientated perpendicular to the cells edges, often showing a preponderance of accumulation within the advancing edges of pseudopodia (Silletti et al., 1995). In addition an anti-gp78 mAb was able to mimic the motility stimulating effect of the natural ligand but only in high-metastatic sublines of a series of melanoma cell lines as indicated by an increase lung colonization (Silletti et al., 1994)

In the course of more thorough investigations addressing motility factors, it became evident that motility factors often functioned as growth factors and vice versa. For example scatter factor (SF) was later shown to be identical to hepatocyte growth factor (HGF) (Weidner et al., 1991) and vascular permeability factor (VPF) identical to vascular endothelial growth factor (VEGF; Leung et al., 1989). Now there exists a large repertoire of growth factors such as PDGF, FGF, EGF, IL-6, NGF, or IGF-1 that also affect cell motility (see review by Stoker & Gherardi, 1991).

Hepatocyte growth factor (HGF) and scatter factor (SF) were independently identified by their abilities to induce the proliferation of primary hepatocytes and dissociation/motility of epithelial cells ("scattering"), respectively (Nakamura et al, 1986; Stoker et al., 1987; Zarnegar & Michalopoulos, 1989; Weidner et al., 1990). The soluble pleiotropic mesenchymal cytokine HGF, is capable of promoting a highly integrated biological program leading to and including proliferation, dissociation and migration, cell polarization and tubule formation, prevention of apoptosis and invasive growth in epithelial cells (Medico et al., 1996; Bardelli et al., 1996). This phenotype results from the integration of many distinct HGF responses that are initiated upon binding to its receptor. The large (100 kDa) single chain heterodimeric HGF protein requires proteolytic cleavage for activation, resulting in the generation of two subunits (α and

β), being 60 kDa and 30 kDa respectively (Hartmann et al., 1992; Lokker et al., 1992). The cell membrane receptor for HGF (c-Met) belongs to the tyrosine kinase receptor superfamily and is the product of the c-Met proto-oncogene (Bottaro et al., 1991; Naldini et al., 1991). This 190 kDa heterodimeric receptor, like the ligand, undergoes proteolytic cleavage to yield two mature subunits, α and β of 50 kDa and 145 kDa respectively, both of which are required for biological activity (Giordano et al., 1989). Inactivation of either HGF or c-Met in the mouse causes embryonal lethality (Schmidt et al., 1995; Maina et al., 1996), as a functional HGF/c-Met pair is essential for morphogenic migration of epithelial cells to the surrounding mesenchyme and migration of myoblasts from the somites into the limb buds (Bladt et al., 1995).

Constitutive overexpression of the c-Met receptor has been demonstrated in several tumors of epithelial origin and even amplified in the derived metastatic cells (Liu et al., 1992; Rong et al., 1993). The ability to grow independently from a substrate attachment and to invade the ECM is considered a hallmark of tumorigenic cells, and these properties can be conferred to normal cells by transfection of the activated HGF receptor (Giordano et al., 1993; Rong et al., 1994). In addition, HGF/SF stimulates the *ras*-guanine nucleotide exchanger (Graziani et al., 1993), which is required for the scattering activity of SF (Hartmann et al., 1994). In agreement with these results, *ras* activity is continuously required throughout the process of endothelial cell motility (Fox et al., 1994). These data would suggest that *ras* activity, stimulated by either growth factor binding or integrin engagement, might be a central regulator of cell motility. Interestingly, other small GTP-binding proteins *rho* and *rac* have been implicated in growth factor-induced actin reorganization (Ridley & Hall, 1992; Nobes & Hall 1995), in that *ras* and *rac* function downstream of c-Met to mediate cell spreading but not scattering. When full-length c-Met cDNA was transfected into NIH 3T3 fibroblast, stable transfectants responded to HGF/SF factor stimulation as determined by c-Met receptor phosphorylation, changes in cell shape, migration in Boyden chambers, and invasion of collagen matrices *in vitro.* The motile and invasive phenotype was strictly dependent on the presence of HGF/SF. The factor did not stimulate either cell growth or thymidine incorporation in transfected cells. These data conclude that the c-Met receptor induces cells to pursue a motogenic-invasive rather than a proliferative program (Giordano et al., 1993). Later Matsumoto et al. (1994) could show that the migratory response of fibroblasts to HGF/SF proceeded through the initial recruitment of certain intergrins, cytoskeletal proteins and p125 FAK into focal adhesions that are dependent on tyrosine kinase activity (Matsumoto et al., 1994). The recent identification of a number of germline and somatic mutations in the tyrosine kinase domain of c-Met in cells from patients affected by papillary renal carcinomas, represented a milestone in the field of research involving this oncogene (Schmidt et al., 1997). In addition, c-Met overexpression had been observed in carcinomas derived from follicular epithelium (Di Renzo et al., 1992) of the thyroid. In ovarian carcinomas, an increased expression of c-Met correlated with a particular aggressive phenotype. In colorectal cancers, the c-Met oncogene is overexpressed from 5- to 50-fold in about 50 % of primary lesions, and in 70 % of liver metastasis (Di Renzo et al.,

1995). Moreover, is has been shown that HGF/SF is expressed in breast carcinomas, and that the level of expression correlated with the state of tumor progression (Yamashita et al., 1994). All of the above observations strongly implicates that the c-Met oncogene can and does play a relevant role in the ability of tumorigenic cells to invade and cause distant metastasis.

1.4.9 Patterns of Metastatic Spread of Tumorigenic Cells

Lymphatic and vascular drainage at the primary site initially determine tumor cell access to secondary sites, and much of the organ distribution can be explained solely by anatomical location of the primary tumor (Weiss, 1985). This explanation, while sufficient for the majority of early metastatic patterns, fails to account for the observations made in late-stage disease or in instances of marked aggression where widespread dissemination occurs early in tumor development. It is a well established observation that a malignant tumor of specific histological type often metastasize preferentially to specific organs (Pauli & Lee, 1988). This surprisingly, was already proposed in 1889 by Paget. He questioned whether the distribution of metastases was due to chance and thereby analysed the autopsies of a large number of patients with metastatic breast cancers (Paget, 1889). The non-random pattern of visceral metastasis suggested that metastasis to a particular site was not due to chance, but rather that certain tumor cells (the seed) had a specific affinity for a certain organ (the soil). In the last few years, Paget´s hypothesis has received considerable experimental and clinical support (Price, 1994, Fidler, 1995). Studies with B16 melanoma cells injected intravenously into syngeneic mice produced metastases in the lungs and ovarian tissues but not in kidneys even when implanted intramuscularly (Hart & Fidler, 1981). The introduction of peritoeovenous shunts for palliation of malignant ascites provided similar data in humans. The autopsy findings in 15 of these patients substantiated the clinical observation that the shunts did not increase the incidence of visceral organ metastasis. In fact, despite continuous entry of billions of tumor cells into the circulation, metastases in the lung (the first capillary bed encountered) were rare (Tarin et al., 1984).

It is therefore evident that that development of distant metastases in a particular target organ depends on characteristics of the tumor cells and the target organ (Zetter, 1990). Tumor cell characteristics responsible for organ preferential metastasis likely include cell adhesion molecules and the ability to respond to tissue-specific factors (chemotaxis and proliferation) (Cavanaugh et al., 1991; Doerr et al., 1989; Rossi et al., 1992; Zetter, 1990). Target organ factors that influence tumor cell population include microvascular endothelial cell adhesion molecules (Auerbach et al., 1987; Pauli et al., 1992) and local growth factor production by target organ stromal cells (Nicolson, 1993). For example, Sierra et al. (1997) could show that astrocyte-derived cytokines, namely IL-6 enhanced the growth and adhesion of the 435-Br1 cell line (derived from a brain metastases in a nude mouse previously inoculated subcutaneously (s.c) with the breast cancer cell line MDA-MB-435) relative to the parental

MDA-MB-435 line, when seeded onto primary astrocytes in culture. These results hint to a plausible scenario whereby metastatic cells respond to exogenous cytokines in a paracrine fashion such that their growth is favoured in certain organs where these factors are present. When two sublines of the Lewis lung carcinoma, H-59 and M-27, which are both highly and preferentially metastatic to the liver and lung respectively, were grown in serum-free conditioned medium from primary mouse hepatocytes, the medium proved highly and specifically mitogenic for H-59, but had no effect on M-27. Furthermore, this mitogenic activity could be blocked with a monoclonal antibody to the insulin-like growth factor 1 (IGF-1). This would indicate that hepatocyte-derived IGF-1 is a potential mechanism of selection in the process of liver colonization by these carcinoma cells (Long et al., 1994). More recently, transfection of full length insulin-like growth factor receptor (IGF-1R) cDNA into the M-27 subline (which normally has low levels of the receptor) resulted in multiple tumors of the liver when inoculated via the splenic/portal route *in vivo* (Long et al., 1998).

Knowledge of the expected metastatic pattern of a particular tumor type is of clinical importance as the pattern data may contribute to the development of innovative strategies aimed at inhibiting metastasis. For example, breast cancer in women has a predilection for metastasis to regional lymph nodes, skeleton and lung. Paracrine stimulation of cell proliferation by insulin-like growth factor-I (IGF-I) is a feature of many mammary carcinoma cell lines (Cullen et al., 1992), and recently tamoxifen, a partial antagonist to the oestrogen receptor now widely used in the treatment of breast cancer, was demonstrated to decrease IGF-I production by lung stromal cells (Huynh et al., 1993). Because IGF-I is a potent mitogen for breast cancer cells (Doerr & Jones, 1996), reducing the expression of IGF-I in common target organs for breast cancer metastasis may contribute to its antineoplastic properties. Thus, suppression of organ specific local growth factors opens up a new avenue of potential therapeutics because it recognizes the role and importance of target organ microenvironment in the development of metastatic disease.

1.5 Genetic Aspects of Invasion and Metastasis

The term tumor progression encompasses a range of incremental biological changes that typically occur in tumors over time (Mareel et al., 1990). It is proposed instead that the genomic instability inherent in the tumor cell leads to disruption of only a relatively small number of critical regulatory genes common to several cellular processes (Egan et al., 1991). This view is supported by the demonstration that metastasis may be controlled in experimental systems by altering the expression of oncogenes which are known to have critical regulatory roles in cellular growth. Two genes that have recently been reported to be associated with metastasis, namely nm23 and MTS1 will be considered further.

1.5.1 NM23 in Metastasis and Oncogenic Development

A central hypothesis in metastasis research proposes that some of the phenotypic changes noted are mediated, at least in part, by increased or decreased tumor cell expression levels of specific genes. A number of techniques have been used with success to identify differentially expressed genes that accompanies a metastatic phenotype and are all founded on the analysis of mRNA from usually highly versus non-or low metastatically competent rodent cell lines. Using differential colony hybridization between a low and high metastatic K-1735 murine melanoma cell line, one clone was identified that exhibited a pattern of expression that was consistent with the tumor metastatic potential of all seven cell lines in the K-1735 system. This lead to the isolation of clone nm23, whose mRNA levels were ten-fold higher in the two low metastatic cell lines (Steeg et al., 1988a/b) that is there was an inverse relationship of gene expression verses metastatic capacity.

Subsequent cloning revealed that nm23 encodes a 17 kDa cytoplasmic metabolic enzyme, nucleoside diphosphate kinase (NDPK), whose principal function is to provide nucleoside triphosphate precursors for DNA and RNA synthesis (Lascu, 1996). There exists two isoforms denoted nm23-H1 and nm23-H2, the later being identical to the previously described c-myc transcription factor PuF (purine-binding factor; Postel et al., 1993). Both the murine genes nm23-M1 and -M2 (Urano et al., 1992) and the rat ndpk-β and -α (Shimada et al., 1993) are 80 % and 90 % homologous, respectively, to human nm23-H1 and -H2. Validation of nm23-H1 has proceeded by examination of its expression in other metastatic model systems. Reduced expression, either at the protein or RNA level, was correlated mouse mammary tumor virus induced mammary tumors (Caligo et al., 1992), *ras* or *ras* plus adenovirus E1a transfected rat embryo fibroblasts (Steeg et al., 1988b) and B16 murine melanomas (Lakshimi et al., 1993). In other systems, no correlation was found. Transfection studies of nm23 cDNAs into a paneal of murine, rat and human cell lines resulted, in each case, in a reduction of *in vivo* metastatic potential using experimental (tail vein) or spontaneous (fat pad) injection routes. (Leone et al., 1991, 1993; Baba et al., 1995; Parhar et al., 1995). These data confirm nm23 as a metastatic suppressor gene in a variety of model systems. The potential relevance of nm23 to human cancer metastasis has been investigated by examining nm23 expression in tumor cohorts. A decreased expression has been significantly correlated with either decreased patient survival or the presence of lymph node metastases in women with breast carcinomas (Tokunaga et al., 1993; Cropp et al., 1994; Noguchi et al 1994). In other tumor cohorts, such as lung, colon, prostate and kidney, no trend of nm23 expression and tumor metastatic potential has been evident (reviewed by De La Rosa et al., 1995). Perhaps these systems indicate that the role of nm23 in tumor progression is rather restricted to tumor types or that nm23 is itself victim to deregulation. Of interest is the fact that the kinase activity of nm23 appears not to be required for its metastasis-repressing ability (MacDonald et al., 1993), but rather serine phosphorylation of the protein was correlated with its repressing function. This may suggest that particular facets of intracellular signalling may be involved in nm23 function.

The possibility of nm23 functioning as a transcription factor concerns the nm23-H2 isoform. whereby Postel could show that nm23-H2 is indeed a DNA-binding protein capable of stimulating transcription of, amongst others, the human c-myc gene *in vitro* (Postel et al., 1993), and that it can transactivate c-myc in a cell transfection assay (Berberich & Postel, 1995). The role of nm23-H2 in c-myc activation was recently confirmed by Ji et al. (1995). They observed that nm23-H2 is involved in the deregulation of the c-myc gene in Burkitt's lymphoma, and thus, in the pathogenesis of this tumor. A possible nm23/PAI-1 connection is intriguing, since PAI-1 is elevated in breast tumors (Schmitt et al., 1992). A model whereby nm23-H2 is responsible for the activation of PAI-1 expression, by blocking the proteolytic pathway that results in matrix degradation and metastasis, is proposed. PAI-1 requires a PuF-like sequence element (TGGGTGGGGC) at -78 to -69 for promotor activity in cell transfection assays (Descheemaeker et al., 1992; Amarosa, 1993). Since urokinase (uPA) inhibitors such as PAI-1 are implicated in tumor progression, it is possible that nm23-H2, PAI-1 and c-myc (which also regulates PAI-1; Prendergast et al., 1990) may all be involved in a common regulatory pathway.

1.5.2 Metastasis-Related mts-1 Gene

As with nm23, transcriptional comparison between two murine mammary carcinoma cell lines one of high metastatic capacity, CSML-100 and its non-metastatic counterpart CSML-0 was performed to generate a subtracted library. Upon differential screening of the library, one clone designated mts-1 hybridized only with cDNA probes derived from the highly metastatic CSML-100 cell line (Ebralidze et al., 1989). Sequence analysis revealed mts-1 to be the murine homologue to p9Ka (Barraclough et al., 1987), and CAPL (Engelkamp et al., 1993) which represent the rat and human homologues respectively. The mts-1 gene encodes a 101-amino acid cytosolic protein belonging to the S100 subfamily of Ca^{2+}-binding proteins, which are reported to be involved in the regulation of cell growth and differentiation by conversion of Ca^{2+}message signals into intracellular responses (Kretsinger et al., 1991; Heizmann & Hunziker, 1991).

Demonstration of a role for mts-1 in metastasis was obtained through transfection studies whereby mts-1 cDNA in antisense orientation was transfected into the CSML-100 cell line, resulting in a marked reduction of metastatic ability (Grigorian et al., 1993). Conversely, forced expression in CSML-0 cells gave rise to clones of increased metastatic capacity although not all clones metastasized, suggesting clonal variation. Davies and co-workers, transfected the nonmetastatic rat mammary epithelial Rama 37 cell line with p9Ka (Davies et al., 1993) which upon subsequent inoculation into rats produced tumors which metastasized to the lung and lymph nodes. In light of the above, it would appear that mts-1 has the capacity to modulate the metastatic phenotype of several tumor cell lines. Several attempts were made to find proteins that interact with mts-1, one of which was tropomyosin (TM; Takenaga et al., 1994). The binding was specific and dependent on the presence of Ca^{2+}. Although the function of

S100 proteins remains still unanswered, it is tempting to speculate that mts-1 might be involved in the Ca^{2+}-dependent regulation of the cytoskeleton (Bianchi et al., 1993) and thereby influence cell motility as a result.
The human connection has come from a different angle. It has been appreciated for some time that the long arm of chromosome 1 is characterized by a high degree of anomaly in human hematological and solid tumors. For instance, duplication of the chromosomal segments 1q11 and 1q32 corresponded to increased proliferation and metastasis formation in human leukemia B cell clones (Ghose et al., 1990). Human gastric adenocarcinomas frequently undergo a loss of heterozygosity on chromosome 1q (Sano et al., 1991) and genetic alterations in breast cancer are often associated with locus 1q21 (Devilee et al., 1991), 1q23-32 (Chen et al., 1989) and 1q42-43 (Gendler et al., 1990). When Pedrocchi et al. (1994) examined expression of four members of the S100 family, all of which are clustered on chromosome 1q21, in a battery of different human breast cancer cell lines and human breast cancer tissues, only expression of CAPL/mts-1 was specifically correlated with the tumorigenesis of human mammary cells.

The biology of cancer extends far beyond the physiology of cell growth control and while we are just beginning to unravel the intricate complexities that predisposes normal cells to shift their phenotypic behaviour to one capable of uncontrolled growth and insensitivity to surrounding growth promoting/inhibiting signals, this shortfall in understanding represents the major drawback in current cancer genetics. The last two decades has seen the rapid increase in number of genes thought to be functionally involved in tumor progression, and beyond this may lie yet other genes about which we know little or nothing at present. New cloning techniques for example subtractive suppression hybridization (SSH) may have a role here as they hold promise concerning the isolation of these novel genes whose deduced function may open up new avenues that can be exploited for the prevention of cancer.

PART TWO

1.6 Current Strategies and Methodologies that Allow Identification of Differentially Expressed Genes

There exists a large repertoire of techniques that aim at producing an inventory of differential transcripts based between two populations of mRNAs. The isolation and identification of differentially expressed transcripts is generally achieved by one of the following methods: Differential Display and related techniques (Liang & Pardee, 1992; Sokolov & Prockop, 1994), Representational Difference Analysis (cDNA-RDA; Lisitsyn et al., 1993), Enzymatic Degradation Subtraction (EDS; Zeng et al., 1994), Linker Capture Subtraction (Yang & Sytkowski, 1996), and techniques involving physical removal of common sequences (Akopian & Wood, 1995; Deleersnijder et al., 1996). Despite the fact that all these methods have proven successful in the isolation of differentially expressed genes they all possess some specific intrinsic drawbacks. For instance, differential display restricts the analysis to differences of the 3´end of cDNAs, so that differences in the 5´portion of cDNAs (e.g. variants of alternatively spliced genes) are often not detected. Additionally, variable reproducibility of the differential band patterns and the significant incidence of false-positives makes it difficult to isolate rare transcripts that are truly differentially expressed. Another common feature of the methods mentioned above and represents also an obstacle in the isolation of rare transcripts, is the disproportion of concentrations of differentially expressed genes which is maintained in the subtraction. For this reason, RDA requires multiple rounds of subtraction as the method fails to take into account the large differences in relative abundance of individual mRNA transcripts. The ideal system for subtractive cloning would generate an equalized representation of differentially expressed genes irrespective of their initial relative abundance, would permit the monitoring of subtraction efficiency prior to the time consuming screening work, and would minimize if not completely exclude the isolation of false positive clones.

Recently, a novel technique called Subtraction Suppression Hybridization, (SSH; Diatchenko et al., 1996) has been described that combines a high subtraction efficiency with an equalized representation of differentially expressed sequences: The method is based on a specific form of PCR that permits exponential amplification of cDNAs which differ in abundance, whereas amplification of sequences of identical abundance in two populations are suppressed. The tabulation of this technique became a central piece of the work in this Ph.D project

1.7 Project Outline and Specific Aims

The principal aim of this thesis was to address the question **"What are all the global changes in gene expression that accompanies the transition from a benign to a metastatic phenotype in a defined cellular system"?** Although simplistic enough, the question is of great importance as it recognizes the fact that metastasis is not the result of dysregulation of a single gene, whether it be loss or overexpression, but rather the result of a cohort of genes that have pivotal roles in cell regulation. To date there exists no such study, as to answer such a question is of some technical challenge and was not previously possible up until a few years due to technical limitations. Most reports have instead focused on one particular gene whose expression coincided with the metastatic phenotype. This in itself is of limited use as it fails completely to consider the functional actions of such a gene in the context of other dysregulated genes.

The model system which I chose was the Bsp73-AS pancreatic adenocarcinoma cell line system that comprises of two subclones of increasing metastatic capacity (Bsp73-10AS and Bsp73-ASML respectively) that originate from the non-metastatic parental line Bsp73-1AS. The ASML cell line was reported to be highly metastatic *in vivo* (Matzku et al., 1983) when compared to 1AS. Previous studies on these two cell lines demonstrated that there were intrinsic differences between ASML and 1AS at the molecular level by using a monoclonal antibody (MAb1.1ASML) raised against a surface glycoprotein of the metastasizing rat pancreatic carcinoma cell line BSp73-ASML. With the help of this antibody, one clone was isolated that encode an additional extracellular domain inserted into the rat CD44. This new variant was only expressed in the metastasizing cell lines of two rat tumors, and further more, overexpression of this variant in the non-metastasizing BSp73-AS cells sufficed to establish full metastatic behaviour (Günthert et al., 1991). This variant form of CD44 was then subsequently shown to be also expressed in human tumors (Hofmann et al., 1991). As a result CD44 became the focus of intense study in relation to human tumors. So while there existed documented evidence that a variant form of CD44 is necessary and indeed sufficient to endow metastatic properties to recipient cells, it was clear that a complex phenotype such as metastasis cannot be the product of genetic dysregulation of a single gene. Hence it seem appropriate that the Bsp73-AS system would offer a suitable platform from which other genetic alterations could be identified by subtraction at the nucleic acid level.

Specific aims of the project where as follows:-

1) Isolation and subsequent identification of the majority of genetic dysregulations in the form of upregulation that accompanies the transition from a non-metastatic to a metastatic phenotype.

2) Selective screening of all identified differentially expressed genes against a large paneal of rodent and human cell lines of varying tumorigenicity. This would identify

those clones that appeared to have a more significant role in tumor progression and exclude those that were specific to the pancreatic system from which they were obtained.

3) Screening of human tumor material against all individual differentially expressed clones in order to identify those clones that not only cross hybridize and therefore may possess high homology, but to identify clones specific to the tumor and therefore absent in normal matched tissue.

4) Selective full length cloning of potential genes involved in tumor progression as defined from the two screening procedures.

5) Functional analysis of the full length clones in vivo through overexpression and /or interference with normal function by ectopic expression of a dominant negative mutant in non-metastasizing and metastasizing cell lines respectively.

2 MATERIALS AND METHODS

2.1 MATERIALS

All general chemicals were of the highest purity grade, the majority of which were supplied from *Merck*, Darmstadt, *Carl Roth GmbH* & *Co* Karlsruhe and *Sigma Chemie GmbH*, Deisenhofen. Aqueous solutions where made with water previously purified (bi-dest) using a Milli-Q water purification system.

Oligos

All oligos were obtained from either MWG Biotech GmbH or from Birsner & Grob-Biotech GmbH and were all of a HPLC purified grade.

Sequencing

5`- TCAGCGATGAGATCTACTGCC-3´	For (Ezrin)-Texas red
5`- AACGCGGAGGCACGGAGGAGGTCG-3´	Rev (Ezrin)-Texas red
5`- TTGGCCTCCGTGCCTCCTCCAGC-3´	3´-Ezrin-Texas red
5`- GTTTTCCCAGTCACGAC-3´	PUC/M13 For(-40)-Texas red
5`- CAGGAAACAGCTATGAC-3´	PUC/M13 Rev-Texas red
5`- TAATACGACTCACTATAGGG-3´	T7-Texas red
5`- ATTAACCCTCACTAAAG-3´	T3-Texas red

Colony PCR (SSH nested primers)

5`- TCGAGCGGCCGCCCGGGCAGGT-3´	PN1
5`- AGGGCGTGGTGCGGAGGGCGGT-3´	PN2

uPAR

5`- TGTATTGAAGTGGTGACGCTCC-3´	Rat uPAR (457s)
5`- AGGCAATGAGGATAAGATGAGC-3´	Rat uPAR (958as)

Full length cloning of Ezrin (rat)

5`- CTCGGAAGCTTAGCCACCAACCAGCCA AGATGCC-3´	5`- Ezrin (Hind III)
5`- GCCATGAATTCCTAGCCCGCATAGTCAG	3´- Ezrin (HA/EcoRI)

GAACATCGTATGGGTACATGGCCTCAAA
CTCGTCGATGCG-3

5`- GCCATGAATTCCTAGCCCGCATAGTCAG 3´-Ezrin (HA/EcoRI)N
GAACATCGTATGGGTACTGGGCCTTCAT
CTGCTGCACCTC-3´

Antibodies

Anti-Ezrin (mouse IgG1 cross-reacts to rat, human and dog. Also reported to cross-react to two related proteins, moesin and radixin. Peptide corresponds to positions 575-585. (Transduction Labs. Kentucky. USA)

Anti-HA (mouse monoclonal to an epitope derived from hemagglutinin protein of human influenza virus (HA-tag). Recognizes peptide sequence YPYDVPDYA. Boehringer Mannheim)

Anti Erk (K-23, a goat polyclonal to an epitope corresponding to amino acids 305-327. Reactivity to human, mouse and chicken. Santa Cruz Biotech)

2.2 General Methods

A number of protocols and receipts for commonly used buffers used in this project were taken from the laboratory manual of Maniatis et al (1989) and Current protocols in Molecular Biology (Ausubel F.M.,et al 1989) unless otherwise stated.

Phenol/Chloroform extraction of nucleic acids

To remove unwanted protein contaminants from nucleic acids, an equal volume of Tris-buffered phenol, chloroform and isoamylalcohol (2-propanol) at a ratio of 25:24:1 was added and the mixture vortexed until the solution had a milky appearance. The two phases were separated by centrifugation at 13000 x g for five minutes after which the upper aqueous nucleic acid containing phase was transferred to a new reaction tube and subjected to a further round of extraction with chloroform/isoamylalcohol (24:1).

Isolation/Purification of DNA from agarose gels

A number of methods were employed to isolate DNA (e.g.,Oligos, PCR fragments, vectors, plasmid clones or ds cDNA) from agarose gels once eletrophoresis was complete.

(a) Electrophoretic isolation of DNA

Once the DNA had migrated the desired distance, as determined by visualising the gel under UV light, a slit was cut into the gel with a scalpel just below the chosen DNA band. A strip of DE81 DEAE-cellulose membrane (Schleicher & Schuell, Dassel) was inserted into the slit and electrophoresis continued until the DNA fragment had run into the membrane. At this point the membrane was removed, rinsed briefly with distilled H_2O and the DNA eluted by incubation in 400 µl 1.5 M NaCl, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA for 30 minutes at 65 °C with shaking. After two extractions with phenol/chloroform, the DNA was precipitated with ethanol, air dried and dissolved in an appropriate volume of water.

(b) Direct isolation from agarose gels

The DNA band of choice was cut out from the gel, under long wave UV light with the aid of a scalpel. After trimming excess agarose the DNA containing gel strip was placed inside a pierced 0.5 ml reaction tube containing glass wool which in turn was placed inside a 1.5 ml reaction tube and centrifuged at high speed for 5 minutes. The DNA would be collected in the reaction tube, leaving the agarose caught in the glass wool. The DNA was then extracted with phenol/choloroform and precipitated with ethanol.

(c) DNAeasy kit (Biozyme) DNA isolation from agarose gels.

As above with the exception that the gel strip containing the DNA is added to 3 x it's weight to volume of "salt buffer" (all reagents provided in the kit). The gel piece is melted in the buffer by incubation at 65 °C before the binding resin is added. After two subsequent washing steps the resin with bound DNA was air dried and the DNA eluted by addition of bi-dest H_2O.

Precipitation of nucleic acids

The precipitation of both RNA or DNA was performed by adjusting the final salt concentration of the reaction mixture to 300 mM with 3M Na-acetate (pH 4.8-5.2) and adding 2.5 volumes of 100% ethanol or 1 volume of 2-propanol (Isopropanol) followed by a 30 minute incubation at -20 °C. The precipitate was pelleted by centrifugation at 13000 x g, at 12 °C for 30 minutes after which the aqueous phase was discarded. The pellet was washed twice with 80 % ethanol to remove excess salt and allowed to air dry for 10 minutes.

Determination of nucleic acid concentration

The concentration of nucleic acids in aqueous solution was determined by spectroscopic measurement of their extinction coefficient at 260nm and 280nm. An optical density (OD) of 1 at 260 nm is equivalent to 50 mg/ml of double stranded DNA, 40 mg/ml of

RNA or 20 mg/ml single stranded oligonucleotide. The OD_{280} is used as an indication of purity and should be approximately 50% of the OD_{260} value. Pure RNA and DNA should have a OD_{260}/OD_{280} ratio of 1.8-2.0 and 1.6-1.8 respectively in bi-dest water.

Restriction endonuclease digestion of DNA

Depending on the restriction enzyme used, 2-5 units (U) of the enzyme was added for each µg of DNA taken. The digestion was performed using the appropriate buffer and incubation temperature as indicated by the supplier. Reactions were incubated for ≥ 2 hours at a final DNA concentration of 1-5 mg/ml after which the reaction was terminated by either heat inactivation of the enzyme (65 °C for 10 minutes) or by phenol/chloroform extraction.

DNA Ligation

All ligation reactions were performed in a total volume of 10 µl and incubated overnight at 14 °C, followed by heat inactivation of the ligase at 70 °C for 5 minutes before storing at -20 °C

(a) Ligation of linkers / adaptors

Approximately 100-200 ng of ds cDNA was ligated to a 3-4 molar excess of linkers or adaptors using the supplied ligation buffer 1-3 units of ligase.

(b) Ligation of PCR fragments

After purification of the PCR fragment, the DNA was directly cloned via the T/A overhangs into the T/A cloning vector pCR2.1 kit (Clontech Laboratories, Inc. USA) according to the suppliers protocol and using the kit´s components.

(c) Sub-cloning

The cloned fragment of DNA was released from the vector using appropriate restriction endonucleases, purified by agarose gel electrophoresis and subsequently cloned into the new vector using compatible sites or through blunt end ligation.

Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a total volume of 25-100 µl, in the presence of 250 µM dNTPs, 1-2 pmol of primer(s), 0.25U to 1U of Taq and 1X suppliers buffer and 1.5-2.0 mM f.c $MgCl_2$ (EuroBio Taq buffer is Mg^{2+} free). The reactions were carried out in a number of commercially available PCR machines (e.g., Perkin Elmer 9600/2400 and DNA Engine MJ Research), using specific cycling parameters depending on the application. All individual parameters are noted for each PCR reaction in the results section. Analysis of the PCR reaction was performed using gel electrophoresis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

All reactions were performed as described for standard PCR with the exception that 5 µl of a previously diluted (1:10) first-strand cDNA reaction was taken as template DNA and the primers used were specific for the gene of interest only (as determine through BLAST). Again, specific cycling parameters were dependent on the primers chosen and size of the expected product. Evaluation of the RT-PCR reaction was performed by gel eletrophoresis, and were cloning was concerned, the product was sequenced.

Radioactive labelling of DNA/cDNA probes

Approximately 25 ng of ds cDNA in a volume of 45 µl was denatured by heating to 100 °C for 1-2 minutes then rapidly cooled on ice. The entire solution was then added to a ReadyPrime reaction vial (*redi* Prime kit, Amersham Life Science) to which was added 5 µl of [α^{32}P]-dCTP (Amersham Buchler GmbH, Braunschweig, 370 MBq/ml, 10 mCi/ml). After incubation for 15-30 minutes at 37 °C, or one hour at room temperature, unincorporated nucleotides were remove from the labelled DNA using Elutips (Schleicher & Schuell, Dassel) according to the manufactures guidelines. The labelled DNA was eluted in a volume of 600 µl and denatured at 100 °C for 2-3 minutes immediately before use.

Isolation of total RNA from tissue or human tumor material

Approximately 200-500 mg of frozen tumor material was placed in a pre-chilled stone pestor and ground to a fine powder under liquid nitrogen. The powdery contents were then immediately poured with the nitrogen into a falcon 50 ml tube and allowed to stand briefly until all the nitrogen had dissipated upon which 5 ml of a mono-phasic solution (peqGOLD RNA Pure™, peqlab GmbH) was added. For fresh wet tissue, the above step was omitted and instead the tissue was homoginized directly in 5 ml of the solution. The entire contents were then homoginized at 20000 rpm using an ultra-Turrax T25(IKA®-Labortechnik) for 5-10 minutes followed by an incubation period of 3-10 minutes at RT to allow the nucleic acids to dissolve into the solution. Following this, 1 ml of pure chloroform (0.2 ml of chloroform per 1 ml of peqGOLD used) was added to the homogenised mix and vortexed vigorously until the solution took on a milky white appearance. Again following a brief incubation at RT, the mixture was transferred to a 14 ml polypropylene tube and subsequently centrifuged for 15 minutes at 10000 x g in a cooled 4 °C swing-out rotor. Once completed, the tubes were carefully removed and the top aqueous RNA containing phase was removed and transferred to a clean polypropylene tube containing 3 ml of pure choloroform before vortexing the contents. Again the tubes were centrifuged as before and the top phase removed for a further round of choloroform extraction. Lastly, the top phase was precipitated with an equal volume of isopropanol and the nucleic acid pellet was washed twice with 75 % ethanol before

resuspending in 150 ml of bi-dest water. The integrity of the isolated total RNA was monitored by size fractionation over a 1 % formaldehyde/MOPS agarose gel.

Isolation of Poly (A)$^+$ RNA

Cells were grown to a confluency of approx 70-80% in 15 cm petri dishes (Greiner) after which the medium was removed and the cells washed briefly in PBS. The cells were then immediately lysed in 20 ml of STE-SDS (20 mM Tris-HCL pH 7.4, 100 mM NaCl. 10 mM EDTA and 0.5% SDS) containing 300 mg/ml proteinase K (Sigma, München). Using an Ultra-Turax™ homogenizer, the high molecular weight DNA was sheared followed by incubation at 50 °C for 30 minutes in order to degrade cellular proteins by the action of proteinase K. After adjusting the final concentration of NaCl to 0.5M, 100-200 mg of oligo-dT cellulose (type VII, Pharmacia Biotech or NEB) was added and the resulting mixture rotated over night (o/n) to allow binding of the poly A$^+$ RNA to the oligo-dT cellulose. The oligo dT cellulose was then washed by pouring the contents into a 20 ml Biorad column, allowing the liquid to drain out while retaining the oligo-dT cellulose, then applying 20 ml washing solution (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM EDTA and 0.2 % SDS). The poly A$^+$ RNA was eluted from the oligo-dT cellulose by addition of 4 ml of elution solution(1 mM Tris-HCl pH 7.4, 1 mM EDTA and 0.2 % SDS) and the contents collected in a 14 ml polypropylene tube on an ice bed. The RNA concentration was estimated from 400 µl of the eluent, the remainder precipitated by adding 350 µl of 3 M Na-Acetate and 0.8-1 vols of isopropanol (2-propanol) followed by centrifugation at 12000 x g for 30 minutes at 4 °C. Once the centrifugation was completed, the aqueous phase was decanted off and the RNA pellet washed twice with 70 % ethanol, before being air dried for 10 minutes. The pellet was then resuspended in bi-dest H_2O, usually at a concentration of 0.5-2.0 mg/ml before being transferred to a 1.5 ml reaction tube and stored at -80 °C.

First and second strand cDNA synthesis

(a) First strand synthesis using SuperScript™ II for RT-PCR

For most applications, 2 µg of high quality poly (A)$^+$ RNA together with 500 ng of oligo $(dT)_{12-18}$ primer in a total volume 11 µl was heated to 70 °C for 10 minutes in a thermal cycler (Perkin Elmar 9600/2400) before placing the reactions rapidly on ice. The reaction mixture was then subsequently made up to 20 µl by adding 4 µl of 5 x first strand reaction buffer (provided with the reverse transcriptase), 2 µl of 0.1 M DTT and 1 µl dNTP mix (10 mM each dATP, dCTP, dGTP and dTTP). Reverse transcription was started by addition of 2 µl of SuperScript™ reverse transcriptase (Gibco BRL) to each reaction before incubation for 1-1.5 hours in an air incubator previously equilibrated at 42 °C. After which the reverse transcriptase was inactivated by rapidly heating the contents to 94 °C for 3 minutes then placing the tubes

on ice. The first strand reactions were stored at -20 °C once their volume had been adjusted to 200 µl by the addition of 180 µl bi-dest H_2O.
In order to monitor the efficiency of the first strand reaction, 1 µl was removed directly after the addition of the reverse transcriptase and added to 1 µl of a labelling mixture containing 0.3 µl[^{32}P]dCTP and likewise incubated at 42 °C. This so called *tracer reaction* was stopped by the addition of EDTA to a f.c. of 20 mM. Specific incorporation of dCTP into high molecular weight nucleic acid was determined by the TCA precipitation procedure (Ausubel et al., 1987). Typically, ~28 % of all poly(A)$^+$ RNA transcripts were converted into first strand cDNA. Note: first strand cDNA synthesis performed for the generation of a subtracted library was done exactly as described above with one exception. The oligo (dT) primer used for cDNA synthesis contained an Rsa I restriction site and a `dT´ tail of 30 nucleotides in length. This primer was provided in the PCR-Select™ cDNA subtraction kit (Clontech Laboratories, Inc. USA).
In the case of performing second strand synthesis, the heat inactivation and dilution steps were omitted and instead the reaction vials were placed on ice after incubation at 42 °C.

(b) One-tube second strand synthesis

Following first strand synthesis, second strand synthesis was performed by adding 92 µl of sterile, bi-dest water, 32 µl of 5x second strand buffer [94 mM Tris-HCl, pH 6.9, 453 mM KCl, 23 mM $MgCl_2$, 750 µM ß-NAD and 50 mM $(NH_4)_2SO_4$], 3 µl of *Escherichia coli* DNA ligase (7.5U/µl), 4 µl *E.coli* DNA polymerase I (10U/µl) and 0.7 µl of *E.coli* RNase H (2U/µl). The contents were mixed and 5 µl removed for the radioactive incorporation assay (as above). Both reactions were incubated at 16 °C for 2.5 h. The double stranded cDNA in the unlabelled reaction was blunted by the addition of 2 µl of T4 polymerase (10U/µl, using 10U per µg of mRNA taken in the reaction), followed by further incubation at 16 °C for 20 minutes before both reactions were stopped by addition of EDTA. As before, labelled second strand cDNA synthesis was monitored by TCA precipitated and resolved on an alkaline agarose gel as described by Maniatis (1989). The unlabelled fraction was P/C/I extracted and precipitated in the presence of 10 µg of glycogen before resuspending the ds cDNA in bi-dest to a concentration of 0.1-0.5 mg/ml.

Mini-prep plasmid preparation from bacteria

(a) Standard method.

Individual colonies were picked from a LB agar plate and used to inoculate 3 ml of LB medium (10 g/lt tryptone, 5 g/lt yeast extract, 5 g/lt NaCl), containing 100 mg/ml ampicillin. The inoculated bacteria were then incubated with shaking (220 RPM) o/n at 37 °C until a stationary phase had been reached upon which 1.5 ml was removed and the bacteria pelleted by

centrifugation at 4000 x g for 5 minutes. The pellet was resuspended in 100 µl of solution I (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA and 300 mg/ml RNase A) and left at RT for 5 minutes before addition of 200 µl of solution II (0.2 M NaOH and 1.0 % SDS). Once the resulting mixture appeared clear, it was neutralised with 150 µl of solution III (3 M Na-Acetate pH 5.2) and mixed by gentle inversion of the reaction tube. Following 15 minutes incubation on ice, the precipitated protein and chromosomal DNA was pelleted by centrifugation at 10000 x g for 10 minutes before the aqueous supernatant was removed. Extraction of the supernatant with P/C/I was followed by precipitated of the plasmid DNA with ethanol and the resulting DNA pellet resuspended in 50 µl of bi-dest H_2O.

(b) Wizard Mini-prep kit (Promega)

Resuspension, lyses and neutralising of the bacterial pellet was carried out according to and using the manufacturer's provided buffers. The plasmid DNA in the retained supernatant was isolated using the supplier's DNA-binding resin and suction manifold. The resulting plasmid DNA was then eluted in 50 µl of bi-dest H_2O. This method was primarily used for the production of sequencing-grade plasmid DNA.

Large scale plasmid preparation from bacteria

Usually, a volume of 200-250 ml of LB or 2TY (16 g/lt tryptone, 5 g/lt yeast extract, and 5 g/lt NaCl) medium supplemented with the relevant antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37 °C overnight until the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 4000 x g for 10 minutes and the pellet resuspended in 10 ml of solution I (10 mM EDTA, 50 mM Tris-HCl pH 8.0 and 400 mg/ml of RNase A). Following 5-10 minutes incubation at RT, the cells were lysed by the addition of 10 ml of solution II (200 mM NaOH and 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralised with 10 ml solution III (3 M potassium acetate pH 4.8) the entire contents gently inverted to aid mixing of the solutions. After an additional 10-20 minutes on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 13000 x g for 20 minutes at 4 °C in a fixed angle rotor. The retained supernatant was then added directly to a pre-equilibrated Qiagen-tip 500 column (Qiagen Inc.) and the plasmid DNA was recovered according to and using the manufacturer's supplied buffers. The purified DNA was precipitated using 0.8-1.0 vols of isopropanol, washed twice in 70 % ethanol before resuspending the DNA to a final concentration of 1-3 mg/ml in bi-dest H_2O and stored at -20 °C.

Preparation of competent bacteria (E.Coli)

(a) Chemical competent E.Coli (calcium chloride method)

A single colony of *E.coli* DH5α was taken to inoculate 5 ml of LB medium (10g/lt tryptone, 5 g/lt yeast extract, 5 g/lt NaCl) and allowed to grow o/n at 37 °C with shaking (220 rpm) before 4 ml was removed and added directly to 400 ml of LB medium. The bacteria were grown to an OD_{590} of 0.375 before incubating the bacteria on ice for 10 minutes. The bacteria were sedimented by centrifugation without brake at 3600 x g for 7 minutes at 4 °C and the pellet carefully resuspended in 20 ml of ice cold 0.1 M $CaCl_2$ and allowed to stand on ice for a further 10-15 minutes. The cells were centrifuged again under the same conditions and resuspended in a further 20 ml of ice cold $CaCl_2$. This process was repeated once more and the final pellet resuspended in 2 ml of ice cold $CaCl_2$.with 10 % glycerol After a short period on ice, the bacteria were dispensed in 100 µl aliquots in pre-chilled reaction tubes and snap-frozen in liquid nitrogen before storing at -80 °C.

(b) Electrocompetent E.Coli

As above, a single colony of *E.coli* DH5α was taken to inoculate 1 ml of SOB medium and the culture grown o/n at 37 °C with shaking. This in turn was used to further inoculate 300 ml of SOB medium and the bacteria grown at 18-20 °C until and OD_{600} of 0.4-0.5 had be reached. At this point the entire culture was heat-shocked at 37 °C for 5 minutes before subjecting the bacteria to a further 20 minutes at 18-20 °C. The bacteria were then gently sedimented by centrifugation at 3600 x g for 10 minutes before the pellet was resuspended in 50 ml of ice cold bi-dest H_2O. The process of pelleting and resuspending was repeated 4-5 times in order to remove trace amounts of salt from the original medium. Finally, the cells were taken up in 800 µl of ice cold H_2O containing 7 % DMSO. Aliquots of 40 µl were then snap-frozen and stored at -80 °C until use.

Transformation of bacteria

(a) Chemically

Depending on the application, 5 ng of super coiled plasmid DNA or 1 µl of a ligation mix (usually a 1/10 of the ligation) was added to 200 µl of competent cells and left on ice for a period of 30 minutes. Following this, the cells were heat-shocked at 42 °C for 90 seconds before rapidly returning the tube to ice for a few minutes. After addition of 1 ml of SOC medium (2.0 % tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, and 20 mM glucose) the bacteria were transferred to a shaker and incubated for 45 minutes at 37 °C. The cells were then pelleted lightly by a short centrifugation (3 min at 1000 x g) and 1 ml of the supernatant was removed before resuspending the cells in the remaining 200 µl. A volume of

50-200 µl was plated out on LB-agar plates supplemented with the correct antibiotic and the plates incubated 18-24 hours at 37 °C.

(b) Electroporation

Usually, 1-2 µl of a ligation mixture was added to 40 µl of electrocompetent cells previously aliquoted in pre-chilled reaction tubes. The cells were then immediately transferred to a pre-cooled electroporation cuvette (0.1 cm gap, BioRad) and pulsed at 1.8 Kv (Gene pulser, BioRad). Once completed, 1 ml of SOC medium was immediately added to the cuvette and retrieved with the cells and placed in a 15 ml falcon tube before incubation at 37 °C for 45 minutes. Again the cells were lightly pelleted before plating out on appropriate LB-agar plates.

Sequencing of double-stranded template DNA

(a) [a-^{33}P]-ddNTP method

The Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham) was used for the sequencing of cloned templates. The kit combines two revolutionary innovations for sequencing DNA. First, the label is incorporated into the DNA reaction products by the use of four [α^{32}P]-ddNTP terminators and secondly, the use of a engineered Thermo Sequenase DNA polymerase which allows for efficient incorporation in cycling sequencing protocols. Sequencing of desired templates was carried out according to the manufacturer's guidelines. Approximately, 0.5 µg of plasmid DNA was taken together with 1-2.5 pmol of an appropriate primer (usually M13 Forward or Reverse universal primers) with 8 U of the Thermo Sequenase polymerase in a total volume of 20 µl. From this mixture 4.5 µl was aliquoted into each termination vial (`G', `A', `T' and `C') and cycled in a Perkin Elmar 9600 PCR machine using the following cycle parameters: 95 °C, 30 sec, 55 °C, 30 sec and 72 °C, 1 minute for a total of 35 cycles. Upon completion, 3 µl was loaded in each lane and resolved over a 6 % polyacrylmide 6 M urea TBE gel. Once the run had reached the desired length, the gel was removed, dried on Whatman 3MM paper at 80 °C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

(b) ™Vistra DNA sequencer 725

For the use of automated fluorescent sequencing of template DNA, a commercially available kit was used (Thermo Sequenase pre-mixed cycle sequencing kit, Amersham) together with the appropriate Texas Red labelled primers. All reagents were taken from and used following the supplier's protocol. The thermocycling conditions for the sequencing reactions were set at: 94 °C 1 minute, then 22 cycles of 94 °C, 30 sec; 50 °C, 15 sec; and 72 °C for 30 sec. Following completion, 3 µl of loading dye was added to each reaction vial and mixed before placing the reactions in a vacuum centrifuge until the desired volume had been

reached (usually 3-4 µl). After the gel had been pre-run for 30 minutes 3 µl of each reaction sample was loaded onto the gel and allowed to run over night. Sequences were base called from the gel using the provided software.

Generation of a subtracted library by SSH

Suppression Subtractive Hybridisation was performed between Bsp73-1AS ("Driver") and Bsp73-ASML ("Tester") using the PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc. USA) according to the manufacturers' recommendations, except for modifications of the PCR and hybridisation conditions. All PCR and hybridisation steps were performed on a Perkin-Elmer 2400 thermal cycler. Approximately 6-8 µg of high quality poly(A)$^+$ RNA from both cell lines was converted to first strand cDNA as described previously using the oligo $(dT)_{30}$ primer in 3-4 separate reactions. Upon completion, the first strand reactions were converted directly to double strand and blunted with T4 DNA polymerase as previously described. Like samples were pooled and both batches of ds cDNA were then restricted as described in the original protocol. All further manipulations were performed in accordance to the original protocol.

For the first hybridisation the mixture of driver and tester cDNAs was denatured at 100 °C for 20 seconds and then cooled over one minute to 68 °C and maintained at that temperature for approximately 8 hours. For the second hybridisation, driver cDNA was denatured at 100 °C for 20 seconds then added directly to the pooled mix of the two previous hybridisation's and allowed to incubate at 68 °C for 20 hours. It was necessary to alter the PCR conditions (see below) such that amplification of unwanted sequences was kept to a minimum. All other procedures for the generation of the subtracted library were done according to the manual provided with the cDNA subtraction kit (Clontech).

Evaluation of subtraction efficiency

Double stranded cDNA from the tester (Bsp73-ASML) and the Driver (Bsp73-1AS) were separately digested with Rsa I (a four base cutter-as used for the construction of the initial subtracted library). Hind III linkers were added to the tester cDNA and EcoRI linkers to the Driver cDNA [Hind III linker 5´-ATCGTCAAGCTTCAAGTTACATCG-3´, 5´-GCTAACTT GAAGCTTGACGAT-3´. EcoRI linker 5´-TAGTCCGAATTCAAGCAAGAGCACA-3´, 5´-CTCTTGCTTGAATTCGGACTA-3´]. Free linkers were removed by preparative agarose gel electrophoresis and the cDNA mixture was amplified as follows. 1 µl of adaptor-ligated cDNA was diluted into 1 ml of H_2O and amplified in a 50 µl reaction using the appropriate primers (Hind III and EcoRI primers), a standard PCR buffer (Pharmacia), 200 µM dNTPs and 2 units of Taq-Polymerase (Pharmacia). Cycling parameters were as follows: 30 cycles of 94 °C 20 seconds, 52 °C for 20 seconds and 72 °C for 2 minutes. For the subtracted cDNA, PCR conditions were as follows: 27 cycles each of 94 °C for 30 seconds, 68 °C for 30 seconds and

72 °C for 2 minutes. Only the subtracted cDNA was subjected to a second round of PCR (nested) using the same PCR conditions with the exception that 12 cycles were performed. Equal amounts of amplified cDNA from the Driver, the Tester and of the subtracted library cDNA were resolved on a 1.5 % agarose gel, blotted and transferred onto nylon membrane. This was performed in order to monitor the degree of subtraction efficiency as determined b y the removal of genes common to both population (e.g., GAPDH) and the subsequent enrichment of genes specific to the tester population (e.g., uPA). This is discussed further under the results section. Hybridisation's were carried out under stringent conditions in 0.5M Na_2PO_4 pH7.2, 7% SDS at 65 °C (Church & Gilbert, 1984). Filters were washed twice in 2 x SSC, 0.5 % SDS at 68 °C, then once in 0.1 x SSC, 0.1 % SDS at 68 °C.

Cloning into TA vector

After evaluation of the subtraction efficiency the subtracted library cDNA was cloned directly into pCRII.1 vector (TA cloning kit, Invitrogen). Prior to ligation, the subtracted PCR cDNA mix was incubated for a further 15 minutes at 72 °C with additional dATP and Taq DNA polymerase (Pharmacia) to ensure that most of the cDNA fragments contained 'A-overhangs' (known as forced `A´ tagging). Approximately 100 ng of PCR-amplified cDNA was ligated without further purification into 50 ng of vector and the ligation mixture was introduced into ELECTROMAX bacteria strain DH10B (Gibco/BRL) by electroporation (1,8 kV) using an E.coli pulser (BioRad). The library was plated onto 22 x 22 cm ampicillin containing agar plates and bacteria were grown until colonies were visible. Bacteria were then washed off in LB medium, aliquoted and frozen in 10% DMSO. For library screening the titer was determined and bacteria were plated onto 22 x 22 cm agar plates containing 100 µg/ml ampicillin, 100 µM IPTG and 50 µg/ml X-Gal. Plates were incubated at 37 °C until small colonies were visible then incubated further at 4 °C until blue/white staining could be clearly distinguished.

Reverse-northern high density blots and screening

A total of 5000 individual recombinant clones were picked from the subtracted library and used to inoculate 52 sterile 96-well microtiter plates containing LB medium and ampicillin at 100 µg/ml. After incubation of bacteria on a gyratory shaker for 4 hours at room temperature, 5 µl of bacterial culture were transferred into 15 µl of sterile water in PCR tubes. [This part of the protocol was done in 96 tube format using a Perkin-Elmer 9600 thermal cycler. Pipetting of PCR mixes was done using a multi-channel pipette.] The bacteria were lysed by heating to 100 °C for 5 minutes. 5 µl of the bacterial lysate was used to PCR-amplify cloned inserts in 50 µl reactions using standard PCR buffer (Pharmacia), 200 µM dNTPs, 2 units Taq-Polymerase (Pharmacia), and 10 µmol of M13 rev and M13(-20) primers (which flank the multiple cloning site of pCRII.1) under the following conditions: 30 cycles each of 94 °C for 20 seconds, 48 °C for 20 seconds and 72 °C for 45 seconds.

After amplification, 12 µl were loaded onto high density gels (Centipede™ gel electrophoresis chambers, Owl Scientific, Woburn, USA). PCR products were denatured and alkaline blotted onto nylon membranes. The filters were hybridised under stringent conditions (7% SDS in 0.5 M $NaPO_4$ pH 7.2) with equivalent amounts (20-30 ng) of ^{32}P-labelled double stranded cDNA of approximately equal specific activity derived from driver and tester mRNA, respectively. Filters were washed under stringent conditions (see above), and exposed to phosphoimager plates overnight (equivalent to 8 days exposure to conventional film). In addition, the filters were exposed to conventional film up to 12 days at -80 °C and the signals of like clones compared.

Subtracted clone amplification and reverse northern screening

From the subtracted library all differentially expressed genes identified, were screened via reverse northerns against Rsa I restricted ds cDNAs obtained from a large paneal of both rat and human tumorigenic cell lines as mentioned above.

Cloned inserts were amplified using the nested primer sequences from the original subtraction protocol in a total volume of 100 µl. Approximately 10 ng of plasmid template harbouring the insert was taken together with the standard PCR components (see PCR conditions) and 1 µl of a 10 pmole stock of the nested primers. Cycling parameter were set at 94 °C for 40 sec, then 30 cycles of:. 94 °C 20 sec, 68 °C 12 sec and 72 °C 30 sec. Using a multichannel pipette, 12 µl of PCR product was loaded onto the high-density gels and once electrophoresis was complete, the gels were blotted over onto Hybond membrane as described below (see protocol for southern blotting). In this manor, 40-50 membranes each containing the predefined complete set of differentially expressed genes as identified from the original library could be hybridised to ds cDNA from differing sources.

Coupled transcription/ translation in vitro (TNT)

The TNT® (Promega) coupled rabbit reticulocyte lysate system was used to determine the authenticity of the cloned full length rat ezrin and the dominant negative mutant generated by PCR. All reagents used were supplied in the kit and performed according to the accompanying outlines provided. Briefly, 1 µg of clean plasmid DNA was taken and added to a reaction vial containing: 25 µl of TNT lysate, 2 µl TNT reaction buffer, 1 µl of an amino acid mix (minus methionine), 1µl RNasin (40U/µl), 4 µl of [^{35}S]-methionine, 1 µl T7 RNA Poly (10 U/µl), in a total vol of 50 µl. For cold protein products, the labelled methionine was omitted and replaced with normal methionine. All reactions were incubated at 30 °C for 1 hour before the product was heat denatured and loaded on a 12 % polyacrylamide gel. The gel was dried and exposed to autoradiography.

Cell Culture and Transfections

Cell lines.

The rat BD 10 pancreatic cell lines Bsp73-1AS, Bsp73-10AS and Bsp73-ASML were cultured as described. (Matzk et al., 1983). Fisher rat F344 prostatic tumor cell lines (Dunnings cell lines. Isaacs et al., 1986) AT1, AT3, Mat-Lu and Mat-LyLu were cultured according to the conditions originally described. Rat 13762NF mammary carcinoma lines MtPa, MTC, MTLy, MTLN-2 and MTLN-3 were cultured according to the authors recommendations (Steck & Nicolson, 1983). All human pancreatic adenocarcinoma and carcinoma cell lines were obtained from the German collection of micro-organisms and cell cultures (Braunschweig, Germany) and maintained according to their recommendations. AR42J a rat pancreatic carcinoma cell line was maintained in RPMI1640 medium supplemented with 10 % Fetal calf serum and antibiotics. MTW9B and MT450, a non-metastasising and metastasising rat mammary carcinoma cell lines respectively, were both maintained in DMEM medium supplemented with 10 % fetal calf serum and antibiotics.

Cell culture

All cells were maintained at 37 °C in an incubator (Forma Scientific, Labotect GmbH, Göttingen) in 5% CO_2 and 95 % air humidity. All cells were grown in petri dishes (Greiner Labortechnik, Flikenhausen) of varying sizes depending on the application. The cells were allowed to grow until a confluency of 80-90 % had been reached, whereupon, the cells were subsequently split by trypsinization and reseeded at a lower density.

Tyrpsin treatment of cells was performed by removal of the culture medium from the cells, followed by one wash with 20 ml of Ca^{2+} / Mg^{2+} free PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 x 2 H_2O, 1.5 mM KH_2PO_4). After removal of the PBS, 1.5-2.0 ml of 0.25 % trypsin was applied to the cells (amount depending on petri dish size) and the cells incubated at 37 °C until cells had become detached as seen under a low-powered microscope. Fresh medium was then directly applied and the cells replated at the desired density in new petri dishes.

Storage of cells was done by trypsinizing logarithmically growing cells followed by addition of 10 ml of medium before the cells were pelleted with light centrifugation at 300 x g. The medium was removed and the cells resuspended in 1 ml of modified medium containing 20 % FCS, without antibiotics and 10 % DMSO (Fluka Chemie AG. Switzerland) and placed in a cryo-vial. After incubation on ice for 1 hour, the cells were transferred to -80 °C for several hours before finally to liquid nitrogen. To re-propagate cells, the vials were removed form nitrogen placed at 37 °C until most of the cells had thawed. The cells were then removed and mixed with 10 ml of fresh medium (to remove DMSO) followed by light centrifugation, before being replated on petri dishes in fresh medium.

Stable transfection of cells

(a) DOTAP liposomal transfection protocol

Cells used for transfection were split and reseeded 24 hours before transfection commenced. Usually, transfections were performed in 10 cm petri dishes containing approximately 2 x 10^6 cells, giving a confluency of 60-80 %. Transfection was performed using the liposomal transfection reagent DOTAP (Boehringer Mannheim) exactly as described in the protocol provided, using 8-10 µg of maxi-prep plasmid DNA per transfection. The cells were reseeded 24-36 hours post-transfection and placed under appropriate antibiotic selection to generate stable clones. After a period of 1-2 weeks, visible clones were picked and placed in 24 well petri dishes and propagated further under selection until sufficient cell number had been reached, whereupon, the clones were expanded further in 10 cm dishes.

In vivo inoculation of eucaryotic cells

Before cells were taken for *in vivo* experiments, they were monitored for the presence of mycoplasma in the medium of the growing cells. Detection was carried out according to and using the reagents provided in a commercial mycoplasma detection kit (Boehringer Mannheim). Once cells had been confirmed as negative, they were grown to about 70 % confluency, before being lightly trypsinized, washed once in fresh medium (to inactivate the tyrpsin) and twice in sterile PBS. Cells were resuspended at a density of 5 x 10^6 cells/ml. Male BDX-10 rats (9-16 weeks of age) were then inoculated subcutaneously (s.c.) with 5 x 10^5 cells in the left flank before the hind leg. Measurement of tumor growth began at the point were the tumor was easily palatable and continued, twice weekly until the animals were sacrificed (before the tumor had reach a size of 5 cm in one dimension). Tumor samples and metastases of the lymph and lung were removed and immediately snapped-frozen in liquid nitrogen.

Human tumor samples

All human tumor samples and corresponding matched normal tissue were obtained with consent from Dr Kurt Zatloukal, Institute for Pathologie, Graz University Austria For each tumor specimen received exists a record describing the pathological staging of the sample as judged by histological means, sex and age of the poor barstard.

2.3 Analytical Methods

Northern blot hybridisation

Usually between 2-4 µg of poly $(A)^+$ RNA was mixed with DEPC treated bi-dest H_2O to give a final volume of 15 µl. To this was added 7.5 µl of formaldehyde denaturing buffer (50 % crystallised formamide, 1 x MOPS, 3 µg/ml ethidium bromide and 6.5 % formaldehyde) and the samples denatured at 65 °C for 10 minutes before snap-cooling the tubes on ice. To the denature mRNA was added 3 µl of 10 x RNA loading buffer (50 % glycerol, 1nM EDTA and 0.1 % bromophenol blue). The mRNA samples were then size fractionated on a 1-1.4 % formaldehyde/MOPS agarose gel (1-1.4 g of agarose dissolved in 79 ml of bi-dest H_2O and allowed to cool to 60 °C before addition of 16 ml of 37 % formaldehyde and 5 ml of 20 x MOPS) in 1 x MOPS running buffer. Once the migration of the bromophenol blue band had reached the desired distance the gel was removed and the integrity of the mRNA visualised under 302 nm UV light source. Providing the mRNA was of sufficient quality and that no signs of degradation were seen, the mRNA was blotted onto Hybond N^+ membrane (Amersham).
For this purpose it was first necessary to incubate the gel in 5 x SSC for 30 minutes before the mRNA was transferred to Hybond N^+ membrane as described by Maniatis et al. (1989) using 10 x SSC as the transfer buffer. Following transfer , the mRNA was covalently cross-linked to the active OH groups in the membrane using a UV stratalinker 2400 (Stratagene, La Jolla, CA)

Southern blotting of ds cDNA and PCR products

The desired ds cDNA was loaded onto an agarose gel and run in 1 x TBE buffer until the DNA had migrated sufficiently as determined by visualisation under UV-light. The gel was then removed and allowed to incubate for 30 minutes in a solution of 0,4 M NaOH at RT with mild rocking. During this time, a blotting chamber was set up in a manor identical to that for a northern blot. The gel was removed and carefully laid down on 3-4 pieces of 3MM Whatman paper soaked in 0,4 M NaOH After ensuring no air bubbles under the gel were visible, a piece of Hybond N^+ membrane (Amersham) was placed on top of the gel followed by one piece of pre-wetted (in NaOH) Whatmann. A stack of Kimwipes was placed on top and weighted down with a suitable weight. Blotting was allowed to continue o/n upon which the membrane was removed, the position of the wells marked clearly before washing the membrane for 5-10 minutes in 5 X SSC. Membranes were stored dry until use.

Immunoblot analysis of proteins (Western blot)

(a) Separation of proteins by polyacrylamide gel electrophoresis (PAGE)

For most applications a polyacrylamide separating gel of 10-12 % was made and a 5 % stacking gel. Reagents for the stacking gel were 8.3 ml of acrylamide/*bis* -acrylamide (30:0.8, Carl Roth GmbH & Co, Karlsruhe) 6.25 of 1.5 M Tris-HCl pH 8.8, 0.125 ml of 20 % SDS, and 10.05 ml of H_2O (for a 12 % separating gel, the amount of acrylamide/*bis* -acrylamide added was 10 ml and the H_2O reduced accordingly to give the same end volume) To the mixture was added 250 µl of 10 % ammonium persulphate (APS) and the reaction initiated with 20 µl of TEMED. The gel mix is poured between two glass plates with spacers between and allowed to polymerize. Upon completion, a stacking gel is poured on top. This was made up of 1.7 ml of acrylamide/*bis* -acrylamide, 2.5 ml of 0.5 M Tris-HCl pH 6.8, 50 µl of 20 % SDS, 5.65 ml of H_2O, 100 µl of 10 % APS and 7.5 µl of TEMED. The gel was then run in 1 x Laemmli-running buffer (25 mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1 % SDS) until the desired distance had been reached.

(b) Preparation of protein probes

Cells of confluency between 60-80 % in 10 cm petri dishes were washed twice in 20 ml of fresh PBS before 0.75 ml of lysis-buffer was added (160 mM Tris-HCl pH 6.8, 4 % SDS, 16 % glycerol, 0.57 M β-mercaptoethanol, and 0.005 % bromophenol blue). The slurry was transferred to a 2 ml reaction vial and the genomic DNA sonified (Branson cell disruptor B15. output 6) to reduce the viscosity. Before loading the samples, the proteins were denatured by heating for 3 minutes at 95 °C.

(c) Coomassie blue staining of resolved proteins

That part of gel that was not used for transfer was cut away and incubated with mild shaking for 1-2 hours in Coomassie staining solution (0.25 % Serva Blue G250, 46 % methanol, and 8 % acetic acid). After this the gel was destained in two steps. Firstly in 50 % methanol and 8 % acetic acid and then incubated in 10 % methanol with 7 % acetic acid until blue clear bands were visible. At this point, the gel was removed, placed on whatmann paper and vacuum dried.

(d) Electrophoretic transfer of resolved proteins onto membrane and subsequent antibody detection

The section of gel containing proteins of interest was isolated and a piece of pre-soaked (in methanol) Immobilon membrane (Millipore, type PVDF) placed on top of the gel and the proteins transferred in 25 mM Tris-HCl pH 8.3, 250 mM glycine, 20 % methanol using a

BioRad Trans-Blot Cell transfer chamber. Transfer was usually carried out at 350 mA for 4- 16 hours. Once complete, the membrane was incubated for 30 minutes with in blocking buffer (10 % low-fat milk in PBS with 0.3 % Tween 20) to reduce unspecific binding of the antibodies to the membrane. For detection, the membrane was further incubated in fresh blocking buffer containing the appropriate primary antibody (usually, at a dilution of 1:500 to 1:10,000) for one hour. After extensive washing in PBS/0.3 % Tween 20, the membrane was incubated again in blocking buffer containing a 1:3000 dilution of a peroxidase-coupled secondary antibody (anti-rabbit or anti mouse depending on the primary Ab used). Once the membrane had been washed in PBS/0.3 % Tween 20, detection of specific proteins was achieved by enhanced chemifluoresence using Amersham ECL western blotting detection reagents following the manufactures recommended guidelines.

Immunoprecipitation of proteins

For the immunoprecipitation of cytosolic proteins, cells were grown to 60-80 % confluency in 10 cm dishes, washed once in ice-cold PBS before lysing the cells on ice with 1 ml of lyses buffer (NaCl 150 mM, Tris-HCl pH 7.4 30 mM, EDTA 1.0 mM, Triton X100 0.5 %, Na-deoxycholate 0.5 %, PMSF 1 mM, Aproptinin 1 µl/ml (10 mg/ml), Leupeptin 1 µl/ml (10 mg/ml). After a 10 minute incubation cells were scraped together. To the lysed cell mix was added either 30 µl of protein A-sepharose (if a rabbit antisera is used) or 30 µl of protein-G agarose (if a murine monclonal antibody is used). The entire mix was rotated at room temperature for 2 hours before being washed 3 times with 600 µl of lysis buffer. The immunoprecipitation reaction was the washed once with 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, briefly centrifuged and the supernatant removed and kept for analysis. To the pelleted product 50 µl of sample buffer was added, heated to 100 °C followed by a short centrifugation and reheating to 100 °C. The reaction is then loaded on a SDS-PAGE gel of appropriate percentage.

3. RESULTS

PART ONE

3.1 The Model System

The rational for using a cell line system as compared to a direct subtraction between a specific human tumor type and its matched normal tissue rests on the fact that tumor material is limited and the quality of RNA transcripts cannot be guaranteed. Moreover, a tumor represents an additional level of complexity due to the presence of other cell types in the tumor mass (e.g., infiltrating leucocytes). Furthermore, tumor cell population is composed of multiple heterogeneous cells. When subtracted against normal tissue, there is considerable risk that many potential differentially expressed clones are not derived from the tumor cells, but represent transcripts originating from other cell types.

As described in the introduction, the model system chosen for the generation of a subtracted library and the subsequent isolation of differentially expressed genes, was a pancreatic adenocarcinoma cell line system (Bsp73) composed of a parental line and two sub-lines of increasing metastatic potencies. Bsp73 arose spontaneously in 1979 (Matzku et al., 1983) as intraperitoneal nodules together with ascites. Histologically, the nodules were classified as adenocarcinoma of the pancreas and through serial transplantation of ascites cells to a s.c. site, two variants appeared being Bsp73 (AS) non-metastasizing and Bsp73 (ASML) metastasizing. The AS cell line form solid tumors when inoculated subcutaneously (s.c) the cells actively invade neighbouring normal tissue, but do not form metastases in distant organs. The ASML line when inoculated only form rather loose accumulation of tumor cells but rapidly start to colonize in the ipsilateral region lymph nodes, spreading then through the lymphatic system to finally form numerous metastases in the lung. When inoculated intravenously, both tumor cell types develop nodules in the lung which in turn results in metastases to other organs (Knierim et al., 1986b; Paweletz et al., 1986). Other than their metastatic potencies, the two cell lines also differ in other respects such as morphology, adherence characteristics, sensitivity to trypsinization amongst others (Weling. et al., 1985; Knierim et al., 1986a; Paku et al., 1986; Raz et al., 1986). As mentioned in the introduction, Günthert et al. (1991) could show that a specific variant form of the cell surface glycoprotein CD44 was necessary and sufficient to promote metastases upon transfection into non-metastatic Bsp73-AS recipient cells. Furthermore the *in vivo* effects of transfected variant CD44 could be abolished by antibodies directed against the variant. What is also evidently clear is that the requirement of CD44 in metastasis is not absolute. That is, the functional relevance of CD44 for metastasis is apparent for some tumor cell types such as Bsp73-ASML (Günthert et al.,1991) but not for other cancer types, such as endometrial cancer (Tokumo et al., 1998). It is therefore likely many other genetic dysregulations in ASML cells that predispose them to a metastatic phenotype. The isolation and identification of these "other" genetic alterations in respect to the metastatic cell line Bsp73-ASML became the primary aim of my work.

3.2 Confirmation of the Metastatic Potential of the Bsp73 Cell Lines

As a large part of the project centred in the systematic isolation of genes whose up-regulation coincided with a metastatic phenotype, it was foremost that the cell system chosen responded *in vivo* as previously described (Matzku et al., 1983). For this purpose all cell lines used were passaged in male BD 10 rats.

The rat pancreatic adenocarcinoma cell lines Bsp73-1AS (1AS), Bsp73-10AS (10AS) and Bsp73-ASML (ASML) were reported to form local tumors at the site of injection when inoculated subcutaneously (s.c.) in syngeneic rats (Paweletz et al., 1982). In this series, the 1AS represented the non-metastatic parental cell line from which the other two are derived subclones of differing metastatic competence. They are therefore isogenic in origin. The 10AS gives rise to a low number of lung and lymph metastases, whereas the ASML is highly metastatic, producing secondary neoplasms in lymph and lung tissue. All cell lines were inoculated s.c. into male BD 10 rats (eight animals per line) and their growth rates recorded based on relative tumor volume. Figure 3, indicates the growth rates of the cell lines plotted as tumor volume against time. What is evident from the graph is that the relative growth rates of the primary tumor shows an inverse correlation to metastatic capacity of the cell line.

Figure 3

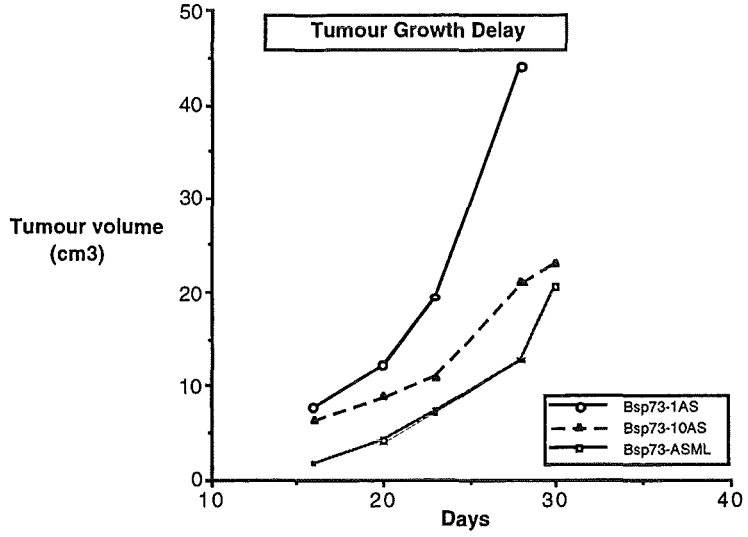


Figure 3. Relative *in vivo* growth rates of three isogenic pancreatic adenocarcinoma cell lines. The 1AS cell line is non-metastatic in syngeneic rats. 10AS is of low metastatic capacity, and the ASML line is highly metastatic. 5 x 10^5 cells in sterile PBS were injected s.c. in BD 10 male rats and measurements taken twice weekly until sacrificed. Tumor volumes were calculated and have been plotted as a function of time.

This is in accordance with original observations made (Matzku et al., 1983), describing the *in vivo* characterisation of these cell lines, namely the non-metastatic being the fastest growing. The 1AS produced tumors of comparable volume to ASML, however in half the time period required for ASML. Measurements are depicted after day 14 post-injection (point at which

tumor was measurable). Upon dissection of the animals, the Bsp73-1ASML cell line gives rise to multiple metastases (through the lymphatic and venous routes) in both lymph and lung tissues. These *in vivo* data demonstrates that the metastatic properties of the Bsp73 cell system are as expected in that they have not altered their intrinsic metastatic capacity and confirm the original observations made with these cell lines (Matzku et al., 1983).

3.3 Subtractive Suppression Hybridization (SSH)

The art of obtaining differentially expressed clones from a cell or tissue system has been a much pursued avenue of research. This is reflected by the large number of documented methods that now exists enabling such aims to be achieved. However, no method is infallible as they all have some intrinsic limitation that must be taken into consideration. One particular method (Diatchenko et al, 1996) seemed to offer a high degree of sensitivity over other existing methods, and is composed of a normalisation step coupled with a PCR enrichment step that would enable the isolation of the majority of differentially expressed transcripts, irrespective of their initial relative abundance in a given system. The method is known as Subtractive Suppression Hybridization (SSH) and functions in the following manner.

It starts with the two populations of mRNAs that are to be subtracted (see figure 4). The "Driver" is defined as the population of mRNAs that will be eliminated during subtraction whereas the "Tester" population of mRNAs contains, in addition, the differentially expressed genes of interest. Both mRNA populations are reversely transcribed, producing double-stranded (ds) cDNA that are then restricted with a four-base recognition endonuclease RsaI (cuts theoretically every 256 bp). This ensures that the majority of cDNAs have an average length of 200-800 bp which in turn aids hybridization of compatible strands as the complexity has been reduced via the restriction. At this point, the "Driver" cDNA population requires no further modification.

The cDNA originating from the "Tester" population is split into two equal aliquots, and ligated to two different linkers to produce two sub-populations that differ only in their respective linkers. These two sub-populations are subsequently denatured and each separately hybridized in the presence of a 30-fold excess of "Driver cDNA". This step is critical as it normalizes the cDNA populations originating from the driver and the tester mRNA, and thereby prevents over-representation in a library. That is, a transcript whose initial abundance is high in relation to the driver, has a high probability of hybridizing to compatible driver sequences such that the concentration of single stranded cDNA of that gene is reduced. Conversely, a transcript of low initial abundance will not readily hybridize to compatible driver sequences (as the probability is proportional to the initial concentration) thereby the concentration of single-stranded cDNA will remain largely unchanged. So in effect the high abundant differentially expressed cDNAs are reduced in concentration, and those of low abundant differentially expressed cDNAs are unchanged.After the normalization, both hybridizations are pooled and hybridized again to a

30-fold excess of denatured driver (note: only the driver is denatured). From the first hybridization only the remaining single-stranded cDNA can form hybrids with complementary sequences derived from the driver. Those cDNAs that do not have complementary sequences in the driver will anneal to complementary tester sequences instead. It is exactly these hybrids (one strand from the tester sub-population with one type of adaptor annealed to a complementary strand from the other tester sub-population with the other adaptor) that form the bases of subtraction as they are the only form of hybrid capable of exponential amplification with two different primers that are complementary to the two different linkers used.

Figure 4

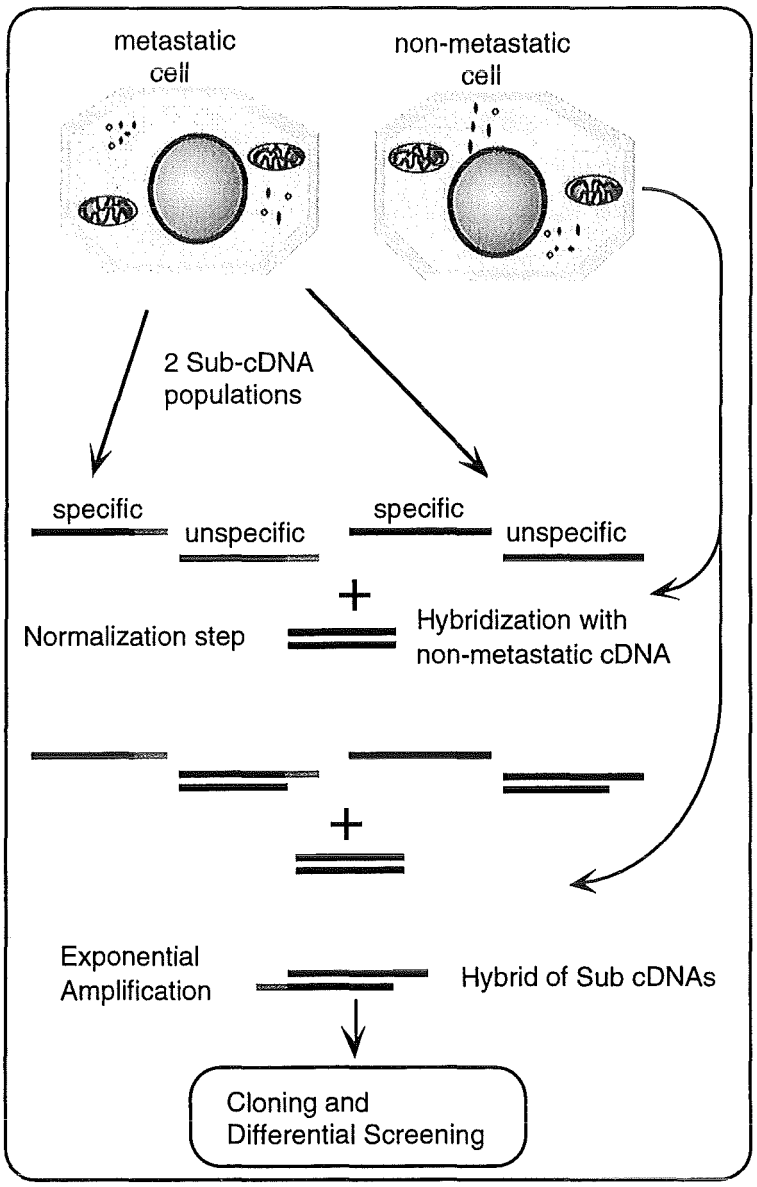


Figure 4. Schematic diagrame of SSH and how it functions Upon isolation of poly $(A)^+$ RNA from the non-metastatic and metastatic cell lines, cDNA is made and restricted such that the average length of cDNAs is reduced. For the non-metastatic cDNAs no further steps are required. cDNAs derived from the metastatic line are split into two pools, each of which receives a specific but different adaptor (depicted as black and green ends of cDNA molecules). The red bars indicate sequences that are specific to the metastatic cell line whereas, the blue bars indicate unspecific sequences present in both cell lines (e.g., GAPDH). Upon hybridization with an excess of driver cDNAs, unspecific sequences from the metastatic line anneal to complementary unspecific sequences derived from the non-metastatic cell line (indicated by the double-stranded blue cDNA molecules). Specific sequences find no complementary partner and remain single-stranded (shown by the two red bars). During the last hybridization step these two red cDNAs will anneal together (providing they represent complementary strands of the same cDNA) to produce a double-stranded cDNA molecule, having having a different adaptor sequenced on either end (shown as double red bars). These molecules are subsequently exponentially amplified through PCR using a primer pair that anneals to the two adaptor sequences.

Suppression of PCR amplification occurs with those hybrids that have the same linker on either end of the single-stranded cDNA molecule due to the internal sequence complementarity (inverted terminal repeats). During each primer annealing step, the hybridization kinetics strongly favour (due in part to the fact that the adaptor is twice the nucleotide length as that of the primer) the formation of a pan-like structure (see figure 5) that prevents successful primer annealing and thereby, the template is not amplified. Should a primer anneal the newly amplified template will contain again the inverted tandem repeats so a pan-structure is again possible. Thus, during the course of PCR, non-specific amplification is efficiently suppressed while amplification of specific sequences having different adaptors at both ends precedes as normal.

The net out come is selective amplification and enrichment of cDNAs that originate from the tester population only. The differentially expressed cDNAs are then be subsequently cloned into a suitable vector cloning system such that a library is constructed that now contains an enriched proportion of cDNAs derived from the tester population.

Figure 5

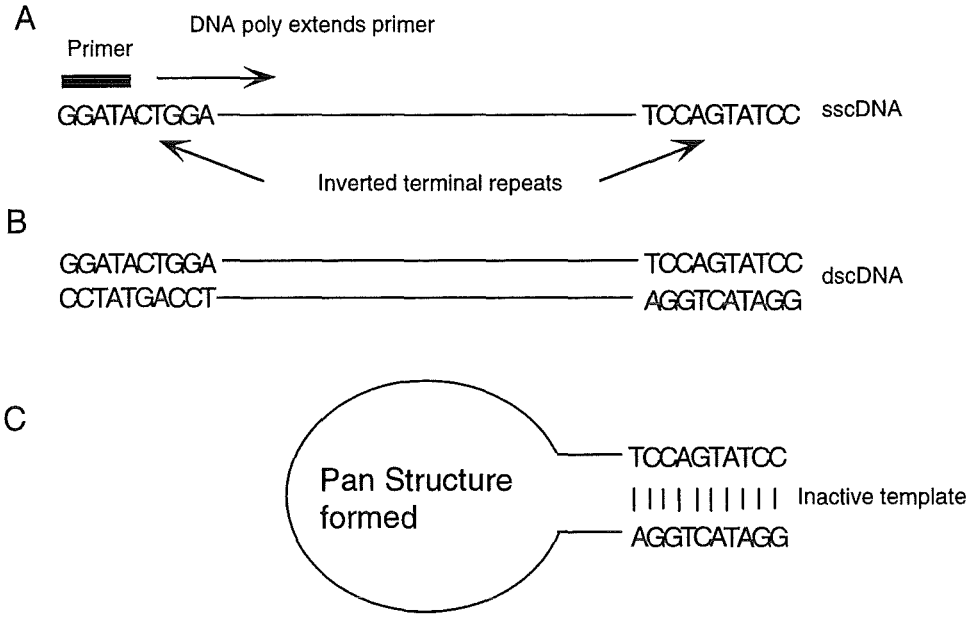


Figure 5. Suppression PCR. (Siebert et al., 1995).
(A) If primer anneals to one of the complementary sequences (adaptor) present on each end of the ss cDNA molecule, and is extended through the action of the Taq polymerase, such that the resulting double stranded cDNA molecule (B) has now also inverted terminal repeats (as in the case for the original template (A)). Upon the denaturing cycle of a PCR protocol, the two strands dissociate and during the annealing step form a pan-like structure (C) thereby preventing the successful annealing of a much shorter primer sequence. In this fashion such template amplification is suppressed, Consequently, only the amplification of single-stranded DNA templates, having two different adaptors on their ends is possible as no inverted repeats are present to form the pan structure. In the SSH protocol, these can only originate from the tester population.

Despite the low number or reports that have utilized this method (due in part to the technical demands) it has nevertheless, when coupled with a sensitive screening protocol, proven to be extremely versatile and powerful were identification of differentially expressed genes is required (von Stein et al., 1997).

3.4 Generation and subtraction efficiency monitoring of the Bsp73-1AS/ASML subtractive library

The construction of the subtractive library was performed as outlined in the methods section. Prior to screening the library it was of utmost importance to monitor the level of efficiency of subtraction between the two cell lines. This is done by monitoring the depletion of a transcript (negative control) that displays no differential expression pattern between the two cell lines (e.g., usually the common housekeeping gene, GAPDH). The retention and subsequent enrichment of a gene whose expression is exclusive to the metastasizing cell line (positive control) can be determined assuming prior knowledge of such a gene. In the Bsp73 system it had been previously shown that the urokinase type plasminogen activator gene (uPA) was expressed in the ASML cell line (Hofmann, Ph.D thesis 1991), showing no detectable expression in the 1AS cell line

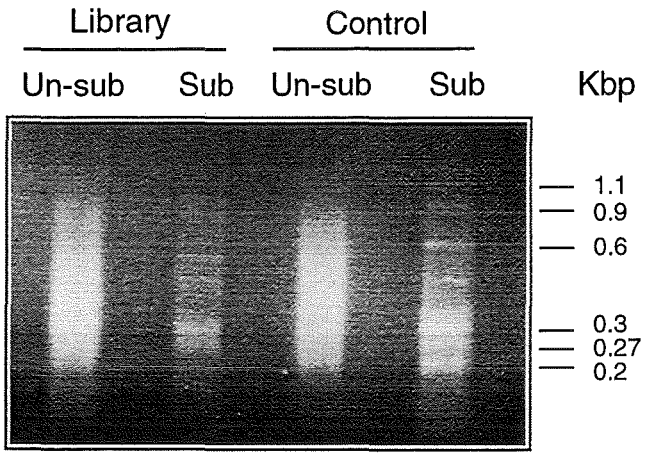


Figure 6. Efficiency monitoring of subtraction ("experimental test with deliberate contamination of plasmid specific sequences").
Lanes denoted as "un-sub" depict PCR amplified cDNAs before subtraction and those marked "sub" indicate PCR amplified cDNAs after subtraction. In the case of the control, a mock subtraction was performed between human skeletal muscle cDNA against human skeletal muscle cDNA that had been previously contaminated with 100 pg of *Hae* III-digested bacteriophage ØX174 DNA. For the library, Bsp73-ASML was subtracted against Bsp73-1AS. The appearance of bands in the sub-lane of the control lane corresponds to the plasmid fragments used to contaminate the tester. Likewise, bands seen in the sub-lane of the library indicate enrichment of specific transcripts that are derived from the tester only.

In the case were no positive control is known, the appearance after subtraction of specific contaminating plasmid sequences can be determined. In this respect, the contaminating plasmid sequences function as an internal subtraction control. In figure 6, there is no visible

banding pattern in the unsubtracted lane of the control, however, after subtraction bands become clearly visible and represent the correct fragment sizes seen with a pure ØX174 DNA *Hae* III digest. It is therefore obvious that through subtraction, there is a selective amplification of these plasmid sequences (as they do not exist in the driver population), with a concomitant reduction of background sequences. The appearance of bands seen after subtraction in the library is typical, the banding pattern being different to that seen in the control, and indicates again selective enrichment of certain sequences derived from the tester cDNA population.

Figure 7

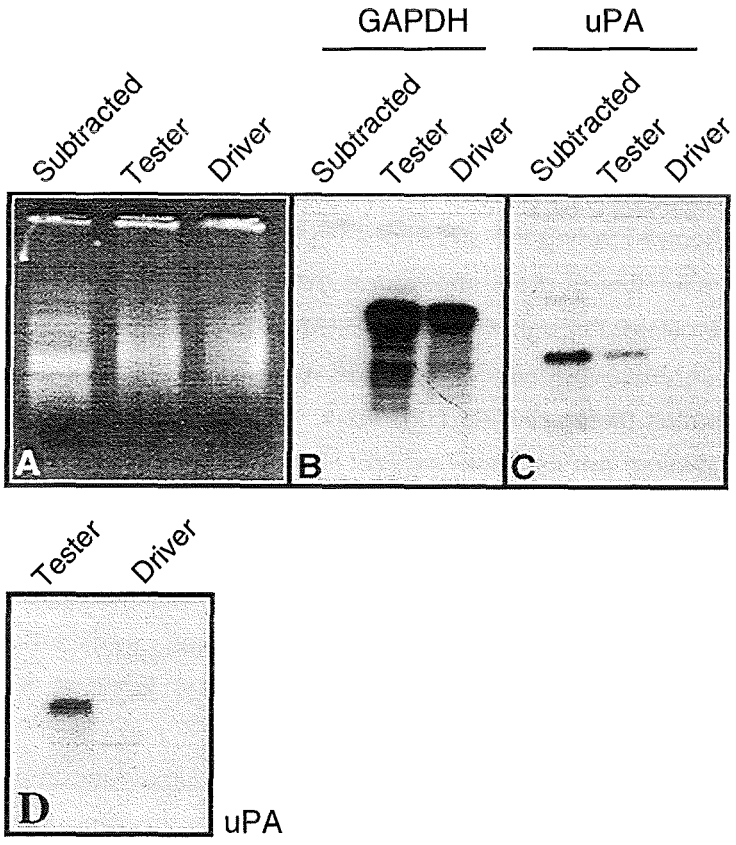


Figure 7. Efficiency monitoring of the 1AS/ASML subtraction (using positive and negative controls, see Materials & Methods)
(A) Following the nested PCR amplification (using primers complementary to the adaptors used in the initial subtraction) as outlined in the subtraction protocol, 200 ng of amplified ds cDNA from the tester (Bsp73-ASML), driver (Bsp73-1AS) and subtracted cDNA (tester minus driver) was loaded in duplicate on a 1.4 % TAE agarose gel and transferred to Hybond-N+ membrane under alkaline conditions. Both membranes were probed with ^{32}P-dCTP labelled GAPDH (mouse probe, nucleotide positions 489-723) and rat uPA, **(B)** and **(C)** respectively. **(D)** 2 µg of poly(A)$^+$ RNA was size fractionated on a 1.2 % formaldehyde agarose gel and transferred to membrane. The membrane was probed with uPA as in panel (C).

Figure 7 again demonstrates the efficiency of subtraction in the context of two specific genes. The negative control (panel B) indicates the presence of GAPDH in both cell populations prior to subtraction. However it is no longer detectable after subtraction indicating a very efficient

removal of the gene, and thereby one can assume that other sequences that also exhibit no differential status between the two cell lines will be also efficiently removed. For the positive control (panel C), the uPa gene is present exclusively in the tester cDNA population, reflecting its true differential status as seen in direct northern analysis, (panel D). This gene and others of similar differential status becomes successfully enriched during the subtraction procedure. Consequently, there is a specific amplification for sequences originating from the tester (uPA) with a concomitant suppression of sequences common in both cDNA populations (GAPDH). After positive evaluation of subtraction efficiency, the subtracted cDNAs were cloned and screened as described in Materials & Methods (see also; von Stein et al. (1997).

3.4.1 Reverse Northern Screening of the Bsp73-1AS/ASML Subtracted Library

A total of 5000 clones were picked under blue/white selection and amplified by colony PCR in 96 well-format before loading the amplicons via the use of a multi-channel pipette onto high-density gels in parallel. It is of great importance that each gel is loaded equally such that potential differences identified through differential screening reflect true differences and not variations as a result of unequal loading. The gels were run in parallel (see figure 8) then transferred onto membranes under alkaline conditions. This resulted in the generation of two membranes each containing an identical set of clones that are subsequently used for differential screening. Screening takes place in the form of ^{32}P-dCTP radiolabeled cDNAs derived from the tester and driver cell lines. Again, it is of importance that the ds cDNAs are restricted with Rsa I prior to labelling such that the fragments produced match those as cloned inserts. Results obtained with unrestricted probes are vastly less sensitive than those seen with resticted probes (data not shown). For screening, two identical membranes were hybridized under stringent conditions with labelled ds cDNA from both tester and driver respectively. The membranes were then exposed and the two films superimposed to allow for direct comparison of like clones. Signals specific for the tester probe represent potential cDNA clones originating from the metastatic cell line. Conversely, like signals seen with both probes are obviously not differentially expressed and are ignored. In figure 9 two identical membranes have been hybridized with tester and driver ds cDNAs respectively. What is immediately obvious is the increase in signal number seen on the filter hybridized with cDNAs derived from the metastatic cell line (ASML blot 2). Signals present on both membranes act as internal positive controls with respect to labelling efficiency, hybridization and amount of insert cDNA; in other words, like signals of equal intensity indicate equal conditions. All clones that are potential differentially expressed are marked with a straight bar and in addition, not all differential signals are of equal intensity. This may be indicative of the initial relative abundance of these cDNAs within the tester population. Those clones which fail to hybridize to either probes may represent cDNAs whose abundance is too low and thereby falls outside the limit of sensitivity of reverse northerns.

Figure 8

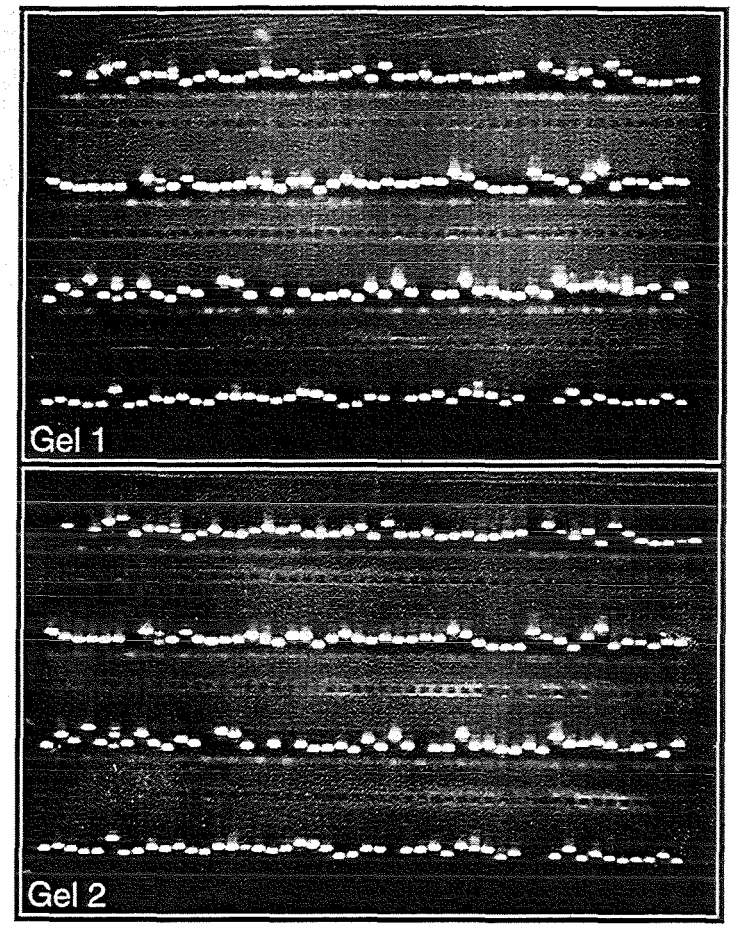


Figure 8. Simultaneous analysis of 200 clones on duplicate high-density gels. The above figure displays the appearance of the gel format used to screen the subtractive library. Each gel has a total capacity of 200 individual clones that can be run in one setting. The clones were PCR amplified in 96 well-format in a total volume of 100 µl under the following cycle parameters (94 °C, 1 min, then 30 cycles of 94 °C, 20 sec; 68 °C, 12 sec; 72 °C, 30 sec) using a Perkin Elmer 9600 PCR machine. 12 µl of amplified product was loaded into each well of a 1.2 % TAE agarose gel and run until the cDNA had migrated roughly half-way between the rows of slots. The gels were then alkaline blotted and probed as outline in Materials & Methods.

Equally, not all clones contain inserts (approximately 10% of clones harbour no insert as determined by restriction analysis of plasmid mini-prep DNA). However, reverse northern screening allows for the rapid high-throughput and easy detection of cDNAs that are specific to the metastasizing ASML cell line. It does not, however, allow for the detection of polymorphism of mRNA, or indeed splice variants, or size of transcripts and it is not as sensitive as direct northern analysis These facts highlight the limitation of reverse northern screening. Reverse northern screening is, however, very much more reliable and has a higher sensitivity when compared to standard colony-lifts. Considerable time was invested in attempts to screen the subtracted library via colony lifts, but due to the unacceptable high background (caused in part by the presence of bacterial proteins, chromosomal DNA that had adhered to the membrane) was not pursued further. Additionally, if the criteria for a positive signal is just signal intensity difference between the two probes, colonies of unequal size can often lead to mass isolation of false positives. This in turn necessitates additional rounds of screening. With reverse northerns this is not the case. The amount of DNA can easily be monitored (see figure 8) and due to ist reliability, rescreening is completely unnecessary.

Figure 9

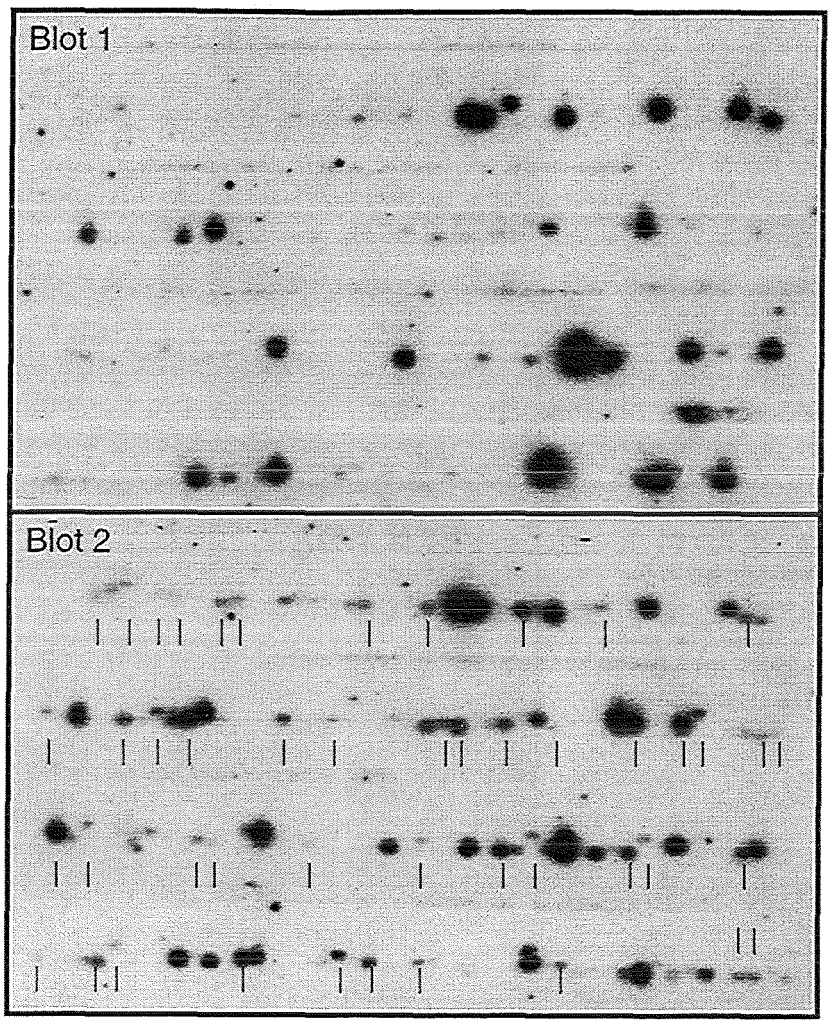


Figure 9. Differential screening of amplified cDNA clones.
Blot 1 and 2 represent identical clones probed with ^{32}P-dCTP labelled cDNAs derived from Bsp73-1AS (Driver) and Bsp73-ASML (tester) respectively. Hybridizations were performed in Church buffer at 64 °C and the membranes subsequently washed once in 2 x SSC, 0.1 % SDS and twice in 0.1 x SSC, 0.1 % SDS at 65 °C. Exposure to film at -80 °C was up to 12 days to allow detection of those clones of lower expression status. Bars indicate position of clones harbouring potential differentially expressed cDNAs with respect to the metastasizing cell line. Signals common to both function as an internal control insofar as like signals of equal intensities indicates identical conditions.

3.4.2 Confirmation of Differential Status by Northern Analysis

Through reverse northern screening a total of 625 differentially expressed signals were identified that demonstrated expression intensities from very strong to very week (seen only after 10-12 days exposure to film). All positive clones were grouped and the inserts re-amplified by colony PCR and gel purified before being labelled with ^{32}P-dCTP. Inserts were hybridized against poly(A)$^+$RNA derived from the non-metastatic (Bsp73-1AS), low metastatic (Bsp73-10AS) and the highly metastatic (Bsp73-ASML) cell lines. A selective panel of 19 clones and a loading control (GAPDH) are shown in figure 10.

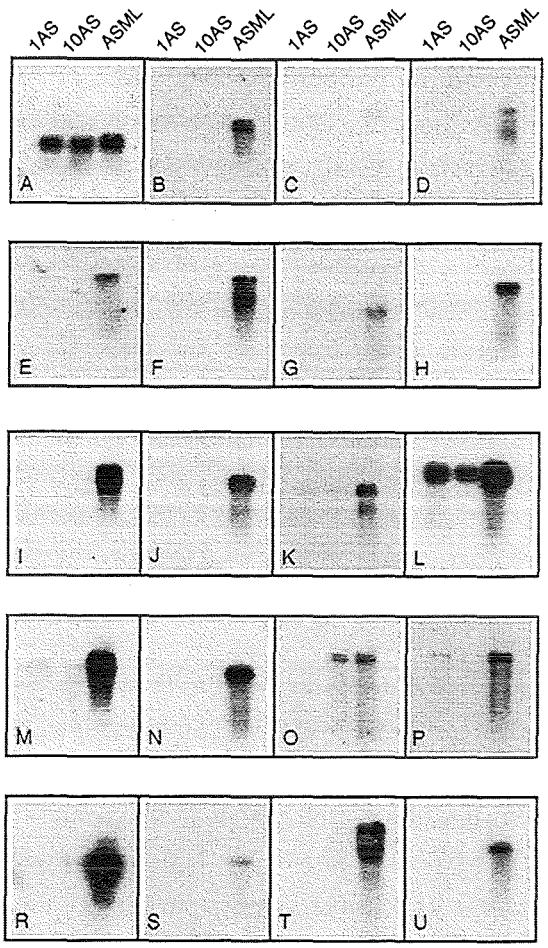


Figure 10. Northern analysis of a number of positively identified clones.
The above figure represents a selection of known and novel cDNAs identified through the subtracted library (panels **B-U**). For each blot, 4 µg of poly $(A)^+$ RNA isolated from 1AS, 10AS and ASML cell lines were loaded and size fractionated on a 1.2 % formaldehyde agarose gel before transfer to Hybond N^+ membrane. Each blot was hybridized under stringent conditions with a different clone (**B-U**) and exposed to film (between a few hours up to 4 days at -80 °C). Putative clone identities are given in parenthesis. The blot indicated with an (**A**) represents GAPDH and is used as a loading control. All other probes were as follows: (**B**) clone # 75 (melanoma growth stimulatory activity); (**C**) clone #128 (transcript differentially expressed after nerve growth factor treatment); (**D**) clone#135 novel; (**E**) clone #29 novel; (**F**) clone #94 rat testin; (**G**) clone #86 novel; (**H**) clone #72 novel; (**I**) clone #115 (CDK4/6 inhibitor $p16^{ink4a}$); (**J**) clone #43 HMG-1; (**K**) clone #18 (SrcSH3 binding protein); (**L**) clone #126 calgizzarin; (**M**) clone #79 (fragile X mental retardation protein 1); (**N**) clone #45 caveolin; (**O**) clone #62 ezrin; (**P**) clone #85 cMET receptor; (**R**) clone #91 AHNAK; (**S**) clone #100 novel; (**T**) clone #71 novel; (**U**) clone #67 novel.

It is clear that from the panel of northerns indicated in figure 10, there are clones whose relative abundance approaches the order of "rare" transcripts (e.g., panels C, G and S) as these clones require a few days exposure before a signal is observed. Others clones such as caveolin, HMG-1 and testin (panels N, J, and F respectively) are all highly abundant in the metastatic cell line. Panel (L) demonstrates a clone whose expression is upregulated some 3-fold from basal level, indicating genes of moderate differential expression can also be isolated. That is the protocol of SSH is not biased for the isolation of differential transcripts that differ

dramatically between to populations, but rather allows the identification of transcripts that cover the whole spectrum of different initial abundances. In conclusion, the differential expression profile seen with the reverse northern screening analysis reflects true differences as confirmed in straight northerns. A total of 70 clones were monitored for differential expression with respect to the metastasizing cell line ASML, four of which appeared to be false positives. This equates to a true positive rate from the reverse northern screening to standard northern analysis of over 90 %. This value also indicated that positive signals as seen through reverse northern analysis used to screen the library, obviously reflected to a very high percentage the true expression status of the clones when analysed by standard northern analysis. This would equate to the fact that it is probably not neceassary to screen each identified clone for differential expression via standard northern blots, as a differential signal with reverse northern screening can be considered as being truely differential.

3.4.3 Sequence Identity of Isolated Differentially Expressed Clones

From all clones identified as being potential differentially expressed, plasmid DNA was isolated and the inserts sequenced. A large proportion of sequencing was done commercially (4 base labs GmbH Reutlingen), and the obtained sequences edited to remove flanking vector sequences before blasting them through a number of public sequence data bases via the internet (Ebeling & Suhai, 1997; Altschul et al., 1997). Retrieved matches were analysed on the strength of homology and a table constructed that depicts either the true identity (homology > 94 % to known rat sequences), putative human homologous (homology ≥ 56 % to known human genes) or novel sequences (homology ≤ 56 % to any call). Sequencing analysis indicated that the initial 625 cloned cDNAs represented 126 individual cDNA fragments. Their identity, strength of match, redundancy and size in bp is given in table 1.

The accession number listed for most clones represents the data base identifier for the sequence that exhibited highest homology to the query sequence. Homology is given as a percentage identical match over the entire length of the query sequence. It should however be noted that the highest match does not mean closest identity as all searches were performed initially using the nucleotide sequence and not the translated protein sequence, which is generally more sensitive (Henikoff & Henikoff, 1994; Henikoff et al., 1998). Therefore those matches were the homology is questionable, the suggested identity should be only regarded as an indication. In cases were the matched homology is high > 90 % then the identity is probable (matched to rat), relatively high to high homology to a mouse or human sequence may indicate a rat homologue, but not necessarily. Lower homology 60-80 % might indicate a new family member. If the returned matches are low the clone could be novel. Additionally, some of the clones identified are too short to allow a match of any certainty possible, moreover clones that lie in the untranslated regions may be mistaken for novel.

The redundancy for each clones is listed and provides an indication to relative abundance in the initial subtracted library. For example, caveolin (clone # 45) was represented by 47 clones from an initial 5000 screened. This equates to a redundancy of 0.9 % in the original library. However, from all clones identified as being potential differentially expressed (625) caveolin represents a redundancy of 7.5 %. So there is a small degree of redundancy for a number of clones but it is marginal and indicates respectively that the normalization step in SSH (e.g., the first hybridization) had worked with high efficiency (Gurskaya et al., 1996; Bonaldo et al., 1996).

Table 1

Clone	Accession.#	Size (bp)	% ID over bp	Red	Identity/ highest homology
001	-	121	no EST	2	novel
002	gb:M33534/gb:M590	193	95%, 183bp	2	5' / 3' LTR, NICER element (R)
003	-	123	no EST	2	novel
004	gb:X82180	161	78.3%, 143bp	1	ribosomal protein L4 (R)
006	-	131	no EST	1	novel
007	gb:U16741	201	95.9%, 172bp	3	capping protein, alpha 2 subunit (M)
008	gb:U72141	204	83.8%, 142bp	1	multiple exostoses 2 gene EXT2 (M)
009	-	102	no EST	1	novel
010	EST (M) gb:AA168416	124	88.1%, 118bp	1	novel
011	-	126	no EST	1	novel
013	gb:L10377	294	88%, 283bp	2	clone CTG-B37 (H)
014	-	270	no EST	4	novel
015	-	92	no EST	1	novel
018	gb:U92478	259	92.7%, 259bp	22	SrcSH3 binding protein (M) (2 separate clusters)
021	-	84	no EST	1	novel
022	EST (H) gb:W01116	251	77.6%, 161bp	6	novel
023	EST (H) gb:AA304806	447	91 %, 276bp	2	novel
024	-	122	no EST	1	novel
025	emb:X59993	192	93.8%, 113bp	1	probable Zinc finger protein (R)
026	EST (M) gb:AA09131	44	86%, 44bp	2	novel
029	EST (M) gb:AA62735	161	83.1%, 169bp	8	novel
031	-	102	no EST	1	novel
032	EST (M) gb:AA124683	541	83.4%, 381bp	2	novel
033	-	242	no EST	7	novel
034	dbj:AB001349	210	98%, 210bp 3' UTR	3	steroidogenic acute regulatory protein (R)
035	dbj:D63477	174	65.4%, 174bp	13	KIAA0143 (H)
036	-	105	no EST	1	novel
037	gb:U83411	155	83.9%, 155bp	1	carboxypeptidase Z (H)
038	gb:L13039/X66871	50	100%, 50bp	1	annexinII/calpactin I heavy chain (R)
040	-	91	no EST	1	novel
041	emb:Y00345	223	97%, 223bp	1	polyA binding protein (H)

Clone	Accession.#	Size (bp)	% ID over bp	Red	Identity/ highest homology
042	EST (M) gb:AA409131	498	86.7%, 330bp	3	novel
043	emb:X62875	66	100%, 66bp 3' UTR	42	high mobility protein I (R)
044	emb:X13835	181	99%, 174bp	1	calmodulin II (R)
045	gb:U32114	454	85%, 426bp	47	caveolin-2 (H)
046	EST (R) gb: H32003	197	97%, 153bp	1	transcript diff. expressed after nerve growth factor treatment
048	gb:Z19552	256	97%, 253bp	1	Topoisomerase II (R)
049	-	247	no EST	1	novel
051	EST (H) gb:T91167	221	79.8%, 198bp	1	novel
052	-	104	no EST	12	novel
054	dbj:D88313 gb:X08004	148	96.6%, 148bp 92.6%, 148bp	1	rap1B (R) rap1B (H) ras-like GTP exchange factor
056	gb:X81059 gb:L76702	65	98.5%, 65bp 89.2%,65bp	1	TEX-271, protein phosphatase 2A (M) B56 delta (H)
057	EST gb:AA161810 (M) gb:T10577 (H)	255	90.7%, 108bp 78.8%, 156bp	1	novel
058	gb:X03212	46	82.6%, 46bp	1	keratin-7 Type II (H)
059	gb:M86564 gb:M14630	159	91.3%, 161bp 87.6%, 161bp	1	alpha-prothymosin (R) alpha-prothymosin (H)
060	gb:D14695	115	87.9%, 99bp	1	KIAA0025 gene (H)
061	gb:M76124 gb:M26481	242	76.8%, 142bp 67.4%, 141bp	3	EPG314 precursor (M) KS1/4 antigen (H)
062	gb:X60671	135	92.6%, 135bp	2	ezrin (M)/ (2 separate clusters)
063	EST (H) gb:AA186686	228	76%, 91bp	1	novel
065	gb:S72008 gb:U08103	256	89%, 264bp 74%, 171bp	1	CDC10 homolog (H) D. melanogaster peanut
066	-	137	no EST	1	novel
067	-	265	no EST	6	novel
068	gb:M92340	84	82.9%, 82bp	1	gp 130/IL-6 signal transducer protein (R)
069	gb:D84068 gb:U58516	309	95.9%, 293bp 73.4%, 293bp	2	O-Acetyl GD3 ganglioside synthase (R) breast epithelial Ag B46 (H)
070	-	96	no EST	1	novel
071	-	165	no EST	2	novel
072	EST (M) gb:AA426984	129	91%, 61bp	6	novel
073	EST (H) gb:R13346	64	87%, 64bp	1	novel
075	emb:X12510 gb:M57731	316	69.4%, 186bp	3	melanoma growth stimulatory activity (H) 3'UTR
076	emb:X74215	90	81%, 90bp	1	Lon-like protease (H)
077	EST (H) dbj:D62588	228	90.4%, 228bp	1	novel
078	gb:M10068	83	100%, 83bp	1	NADPH cytochrome P450 reductase (R)
079	emb:X90875	217	96.3%, 217bp	1	fragile X mental retardation protein 1 (M)
080	EST (M) gb:AA162657	172	97%, 172bp	1	novel
081	EST (M) gb:AA033067	95	92.3%, 91bp	1	novel
082	EST (M) gb:AA174699	243	95%, 243bp	2	novel

Clone	Accession.#	Size (bp)	% ID over bp	Red	Identity/ highest homology
083	emb:Z63378	125	77.1%, 83bp	1	cpG island DNA fragment (H) 64.5 % amino acid id.with unknown ORF
084	gb:U00594	193	75.9%, 195bp	41	TGF-beta regulated transcript 1 (TRT1),
	gb:T84080		66.2%, 183bp		Mustela vison human EST (3 separate clusters)
085	gb:U65007	269	97.4%, 269bp	5	hepatocyte growth factor (R) (2 separate clusters)
086	EST (H) gb:AA075872	233	67.3%, 220bp	12	novel
087	gb:U30877	148	71%, 141	1	Urechis caupo cathepsin B-like protease
088	EST (M) gb:AA032965	179	72.7%, 154bp	1	novel
091	gb:M80899	221	86.9%, 221bp	1	AHNAK, desmoyokin (H)
093	gb:X95082	88	92.1%, 89bp	2	parathyroid hormone regulated sequence (R)
094	gb:x78989	240	90.6%, 234bp	25	testin 2 (M) (3 separatre clusters)
095	-	252	no EST	2	novel
096	-	286	no EST	1	novel
097	gb:M74067	131	96.2%, 131bp	1	RAT ORF induced by androgen withdrawl and apoptotic cell death (R)
098	gb:X65651	131	99.2%, 131bp	1	uPA (R)
099	-	109	no EST	1	novel
100	EST (M) gb:W11916	272	81.8%, 279bp	3	novel
101	-	81	no EST	1	novel
102	emb:X51538	96	95%, 96bp	1	ribosomal protein S24 (R)
103	gb:U31867	108	92%, 88bp	3	Tclone 15, calcycline (R)
	gb:X52278		84%, 100bp		calcycline (M)
104	-	60	no EST	1	novel
106	-	60	no EST	1	novel
107	emb:V00680	60	96.7%, 60bp	1	mitochondrion 16S ribosomal RNA (R)
108	gb:M69055	55	92.6&, 54bp 3' UTR	1	Insulin-like growth factor binding protein (R)
109	gb:M19967	228	99 %, 228bp	19	calpactinII/lipocortinI
	emb:X05908		85 %, 228bp		
110	gb:M63482	118	96%, 116bp	2	cytokeratin 8 (R)
111	-	81	no EST	1	novel
112	gb:U05341	153	97.7%, 131bp	2	p55CDC (R)
113	-	101	no EST	1	novel
114	gb:D85904	234	90.3%, 237bp	1	apg-2 (rel. to HSP 110) (M)
	gb:L12723		85.8%, 232bp		hest shock protein 70 (H)
115	gb:L76150	149	88.6%, 146bp	1	CDK4/6 inhibitor p16ink4a (M)
116	gb:M19635	78	96.4%, 56bp	1	large subunit ribosomal protein L36a (R)
117	gb:X97443	216	97.7%, 216bp	2	Tmp-21 (R)
	gb:X97442		87.9%, 215bp		Tmp-21 (H) /transmembrane protein
119	EST (H) gb:AA374085	211	80.5%, 149bp	1	novel
121	gb:X14671	270	98.1%, 258bp	2	ribosomal protein L26 (R)
122	-	81	no EST	3	novel
125	EST (M) gb:AA214794	167	81% , 82bp 3'	1	novel

Clone	Accession.#	Size (bp)	% ID over bp	Red	Identity/ highest homology
126	gb:U41341 gb:D38583	106	94%, 55bp 75.2%, 105bp	1	endothelial monocyte-activating polypep.I (M) calgizzarin (H)
127	EST (M) gb:AA254302	218	90.8%, 142bp	1	novel
	EST (R) gb:H32315	204	97%, 195 bp	1	transcript diff. expressed after nerve growth factor treatment
129	EST gb:W55478 (M) gb:R71504 (H)	245	 100%, 104bp 90%, 105bp	1	novel
130	EST (M) gb:W89336	290	94%, 152bp	1	novel, Zn-finger motif (partial)?
131	EST (M) gb:AA143958	109	81.5%, 86bp	1	novel
132	EST (M) gb:AA102862	164	80.2%, 164bp	1	novel
133	gb:D10729	106	95.3%, 106bp	1	proteasome subunit RC1 (R)
134	-	76	no EST	1	novel
135	-	290	no EST	2	novel
136	-	49	no EST	1	novel
137	-	288	no EST	1	novel
138	gb:W89336	243	40% aa id. 50% aa id. with KIAA0041	1	Protein motif found in yeast GCS1, GLO3, human KIAA0041 and KIAA0167
139	gb:M24361	100	98.0%, 100bp	1	pyruvate kinase (R)
140	-	131	no EST	1	novel
141	gb:L38644	183	98.4%, 183bp	1	karyopherin beta (R)
142	-	107	no EST	1	novel
144	-	131	no EST	2	novel
145	gb:M84340	268	87.1%, 272bp	1	IL-10 (within intron sequence) (M)
147	-	175	no EST	1	novel
148	gb:U59289	33	81.5%, 27bp 60%, 10 aa	1	h-Cadherin (H)
151	EST (H) gb:AA354227	106	79.2%, 125bp	1	novel
152	EST (R) gb:H34895	145	93.7%, 127bp	3	novel

Table 1. Key: (R) Rat, (M) mouse, (H) human, EST expressed sequence tag.
The number of each respective clone is listed in the left-most coloumn. Accesion numbers are coded identifiers for a particular sequence that allow these sequences to be addressed dirctly from the public data bases. The percentage identity is given together with length of nucleotide match of the clone. Redundancy (Red) indicates how many times an identical cDNA fragment was isolated from the subtracted library and clones identified numerous times reflect those that were highly overexpressed as seen on direct northern analysis (for example, clone # 43 and 45). The identity of each clone is given as the most probable identity and in some cases it is not possible to know whether a particular clone is novel or not, just based low homology, particularly across species. Note: the table depicts 152 gene fragments or clones when the stated number is 126. This is because some clones upon closer inspection represented the same target gene and were removed. The result is a list of clones numbered 1-152, with numbers missing, representing those clones removed (e.g., there is no clones with numbers 5,12,16,17 etc)

Had the normalization procedure not been included then the proportion of abundant differentially expressed transcripts would have been over-represented in the library and may have masked those transcripts of low abundance.

In table 2 are the overall statistics of the library as determined by sequence homology to known genes deposited in public data bases. Sequences that exhibited homology greater than 94 % were classified as being identical (as a 6 % difference is around the expected limit of of errors generated by automated sequencing). Those sequences were the homology has been ranged between 56-86 % are classified as homologues. This range is arbitary as the border-line of homology, between a homologue or new family gene member to a novel sequence cannot be known. Therefore sequences that have homologies less than 50 % have been catagorized as novel. It is also of importance how the homology is desplayed and how many nucleotides are involved.

Table 2

Subtractive Library Analysis

A — 625 clones representing a total of 126 genes

	Perfect/near perfect match > 94 % id	Homologues to known h/m genes 56-86 % id	No match. Novel
Individual genes	23	69	59
Total Nr of cDNA clones	92	281	253
Total % of clones	15	44	40
Total % of genes	15	46	39

B — Number of genes represented by `X´ number of clones

`X´	1	2	3-5	6-10	11-20	21-40	41+
genes	44	22	18	13	7	2	3

Table 2. (a) Statistical sequence analysis of 625 cloned cDNAs identified in the Bsp73-1AS/ASML subtracted library. Clones were grouped according to their degree of homology to matched target sequences from the data bases. (b) The identified genes are group according to the number of times picked. For example, 44 genes were isolated each as a single clone, 22 genes were pick twice only etc. Key h (human), m (murine).

A high match consisting of a block of nucleotides with no homology on either side might indicate the presence of a homologous domain, but is otherwise novel. Conversely, a reasonable (50-

60 %) match over the entire length of the sequence in question to a human sequence may indicate the homologue or might be indeed novel. So the distinction between known and novel is often a very fine line and should be regarded as so.

What is apparant from table 2 (A) is that the large majority of clones, being 44 % and 40 % represented homologues and novel sequences respectively, with only a small proportion exhibiting perfect of near perfect match. (B) Many individual genes were identified by one clone only (being 44 genes, see figure 11) and therefore may indicate that the screening of 5000 clones maybe under-representative of the complexity of the library. That is, the point at which no further addition of new genes (identified by picking more clones) had not been reached. As most genes were represented by one clone suggests the presence of more potential differentially expressed genes. Had all genes been picked more than 2-3 times each would indicate saturation and that the number of clones analysed exceeded the number of differentially expressed genes present in the library. Nevertheless, the analysis of 5,000 clones would approach the upper limit of such a library and that doubling the number to 10,000 may only bring a few additional clones.

Figure 11

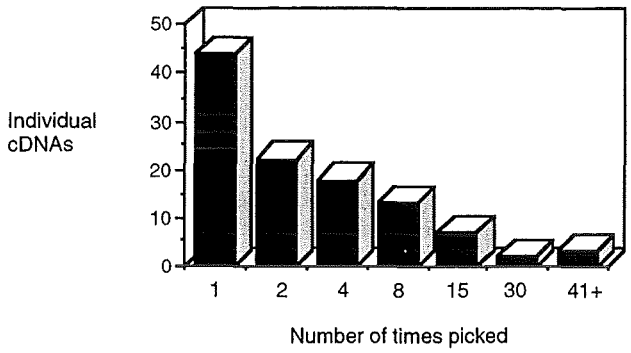


Figure 11. The above graph indicates the relative number of individual cDNAs against a given number of times picked (e.g., how many times each gene was picked), from the library.

Figure 12

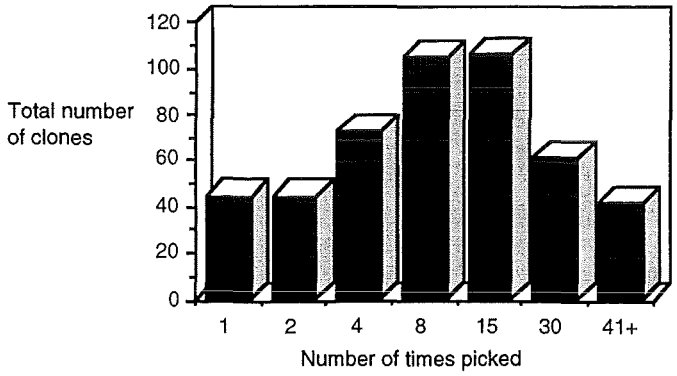


Figure 12. Majority of clones were picked in the range from >2 times to <40 times as depicted in the graph above

On the other end of the scale are those genes which were picked more than 40 times, such as clone # 45 (caveolin). In the library were a total of 3 such genes (see table 1) and this over-representation reflected their high abundant status as seen by northern analysis (see panel N, figure 10). A large proportion of genes were picked once only (see figure 11) and the number of inidividual genes represented by two of more picked clones progressively decreased until a point was reached were 40 or more clones represented only one gene. Conversely, most clones were picked in the order of more than twice but less than 30 times as depicted in figure 12. The positive control for subtraction was as mentioned the uPA gene. This in the library was represented by one clone only but nevertheless was identified despite it's low abundance in the ASML line. By logical inference, genes of relatively high initial abundance in the ASML line will be represented by more than one clone as appose to those of lower abundance (e.g., uPA). However the finding that the positive control gene uPA was identified by one clone speaks for the fact that the reverse northern screening is sensitive enough to detect genes of low differential expression.

3.4.4 cDNA Subtraction of Bsp73 AS versus ASML Identifies Known Tumor Progression Genes

Despite the fact that nearly half of the genes isolated were novel (based on the assumptions previously stated) or exist as ESTs, many genes identified through subtraction were reported to be associated with or even play a functional role in tumor progression. This in itself stands as proof of concept in that as a metastatic cell line was subtracted against a non-metastatic line, then it stands to reason that the differences must at least to some degree dictate the phenotype. Over 20 genes identified from the subtracted library are documented to be directly or indirectly involved in tumor progression. Some well known examples are given in table 3. Hepatocyte growth factor receptor (c-MetR) as been documented to be expressed in human prostatic cancers (Kurimoto et al., 1998) and in addition has been shown to induce an motogenic-invasive phenotype (Giordano et al., 1993). The urokinase type plasminogen activator (uPA) gene is overexpressed in a number of human tumors (reviewed by Duffy et al, 1990) and together with its inhibitor PAI has become a prognostic indicator in a number of malignancies, including breast (Jänicke et al., 1993). Table 3 above depicts some of the identified genes obtained from the subtracted library that are (A) functionally described in tumor progression and (B) only associated in the development of certain tumors but the functional role remains largely unknown. Nevertheless it indicates without any doubt that this subtractive approach produces an inventory of known genes involved in tumor progression and novel genes that are possibly involved, and that potentially reflect the the genetic program mediating malignant properties of Bsp73-ASML cells.

(A) **Table 3**

Clone #	Identity	Reference
38	Annexin II	Nygaard et al., 1998
85	c-Met receptor	Di Renzo et al., 1995
98	uPA	Ferno et al., 1995
108	IGF-binding protein	Gill et al., 1997
115	CDK4/6 ink4a	Pomerantz et al., 1998
126	Calgizzerin	Tanaka et al., 1995

(B)

Clone #	Identity	Reference
08	EXT2	Hecht et al., 1997
43	High mobil prot I	Xiang et al., 1997
50	IAP	Wang et al., 1997
69	alpha Prothymosin	Wu et al., 1997
79	FRX prot I	Fulchignoni-Lataudt et al., 1997
94	Testin	Kondoh et al., 1997
103	Calcyclin	Berta et al., 1997

In other words, using SSH, known genes of clear documented function in cancer progression have been isolated as one might expect, however in addition, novel genes that may represent putative new tumor progression markers have also been identified. This in itself stands as proof of concept in that SSH as a tabulated method is able to isolate from a specific tumor cell system (or indeed any system) a defined set of genes of known relevance in tumor progression.

3.4.5 Functional Classification of Identified Genes

From the sequence identity of the known clones (see table 1) one can group them according to their functional roles in eucaryotic cells. This provides an overview concerning the dysregulation of certain functional classes of genes that accompanies a metastatic phenotype as depicted in figure 13. For every group listed in the pia chart, exists molecules that fall into these catagories which are also functionally involved in tumor progression. The functional groups are however not absolute as there is considerable overlap and many proteins have multiple functions. However it serves as a guide and a number of examples of isolated genes from the subtracted library that fall into these groups are given. Without question most of the cDNA clones from the subtracted library fall into the first three catagories, namely novel, existing as ESTs, or genes with ill defined functions (segments 1,2 and 3 respectively). The

next largest group (group 4) covers those molecules involved in cytoskeletal organization and transduction of signals from cell surface receptors to various cytoskeletal components. Such cellular components would be required for cell motility. For example, cytokeratin 8 (clone # 110), identified as being overexpressed in the metastasizing line, is reported in transfection studies to increase the invasive potential and migratory behavior of recipiant human melanoma cells as determined *in vitro* gelatin migration assays (Chu et al., 1996). Group 5 covering growth factor receptors relates to a large group of molecules, many of which have been implicated in tumor progression. For example hepatocyte growth factor receptor (c-MetR) has been associated with tumor progression in different human carcinomas. It is overexpressed in osteosarcomas suggesting a role for the met proto-oncogene in the pathogenesis of this tumor type (Scotlandi, et al., 1997). In human gastric carcinomas c-Met overexpression is also observed (Kuniyasu et al., 1993). Increased levels of annexin II are observed in various cancer cells and tissues, and the molecule has been proposed as a marker of malignancy in vivo (Nygaard et al., 1998).

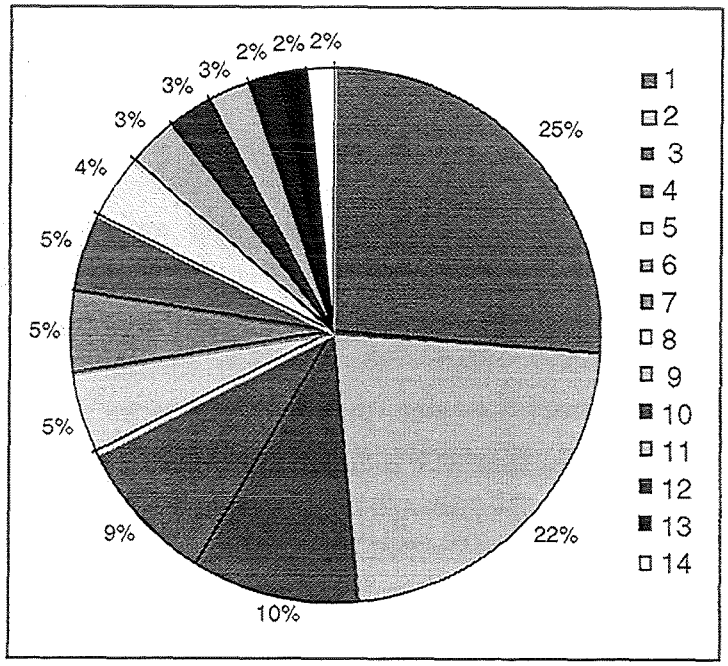


Figure 13. Categoric grouping of clones. The pia chart indicates the functional grouping of all 126 individual cDNAs identified as being upgerulated with respect to the metastasizing cell line Bsp73-ASML and have been grouped according to their documented function. Values depict percetages of clones that fall into the 14 functional catagories listed. The groups are as follows: (**1**) Novel; (**2**) ESTs; (**3**) Known identity but function not clear; (**4**) Cytoskeletal associated molecules and intermediate filaments; (**5**) Growth factor receptors/growth factors; (**6**) Intracellular signalling molecules; (**7**) Proteins of metabolic function; (**8**) Ribosomal proteins; (**9**) Cell cycle regulatory proteins; (**10**) Transcription factors/ modulators; (**11**) Proteases; (**12**) Adhesion molecules; (**13**) Proteins involved in DNA replication/ stability and topology; and (**14**) Transposable elements.

Annexin, a calcium-dependent, phospholipid binding protein involved in calcium signalling belongs to group 6 depicted in the pia-chart and was also isolated from the subtracted library. Proteins involved in cell cycle regulation (group 9) such as the cyclins have been well documented in tumorigenesis. The cyclin D1 protein is found to be amplified and is a reliable prognostic factor and post-translational modification of this gene may play a functionally important role in development of primary esophageal cancer (Inomata et al., 1998). Overexpression of cyclin E protein has been

reported in several solid tumors (Porter et ai., 1997) and in acute myelogenous leukemia (AML; Iida et al., 1997). Clone # 65, a rat homologue of the yeast protein CDC10 encodes a component of the DSC1Sp/MBF transcription complex, which is required for cell-cycle regulated expression at G1-S of several genes via cis-acting MCB (MIuI cell cycle box) elements (McInerny et al., 1995). This clone is overexpressed in the metastasizing line and might therefore be responsible for the activation of certain genes which might of otherwise remained at a basel level and consequently affect cell cycle. An interesting finding is clone # 115, identified as p16INK4a (inhibitor of CDK4). This tumor suppressor protein represents one of the most direct links between cell-cycle control and cancer. The p16INK4a gene is frequently inactivated in human tumors, and inheritance of mutant alleles results in susceptibility to several types of cancer (reviewed by Serrano, 1997). Inactivation of p16INK4A in tumors expressing wild-type Rb is thought to be required in order for many malignant cell types to enter S phase efficiently or to escape senescence (Shapiro et al., 1998). This gene is inactivated by intragenic mutation or by homozygous deletion in almost 100 % of all pancreatic cancers (Schutte et al., 1997) but is overexpressed in the Bsp73-ASML pancreatic metastatic cell line. This might suggest that the overexpression of p16 might be the result of a dominant negative mutant that inadvertantly confers growth advantages to the ASML cells. Lastly, group 14 that covers transposable elements is also of interest. Intracisternal A-Particle (IAP) sequences are endogenous retrovirus-like mobile elements, present in the mouse genome. These elements transpose in a replicative manner via an RNA intermediate and its reverse transcription. Blumenstein et al. (1998) found that FDC-P1 cells undergo leukemic transformation when injected into sublethally irradiated DBA/2 mice. Transformation was related to Intracisternal A-type particles (IAP) aberrant activation of growth-regulatory genes by insertion of IAP genomes. In *c-myc* transgeneic mice IAPs are upregulated (Dupressoir & Heidmann, 1997) indicating that *c-myc* might regulate transcriptional status of IAPs which inturn could activate other downstream genes depending on their intergration site. So it is evident that many of the genes isolated do possess functions that may contribute to the manifestation of tumorigenesis or tumor progression.

3.5 Identification of subtracted genes in other cell systems

Now that a list comprising of 126 individual differentially expressed genes that accompanies the metastatic phenotype of the Bsp73-ASML cell line had been defined, came the inherent problem that the shear number of clones prohibited functional analysis. That is, to functionally elude the role of each gene in the context of a metastatic phenotype by either overexpression or antisense studies would be an astronomical task and is not feasable. That some of the isolated genes play a significant role in the tumorigenic process is highly likely, however there exists no suitable experimental setting that allows the independent functional analysis of so many genes, such that the important players can be rapidly identified. What remains is correlation as the coordinated action of more than one gene might be required and

this is difficult to address in single cDNA transfection experiments. In this context, correlation is the expression of certain sets of genes that accompanies a given characteristic trait in a defined system or in more than one system. The identified genes from the subtracted library correlate to the expression differences between a non-metastatic cell line and one that is able to metastasize, yet are these differences also typical in other cell line systems that comprise of cell lines of differing metastatic potencies?
In other words, does the correlation extend to metastatic cell lines of different tumor progression models? This question is based on the asumption that general expression programs underly metastasis or, at least a limited number of such metastatic expression programs exist. To answer this question would identify those genes whose overexpression is seen in other cell line systems and therefore the likelihood of that particular gene, being of any functional relavance, would increase. These genes would represent promising candidates in this respect because their overexpression is not limited to just the one cell line system from were they were originally identified, but was rather more general concerning the metastatic potential in other tumor progression model system.

3.5.1 Reverse Northern Screeing Identifies Common Genes in other Tumor Progression Cell Systems

In order to reduce the large initial number of genes to a smaller number of more potentially valuable genes, required the analysis of correlation in other systems. Three other rat tumor progression models were chosen for this purpose, two originating from the mammary gland and the third from the prostate. In addition, cross hybridization to human cDNAs was also addressed by generating cDNA probes from three human pancreatic adenocarcinoma cell lines PATU-8902 (Elsässer et al., 1993), PATU-8988s (Elsässer et al., 1992), and CAPAN-1 (Kyriazis et al., 1986) and two human pancreatic carcinoma lines HUP-T3 and HUP-T4 (Nishimura et al., 1993). Their tumorigenicity in nude mice has only been reported for PATU-8902 (Elsässer et al., 1993) and for CAPAN-1 (Kyriazis et al., 1986).
All differentially expressed clones from the Bsp73-1AS/ASML library were re-isolated, amplified by colony PCR and membranes constructed each containing a complete set. These membranes were subsequently screened in reverse northern fashion against three rat tumorigenic cell lines systems originating from the protate and mammary as mentioned above. By screening in this manner it was possible to analyses the expression profiles of all clones simultaneously in each cell line used. The prostatic cell system (Isaacs et al., 1986) comprises of four tumorigenic cell lines of differeing *in vivo* metatstatic potencies. The AT.1 line produces no metastases upon inoculation into Fisher F344 rats, whereas the AT.3 subline is moderately metastatic. MatLu and MatLyLu both metastasize preferencially to the lymph and lung/lymph respectively. Concerning one mammary cell line system (Nicolson et al., 1988b; Nicoloson et al., 1988c) the parental line MTPa was reported not to metastasize *in vivo* whereas MTC

exhibts none to low metastatic potential. The lines MTLN-2, MTLN-3 and MTLY were all demonstrated to produce metastases to varying degrees (Nicoloson et al., 1988b).

Figure 14a

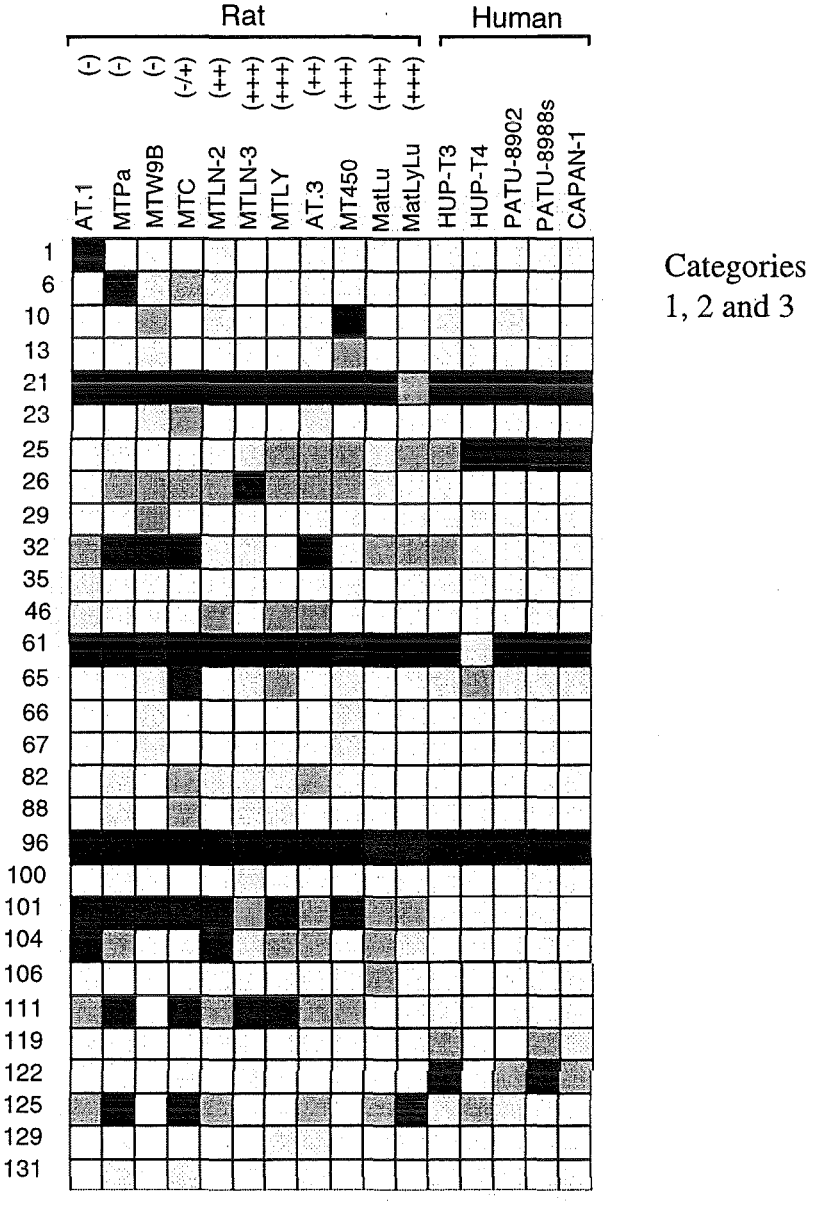
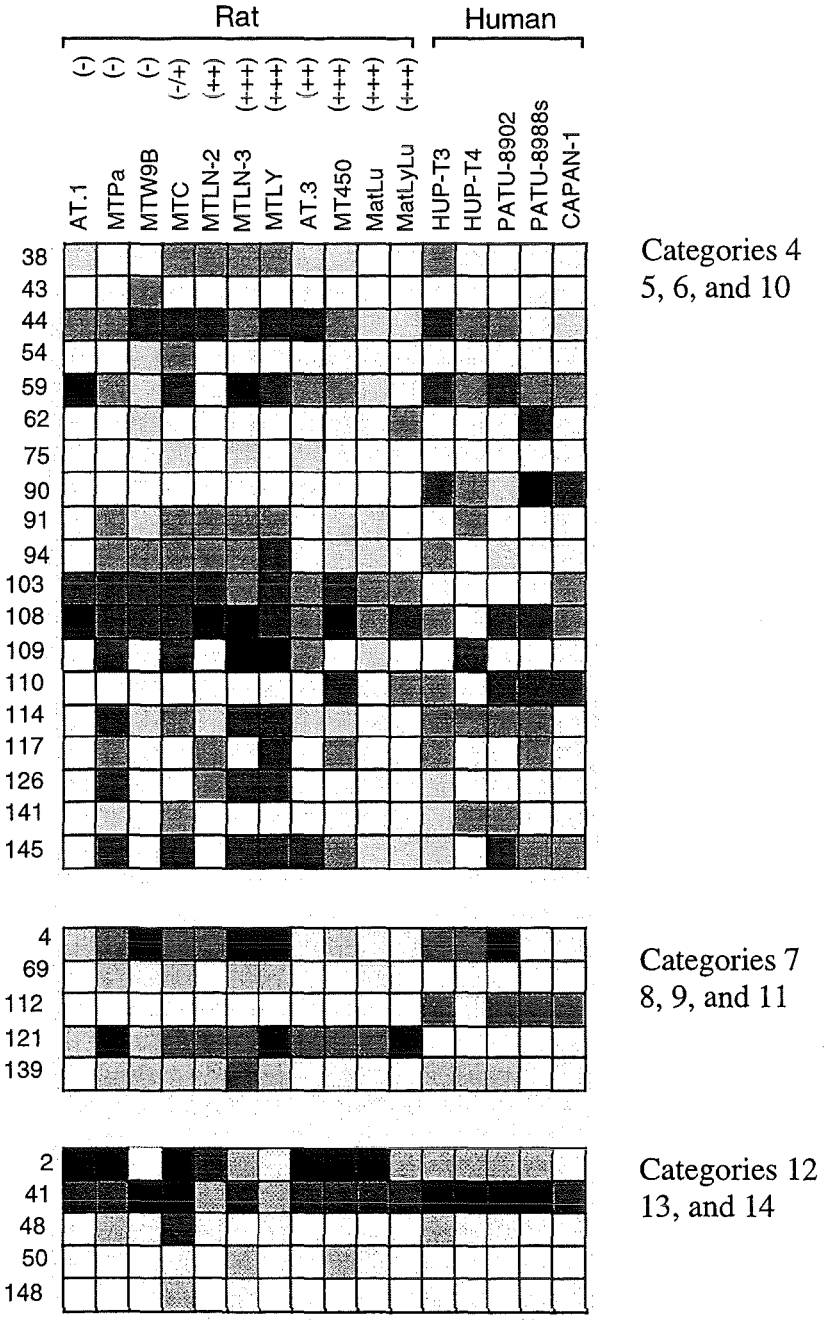


Figure 14a. Expression profile of clones indentified as novel, having ESTs or whose function is ill-defined.

From each cell line indicated, poly $(A)^+$ RNA was isolated, ds cDNA synthesized and the cDNA restricted with Rsa I before labelling with ^{32}P-dCTP. For probes obtained from rat cell lines, hybridization conditions were as described for standard reverse northern screening. Concerning cDNAs derived from human lines the hybridization stringency was reduced (hybridization performed at 52 °C in Church buffer with subsequent washing steps of twice in 2 X SSC, 0.1 % SDS and once in 1 X SSC, 0.1 % SDS at 54 °C) in order to detect clones of homology. The colour intensity indicates expression level of that particular clone seen in the different cell lines. Dark red depicts highest expression, light red lowest and absence of colour no expression. The respective clone numbers are listed on the left. Cell lines are listed on top. (-), (-/+), (++), and (+++) indicates the metastatic potential of the cell lines, being non-metastatic, non-to-low metastatic, moderately metastatic, and highly metastatic respectively.

Figure 14b. The three blocks blue, green and brown shows expression profiles of the listed clones (see numbers left of figure) in all cell lines.
The large blue block covers clones in the following functional groups: Cytoskeletal associated molecules, growth factor receptors/growth factors, intracellular signalling molecules, and transcription factors/modulators. The green block covers clones of metabolic function, ribosomal proteins, cell cycle regulatory proteins, and protases. The brown coloured block indicates clones of adhesion molecules, proteins involved in DNA replication/stability/topology, and transposable elements. Again, darkest colour indicates strongest expression, lightest colour lowest expression and no colour no detectable expression at the reverse norther level. The respective clone numbers are listed on the left. Cell lines are listed on top. (-), (-/+), (++), and (+++) indicates the metastatic potential of the cell lines, being non-metastatic, non-to-low metastatic, moderately metastatic, and highly metastatic respectively.

Figure 14b



The other mammary cell line system composed of just two lines MTW9B (non-metastatic) and MT450 (metastatic) were a kind gift from Prof. U. Kim (Kim & Depowski, 1975). Reverse northerns were performed with all cell lines and the intensity of positive signals graded according to the signal strength. The signal intensity gives an indication as to the relative abundance of that clone in a population of complex cDNAs against other positive clones that show significantly weaker signals. The results of such a screening are shown in figures 14a/b. From the reverse northern screening of all cell lines depicted in figures 14a/b (see top of figure), clones that were identified as positive were noted and are shown as the numbers on the left of the figures. The colour intensity serves as a indication of expression level of that particular clone with respect to others, that is the strongest signal was taken as 100 % and correlates to the strongest colour. Weak colouring is indicative of weak expression and those boxes that are white represent no detectable expression.

From figures 14a/b there are a number of clones that on the reverse northern level appear to be correlative in that their expression was also seen in other cell lines of differing tissue origin and metastatic competence. About 13 clones were positive for differential expression in the two rat tumor progression models and are listed in table 4 (their identity is given in table 1). Concerning the human cDNAs, reverse northern analysis (see figures 14a/b) showed that a large proportion of clones cross-hybridized to the human cDNAs. Moreover, many clones demonstrated differential expression across the five human pancreatic cell lines, that is some clones were only expressed in some of the human cell lines and not in others (see figure 14a, clones 10, 119 and 122), however only three of the 13 rat clones, defined as differentially expressed in both a rat mammary and a rat prostatic tumor progression model, cross-hybridized to the human cDNAs.

So by simply addressing the question of correlation in other cell line systems through reverse northern screening, the original number of differentially expressed clones identified from the subtraction between Bsp73-1AS and Bsp73-ASML, being 126, has now been reduced to approximately 13. That is, 13 clones exhibit differential expression in two independent cell line systems. One could therefore speculate that these clones represent potential candidates concerning tumor progression markers as their dysregulation is not limited to the pancreatic system from which they were derived. In addition, three of these clones (clones 25, 62 and 110) could be of potential value as they also cross-hybridized to human cDNAs indicating the possible presence of a human homologue for clone 25 (novel with putative homology to a zinc-finger protein). Clones 62, and 110 are ezrin and cytokeratin 8 respectively.

Table 4

Clone Number	Differential Expression Prostatic system	Differential Expression Mammary system	Cross-Hybridization to Human Cell Lines
23	yes	slightly	no
25	yes	yes	yes (in all lines)
38	yes	no	no
43	yes	yes	no
46	yes	yes	no
62*	yes	yes	yes (in PATU-8988s)
75	yes (weakly)	yes	no
82	yes	no	no
85*	yes	yes (weakly)	no
100	yes	slightly	no
110	yes	yes	yes (not in HUP-T4)
147*	yes	no	no
148	very weak	no	no

Table 4. Expression of Bsp73-1AS/ASML subtracted clones in other progression models and cross hybridization to human tumor cell lines. The list of clones in the table above depicts those that demonstrate differential expression at the reverse northern level in the two rat tumor progression model systems. In addition, cross-hybridization of these particular clones to human tumorigenic cell lines is shown. The asterics (*) indicates that differential expression was seen with direct northern analysis only.

3.5.2 Direct Northern Analysis Prooves Correlative Expression of Subtracted Clones

As these 13 clones were identified again by reverse northern screening it was nevertheless important to determine their expression directly at the mRNA level as gradual differential expression levels between cell lines is hard to determine in reverse northern analysis. Direct northern analysis is more sensitive and provides additional information (e.g., transcript size). For this purpose the clones were hybridized to membranes containing poly(A)$^+$ RNA isolated from all cell lines present in the three cell systems as is shown in figures 15-17.From figure 15, the differential status of all clones is clearly visable with respect to the three cell line systems used to determine the extent of correlation. For additional comparison three genes that have been described as tumor markers or tumor progression markers, respectively, were also analysed. Firstly, c-Met (identified in the subtracted library) was analysed for correlative expression is a classical receptor tyrosine kinase involved in cellular motility and invasiveness (Giordano et al., 1993) and can even confer invasive properties to recipient normal cells (Rong et al., 1994).

Figure 15

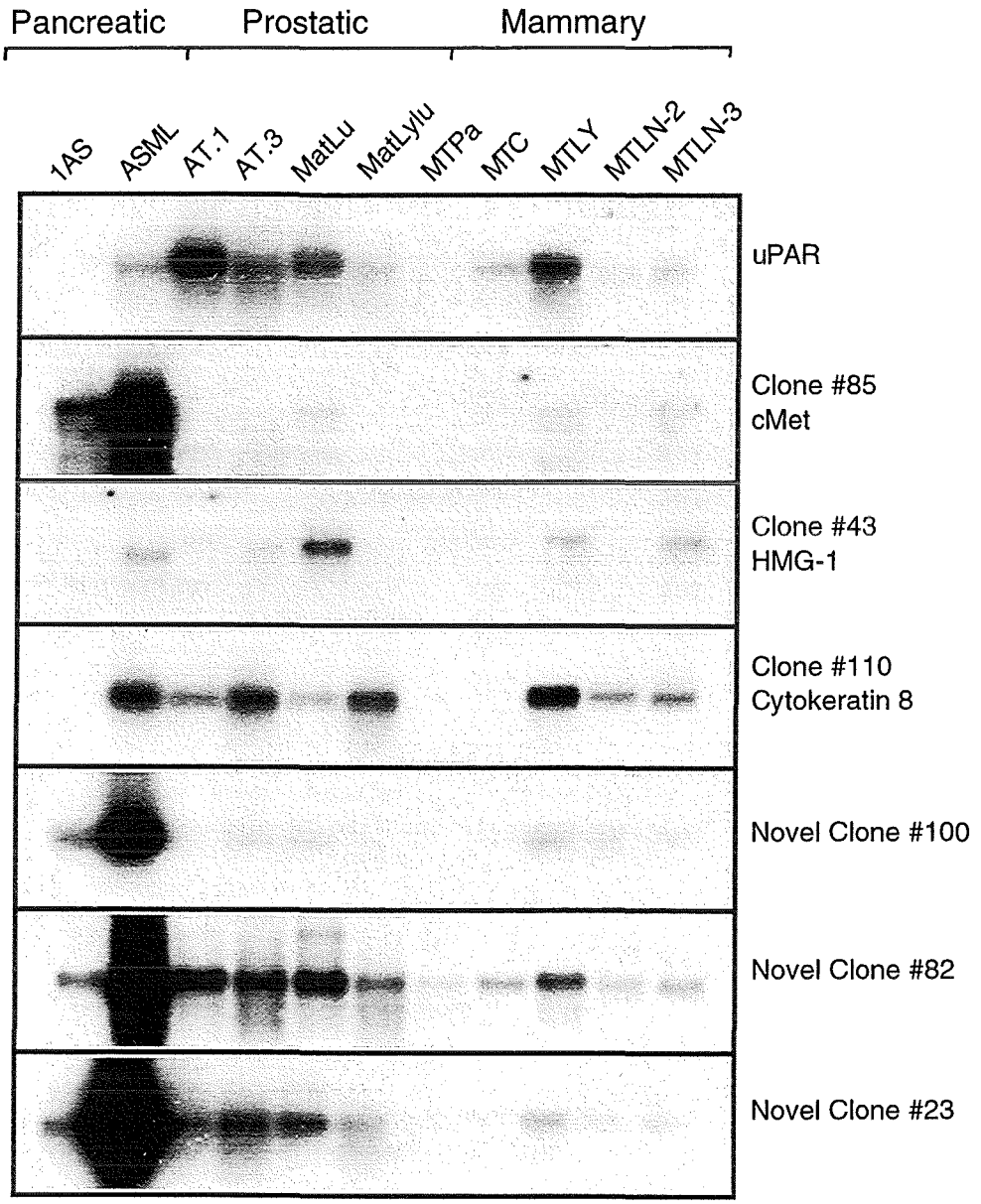


Figure 15. Comparison of expression profiles of novel genes and known tumor associated molecules in cell line systems of differing metastatic potential. The 1AS and ASML lines represent the cell lines from which the subtraction was made. From the prostatic cell lines, AT.1 does not metastasize in vivo, whereas AT.3, MatLu and MatLyLu are all capable of producing metastases in vivo, MatLyLu being the most potent. In the mammary cell lines, MTPa also shows no metastatic phenotype in vivo, MTC exhibits non-to-low metastatic potency, whereas MTLY is highly metastatic. MTLN-2 and MTLN-3 are both metastatic, the latter having a somewhat greater potential. For northern analysis, 3 μg of poly $(A)^+$ RNA was taken per cell line and run on 1.2 % agarose/formaldehyde gels in multiples such that all clones could be hybridized each to a seperate membrane. Clone fragments were amplified by PCR. Conditions of cycling were 94 °C, 1 minute, then 30 cycles of: 94 °C, 20 sec; 68 °C, 12 sec; 72 °C 30 sec. Products were gel purified before extraction from the gel using DNAeasy (Biozyme). Approximalely, 25-50 ng was used for labelling and hybridization was carried out under stringent conditions at 64 °C in Church buffer. Membranes were washed once in 2 X SSC, 0.1 % SCS and twice in 0.1 X SSC, 0.1 % SDS at 65 °C.

Secondly, uPAR gene was also analysed for correlation as its overexpression is an important factor in tumor progression (see introduction, section 1.3.5 part (B)) and as it also represents a gene not identified through the subtracted library. Lastly, the gene HMG-1 was analysed for correlative expression. It has only been demonstrated to be associated with tumors (Chiappetta, et la., 1995) but was identified many times in the library. While it is clear that c-Met shows pronounced differential expression in the pancreatic lines, it is only weakly detected in the prostatic line MatLu. Likewise there is only weak expression in the MTLY and MTLN-3 lines both of which are very metastatic. For uPAR there is clear expression in both the ASML and MTLY (both highly metastatic) lines, however, in the prostatic system there is even a reduction of expression from the transition of non-metastatic (AT.1) to a metastatic line (AT.3). This for a gene of classical tumor progression status is somewhat surprising. HMG1 (clone # 43) by contrast shows clearly differential expression in all systems conerning metastatic potential and in this respect shows a better profile. The case for cytokeratin 8 (clone # 110) is the same were again there is very pronounced differential expression in both the pancreatic and mammary systems. In the prostatic lines there is an obvious increase of some 2-3 fold between AT.1 and AT.3. With respect to the novel clones shown (clones 23, 82 and 100), their differential status is extremely strong for the ASML line and for clones 23 and 100 there is clear differetial expression in both mammary and prostatic cell lines of metastatic capacity. This classifies them with respect to expression profile equivalent to c-Met and even better than uPAR (which does not exhibit increased expression in the prostatic cell lines capable of metastasizing). The expression picture of uPAR is more similar to that of clone # 82, being only correlative in two systems.

The northern analysis of a further four novel clones is shown in figure 16. Here again there is a perfect correlation of expression with metastatic capacity as is seen clearly with novel clone (clone # 46). Is clearly not expressed in the two non-malignant lines AT.1 and MTPa, but is present in those lines highly capable of producing metastases namely AT.3 MatLyLu and MTLY. The signal is not as defined as those seen with other clones but the expression pattern is obvious and represents a possible candidate gene in tumor progression.

So already it is becoming evident that the subtraction between a non-metastatic cell line and its metastatic counterpart identifies clones like for example clones 23 , 100 and 46 (see figures 15 and 16 respectively) that under the criteria of differential expression in other metastatic cell line systems, are as good if not better than the known tumor progression genes, as for example c-Met and uPAR.

Clones 75 and 148 both exhibit differential expression (as might be expected) in the pancreatic ASML line and additionally in the prostatic AT.3 and MatLu lines (both metastatic), with no detectable expression in the corresponding non-metastatic lines. Both clone 75 and 148 exhibit very weak expression in MTC and MTC/MTLY respectively. Clone 147 apart from being expressed in the ASML line is also upregulated in MTLY by some 5-6 fold but shows no change in mRNA levels in the prostatic cell system. In general these four clones

have an all together weaker expression status in any of the rat tumor progression cell line systems, as compared to those clones depicted in figure 15, whereby all showed significantly stronger expression. They therefore represented a class of abundant differentially expressed clones, and those seen in figure indicate a class of clones of lower differential abundance.

Figure 16

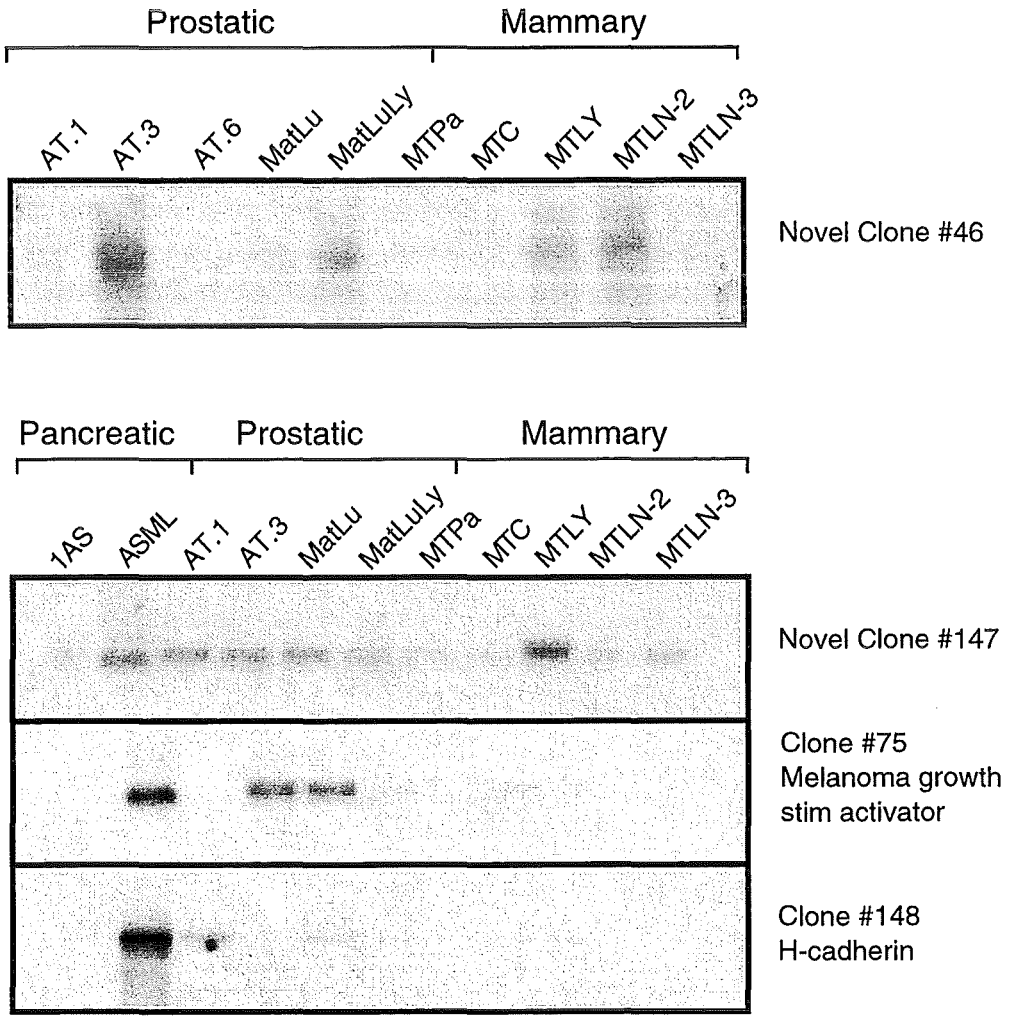


Figure 16. Direct northern analysis of four clones identified as being differentially expressed at the reverse northern level. The four listed clones previously identified as being differentially expressed determined through reverse northern analysis, were checked for differential expression against the cell lines listed on top of the figure. For northern analysis, 3 µg of poly $(A)^+$ RNA was taken per cell line and run on 1.2 % agarose/formaldehyde gels in multiples such that all clones could be hybridized each to a seperate membrane. Clone fragments were amplified by PCR. Conditions of cycling were 94 °C, 1 minute, then 30 cycles of: 94 °C, 20 sec; 68 °C, 12 sec; 72 °C 30 sec. Products were gel purified before extraction from the gel using DNAeasy (Biozyme). Approximalely, 25-50 ng of PCR amplified probe was used for labelling and hybridization was carried out under stringent conditions at 64 °C in Church buffer. Membranes were washed once in 2 X SSC, 0.1 % SCS and twice in 0.1 X SSC, 0.1 % SDS at 65 °C.

Figure 17 shows the results for the last two clones that were identified by reverse northern analysis as being upregulated in the metastasizing lines in more than one cell system. The novel clone 25 (see figure 17) depicts a differential expression pattern that would reflect a putative gene believed to be involved in tumor progression. It shows expression in every cell line in all three cell systems that is capable of producing metastases *in vivo* and is not detectable in the corresponding benign tumor lines. In this regard it represents a model clone candidate that potentially might be involved in tumor progression and would certainly warrent further investigation.

Figure 17

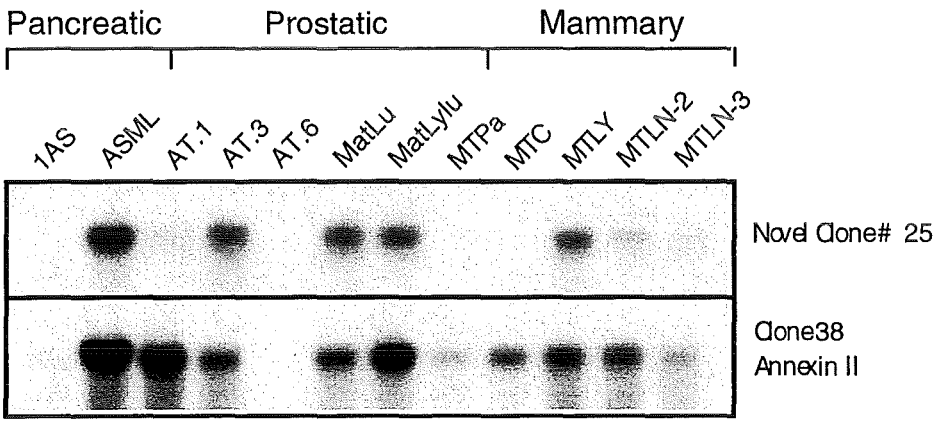


Figure 17. Northern analysis of two clones in three indipendent cell line systems. Clone # 25 and 38 were monitored for their expression pattern in the cell lines indicated. For northern analysis, 3 µg of poly $(A)^+$ RNA was taken per cell line and run on 1.2 % agarose/formaldehyde gels in multiples such that all clones could be hybridized each to a seperate membrane. Clone fragments were amplified by PCR. Conditions of cycling were 94 °C, 1 minute, then 30 cycles of: 94 °C, 20 sec; 68 °C, 12 sec; 72 °C 30 sec. Products were gel purified before extraction from the gel using DNAeasy (Biozyme). Approximalely, 25-50 ng of probe was used for labelling and hybridization was carried out under stringent conditions at 64 °C in Church buffer. Membranes were washed once in 2 X SSC, 0.1 % SCS and twice in 0.1 X SSC, 0.1 % SDS at 65 °C.

Annexin II, clone 38 shows also to a convincing degree differential expression in two from three cell systems being the pancreatic and mammary. In the prostatic cell lines, there is a noted reduction from the transition of the non-metastatic line (AT.1) to the metastatic line (AT.3), however it´s expression is seen also in the two metastatic lines MatLu and MatLyLu. In the five cell lines from the mammary system there is a steady increase in expression from a basel level (MTPa) to a 5-6 fold induction (MTLY) that correlates with the transition from a non-meatstatic line to one of high metastatic potency.

In regard of the results from the northern analysis of the 13 target clones, it is clear that many of them share an expression pattern equal in match and in a number of cases better than known tumour progression markers such as the two included. It would therefore seem reasonable to assume that some of these clones may indeed represent possible novel markers or be of significant importance in the progression of cancer. Moreover, these northern data also indicate

that not only those genes that have a high initial differential abundance are isolated but indeed also those of low differential abundance (compare figures 15 and 16).
After the obvious success of identifing which clones from the original Bsp73-1AS/ASML subtraction fullfilled the criteria of correlative expression in two indipendent rat cell line systems, and therefore might be of potential importance, the question was extended further to consider which of the 126 clones originally defined in the subtraction are also differentially expressed in human tumor material. That is, while correlation in the rat systems allows identification of the best clones for functional analysis, cross-hybridization to human cDNAs aims at rapidly identifying clones that may potentially act as markers for clinical use.

3.5.3 Identification of Subtracted Clones Expressed in Human Tumors

In an identical manner to that used in the reverse northern screening with the different cell lines, human adenocarcinomas of stomach and colon origin were taken together with their corresponding matched normal tissue and screened in reverse northern fashion against all clones. Under these conditions it was necessary to reduce the stringency of hybridization and subsequent washing steps such that human sequences representing possible homologs of the metastases associated rat clones are not lost. After suitable exposure, positive clones were identified as those clones that were positive only to the human tumor cDNAs. The results of such a reverse northern are shown in figure 18. What is obvious is that filters that received probes derived from normal tissues (those marked N) show to a high degree the same positive clones, in particular the three most prominent ones, indeed these clones are expressed in all cDNA populations and therefore are suitable as internal contols This sort of mimicy is seen also with both tumor probes in that there is a high degree of overlap between positive clones, regardless of the fact that the tissue origin of the tumors is different. This is perhaps not surprising if one consideres that the subtracted library represents by definition enriched genes that are indicative of a metastatic phenotype in a rat cell line system. In other words, clones that are positive for the tumor probes might reflect subpopulations of cells in the human tumor that are "on their way" to metastasize. The identity of positive clones as seen with the tumor specific cDNAs only is given in table 5. For the majority of those clones listed of known identity, there exists documented evidence of an associated role in human tumor progression, and a number have been directly linked to tumor progression. Moreover, many clones seen positive with human tumor specific cDNAs were also identified as being expressen in many rat tumorigenic cell line systems (e.g., clone 38, (annexin II); clone 43, (HMG-1), clone 25 and 46, (novels) see figures 15-17). Interestingly clone 85 identified as c-Met receptor from the original library was not detected in the reverse northern screenings of the tumor samples despite its documented role in tumor progression (Di Renzo et al., 1995; Schmidt et al., 1997).

Figure 18

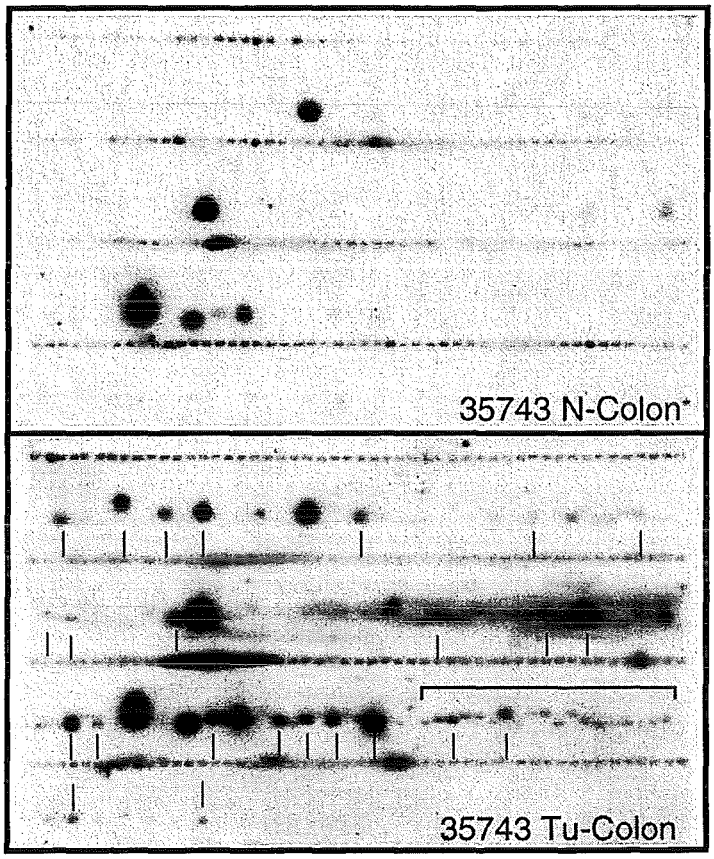
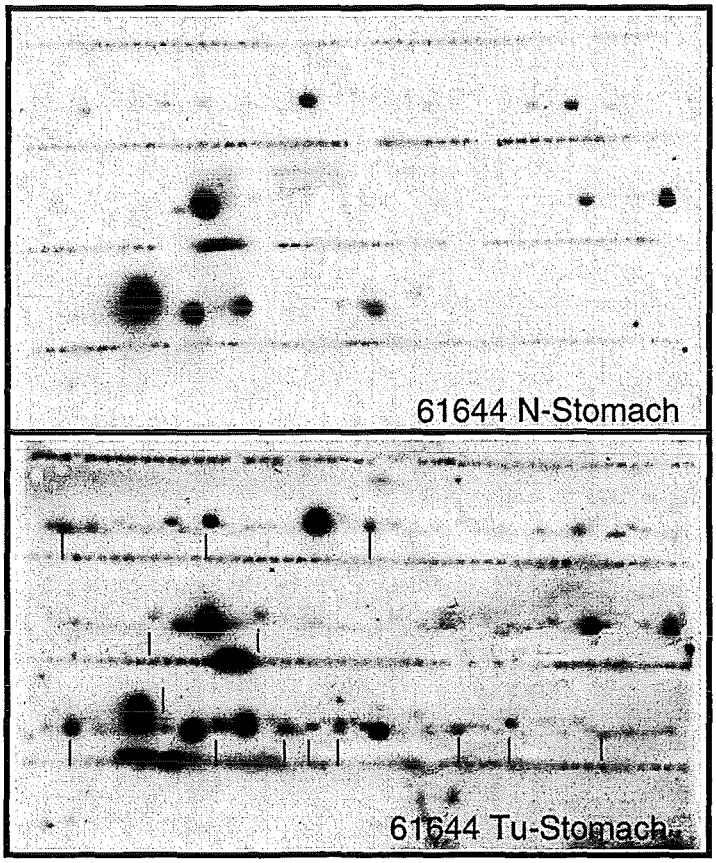


Figure 18. Reverse Northern Screening of Human Tumors. Approximately 0.5 µg of poly $(A)^+$ RNA, isolated from a human colon and stomach adenocarcinoma (TU) and their corresponding matched normal tissue (N), was reverse transcribed to ds cDNA and rescricted with Rsa I. Some 50 ng of the cut cDNA was 32P-dCTP labeled and used to probe two identical sets of differential clones previously identified from the subtracted library. Arrows on the tumor (Tu) membranes indicate clones habouring inserts that are only expressed in the tumor samples. The three strong signals seen with both normal tissue probes act as internal controls as they are also expressed in the tumor samples. Equal intensity of these clones indicates equal conditions.



The bar shown in "35743 Tu-Colon" highlights a sequence of clones (Nrs.125-144) that are not detected in normal colon tissue, (the two arrows) show the two most prominant signals. Hybridizations were performed in Church buffer at a reduced temperature of 52 °C and membranes were washed once in 2 X SSC, 0.1 % SDS, and twice in 0.1 X SSC, 0.1 % SDS at 50 °C. The reduced stringency of hybridization and subsequent washing would allow for detection of clones that cross-hybridized with human cDNAs thus identifying possible homologs. Films were exposed for 3 days at -80 °C before developing.

This could be due to its low abundance of expression as is seen in northern analysis of this gene in rat tumorigenic cell lines (see figure 15), but a more plausible explanation is the low degree of homology seen between human and that of rat (56 % at the nucleotide level according to sequence alignments from the public data bases). This fact demonstrates the limitation of reverse northern screening concerning the identification of putative human homologues from a predefined panel of subtracted clones originating from a different species such as the rat.

Table 3

Colon adenocarcinoma Sample 35743/97-Tu vs N		**Stomach adenocarcinoma** Sample 61664/96-Tu vs N	
Clone number	Identity	Clone number	Identity
2	NICER element	1	Novel
7	alpha capping-prot	**2**	NICER element
10	Novel	**14**	Novel
14	Novel	**25**	Novel
25	Novel	27	Ganglioside synthase
38	Annexin II	32	Novel
41	Poly (A) binding-prot	**44**	Calmodulin
43	HMG-1	45	Caveolin
44	Calmodulin II	46	Novel
46	Novel	**51**	Novel
48	Topoisomerase	57	Novel
51	Novel	**59**	alpha-prothromyosin
59	alpha-prothromyosin	65	CDC10
79	FRX-prot	87	Cathepsin B-like prot
90	Topoelastin	91	AHNAK
99	Novel	97	Apop protein
101	Novel	**99**	Novel
110	Cytokeatin 8	**110**	Cytokeratin 8
115	p16ink4a	**115**	p16ink4a
117	Tmp-21	**117**	Tmp-21
119	Novel	**119**	Novel
122	Novel	121	Ribo protein L 26
128	Novel	**128**	Novel
132	Novel	**132**	Novel
146	Novel	139	Pyruvate kinase
151	Novel	141	Karyopherin-ß
		144	Novel
		146	Novel

Tabel 5. Sequence identity of Bsp73-1AS/ASML subtracted clones that cross-hybridize with human tumor specific cDNAs. Two types of human adenocarcinomas were screened through reverse northern analysis against the defined set of subtracted clones to identify those that cross-hybridized. Clones that failed to be detected with cDNA probes derived from the normal tissue, but were positive for the tumor specific cDNAs were regarded as positive. Clone numbers in bold-case indicate those that are seen in both tumor types.

In light of all facts stated, it is clear that the isolation of known human tumor progression or associated genes and indeed even novel genes that hold potential as being putative new markers, can be readily identified from a subtracted library that is generated not from human tissue material but rather from a rat cell line system. In others words, the identification of novel human genes that are specifically expressed in human tumors identified through a rat subtracted library proves that such an approach is highly valid.

Through reverse northern analysis the initial high number of differentially expressed clones was reduced to 13 concerning correlation in other rat tumor progression models, and to over 35 when these genes were monitored for expression in human tumor samples only. To address the human functional relevance of these genes in the context of tumor progression is difficult, due to the shear number of potential clones. Rather studies should be directed to those clones that on the one hand exhibit differential expression in various rat tumor model systems, yet on the other hand are also expressed (upregulated or exclusively) in human tumor material. Through the large amount of information gained by reverse nothern screening of the original subtracted clones with cDNAs of rat and human origin, a number of clones did fit the criteria mentioned above. One such clone was chosen for functional analysis as it exhibited an expression profile typical to the model clone 25 (see figure 17), being expressed only in metastatic competent cells and in addition was also expressed in human tumor material (see table 5). This clone (clone # 62) was identified as Ezrin and it´s putative function concerning tumor progression was addressed and the results are given in part two.

PART TWO

3.6 Functional Analysis of Rat Full Length Ezrin in the Bsp73 Model System

Ezrin was first characterised as a minor component of intestinal brush border microvilli (Bretscher, 1983). Other related proteins were subsequently identified in various actin-based cytoskeletal structures. These included a 75 kD protein called cytovillin (Pakkanen et al., 1987), and a p81 protein from A 431 human epidermoid carcinoma cells (Hunter & Cooper, 1981; Bretscher, 1989). Through numerous immunological studies and cDNA sequencing have now established that ezrin, cytovillin and p81 are the same protein. Ezrin belongs to the ezrin/radixin/moesin (ERM) protein family and homologues are found in various species. The sequence of their amino-terminal halves is highly conserved (~80 % identity for any pair in mouse or for any pair in human). When the amino and carboxy-terminal halves of ezrin were transfected into cultured fibroblasts, they were targeted to the plasma membrane and actin-filament bundles respectively (Arpin et al., 1994). This indicated that ERM proteins may function as plasma membrane actin filament cross-linkers. Depletion of individual ERM proteins by antisense oligonucleotides has provided direct evidence for their roles *in vivo*. (Takeuchi et al., 1994). Conversely, forced expression of ERM proteins has also profound effects. For example, transfection of ezrin cDNA into normal NIH3T3 firbroblasts resulted in lost of contact inhibition demonstrating its involvement in pathways that negatively regulate contact inhibition (Kaul et al., 1996). Transfection into (NK)-resistant cells induces sensitivity to NK cells through the redistribution of ICAM-2 molecules (Helander et al., 1996).

Moreover, transfection of a truncated ezrin lacking the amino-terminal portion binds with high affinity to actin-filaments under physiological conditions (Turunen et al., 1994). The binding of native full length ezrin to actin filaments has not been established under physiological conditions. Conversely, at physiological ionic strength, full length ERM proteins have a very low affinity to the cytoplasmic domain of CD44 in vitro, whereas, the amino-terminal portions bind to CD44 with high affinity (Hirao et al., 1996). These findings led to the proposal of an intramolecular head-to-tail association model for ERM proteins, the amino- and carboxy-terminal portions may mutually suppress each other's function, namely membrane- and actin-binding respectively. This motion was supported by the observation that the effects of forced expression of the carboxy-terminal half of ezrin in CHO cells (namely the production of numerous thin processes) could be completely abolished by co-expression of just the amino-portion of ezrin (Martin et al., 1995).

Perhaps the most interesting observations concerning ezrin, certainly in the context of the work described in this thesis, is its putative role as a mediator of signal transduction events from certain receptor tyrosine kinases. Krieg and Hunter. (1992) reported the high and transient phosphorylation of ezrin in human epidermoid carcinoma cells upon epidermal growth factor

stimulation. They further mapped the phosphorylation sites to tyrosines 145 and 353. Some three years later, it was shown that hepatocyte growth factor/scatter factor (HGF/SF) a cytokine known to regulate motility, morphogenesis and growth of cells, stimulated the tyrosine phosphorylation of ezrin in human colon epithelial cells. Upon stimulation, ezrin translocated to areas of ruffled membrane, an effect that was abolished by genistein, a tyrosine kinase inhibitor, suggesting that ezrin plays a key role in HGF/SF induced membrane ruffling (Jiang et al., 1995). Most recently, it was demonstrated that ezrin was crucial to HGF-induced morphogenesis in a polarized kidney-derived epithelial cell line. Forced expression of wild-type ezrin in these cells enhanced their motogenic and tubulogenesis formation upon HGF stimulation. Furthermore, a truncated form of ezrin (amino-terminal portion) when expressed impairs the effects of HGF on these cells, thus suggesting a dominant-negative mechanism of action. Site-directed mutagenesis of ezrin at codons Y145 and Y353 (known phosphorylation target sites) also perturbs the motogenic and morphogenic responses to HGF (Crepaldi et al., 1997).

Together these data implicates ezrin as a down-stream effector molecule of the c-Met receptor and that by removing endogenous functional ezrin ablates both motogenic and morphogenic responses induced by HGF.

3.6.1 Full Length Cloning of Rat Ezrin From the ASML Cell Line and the Generation of a Dominant Negative Mutant Form of Ezrin

Through the subtraction analysis of the Bsp73-AS system, c-Met was found to by highly upregulated in the metastasizing ASML line (see figure 10 panel P). This also proved to be the case for ezrin (see figure 10, panel O). Based on the observations made concerning ezrin as a possible mediator of both motogenic and morphogenic responses induced by HGF (Jiang et al., 1995; Crepaldi et al., 1997), and based on the fact that both these genes are highly differential expressed in the ASML line, the tentative assumption was draw that deliberate ablation of endogenous ezrin function (by a dominate negative mechanism) in the ASML cell line could have functional consequences concerning the ability of ASML´s *in vivo* metastatic potential. This seemed reasonable as c-Met is known to induce a motogenic-invasive rather than a proliferative program (Giordano et al., 1993), and any interference of c-Met function by targeting a known down-stream effector molecule, such as ezrin could affect the potential metastatic spread *in vivo.* Moreover it is a more simple task to ablate a signal transduction pathway by the selective inactivation of one particular component in that pathway, than it is to reconstruct a signal pathway where multiple components may be required. That is to say it is technically easier to block signal events from the c-Met receptor by inactivation of ezrin in ASML cells and monitor a reduction of metastatic potential, than it is to reconstruct c-Met signalling in the 1AS cells where multiple components would be required in order to increase the metastatic potential. As ezrin was identified as being differentially expressed in ASML through subtraction, the question addressing whether ezrin exhibited

differential expression in the two rat tumor progression model systems used in the analysis of correlation (see section 3.5.2) was raised. For this purpose direct northern analysed of ezrin expression in all the cell lines of the two model systems was performed as is shown in figure 19. The northern data clearly shows that there is considerable overexpression of ezrin in those lines of metastatic capacity with little or no expression in the non-metastatic lines (1AS, MTPA and AT.1).

Figure 19

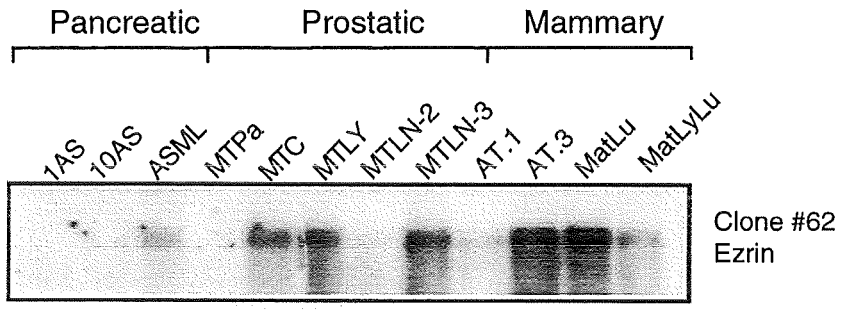


Figure 19. Direct northern analysis of ezrin in three rat tumor progression model systems. Approximately 3 µg of poly(A)$^+$ RNA was isolated from all lines and size fractionated on an 1.2 % formaldehyde agarose gel before transfer to membrane. The membranes were then hybridized with a probe specific for ezrin. Hybridization of membranes were performed in Church buffer at 64 °C followed by washing once in 2 X SSC, 0.1 % SDS and twice in 0.1 X SSC, 0.1 % SDS at 65 °C. Exposure to film was for 1 day at -80 °C.

The degree of differential expression of ezrin in the Bsp37-AS system seen at the northern level was also reflected on the protein level as is indicated in figure 20.

Figure 20

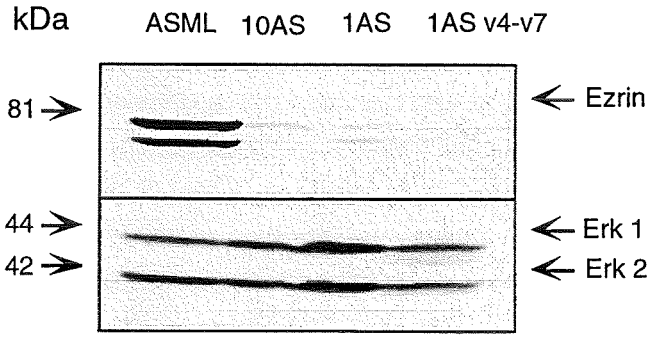


Figure 20. Ezrin protein expression in the Bsp73-AS system. Approximately 20 µg of total cellular protein from the cell lines indicated was run on a 12 % polyacrylamide gel, transferred to membrane and probed with an anti ezrin antibody. For loading control, the bottom part of the gel was separately probed for Erk 1 and 2 expression. ASML and 1AS represent the lines used in the original subtraction, and 10 AS is a subline that exhibits low *in vivo* metastatic capacity. 1AS v4-v7 represents a stable transfected Bsp73-1AS clone and is included as a neo control.

The degree of expression of ezrin in ASML is very high (see figure 20). and both the 10 AS and the 1AS exhibit very low endogenous ezrin expression, seen also in the mRNA level. The presence of a slightly smaller band (78 kDa) is cross-reactivity of the ezrin antibody to other ERM protein members, (as is described for the antibody, see Materials & Methods) radixin which has a molecular weight of around 78 kDa. Radixin was not isolated from the subtracted library, despite its differential appearance on the protein level. Reasons for this discrepancy might lie in fact that the true identity of this second smaller band is not known (would require probing with an anti radixin antibody) and it might represent an entirely different protein that had been isolated in the library as a cDNA clone. Concerning ezrin´s expression profile at both the protein and mRNA level, there is a very close correlation to the metastatic potential in all model systems, indicating it is likely to have functional role in tumor progression.

Before any functional studies could be addressed, it was necessary to obtain the full length rat ezrin cDNA sequence as the clone of ezrin derived from the subtracted library was only about 140 bp long. Through sequence homology searches in public data bases (BLAST program. Internet) a partial 3´- rat ezrin clone was found containing the last 484 bp of the coding sequence of ezrin followed by 1.2 Kb of untranslated sequence (Accession Nr. X67788). This clone when aligned against the full length mouse ezrin cDNA (Accession Nr. X60671) exhibited a match of 94 % at the nucleotide level indicating its authenticity. As only a partial rat ezrin clone existed, a 3-primer was designed from the 3´end of the known partial rat ezrin clone and a 5´primer designed from the mouse sequence as is indicated in figure 21.

Figure 21

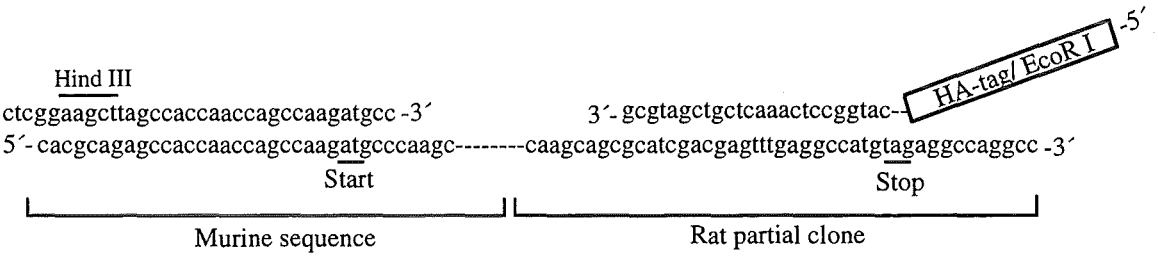


Figure 21. Full length HA tagged cloning strategy of rat ezrin from ASML cells. Two primers were used for the RT-PCR cloning of full length rat ezrin. The 5´-primer sequence was obtained from the sequence of the full length murine ezrin, and the 3´-primer sequence was designed from the partial rat cDNA clone of ezrin. The 3´-primer in addition carried a HA-tag that ablated the internal stop codon to allow translation of the HA specific peptide sequence, required for detection. Both primers contain internal restriction sites to aid subcloning into a suitable eucaryotic expression vector.

The 5´primer (5´- Erzin/Hind III) covered the start codon (as determined form the mouse sequence) and contained an internal Hind III restriction site for the ease of subcloning into an appropriate eucaryotic expression vector. The 3´primer (3´- Ezrin/HA/EcoRI) carried, in addition to an EcoR I restriction site, the coding sequence for a human hemagglutinin protein tag (HA-tag), derived from human influenza virus. This form of "epitope tagging", allows for the detection of an expressed protein against the background of the endogenous protein within

eucaryotic cells by using an antibody that specifically recognizes the tag (Wilson et al., 1984; Herscovics et al., 1994). The sequence of the primers is also given in Materials & Methods. As ezrin was overexpressed in ASML, it was the obvious source of template for the cloning of ezrin. Poly(A)$^+$ RNA was isolated from subconfluent ASML cells and reverse transcribed to cDNA. The cDNA reaction after completion was diluted 10-fold and a 4 µl aliquot used for RT-PCR using the 5´ ezrin Hind III primer and the 3´ezrin EcoRI HA-tagged primer.
After the RT-PCR reaction was completed, the products were analysed on an agarose gel (see figure 23 (A) lane A). The DNA band in lane A had the expected size of 1.8 Kb (murine, bovine and human ezrin sequences have all a length of 1.75-1.8 Kb and an internal homology of around 94 %. data base alignment, NCBI server, Internet) and was removed from the gel before direct cloning via the T/A overhands into the T/A cloning vector pCR2.1 kit. The authenticity of the cloned fragment was confirmed by sequenced analysis of both strands using automated fluorescent sequencing. The cloned full length rat ezrin cDNA matched 100 % to the sequence of the partial rat ezrin cDNA, with an overall match of 93 % to the full length murine sequence.

Figure 22

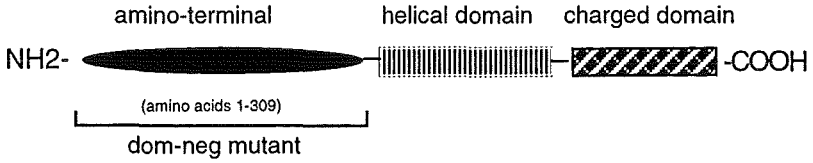


Figure 22. Schematic representation of the functional domains of ezrin. The model structure of the predicted structural organisation of ezrin deduced from its primary structure (Gould et al., 1989). The amino-terminal domain (black oval) is followed by an alpha-helical domain, and by a region rich in charged amino acids. The dominant negative mutant was constructed to include amino acids 1-309 as described by Algrain et al. (1993).

As a truncated form of ezrin (amino-terminal portion) was reported to function in a dominant negative fashion when transfected into kidney derived epithelial cells by abolishing all stimulatory effects of HGF (Crepaldi et al., 1997), a HA-tagged truncated dominant negative mutant form of ezrin was constructed through RT-PCR as described by Algrain et al. (1993). Using the same 5´primer as that used to clone full length ezrin with a 3´- HA-tagged primer that annealed to the end portion of the amino-terminal domain (nucleotide position 1-1019, spanning amino acids 1-309 see figure 22) RT-PCR was performed and the products analysed on an agarose gel (see figure 23 (A) lane B). As with the full length clone, the band was excised, cloned and its authenticity confirmed by sequenced analysis of both strands using automated fluorescent sequencing. Now that the full length clone and the mutant clone had been generated, the two cDNAs were subsequently subcloned into pCDNA 3.1 (+) eucaryotic expression vector.

Figure 23

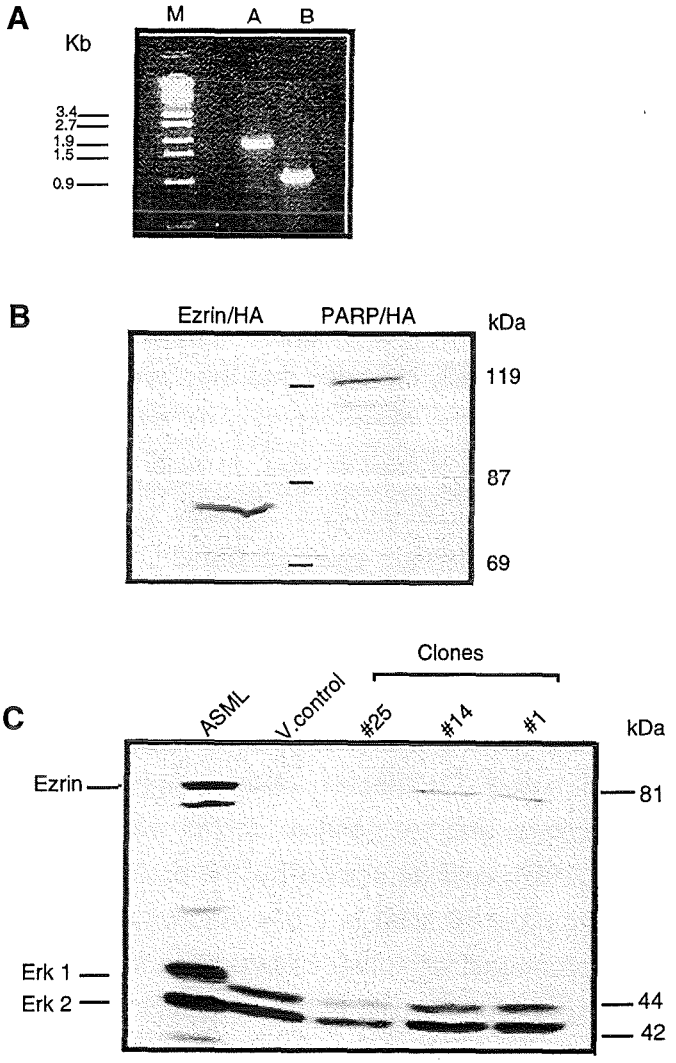


Figure 23. **(A)** The agarose gel depicts the RT-PCR products of full length and dominant negative mutant of ezrin. Lane (A) shows the expected cDNA size of the full length ezrin of approx 1.8 Kb (as determined from murine and human sequence alignments). In lane (B) RT-PCR product of the dominant negative mutant form of ezrin and runs at the expected size of approx 1.0 Kb. Conditions of RT-PCR for the cloning of the full length ezrin. First-strand cDNA synthesis was performed with 2 µg of poly(A)$^+$ RNA isolated from ASML cells, according to the protocol outlined in Materials & Methods. After dilution, a 4 µl aliquot was removed and subjected to PCR in a total volume of 30 µl containing 10 pmole 5´ Hind III ezrin primer, 10 pmole 3´ EcoRI/HA primer, $MgCl_2$ (f.c. 1.5mM), 1 x supplied PCR reaction buffer, dNTPs (10mM each), 2U Taq (Eurobio 5U/µl) and bi-dest H_2O to 30 µl. Cycling parameters were as follows. 94 °C for 50 sec, then 26 cycles of 94 °C, 20 sec; 66 °C, 30 sec; 72 °C , 2 mins. The PCR generation of the dominant-negative mutant of ezrin, all PCR conditions were as described for the full length cloning of ezrin with the exception of substituting the 3´ EcoRI´/HA-tagged primer for a 3´ EcoRI/HA-tagged primer Both bands were cloned and confirmed by sequencing. **(B)** The full length ezrin and the mutant form were both subcloned into a eucaryotic expression vector (pcDNA 3.1 (+), Invitrogen) and maxi-prep plasmid DNA isolated. Full length clone was then subjected to coupled [^{35}S]-methionine labeled transcription-translation as described in Materials & Methods and the products run on a 12.5 % polyacrylamide gel before drying the gel and exposing to film. The positive control (PARP/HA) was an expression vector containing the coding sequence for the gene PARP, known to function in this assay. The translated product of full length ezrin runs at the expected molecular mass of 81 kDa.
(C) 293 cells (E1a/b transformed human fetal embryonic kidney cells) were transiently transfected with the full length construct containing ezrin cDNA (described under Materials & Methods). The cells were harvested 48 hours after transfection, lysed and the proteins (20 µg per lane) separated on a 12.5 % SDS-PAGE gel. The gel was transferred to membrane and later probed for ezrin expression using an ezrin antiboby. As positive control protein lysate from ASML cells was taken. For negative control 293 cells were transiently transfected with an empty expression vector. Lanes marked as #25, #14, #1 represent three different full length clones that were each transiently transfected into 293 cells. Clone #25, contained a premature stop codon (at position 483) which would result in no detection of protein as the antiboby epiotpe is at the extreme carboxy-terminal end. Clones #14 and #1 contain a full length reading frame and both upon transient transfection produce ezrin. In addition, clone #14 was the clone used for the transcription-translation assay. The presence of Erk 1 and 2 serve as loading controls for protein amounts.

(Invirtogen) using the two internal restriction sites (Hind III/EcoRI) present in the primer sequences. Again clones were isolated and sequenced to confirm correct ligation and orientation. Maxi-prep DNA was prepared and the full length clone subjected to *in vitro* coupled [^{35}S]-methionine labeled transcription-translation as described in Materials & Methods. As a control, an expression vector containing the cDNA sequence for the gene PARP known to function in this assay was taken. The products of the reaction were run on a 12.5 % polyacrylamide gel together with middle range standard protein markers (Bio-Rad Labs. CA), before drying the gel and exposing to film (see figure 23 panel B).

The full length HA-tagged ezrin runs at the expected size (as determined through sequence alignment data of murine and human ezrin) and additionally indicates that the clone represents a full length functional reading frame of ezrin. That is, the clone contains no premature stop codons or frame shifts, either of which would result in a change of size. In order to be certain that the full length construct functioned *in vivo*, human fetal embryonic kidney cells, 293 cells were transiently transfected with three cloned constructs (see figure 23 panel C). Clone #25 was found through sequencing analysis to contain an internal premature stop codon (at position 483) that would result in a truncated protein. This protein would not be detected as the ezrin antibody epitope resides in the carboxy-terminal. Clone #14 was the same clone shown to function in the coupled transcription-translation assay (see figure 23 panel B) and clone #1 was another full length clone taken for conformation. Transfection of the empty expression vector in 293 cells functioned as a negative control and the positive control was ASML cell lysate. There is strong expression of ezrin in ASML cells as shown by the presence of a 81 kD band. The second slightly smaller band (approx 78 kDa) is cross-reactivity of the ezrin antibody to radixin (ezrin antibody known to cross-react with other ERM protein members, see Materials & Methods). There is very slight expression of ezrin in both the vector control and those cell which received clone # 25, being due to the presence of endogenous ezrin in 293 cells. Both clones #14 and #1 show increased ezrin expression when compared to the negative controls. The modest increase in expression is due to the fact that these clones were transiently transfected into 293 cells and only a small pool of cells would have taken up the construct and expressed ezrin. Erk 2 indicates that all lanes from the 293 transfectants were equally loaded, whereas there is an apparent reduction in the amount of Erk 1 in those cells transfected with clone # 25. So collectively, the data from figure 23 suggests that the full length rat ezrin clone represents the authentic rat ezrin sequence determined through western blot analysis of transfected clones.

Under an similar experimental setting, the dominant negative clone of rat ezrin was also for protein expression . Using again the *in vitro* couple transcription-translation protocol, without the addition of [^{35}S]-methionine (cold reaction), the dominant negative HA-tagged mutant clone was translated and the product of the reaction used for immunoprecipitation using an anti-HA antibody. The immunoprecipitated was then subjected to western blot analysis and the membrane probed with an anti-HA antibody (see figure 24). Considering figure 24, lane 1

shows the direct products of the coupled reaction of the mutant from of ezrin having the expected size of approximately 40 kDa. In lane 2 shows the translated mutant ezrin after precipitation with an anti HA antibody, and lane 3 indicates that there is still some translated ezrin product in the supernatant of the immunoprecipitation reaction. In regard to these data, the mutant form of ezrin was shown to have a correct functioning HA-tag as demonstrated by the immunoprecipitation experiment and by the fact that the blot was probed with an anti HA antibody. In addition, the mutant form demonstrated the approximate right molecular weight as expected from the length of the coding sequence (1.0 Kb).

Figure 24

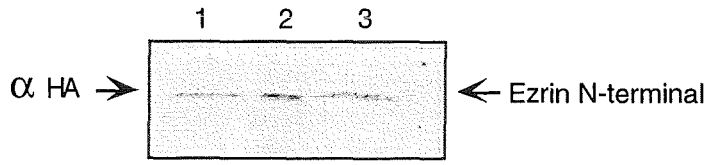


Figure 24. Confirmation of correct translation of the dominant-negative ezrin mutant. Immunoprecipitation was performed on the mutant ezrin clone and the products run on a 10 % polyacrylamide gel, before transfer to immobilon-P membrane (Millipore). The membrane was subsequently probed with an anti HA antibody. Lane 1 represents the initial coupled trascription-translation reaction product of mutant ezrin directly loaded. Lane 2 is the immunoprecipitant of the reaction after immunoprecipitation with an anti HA antibody. Lane 3 represents the supernatant of the immunopreciptation reaction. All reaction protocols are described in the Materials & Methods section.

3.6.2 Generation of ASML Clones Expressing Dominant-Negative Ezrin

Despite the fact that both a full length and muatnt form of ezrin had been constructed, the mutant form of ezrin would offer the most rapid analysis for endogenous function in the context of metastasis, rather than overexpression of the full length in the non-metastasizing Bsp73-1AS cells. It is experimentally easier to abolish a pathway than to reconstruct one whereby multiple genes may be required. For this purpose, stable transfectants of ASML were generated by the transfection of the dominant negative ezrin mutant clone into ASML cells, followed by subsequent selection of putative positive clones under neomycin (G418, Sigma). Once growing clones had been expanded, cell lysates were prepared and subjected to western blot analysis using an anti HA antibody as can be seen in figure 25. From the nine neomycin resistant clones (1-9), three clones demonstrated expression of the HA-tagged dominant negative ezrin (see clones 3, 7, and 8). There was no detection of any 40 kDa band (corresponding to the mutant clone) in either ASML cells alone or in mock transfections with a vector control. This indicates that the detected band in the clones represents true endogenous expression of the transfected mutant ezrin cDNA.

Additionally, there was no change of phenotype of the transfected cells. Now that ASML clones stably expressing the dominant negative form of ezrin were generated, the obvious question was whether this forced expression of a mutant form of ezrin had any functional consequence concerning the tumorigenic potential of ASML cells when compared to ASML cells that had received only the empty vector (negative control). To address this question the three positively expressing clones (clones 3, 7 and 8) were further expanded and prepared for *in vivo* inoculation, together with a vector control, into syngienic male BDX-10 rats (as described under Materials & Methods).

Figure 25

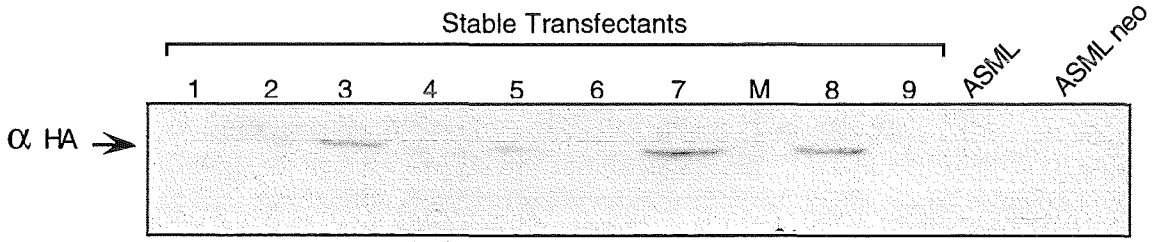


Figure 25. Identification of ASML clones stably expressing dominant negative ezrin. Stable transfectants were generated as described under Materials & Methods. A total of nine neomycin resistant clones were taken and cell lysates prepared and the proteins separated over a 10 % polyacrylamide gel before blotting onto immobilon-P membrane and probing with an anti HA antibody. Stable transfectants are indicated 1-9, M shows the lane used for protein standard markers, ASML indicates pure ASML cell lysate and ASML neo the vector control.

3.6.3 Dominant-Negative Ezrin Reduces Tumor Formation Capacity of ASML Cells *in vivo*

For each clone, and the vector control, eight animals were taken, each receiving approximately 5 X 10^5 cells in phosphate buffered saline (PBS) were subcutaneously injected into the left flank of each animal. This resulted in eight animals per clone and eight animals for the vector control. After a period of 14 days, the animals were inspected for signs of tumor growth on a twice weekly bases. Tumor growth was monitored for each tumor bearing animal until either the tumor had reach the permitted limit, or the animals showed obvious signs of moribund. Each sacrificed animal was autopsied and the internal organs inspected for signs of metastases. The results were plotted as a survival curve (see figure 26). From figure 26 it is clear that those animals that received ASML cell expressing a dominant negative form of ezrin have an obvious prolonged life expectancy when compared to those animals that were inoculated with vector control. After a period of 8 weeks, all control animals were sacrificed, at the same time point there were 6, 7 and 5 animals remaining corresponding to dominant negative clones #3, #8 and #7 respectively. After an addition 4 weeks the number of surviving animals was 6, 5 and 3 for dominant negative clones #3, #8 and #7 respectively. Those animals that were sacrificed from the dominant negative groups did show, in addition to a

primary tumor at the site of injection, presence of metastases in both lung and lymph tissue, however 40-50 % of animals failed to develop even a primary tumor. These animals represented the majority of those that exhibit prolonged survival, as they failed to develop tumors.Others did show signs of tumor formation but at a prolonged rate. The conclusion is that the capacity of ASML cells under forced expression of dominant-negative ezrin to form primary tumors is drastically reduced.

Figure 26

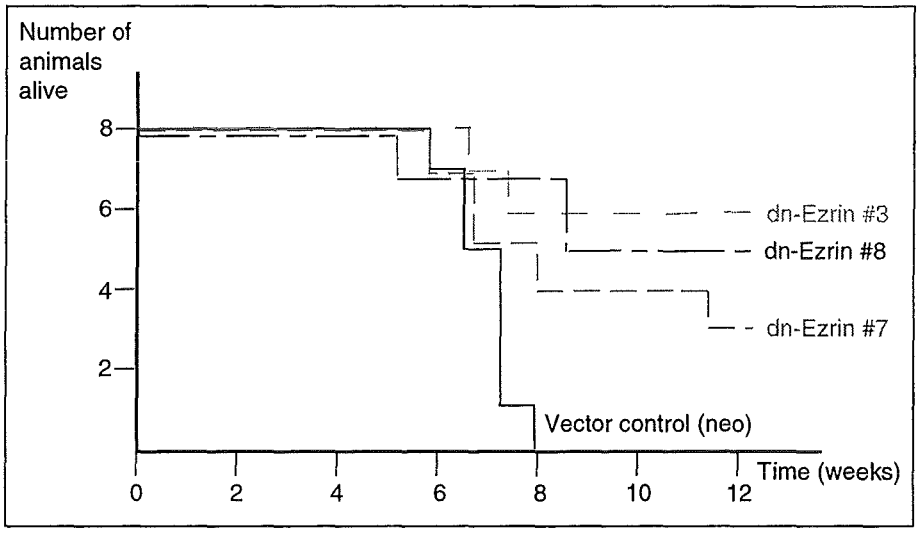


Figure 26. Survival curves of male BDX-10 rats inoculated with ASML cells stably expressing dominant negative ezrin. The three coloured lines indicate the survival curves of animals that were inoculated with three independent dominant negative (dn) ASML clones. The vector control is shown by the black line. Eight animals were taken for each clone.

Dominant negative ezrin in ASML does exhibit profound functional consequences in that inoculated animals either show a dramatic increase in survival or that the domninant clones failed to give rise to a primary tumor. This experiment clearly implicates the functional requirement of endogenous ezrin in ASML cells for tumor development as functional ablation of ezrin perturbs tumor formation *in vivo*. The fact that under *invitro* conditions dominant-negative clones of ASML grow but *in vivo* their growth is drastically reduced suggests a number of things. Firstly, functional ezrin may not be required for in vitro growth in ASML cells but when in the context of an organism functional ezrin is required for growth of these cells. This requirement could be brought about by the presence of a specific factor that is only present in the *in vivo* situation, a factor that requires ezrin to function such that the cells proliferate. In other words, by abolishing ezrin function with the ectopic expression of a dominant-negative mutant, the growth factor dependency of ASML cells is altered. In this respect further investigation should be focused on elucidating the functional role of ezrin in the context of tumor formation as it appears to be of significant importance

This work, the work on ezrin is just one example of a single chosen clone whose functional role in tumor progression has been succsessfully demonstarted. It was the clone of choice as it demonstarted excellent correlation in two other rat tumor progression models, it cross-hybridized to human tunor cNDA probes, and there existed some information concerning its function in eucaryotic cells, for example its dominant-negative effect with c-Met signalling. There is now reason to believe that other clones that also showed excellent correlative expression could also have profound effects on tumor progression like clone 25. However to investigate the function of a novel gene is vastly more work and would have involved the full length cloning of the gene to enable forced expression studies to be made and the generation of antibodies to detect endogenous expression. In the limited time frame left, this would not have been possible. Ezrin appeared a suitable alternative as its correlative expression data suggested general involvement in tumor progression, moreover, there existed antibodies for protein detection and a partial rat clone that aided the full length cloning. The work present here demonstrates the functional relavence of one clone identified from the subtracted library and shows without question that ezrin is functional significance in the context of tumor progression, and should be further investigated.

4 DISCUSSION

4.1 Tumor progression: A Change of Paradigms

At the cellular level, it has long been appreciated that metastasis is an enormous complex pathophysiological event and that this degree of complexity is reflected by an ever-increasing list of molecular alterations which appear to be associated with the process. The powerful techniques of molecular biology are proving themselves able to be focused on a problem of such inherent difficulty as metastasis and tumor formation, however the limitations are obvious. This is most apparent if one considers the early reported attempts to understand metastasis. Such studies were either stuck at a descriptive level (pathology), or (if a molecular genetic approach was persued) produced in the best case single molecules that were usually pleiotropic effectors (e.g. Ras or c-Met) and therefore offered little insight about other potentially involved downstream genes.
The so called "functional" approaches aimed at identifying genes that are involved in metastasis failed mostly due to the inability of these methods (e.g. DNA-mediated gene transfer or retroviral insertional mutagenesis) to activate all the genes that contribute to the metastastic phenotype. Again, as in the case of Tiam-1 (retroviral insertional mutagenesis) or ras (Bernstein & Weinberg, 1985) researchers came up with rather pleitotropic effectors and the molecular mechanisms remained largely unclear.

With the rapid advance of genomics, that is the simultaneous analysis of huge numbers of genes at the nucleic acid level, new methods became available that allowed efficient comparison of the transcriptional repertoire associated with a given phenotype. Despite the fact that these technologies are still in their infancy, they hold great promise as they are capable of analysing enormous numbers of target genes in a single hybridization step. Moreover, which genes are analysed can be predetermined.

4.2 Attempts to Identify Genes Involved in Tumor Progression

4.2.1 At the Genomic DNA level (DNA-mediated gene transfer)

The metastatic spread of cancer is an enormously complex phenomenon that remains largely unsolved, in part because it is difficult to model the entire process using experimental approaches *in vitro.* The ability to transfer DNA into a non-metastatic cells and to observe the induction of metastasis *in vivo* provides a means for identifying DNA sequences that are associated with the development of metastatic capacity. By transfection of human metastatic tumor DNA into Ha-ras transformed NIH 3T3 fibroblasts, resultant colonies produced lung metastasis after subcutaneous inoculation into immunocompetent NFS/NCr mice (Berstein &

Weinberg, 1985). Similar experiments have been performed on rat mammary epithelial cells with fragments of DNA for human metastatic breast tumors and the same observations were made (Barraclough et al., 1998). These experiments demonstrated that the induction of a metastatic phenotype can be transferred via DNA from cell to cell and that the process is entirely DNA dependent. Such approaches can result in the isolation of putative genes but it is a vastly inefficient way as the isolation of the DNA fragment(s) responsible for the phenotype is difficult. In addition, is fault-sensitive as Barraclough et al. (1998) isolated non-transcribed genomic DNA as the metastatsis inducing agent.

4.2.2 At the Protein Level: Differential Antibody Screening.

This approach focuses on identifying cell surface differences between two cell lines that differ in their metastatic capacity. One famous example was the isolation and identification of a variant form of a cell surface glycoprotein known as CD44 as a causative determinant in metastasis formation (Gunthert et al., 1991). Using a monoclonal antibody (MAb1.1ASML) raised against a cell surface determinant of the metastasizing rat pancreatic carcinoma cell line BSp73-ASML, one cDNA clone was isolated that encoded an additional extracellular domain inserted into the rat CD44. This new variant was only expressed in the metastasizing cell lines of two rat tumors, and further more, overexpression of this variant in the non-metastasizing BSp73-AS cells sufficed to establish full metastatic behaviour. This variant form of CD44 was subsequently shown to be expressed in human tumors (Hofmann et al., 1991). So while the use of generating antibodies against metastasizing cells did identify what was later shown to be an important player in metastasis in the cell system used, the fact that only one difference was identified between two cell lines (one capable of metastases, the other not) might suggest that what one is looking at is probably nothing more than " the tip of the iceberg".

4.2.3 At the mRNA/cDNA Level: Subtractive cDNA Cloning

Other investigators have tackled the problem from a different angle but essentially the same ends were met in that again only one gene was identified as being dysregulated. Ebralidze et al. (1989) examined the differences in gene expression in metastatic and non-metastatic tumor lines. Using a subtractive cloning procedure coupled with differential colony screening lead to the identification of one gene (mts-1) expressed specifically in the metastasizing line (Ebralidze et al., 1989). At the same time another group using differential library screening with cDNAs derived from cell lines of varying metastatic potential isolated just one clone that gave a stronger signal with cDNAs derived from low metastatic cells (Steeg et al., 1988a). This clone (NM23) was later shown to be an enzyme, a nucleoside diphosphate kinase (NDPK), whose function is to provide nucleoside triphosphate precursors for DNA and RNA synthesis (Lascu, 1996). Both experimental settings again resulted in the isolation of a single candidate clone and not a inventory of differentially expression genes.

So in retrospect of the above facts there was an obvious problem, despite that these methods were nevertheless useful. The phenotype of tumor progression was very much restricted to the analyses of just single genes, whereas the inherent complexity of metastasis indicates that there are probably many more unidentified genetic alterations that were just "slipping through the net ". How is it then possible to identify more or if possible all the genetic dysregulations that underlie the metastatic phenotype? A simple answer would be just to improve or tighten the "net", and over the last number of years this has been indeed the case.

4.3 Technologies for Expression Profiling and Difference Analysis

The last six years has seen the introduction of novel techniques that are collectively aimed at monitoring differential gene expression as their names suggest, such as differential display (Liang & Pardee, 1992; Sokolov & Prockop, 1994), and representational difference analysis (cDNA-RDA; O´Neil & Sinclaire, 1997). These techniques represent a means to improve the net, although there are still intrinsic drawbacks associated with each method. For example, differential display (DD) restricts the analysis to differences of the 3´end of cDNAs, so that differences in the 5´portion of cDNAs (e.g. variants of alternatively spliced genes) are not detected. Additionally, variable reproducibility of the differential band patterns and the significant incidence of false-positives makes it difficult to isolate transcripts that are differentially expressed. Differential display does however allow muti-comparisons to be made on one gel and additionally, indicates both upregulated and down regulated transcripts. Another common feature of the methods mentioned above represents also an obstacle in the isolation of rare transcripts: the disproportion of concentrations of differentially expressed genes is maintained in the subtraction. cDNA-RDA requires multiple rounds of subtraction as the method fails to take into account the large differences in relative abundance of individual mRNA transcripts. Enzymatic degradation subtraction (EDS; Zeng et al., 1994) relies on discrimination of differential transcripts through a modified form of exonuclease digest followed by PCR amplification. However for efficient subtraction, extensive monitoring of the exonuclease digest is required. In 1995, Vogelstein and collegues, published the tabulation of a new method called "serial analysis of gene expression" (SAGE; Velculescu et al., 1995) which is based on the use of specific sequence-tags (usually 13 bp long), and has the potential to profile the transcriptional status of a given cell type to a sensitivity of single copy numbers. Although powerful, the method is not typically suited for the isolation of novel genes as the sequence-tags are just too small to allow for a confident comparison and subsequent full length cloning.

From the perspective of my work, what was needed was a subtractive cloning procedure that would cater for high sensitivity and produce an inventory of differentially expressed genes (like SAGE), incorporate a normalization step to reduce over-representation of highly abundant genes (unlike that of cDNA-RDA) and not be proned for false positives or variable

reproducibility (as is often the case with DD). All of these requirements appeared to be met b y a another recently introduced technique subtraction suppressive hybridization (SSH) This method, unlike those mentioned, relies on the selective amplification and enrichment of differentially expressed sequences with a concomitant suppression of sequences not differentially expressed, by means of a suppression PCR reaction (Diatchenko et al, 1996). In the course of my thesis, this method was adopted for the differential analysis of messenger RNA transcripts between a non-metastatic and its metastatic counterpart. The importance of the right choice of method, when differential gene expression is to be investigated, is highlighted by the fact that I lost a considerable amount of time trying unsuccessfully to implement another method, Enzymatic Degradation Subtraction (EDS; Zeng et al., 1994).

4.4 Aim of the Work and Choice of the Model System

Of equal importance is the choice of model system. As the aim of my work was to identify putative key players in metastasis it was crucial that a well defined tissue culture cell system was used. One could argue that subtraction could be performed with rat tumor cells injected *in vivo* as appose to tissue culture cells, but this would increase the risk of contamination of other cell types. As a consequence, due to the sensitivity of SSH one might isolate transcripts that are not originally derived from the tumor cells, but rather from contaminating stromal cells present within the tumor mass. The advantages of using a defined rat cell system for the identification of novel molecules that are functionally involved in tumor progression are numerous. Firstly, there is no limitation of the amount of starting material. Secondly, a cell line is composed of a single clone cell type and as such does not exhibit the heterogeneity or indeed the same high degree of complexity as is the case in solid tumors. The 1 AS and ASML cells in this respect represents a genetically stable "frozen state" of expression. Lastly, the possibility of functional studies can easily accommodated by a cell line system, where potential function of tumor associated clones can readily be addressed. Therefore the rat pancreatic adenocarcinoma model system composing of two lines, Bsp73-1AS a non-metastatic cell line and Bsp73-ASML a highly metastatic line was utilized. The ASML line had been reported to produce metastases in both lung and lymph tissue upon subcutaneous injection into BD 10 rats ((Matzku et al., 1983). This I was able to confirm and suggests that despite a period of 15 years, the cells still responded as originally observed as the phenotype was not change, that is the cells were genetically stable. Additionally, the same rat tumor model system had been used to isolate cell surface antigens specific to the metastasizing line, resulting in the identification of a variant form of CD44 (Gunthert et al., 1991). It was therefore reasonable to assume that this tumor model system would, other than a splice variant of CD44, have additional differences that might also contribute to the metastatic phenotype. Moreover, this cell system had been studied in detail and a differentially expressed gene (uPA) that would act as a positive control for the subtraction was already known.

In the colorectal tumorigenic model proposed by Fearon & Vogelstein. (1996), they describe the transition of a locally growing carcimona to metastatses as "other genetic alterations". This statement typifies the present shortcomings of our knowledge concerning the genetic alterations that predisposes a tumorigenic cell to one capable of metastasis. The primary aim of my work was to cast some light into this "black box" by generating an inventory of genes associated the metastatic phenotype.

4.5 Proof of Concept: Subtractive cDNA Cloning as a Rapid Way to Identify Molecular Determinants of Metastasis

A subtraction was performed between the non-metastatic Bsp73-1AS cell line and the highly metastatic Bsp73-ASML cell line. This resulted in the identification of 126 independent differentially expressed transcripts all of which were differentially expressed in the metastasizing Bsp73-ASML line, the majority of which, showed a very high degree of differential expression as confirmed by direct northern analysis. As expected one of the 126 genes isolated was the positive control for the subtractive enrichment of differentially expressed genes (uPA). Upon the analysis of sequence data of all clones, it was obvious that nearly halve represented novel sequences or existed only as ESTs and many of the known clones identified had been already documented as relevant for tumor progression based on published studies.

This observation was taken as a "proof of concept" such that the experimental setting was (totally) valid, moreover, from published reports a large proportion of the known genes were described to be functionally involved with each other. This in turn allowed one to hypothetically construct small "mini-networks" of specific genes whose products functionally interact with each other in a direct manner. Although such a motive is, to a certain degree, speculative, it nevertheless serves as a realistic model and puts single observation into a molecular context.

Consider briefly the mini motive depicted in figure 27. The activation of the c-Met receptor by its ligand HGF/SF is known to increase, through the transcription factor ETS1, the protein levels of both uPAR and its ligand uPA in specific cell types (Jeffers et al., 1996). The uPAR/uPA complex in turn can catalyse the conversion of a pro-HGF/SF single-chain molecule into an active two chain form by proteolysis (Mars et al., 1993). So there is a feed forward loop generated whereby uPAR/uPA activates the c-Met receptor by producing functional HGF/SF, and this in turn up-regulates the expression of both uPAR and uPA. These two molecules couples HGF/SF c-Met signalling to the activation of proteases that are important for the dissolution of the extracellular matrix such that metastatic competent cells can intravasate into the lymphatics or the vasculature. The c-Met receptor upon activation also phosphorylates ezrin (as do other receptor tyrosine kinases like EGF) thereby activating the molecule, which is then able to translocate to the cytoplasmic surface (Jiang et al., 1995) and interact with other surface receptors such as CD44 (Hirao et al., 1996). The function of ezrin is

not well understood but it appears to act as a plasma membrane actin filament cross-linking molecule. However, more recently, it was demonstrated that ezrin was crucial to HGF-induced morphogenesis in a polarized kidney-derived epithelial cell line (Crepaldi et al., 1997). These data places ezrin as a transducer of extracellular signals and implicates it as crucial player in the motogenic and morphological response to HGF/SF. In conclusion, this mini-motive has components necessary for the activation of proteases through c-Met signalling, together with the motogenic-invasive functions of c-Met that have been reported (Giordano et al., 1993) that appear to work through ezrin in some cell types. Although not proven, ezrin is shown in figure 27 to influence protein synthesis, as a motogenic-invasive phenotype almost certainly requires de novo synthesis of certain molecules (e.g., proteases). A dominant-negative mutant form of ezrin can abolish this motogenic-invasive phenotype induced by HGF/SF it is assumed that ezrin must be directly involved.

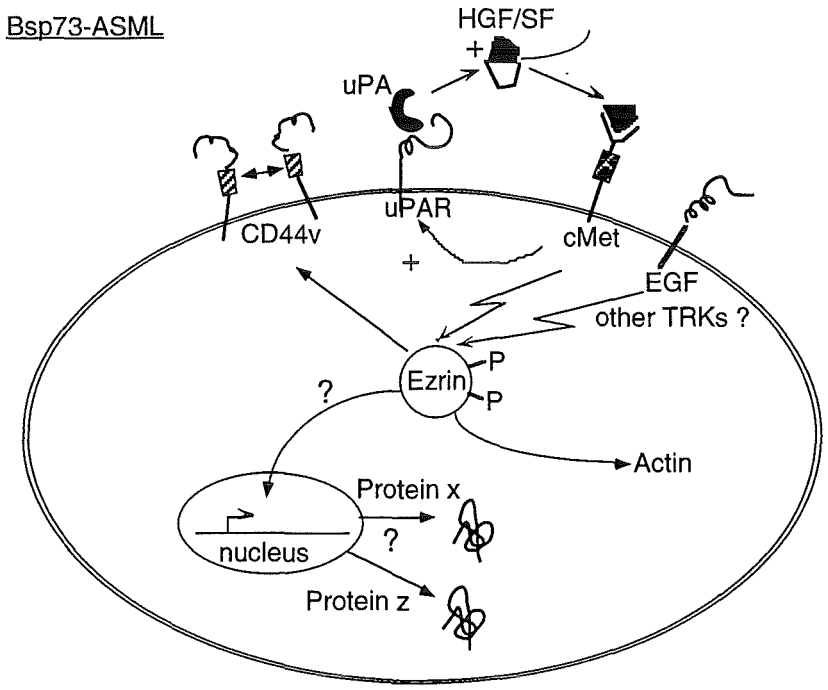


Figure 27. An "expression motive" expressed exclusively in the metastasizing Bsp73-ASML cells. The above diagram depicts a number of genes overexpressed in ASML cells whose products have been reported to functionally interact with each other in a manner that is of significance for tumor progression. An explanation of the motive is given in the text.

Despite the fact that such a model leaves many questions unanswered, it was useful in identifying the central role of ezrin (see part two of results).

Paradoxically the isolation and identification of such a large number of potential tumor progression related clones generated another problem that had in all respects not been

accounted for. Under what criteria are these clones to be segregated such that those of likely importance can be distinguished against those that are perhaps only indirectly involved or even the result of "bystander effect"? This was of particular importance as a large proportion of the clones identified were novel or existed only as an EST. It was clear that by attempting to answer this question on the bases of mass transfection studies (such as overexpression, antisense or dominant-negative experiments) is a task that exceeds the capacity of any individual. This would require the systematic full length cloning of many genes and the subsequent generation of antibodies to detect expression. What was required was a sort of "filter system" that would ascribe relevance to the clones and allow the isolation of those clones of higher probability of being potentially involved in tumor progression. This filter system was achieved through the analysis of correlative expression in other independent tumor progression models, and by cross-hybridization to human tumorigenic cell lines or by hybridization experiments with human cDNAs derived form either a tumor or its matched normal tissue. So while reverse northern screening of the clones with other rat tumor cell lines would indicate clones of likely relevance in tumor progression that could be experimentally addressed, cross-hybridization to human cDNAs would identify clones of potential tumor marker status.

A total of 13 clones were identified through such a screening and further analysed directly at the northern level. All clones, including 6 novel ones (clones 23, 25, 46, 82, 100, and 147) demonstrated excellent correlative expression in the other tumor progression systems. The expression data of clone 25 appeared the clearest, showing expression in all line capable of producing metastases *in vivo*. For comparison, three other genes, uPAR, c-Met and HMG-1, of known involvement in tumor progression were also analysed, the latter two were also identified from the subtracted library. From these three genes HMG-1 showed excellent correlative expression in all systems, while c-Met was only weakly expressed in those lines of metastatic competence in the other two cell line systems. For uPAR, there was even a reduction of expression in the prostatic tumor cell system between the non-metastatic line (AT.1) and the highly metastatic sub-clone (AT.3). So despite their documented involvement in tumor progression, the expression profile of c-Met and uPAR in the three cell systems is not as striking as that of HMG-1. Both uPAR and c-Met represent strong candidates in tumor progression particular for uPAR (Ganesh et al., 1994; Rong et al., 1994, respectively), however HMG-1 that has only been associated with tumor progression (Chiappetta et al., 1995) but exhibits the better profile in these systems. The expression data of the novel clones in comparison to uPAR, c-Met and HMG-1 would indicate that they are all of potential relevance as they follow an almost identical expression pattern and in some cases a more convincing one, in particular clone 25.

By implementation of a suitable "filter system" in the form of correlative expression the initial daunting number of 126 differential expressed clones had been reduced to workable number of 13 promising candidates. The large number of cross reacting clones to human cDNAs is again proof of validity of the experimental setting in that through subtraction of two rat cell lines,

homologues of human cDNAs can be identified that are specific to the tumor status. This means that a direct subtraction between human material may not be necessary and instead a well defined rat tumor model may in fact offer the same potential as it also allows the capacity for functional studies with putative candidate genes.

4.6 From Gene Expression to Protein Function

The finding that several genes were transcriptional up-regulated and their proteins are likely to interact suggested that there should be the option to interfere with these complex networks. The appearance of phenotype-associated transcriptional networks suggests that it is simply nonsense to transcfect one gene (e.g. a protease) and to expect cells to change completely their phenotype. Interference with the functional networks is by far more likely to give insight into the relevance of those networks for the establishment of the phenotype. (at least as long as compensation for the blockage is limited).
Through the identification of the (hypothetical) Ezrin / c-met / uPa / CD44 motive, a basis was provided for an experimental approach aimed at interfering with a crucial element in such a expression motive. For this purpose, a dominant-negative mutant form of ezrin was generated as described by Crepaldi et al. 1997. This mutant form of ezrin was subsequently transfected into ASML cells and stable clones selected. Upon inoculation into male BD 10 rats, it was clear that forced expression of a dominant-negative from of ezrin influences the tumor take rate and thus tumorigenicity of tumor cells and not only the metastatic potential when compared to those animals which had received vector control. As there was no change in *in vitro* growth characteristics of these clones we conclude that expression and function of Ezrin is absolutely necessary for the special conditions and requirements of *in vivo* growth.

This functional approach demonstrates the relevance of interference strategies and marks the endpoint in an experimental system that started with differential analysis between a metastatic cell line and its non-metastatic counterpart and ended with the identification of a gene which was subsequently shown to be functionally involved in tumorigenesis.

5.Resume´

The work presented in this thesis clearly demonstrates the power of a subtractive approach when utilized in the context of identification of novel genes as tumor markers. It also leaves scope for improvement that could take the form of automatization. That is, the complete screening of subtracted libraries could be performed through automatization, coupled with high through-put sequencing with direct links to the public data bases to aid rapid identification of the clones. By adopting a "conveyer-belt" approach, to such an experimental setting, one could rapidly identify from vast numbers of tissue samples, dysregulated genes that

accompany or predispose to a certain disease conditions. This coupled with the human genome project will greatly speed-up the design of novel therapeutic interventions and strategies aimed at interfering with novel target molecules. As our understanding of a complex disease such as cancer improves, so too, will the tools that enable us to intervene such that the quality of life for affected individuals can be improved.

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Note: Cited papers whose author list exceeds more than 14 contributors, have been abbreviated to the first three authors and the last senior author.

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