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Two Genes associated with Motility and Metastasis

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PIP92 and NVM-1: Two Genes associated with Motility and Metastasis

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Pip92 und NVM-1:

Zwei Gene die mit Motilität und Metastasierung assoziiert sind.

Zusammenfasung

Mittels Suppressions-Subtraktions-Hybridisierung (SSH) wurden über ein differentielles Screening Gene identifiziert, die mit Tumorprogression und Metastasierung assoziiert sind. Über hundert Gene werden in der hoch-metastatischen Zelllinie ASML überexprimiert, nicht jedoch in ihrem nicht-metastatischen Gegenpart.

Während meiner Doktorarbeit war es zunächst mein Ziel, unter diesen Genen diejenigen zu identifizieren, die eine Rolle bei der Migration metastasierender Zellen spielen. Dies geschah durch Untersuchung der Expression dieser Gene in mobilisierten und ruhenden Makrophagen, bzw. aktivierten und nicht-aktivierten Lymphozyten. Die hierbei identifizierten Gene wurden in mehreren Tumormodellen weiter auf Metastasen-assoziierte Expression untersucht. Im Anschluß daran wurden zwei Gene für die weitergehende Charakterisierung ausgewählt: Pip92 und NVM-1.

Pip92, das zur Familie der „immediate-early" Gene zählt, wurde bislang nicht mit Tumorprogression und Metastasierung assoziiert. Seine Funktion ist weitgehend unbekannt. Um funktionelle Analysen zu ermöglichen wurden polyklonale Antikörper gegen Pip92 hergestellt. Aus der Literatur war bekannt, daß Pip92 im Zytoplasma lokalisiert ist. Ich konnte in meiner Arbeit zeigen, daß das Pip92 Protein nach Stimulation mit Serum möglicherweise in den Zellkern transloziert. Um Einblick in die Funktion von Pip92 zu erhalten, wurde der Phänotyp von 1AS-Bsp73 Zellen untersucht, die stabil Pip92 überexprimieren. 1AS-Zellen, die Pip92 überexprimieren, zeigen im Vergleich mit Vektorkontrollen in *in vitro* Migrationsassays eine gesteigerte Motilität, was darauf hinweist, daß Pip92 zu der Gruppe von Genen zählt, die für die Regulation der Zellmigration verantwortlich ist. Biochemische Eigenschaften von Pip92 deuten darauf hin, daß es als Transkriptionsfaktor wirken könnte und so wurde nach Genen gesucht, deren Expression in Pip92-überexprimierenden Zellen verändert ist. Die Expression dreier Gene war in Pip92 überexprimierenden Zellen deutlich hochreguliert. Die stärkste Induktion wurde für Osteopontin beobachtet, ein Gen, das bereits zuvor mit Migration und Metastasierung assoziiert worden ist. Gewebsschnitte von Patienten mit invasivem duktalen Mamma-Karzinomen wurden immunhistochemisch mit dem Anti-Pip92-Antiserum untersucht. Eine Färbung wurde nur in Tumorzellen beobachtet, nicht aber in gesunden Normalgeweben.

NVM-1 (novel gene associated with metastasis) ist ein Gen, das zuvor noch nicht beschrieben wurde. Seine komplette kodierende Sequenz wurde isoliert und der vorhergesagte offene Leserahmen wurde durch in vitro Transkription/Translation bestätigt. Die Korrelation der Expression von NVM-1 mit Metastasierung wurde in drei weiteren Tumormodellen bestätigt, zusätzlich zu dem für die SSH benutzten Tumormodell. Nach Abschluß des Human Genome Projects stellte sich heraus, daß das humane Homolog von NVM-1 (hNVM-1) auf Chromosom 14 lokalisiert ist. Die abgeleitete Aminosäuresequenz von hNVM-1 zeigt eine ausgeprägte Homologie im Vergleich mit der Ratten-Sequenz. Die veröffentlichte humane genomische Sequenz ermöglichte es mir, Promoter und Genstruktur von hNVM-1 zu charakterisieren. Die Analyse des hNVM-1 Promoters zeigt eine Reihe von potentiellen Bindungsstellen für Transkriptionsfaktoren innerhalb der vermutlichen Promoter-Sequenz. Das hNVM-1 Gen besteht aus sechs Exons und fünf Introns. Eine umfassende Computer-Analyse von hNVM-1 Genstruktur und EST's zeigt das Vorkommen von zwei Spleiß-Donor-Stellen an der Exon2-Intron2 Grenze, die in verschiedenen menschlichen und Nager-Geweben alternativ genutzt werden. Monoklonale Antikörper gegen rNVM-1 wurden hergestellt und für Western Blot und immunohistochemische Analysen genutzt, wodurch eine zytoplasmatische Lokalisation des rNVM-1 Proteins gezeigt wurde, sowie dessen Expression in Tumoren.

Weitergehende Untersuchung dieser Gene kann möglicherweise zur Entdeckung neuer Zielproteine für die Krebstherapie führen und uns dabei helfen, den Prozess der Transformation nicht-metastatischer in metastatische Tumorzellen besser zu verstehen.

Abstract

The differential screening method of Suppression Subtractive Hybridisation (SSH) has previously been used to compare identify genes associated with tumour progression and metastasis. More than a hundred genes were found to be up-regulated in the highly metastatic cell line ASML in comparison to its non-metastatic counterpart 1AS cells. In my thesis work I have further differentially screened this group of genes to identify those that might play a role in the migration of metastasising cells. This was achieved by analysing the expression of these genes in mobilised and resident macrophages and in activated and non-activated lymphocytes. Those genes identified by these screens were then further screened for metastasis-related expression in multiple tumour models. Following this screening, two genes were selected for further characterisation, Pip92 and NVM-1.

Pip92 belongs to the „immediate early" gene family and has not previously been associated with tumour progression and metastasis. Its function is still obscure. To permit the functional analysis of the Pip92 protein polyclonal antibodies were generated. Pip92 has previously been shown by others to be cytoplasmic. However, the results obtained in my work suggest that the Pip92 protein translocates to the nuclei for example upon serum stimulation. To get an insight into the functional role of Pip92, the phenotype of 1AS-Bsp73 cells stably overexpressing Pip92 protein was studied. 1AS cells ectopically expressing the Pip92 protein exhibit enhanced motility in *in vitro* migration assays as compared to empty vector-transfected cells, suggesting that Pip92 might belong to the set of genes responsible for regulating cell migration. Properties of the Pip92 protein suggest it might act as a transcription regulatory protein and a search for genes whose expression is altered in Pip92-overexpressing cells was therefore performed. The expression of three genes was clearly up-regulated in cells overexpressing Pip92. The strongest induction was observed for osteopontin, a gene whose expression has previously been associated with migration and metastasis. Sections of human tumours dissected from patients with invasive ductal carcinoma were immunostained with the Pip92 antiserum. Positive staining was observed only in tumour cells but not in non-neoplastic healthy tissues.

NVM-1 (novel gene associated with metastasis) is a previously undescribed gene. Its full-length coding sequence was isolated and the predicted open reading frame was confirmed by an *in vitro* transcription/translation. The correlation of expression of NVM-1 with metastasis was confirmed in three tumour progression models in addition to one used for SSH. Upon completion of the human genome sequencing project it became apparent that the human homologue of NVM-1 (hNVM-1) gene is located on chromosome 14. The predicted amino acid sequence of hNVM-1 shows high homology to the rat sequence. The genome sequence allowed me to characterise the hNVM-1 promoter and the gene structure. Analysis of the hNVM-1 promoter revealed a number of potential transcription factor-binding sites within the putative hNVM-1 promoter sequence. The hNVM-1 gene consists of 6 exons and 5 introns. A thorough computer analysis of the hNVM-1 gene structure and ESTs revealed the presence of two splice donor sites at the exon2-intron2 junction which are alternatively used in different tissues of human and rodent origin. Monoclonal antibodies against rNVM-1 protein were generated and proved to be useful for Western Blot and immunonochistochemical analyses, demonstrating a cytoplasmic location for the rNVM-1 protein and expression of the protein in tumours

Further study of these genes may lead to the discovery of the new targets for antitumor drugs and may significantly help us to understand the process of transformation of non-metastatic tumour cells into metastatic ones.

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I am very thankful to all my relatives and especially to my husband and my Mom for their understanding and love. This work would have never been finished without their great help and endless support.

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Abbreviations

A	Adenosine
aa	aminoacids
ABTS	2,2' azino-di-(3-ethyl-benzthiazolin-sulphonate)
AH	Azaserine-hypoxantine
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp(s)	base pair(s)
BRCA	Breast cancer associated
BSA	Bovine serum albumin
C	Cytidine
°C	Degrees celsius
cDNA	Complementary DNA
CHX-1	Cyclohexamide-responsive-1
Ci	Curie
cm	centimetre
cpm	Counts per minute
CTP	Cytidine triphosphate
ddNTP	Di-deoxynucleotide triphosphate
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediamine-N,N-tetracetate
e g	example given
EGF	Epidermal growth factor
EGTA	EthylenGuanidine-N,N-tetraacetate
ELISA	Enzyme linked immunoabsorbant assay

EMT	Endothelial-to-mesenchimal transition
et al	and others (Lat. *et ali*)
ETR101	Early TPA-responsive
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	gram
G	Guanosine
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HBS	HEPES buffered saline
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hr(s)	hour(s)
HRP	Horseradish peroxidase
HSPGs	Heparan sulphate proteoglycans
Pip92	Proline-rich induced protein
hTERT	Human telomerase
TIMP	Tissue inhibitor metalloprotease
TNF	Tumour necrosis factor
TPA	
Ig	Immunoglobulin
IGF	Insulin growth factor
kDa	Kilodalton (10^3 daltons)
l	litre
M	Molar
m	milli- (10^{-3})
mA	milliamper
mab	Monoclonal antibody
mg	milligram
min	minute (s)
ml	millilitre

mM	millimolar
MMP	Matrix Metalloproteinase
MMPI	Matrix Metalloproteinase inhibitor
MOPS	4-morpholinepropanesulfonic acid
mRNA	Messenger RNA
µ	micro- (10^{-6})
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometer
n	nano- (10^{-9})
ng	nanogram
Ni-NTA	Nickel nitrilotriacetic acid
nm	nanometer
NTE	Sodium Chloride-Tris-EDTA buffer
OD	Optical density
o/n	overnight
OPN	Osteopontin
ORF	Open reading frame
p	pico- (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PFA	Paraformaldehyde
pmol	picomols
PVDF	Polyvinylidenedifluoride
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse transcription PCR

s c	subcutaneous
SDS	Sodium-lauryl-sulfate
sec	second(s)
SF/HGF	Scatter factor/Hepatocyte growth factor
SRE	Serum response element
SSC	Sodiumchloride-Sodiumcitrate
SSH	Suppression subtractive Hybridisation
STE	Sodiumchloride-Tris-EDTA
T	Thymidine
TAT	Tumour associated trypsinogen
TBE	Tris-borate-EDTA
TEMED	N, N, N', N' tetramethylene-diamine
TCF	T cell factor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Tris	Tris-(hydroxymethyl)-aminomethane
TTP	Thymidine triphosphate
U	Unit(s)
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
UV	Ultra-violet light
V	Volt
v/v	Volume per volume
W	Watt
w/v	Weight per volume

1 INTRODUCTION

PART ONE

1.1 Introduction

Cancer is one of the leading causes of death in the modern world. It has been long recognised that the process of tumorigenesis is dependent on genetic changes either inherited or accumulated after exposure of DNA to different mutagens (reviewed by Lengauer *et al.*, 1998, Kinzler *et al.*, 1998). There are two major sorts of genetic damages found in cancer cell genomes: dominant, with targets known as proto-oncogenes which typically results in gain of function and recessive with targets known as tumour-suppressor genes which usually causes loss of function. The overexpression of oncogenes and impaired function of tumour suppressor proteins ultimately leads to uncontrolled cell proliferation, escape from apoptotic signals and thus expansion of the transformed cell population (reviewed by Hunter, 1991; Yokota, 2000).

The next step in tumour progression is local invasion. For this, further alterations of cellular features are required. First of all, tumour cells need to change their adhesive properties to allow them to lose contact with neighbouring cells and make new contacts with the extracellular matrix and the host cells they meet on their way of invasion. Secondly, the activation of many types of proteases is required to degrade the extracellular matrix and thereby allow the invading cells to penetrate into surrounding tissues. Finally, in order to migrate away from primary tumour site, the tumour cells generally need to acquire some motility properties. These properties of invading tumour cells also help them in further steps of metastatic dissemination, such as intravasation (penetration into lymphatic or blood vessels), transport in the blood or lymphatic stream, extravasation out of the blood stream into the surrounding tissues and formation of new tumours in distant organs. However, some additional cellular features are required for each of these steps. For example, in order to extravasate, tumour cells should be able to bind to the endothelium and subsequently migrate through the endothelial layer. Within the blood stream they need to overcome shear

stress. To make a new tumour in distant organs tumour cells should be able to survive and proliferate under new "soil" conditions (reviewed by Sleeman, 2000).

Cellular properties are determined at the molecular level, for example through the quantity and quality of genes a particular cell expresses. Identification of the molecular mechanisms underlying progression to malignancy will enable us to predict the development of the disease and to identify possible molecular targets for anticancer therapy.

In this chapter I will attempt to summarise recent advances in cancer research that have provided new insights into the molecular mechanisms underlying transition of normal cells into malignant and subsequently into metastasising cells. Recently published reviews as well as specific articles have been instrumental in this approach.

1.2 Current concept of tumorigenesis

Neoplastic transformation is now recognised to occur because of the progressive accumulation of mutations in genomic DNA within clones of cells. This rule lies at the basis of somatic mutation theory of tumorigenesis. One of the earliest experiment supporting this theory was performed with murine NIH3T3 fibroblasts transfected with genomic DNA isolated from either normal or tumour cells. This experiment showed that tumour DNA causes transformation of the transfected cells, while normal DNA does not (Cooper, 1982). The number of alterations reported in cancer cell genomes is growing with the development of new DNA-array based screening techniques. In a study of colorectal carcinoma, for example, Stoler and co-workers found 11,000 genomic alterations per cell (Stoler *et al.*, 1999). Although only a very small fraction of these mutations affects coding regions, it still represents a significant number of genetic changes, and underlies the generation of a variety of cell sub-populations within the tumour leading to tumour heterogeneity. During the process of tumour progression, genomic instability produces clonal heterogeneity and progressive selection for the more malignant and diverse cells whose inherent heterogeneity enhances their malignant potential occurs (Innaccone *et al.*, 1987). Thus, the process of tumour progression can be compared to the process of evolution in which an increased mutagenicity, gene amplification, chromosomal aberration and alterations in gene expression finally lead to genomic instability and clonal

heterogeneity. Selection pressure is thus the force that drives it from the benign to the malignant state (Poste *et al.*, 1982).

Several groups have attempted to define the minimum number of alterations that lead to the transformation of human cells. The results are equivocal. It was shown that normal human epithelial cells and fibroblasts can be transformed with the combination of SV40 T antigen (to inactivate the two tumour suppressor genes p53 and pRb), human telomerase (hTERT) and activated H-Ras oncoprotein (Elenbaas *et al.*, 2001). However, in previous report Morales and co-workers did not succeed in transforming of human primary fibroblasts with a similar combination of hTERT, human papilloma virus E6/E7 oncoproteins (to abrogate p53 and pRB function), and activated Ras, which they consider to be insufficient for transformation (Morales *et al.*, 1999). The discrepancy between these different results is not clear at the moment, but might be explained if the sequence of events is important, or if the levels of expression of the transforming genes are crucial.

1.3 Molecular mechanisms of tumour progression

1.3.1 PROLIFERATION

An indefinite proliferative capacity is one of the hallmarks of cancer cells. The loss of the proliferation control allows the tumour cell to elude the commitment to terminal differentiation and quiescence that normally regulates tissue homeostasis in the organism. In order to achieve this hyperproliferative ability the tumour cell needs to become independent from external growth stimuli. This can be done in different ways. Some tumour cells, like glioblastoma and sarcoma cells produce their own growth factors, platelet derived growth factor (PDGF) and transforming growth factor α (TGFα) respectively, that are normally produced by stromal cells (Fedi *et al.*, 1997). Similarly, melanoma cells produce high levels of fibroblast growth factor2 (FGF-2) and are dependent on this factor for proliferation (Becker *et al.*, 1989)

Other tumour cells have developed alternative mechanisms to escape proliferational control in which they either by over-expression or by mutation of signal transduction molecules can constitutively activate signalling pathways leading to cell proliferation. Examples are the up-regulated expression of growth-factor receptors, such as epidermal growth factor receptor (EGF) or HER-2/neu (Yarden and Ullrich, 1988). Many tyrosine-kinase receptors, such as EGF receptor, are frequently

found mutated or truncated so that they become constitutively active and are independent of ligand binding (Fedi *et al.*, 1997). Moreover, up to 30% of human tumours are found to have activating mutations in *ras*, resulting in persistent signal transduction via the mitogen associated phosphotyrosine kinase (MAPK) pathway, the (phosphoinositide-3-OH) PI-3 kinase pathway and possibly other downstream effector pathways. It should be mentioned that the transforming potential of *ras* depends on the cell type and the level of its expression. For example, in primary cells introduction of an activated form of *ras* or constitutive activation of the Raf/MAP kinase pathway induces cell senescence instead of the expected proliferation (Serrano *et al.*, 1997; Lin *et al.*, 1998; Zhu *et al.*, 1998). On the other hand, in tumour-prone INK4a-deficient mice expression of an activated Harvey-Ras oncogene is sufficient to induce melanoma formation (Chin *et al.*, 1998). Interestingly, the abolition of *ras* expression leads to reversed tumour growth. Similarly to *ras*, induction of c-*myc* expression in the skin or T lymphocytes leads to neoplasia in a reversible fashion (Felsher and Bishop, 1999; Pelengaris *et al.*, 1999). These experiments suggest that the activation of a single oncogene can, in principle, instruct the cells to proliferate and prevent them from differentiation, growth arrest and apoptosis.

Most signal transduction pathways affect directly or indirectly cell cycle regulation, and tumour cells achieve the capability to indefinitely proliferate also by directly deregulating cell cycle control. These events mainly involve functional inactivation of the tumour suppressor genes such as retinoblastoma (pRb) and p53 proteins and components that are affected by their function. In many cancer types pRB was found to be either mutated, deleted or functionally inactivated by interaction with viral oncoproteins (SV40 T antigen, human papilloma virus E7, adenovirus E1A) (reviewed by Knudson, 2000). Alternatively, the functions of molecules that act in the pRb pathway are frequently impaired. For example, the gene for the cyclin dependent kinase (cdk)-inhibitor p16 is often mutated or deleted, while cyclin D1 and Cdk4 are over-expressed (Chin *et al.*, 1998). These changes allow tumour cells to escape growth-arrest signals and survive.

Inactivation of p53 tumour suppressor gene is one of the most frequent mutations found in tumour cells. Inactive under normal conditions, the p53 gene is activated during cellular stress and DNA damage. Such stressed cells are predisposed to mutations and thus have a greater risk of becoming cancerous. One of the first effects of p53 expression, is a block in the cell cycle division. The p53 directly

stimulates the expression of p21, an inhibitor of cyclin-dependent kinases (CDKs) (reviewed by Vogelstein, 2000). Through its negative effects on various CDKs, p21 inhibits both the G1-to-S and the G2-to-mitosis transitions. Other p53 effector proteins including the most recently discovered Reprimo, can also arrest cells in G2 phase (Ohki *et al.*, 2000). The p53 protein therefore provides a critical brake on tumour development by shutting down the proliferation of stressed cells, inhibiting progress through the cell cycle.

A novel pathway for the onset of tumour cell proliferation has been discovered recently by studying the tumour suppressor gene adenomatous polyposis coli (APC). Normally, the protein product of APC tumour suppressor gene binds to cytoplasmic beta-catenin and exposes it to glycogen synthase kinase 3β (GSK3β) which phosphorylates β-catenin and earmarks it for ubiquitination and degradation in the proteasome pathway (Bullions and Levine, 1998; Eastman and Grosschedl, 1999). However, in most colorectal cancers, there is a loss of function mutation of APC gene leading to an accumulation of β-catenin, which complexes with the DNA binding protein T cell factor (TCF) and activates the transcription of the TCF target genes. These genes include *cyclin D1* and c-*myc* thereby connecting APC mutations to deregulation of the cell cycle (He *et al.*, 1998; Shtutman *et al.*, 1999).

1.3.2 APOPTOSIS

The activation of oncogenes such as *ras* and c-*myc* in tumour cells not only induces proliferation but also suppresses apoptosis (programmed cell death). The apoptotic response to unscheduled proliferation constitutes one of the main defence mechanisms to prevent transformation. Besides its ability to inhibit cell proliferation, the tumour suppressor p53 is also able to trigger programmed cell death. In this case, target genes of transcription factor p53 include the apoptosis inducer *bax* and several other genes such as NOXA and P53AIP1 that are known to directly or indirectly induce apoptosis (Oda *et al.*, 2000a,b). It was shown that up-regulation of c-*myc* induces p19/ARF expression, which subsequently binds and activate MDM2, a proto-oncogene that mediates p53 degradation (Sherr, 1998; Zindy *et al.*, 1998). The MDM2-mediated p53 degradation represents another mechanism of p53 inactivation responsible for tumorigenic transformation in cells where p53 mutations have not been detected.

Another major tumour suppressor gene that is mutated in a large fraction of human cancers, PTEN, is known to inhibit the cell cycle by blocking the PI-3 kinase pathway (Ali *et al.*, 1999). PTEN is a dual specificity phosphotase that is able to dephosphorylate both proteins and phosphotidylinositol phosphates, thereby directly counteracting PI-3 kinase activity (Stambolic *et al.*, 1998).

1.3.3 SENESCENCE

Another major cellular process that needs to be defeated by tumour cells is senescence. Normal cells after a certain limited number of cell divisions become senescent, undergoing crisis and as a result cell cycle arrest. An indicator for the cell doublings number is their telomere length, which becomes shorter and shorter with each replication event. Telomerase, the enzyme that is responsible for maintenance of the proper telomere length, is inactive in normal somatic cells but is up-regulated in 80% of cancer cells (Holt and Shay, 1999). Hence, a gain of telomerase activity allows tumour cells to circumvent crisis and senescence (Bodnar *et al.*, 1998).

1.3.4 GENOMIC INSTABILITY

Not all genes that limit tumour development control cell birth (i.e. cell cycle and proliferation) or death (i.e. apoptosis or senescence) directly. Inactivation of DNA-repair genes which are essential for maintaining genomic stability, indirectly leads to tumour development via accumulation of errors in all genes, including tumour suppressor genes and oncogenes. Confirming this notion, inactivating mutations in genes involved in DNA repair, such as MLH1 and MSH2 (Kuesmanen *et al.*, 1997) or Ku80 and XRCC4 (Difilippantonio *et al.*, 2000; Gao *et al.*, 2000) causes genomic instability and leads to an increased incidence of cancer in many tissues. Microsatellites (highly repetitive DNA sequences that are prone to accumulate errors) have been found within the coding regions of genes that are known to play important roles in cellular transformation, including APC, TGF-β receptor II, IGF-2 receptor, TCF-4, *bax* and the DNA-repair genes themselves (Duval *et al.*, 1999; Schwartz *et al.*, 1999). A failure to repair replication errors within this microsatellites invariably leads to a loss of the tumour suppressing functions of these genes.

Another major type of genomic instability frequently observed in tumour cells is chromosomal instability which is characterised by chromosomal rearrangements, including large deletions, fusions and translocations (Lengauer *et al.*, 1998). Evidence

for an involvement of chromosomal instability in cancer comes from studies on DNA repair in response to double-strand DNA breaks. Such breaks usually induce p53 activation via the kinases ATM/ATR or alternatively of the p53 family member p73 via ATM and c-abl, resulting in growth arrest or apoptosis (Dasika *et al.*, 1999). Several molecules involved in this pathway, such as ATM, or proteins that may be directly involved in the repair mechanisms, such as BRCA-1 and –2, are known tumour suppressor genes that are frequently lost in several types of cancer. There are sites in the genome that show high predisposition to DNA breaks (fragile sites). Another tumour suppressor gene, FHIT, is localised within one of these fragile sites, and errors in repairing DNA breaks are likely the cause of deletions observed in the FHIT gene (Sozzi *et al.*, 1998).

In conclusion, it follows that in order to transform, a cell needs to deregulate many cellular processes such as proliferation, cell survival, apoptosis and senescence. All these processes are mostly mediated by mutations in oncogenes and tumour suppressor genes. Our understanding of the molecular mechanisms of each of these steps towards tumorigenesis is dramatically improved during the last decades and led to discovery of the new targets for diagnostic and prognostic applications. It is believed by many tumour biologists that cancer may eventually be treatable by reversing these mutational alterations or by targeting them to eliminate the tumour cell. There is also a reasonable success in the development of anticancer drugs, which can treat some types of cancer. However, cancer cell genome may be too unstable and the number of genetic alterations found in human tumours might be too numerous to serve as the therapeutic targets for anticancer treatment. Thus, other non-genome-centric approaches have been suggested and can be applied in order to circumvent the problem of genomic instability. These include studies on tumour oxygenation, angiogenesis and epigenetic changes occurring during tumorigenesis (Folkman *et al.*, 2000). These issues are currently under intensive investigations but will not be discussed in this thesis because of the space limitations.

1.4 Acquisition of the metastatic phenotype by the primary tumour cell as a multistage process

Metastasis is the most common and serious, life-threatening complication of cancer. At the time of cancer diagnosis, every second patient has already developed metastasis (Kumar *et al.*, 1997).

The acquisition of the metastatic phenotype by the primary tumour cells is a complex multistage process. As shown in **FIGURE 1.1**, several steps are required for the generation of metastasis. These subsequent changes allow the cell to detach from its contacts with neighbouring cells, break through basement membrane, migrate through the matrix into lymphatic vessels (most human epithelial cancers show a preference for lymphatic spread), reach draining lymphatic tissue and grow out there. Tumour cells can also enter the blood stream either directly or through the lymphatic drainage, adhere elsewhere in the body to the vascular endothelium, extravasate and proliferate under new "soil" conditions.

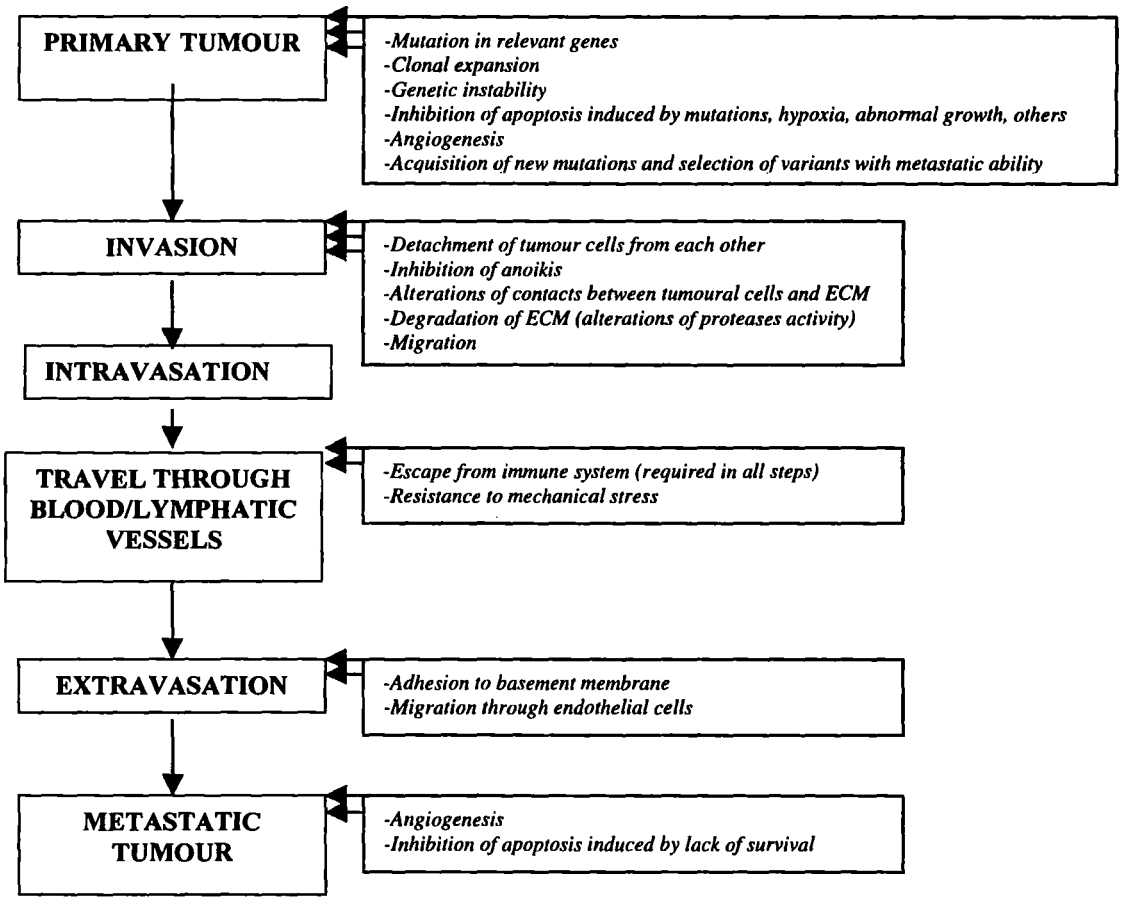


FIGURE 1.1: Consecutive steps in tumour progression towards metastasis.
The basic steps that cells have to complete to give rise to metastasis are boxed. Primary tumours usually contain a heterogeneous mixture of cells, and mutant clones with specific characteristics arise during tumorigenesis. The selection in tumours promotes the overgrowth of only some of the variant clones that have developed a selective advantage. The alterations that cells have to acquire to successfully complete each step are indicated on the right. Taken from Hernandez-Alcoceba *et al.*, (2000).

This sequence of events comprising the metastatic cascade requires the production of different group of molecules for each step. These molecules may be synthesised either by the metastatic cell or by host cells collaborating with metastasising cell. Current knowledge about molecular mechanism of most of the stages mentioned above will be considered here separately and in the sequence they normally occur during metastasis development.

1.4.1 IMPAIRED COMMUNICATION OF TUMOUR CELLS WITH THE NEIGHBOURING CELLS AND EXTRA-CELLULAR MATRIX

The homeostasis of a tissue is maintained by continuous exchange of signals between the cells and the extra-cellular matrix (ECM). Extracellular matrix is a highly complex and specialised, semielastic, continuous structure composed of mainly various collagens, glycoproteins and proteoglycans (Timpl, 1989). The interaction of cells with the ECM is mediated by contacts between cellular surface proteins and components of the ECM.

1.4.1.1 Integrins

The binding of cells to the ECM is mainly provided by transmembrane integrin receptors composed of α and β polypeptide chains. The integrin family comprises about 20 different heterodimeric $\alpha\beta$ combinations that are able to bind to extracellular matrix proteins or cell surface Ig family molecules through short Arg-Gly-Asp (RGD) peptide sequences present in the ligands (Heynes, 1992). Initially found as ECM anchoring proteins, they were later shown to influence dynamic processes in normal and tumour cells such as intracellular signalling and gene expression that lead to cell migration, proliferation, differentiation and survival. Hence, the integrins may play a role in virtually all stages of tumour progression and metastasis and therefore should be discussed here.

The most important role of the integrins in cancer is their participation in signal transduction processes. It has been revealed that ligation of integrins by their ECM ligands leads to the induction of a cascade of intracellular signals that includes tyrosine phosphorylation of focal adhesion kinase (FAK), increase in intracellular calcium levels, inositol lipid synthesis, synthesis of cyclins, and expression of immediate early genes (Guadagno *et al.*, 1993; Juliano *et al*, 1993; Varner *et al.*, 1995b).

A role for integrins in tumour cell proliferation was proposed when it was found that transformed cells exhibit anchorage–independent growth (unlike normal cells, tumour cells do not become quiescent or apoptosise when placed in suspension). It was suggested that anchorage-independence of tumour cells may result from a transformation-associated uncoupling of cell cycle dependence on signals that are transduced by integrin-mediated attachment to the substratum (Guadango *et al.*, 1993). Indeed, inability of tumour cell to attach to the one of the major ECM proteins fibronectin was found to be caused by the loss of the fibronectin receptor α5β1 integrin from the cell surface or its functional inactivation in transformed cells (Plantefaber *et al.*, 1989; Hirst *et al.*, 1986). Overexpression of α5β1 integrin in chinese hamster ovary cells resulted in loss of tumorigenicity and reduced proliferation of transfected cells *in vitro* (Giancotti *et al.*, 1990). Other integrins like α2β1 were also found to negatively regulate cell proliferation of breast epithelial cells (Zutter *et al.*, 1995). In contrast, overexpression of αvβ3 integrin leads to increased proliferation and tumorigenicity of melanoma cells (Felding-Habermann *et al.*, 1992). The elevated levels of α3 and α6 integrins were shown in many metastatic cells (Liebert *et al.*, 1993; Costantini *et al.*, 1990). The molecular mechanisms by which integrins regulate cell proliferation are currently under extensive investigation. Integrin-mediated activation of protein kinase C, MAPK, PI-3-kinase, p21RAS and NF-κB has been demonstrated and may play important roles in integrin-regulated cellular proliferation (Toker and Tankley, 1997; Fashena and Thomas, 2000).

Some integrins such as α5β1 and αvβ3 have been reported to promote cell survival by inhibiting apoptosis. Integrin-mediated cellular attachment to the ECM contributes to the increased survival of human melanoma cells (Montgomery *et al.*, 1994) and prevents apoptosis of Chinese hamster ovary tumour cells overexpressing α5β1 integrin via induction of Bcl-2 expression (Stromblad *et al.*, 1996). Cross-linking of αvβ3 integrin in endothelial cells was shown to suppress p53 activity and to inhibit p21WAF1/CIP1 expression thereby promoting survival (Clarke *et al.*, 1995).

There are three fields of integrin activity in cell invasion and metastasis: cell motility, ECM degradation and angiogenesis. The role of integrins in tumour cell motility will be discussed later in this chapter. Cheresh and co-workers (1996) found the indirect role of integrins in ECM degradation. They demonstrated that αvβ3

integrin can directly bind to the active form of metalloproteinase MMP-2, thereby localising it on the surface of invasive tumour cells and providing migratory cells with co-ordinated matrix degradation, thus facilitating tumour invasion. In agreement with these data, other integrins such as α2β1, α5β1 and α4β1, were reported to induce the expression of metalloproteases (Riikonen *et al.*, 1995; Huhtala *et al.*, 1995).

Probably the most striking role of αvβ3 integrin in cancer is its involvement in the process of angiogenesis. The level of expression of αvβ3 integrin in tumour blood vessel endothelia is found to be significantly higher than that in normal blood vessels (Brooks *et al.*, 1994a, 1994b). Furthermore, a systematic administration of monoclonal antibodies against αvβ3 integrin inhibited angiogenesis in chick CAM assay (Brooks *et al.*, 1994b). It was shown that the abrogation of αvβ3 integrin function by blocking antibodies leads to apoptosis of newly sprouting blood vessels. It appears that αvβ3 exerts its action at a special stage of angiogenesis, namely after induction but before vessel maturation (Varner and Cheresh, 1996).

An important conclusion from the studies reviewed above is that our growing knowledge of integrins has greatly increased our understanding of central biological phenomena, such as anchorage-independence, tumour-induced angiogenesis and tumour metastasis, as well as generating a number of possible approaches to new therapies for cancer.

1.4.1.2 CD44

CD44 is a widely expressed cell surface glycoprotein mostly known for its ability to mediate cell-cell and cell-ECM interactions (Herrlich 1998; Underhill, 1992). A large number of the ECM components such as hyaluronic acid (HA), fibronectin, laminin and collagen, chemotactic cytokines such as osteopontin and serglycin and growth factors have been shown to be important ligands for CD44. The role of CD44 family members has been implicated in a variety of biological contexts such as embryogenesis, haematopoiesis, T cell activation and homing, tumour progression and metastasis (Sherman *et al.*, 1998; Vogt Sionov *et al.*, 1997; Regidor *et al.*, 1996).

The enormous functional mobility of CD44 protein is reflected by its complex structure. The diversity of CD44 isoforms is created by the incorporation of polypeptides encoded by ten alternatively spliced exons that can be inserted into a single extracellular site located close to the transmembrane domain of the shortest

version of CD44 (standard, CD44s), to create CD44 variants (CD44v) (Screaton *et al.*, 1992). Besides alternative splicing, a huge diversity of CD44 isoforms arises from variable post-transcriptional modifications, such as glycosylation and glyconation of the molecule. CD44s is extensively glycosylated at several sites, so that approximately half of its molecular size (about 90 kDa) appears to be due to the N- and O-linked carbohydrates attached to extracellular region of the molecule (Jalkanen *et al.*, 1988; Lokeshwar and Burguignon, 1991).

Positive correlation between CD44 expression and tumour progression has been reported for many types of cancer. Some reports indicate that elevated expression of CD44s in several cancer types such as melanoma (Birch *et al.*, 1991, Guo *et al.*, 1994) and non-Hodgkin's lymphoma (Stauder *et al.*, 1995) leads to endowment of the invasive phenotype by tumour cells. A considerable body of evidence has accumulated implicating role of CD44 splice variants in tumour progression and metastasis (reviewed by Ponta *et al.*, 1994). *De novo* expression of high-molecular-weight splice variants of the CD44 such as v4-v7 isoforms has been shown to promote invasion and metastasis in many types of cancer including cervical carcinomas (Dall *et al.*, 1994; Kainz *et al.*, 1996), pancreatic carcinomas (Hofmann *et al.*, 1991), colorectal carcinomas and adenomatous polyps (Heider *et al.*, 1993, Wielenga *et al.*, 1993). In contrast, in certain tumours such as neuroblastoma, Burrkitt's lymphoma or prostate cancer, down-regulation of CD44 expression seems to correlate with tumorigenic transformation (Gross *et al.*, 2000; Kryworuchko *et al.*, 1999; Verkaik *et al.*, 2000).

Mechanism of action of CD44 proteins has not yet been fully elucidated. However, recent findings obtained by Herrlich *et al.* (2000) and some other groups (Yonemura *et al.* 1998; Bennett *et al.*, 1995) shed light at the molecular functions of CD44 proteins and begot new hypotheses on their function. It has been suggested that CD44 has two opposite modes of function acting either as a tumour-promoting protein or as a tumour-suppressor depending on the biological context.

CD44 has been shown to be capable of binding growth factors and their presentation to their authentic high-affinity receptors, thus promoting cell proliferation (Bennett *et al.*, 1995; Sherrman *et al.*, 1998). Binding of CD44 proteins to growth factors leads to the recruitment of the ERM proteins such as ezrin or moesin to the cytoplasmic domain of CD44 (Yonemura *et al.* 1998). ERM proteins, in turn, are assumed to link membrane proteins to cytoskeleton thereby capable of changing

cellular conformation and promoting cell invasion. This mode of action of CD44 proteins is considered as tumour-promoting one (Herrlich *et al.*, 2000).

CD44 proteins may act completely differently when the cells reach confluent growth conditions. Under these conditions another major ligand of CD44, an ECM component hyaluronic acid (HA) is believed to have its special role. Binding of HA to CD44 leads to the binding of tumour-suppressor protein, merlin, to the CD44 cytoplasmic tail. Subsequent activation of merlin may induce growth arrest thereby inhibiting tumour growth. Thus, according to this hypothesis, the type of action of CD44 proteins is detected by the ligands binding to CD44. This simplified model of CD44 action might be further complicated by the structural differences of each specific CD44 isoform, or by particular signalling pathway activated in a certain cell. Furthermore, post-translational modifications of CD44, mentioned in the beginning of this chapter might play a significant role in CD44 function and still remained to be fully clarified. Nevertheless, current model of CD44 molecular function has a great potential in the development of anticancer therapy. For example, a screen for ligands that lead to the merlin binding to CD44 may identify compounds potent of inhibition of tumour growth.

1.4.1.3 Role of β-catenin/E-cadherin in tumour progression

Cadherins comprise a family of cell surface molecules that generally mediate cell-cell and cell-matrix calcium-dependent homophilic adhesions. Among the different cadherins, E(pithelial)-cadherin is the best characterised in the context of neoplasia and metastasis. E-cadherin is involved in the formation of adherence junctions in epithelia. It is known to be functionally inactivated in virtually all human carcinomas by gene deletion or mutation, promoter silencing and proteolytic cleavage of the protein (Christofori and Semb, 1999). Moreover, germline mutations in the E-cadherins gene predispose patients to diffuse and poorly differentiated gastric cancer (Guilford *et al.*, 1998). It has been demonstrated in a transgenic mouse model for pancreatic β-cell carcinogenesis that the loss of the E-cadherin gene is causally involved in tumorigenic transformation (Perl *et al.*, 1998). One consequence of functional inactivation of E-cadherin in carcinomas is loss of epithelial cell-cell adhesion. This allows cells to move physically from their primary sites and progress from adenomas to invasive carcinomas.

The second consequence of E-cadherin loss in carcinomas is based on their indirect role in cell signalling. It was found that the cytoplasmic domain of functional cadherins requires association with accessory cytoplasmic proteins. These include catenins (α,β,γ), and p120^{cas}, a catenin-related protein whose phosphorylation on tyrosine residues can be induced by a number of classical growth factors, including PDGF, EGF and CSF-1 (Reynolds *et al.*, 1994; Shibamoto *et al.*, 1995). It is at the level of catenins, namely β-catenin which was found to bind specifically E-cadherin, where the two systems, adhesion and signalling, intersect. The cytosolic pool of β-catenin either binds to the cytosolic tail of E-cadherin or heterodimerises with leukocyte enhancer factor (LEF-1), which allows translocation of the complex from the cytosol to the nucleus (Behrens *et al.*, 1996). LEF is an architectural, high mobility group box-containing transcription factor that induces DNA-bending and gene transcription. One of the target genes of this transcriptional complex is the metalloprotease matrilysin (MMP-7) (Crawford *et al.*, 1999). MMP-7, in common with many other proteases, has long been assumed to be associated with the late stages of carcinogenesis, namely in the degradation of ECM proteins and cell invasion. However, recently it has been found that MMP-7 is already expressed in the early stages of human colorectal cancer (Fingleton *et al.*, 1999), linking protease activity with tumour initiation. The role of β-catenin in proliferational control has been already discussed above. Hence, the second consequence of E-cadherin loss is the accumulation of released cytoplasmic β-catenin, which translocates to the nucleus and together with LEF-1/TCF transcription factors induces specific transcriptional programs leading to tumour initiation and progression. Both molecules and their functions are currently under intensive study by both academic and industrial investigators and are likely to yield completely new targets for manipulating cell-cell interactions in the future.

1.4.1.4 Proteases

Tumour growth involves alterations in the stromal extracellular matrix (ECM), and malignant tumours often induce a fibroproliferative response in the adjacent stroma, characterised by increased expression of type I and III procollagens (Kauppila *et al.*, 1996). In order to invade tumour cells must develop sufficient degradative enzymatic capacity to break down the structural barrier represented by the surrounding host tissue.

In general, ECM and basement membranes can be degraded by four classes of proteolytic enzymes: cysteine proteinases, aspartic proteinases, serine proteinases, and metalloproteinases (Birkedal-Hansen *et al.*, 1993). Extensive search for those proteases that are specifically involved in the degradative enzymatic machinery of invasive tumour cells has identified MMPs, plasmin, heparanases, cathepsins, tumour associated trypsinogen (TAT). Here, I shall concentrate on those members of this large family of proteases that have been most extensively studied and are believed to play a crucial role in tumour growth, invasion and metastasis.

Matrix metalloproteinases (MMPs) were found to be overexpressed in most malignant tumours and include the only enzymes known to be capable of degrading fibrillar collagen (Curran *et al.*, 2000). The importance of these molecules in tumour invasion and metastasis is now widely acknowledged, and has led to the search of MMP inhibitors for use as anticancer treatments in the clinical setting.

A member of another class of proteinases, the serine proteinase plasmin, also catalyses degradation of basement membranes and ECM proteins. It is generated from the extracellular zymogen plasminogen. The plasminogen activation enzymatic system appears to be involved not only in migration and invasion of cancer cells, but also in processes such as angiogenesis and desmoplasia when non-cancerous cells such as fibroblasts or epithelial cells need to migrate during metastasis development (Nagy *et al.*, 1989; Hanahan and Folkman, 1996). Because of their great influence on tumour growth, invasion and metastasis this enzymatic system as well as the aforementioned MMPs will be discussed below.

1.4.1.4.1 Matrix metalloproteinases in tumour invasion and metastasis

Matrix Metalloproteases (MMPs) are a family of zinc-dependent neutral endopeptidases, collectively capable of degrading essentially all the components of the ECM matrix. There are currently at least 20 known human MMPs with new members still being discovered (Llano *et al.*, 1999; Kontinnen *et al.*, 1999). A number of studies have demonstrated a positive correlation between MMP expression and metastatic potential of malignant tumours, including colon, lung, breast, prostate, ovarian and gastric carcinomas (Shapiro *et al.*, 1998; Kahari *et al.*, 1999)

All MMP proteins have a characteristic multidomain structure consisting of 1) a signal peptide; 2) a propeptide, which is essential for maintaining the proMMP in a latent form; 3) a catalytic domain containing highly conserved Zn^{2+} binding site; 4) a

proline-rich hinge region that links the catalytic domain with 5) the hemopexin-like domain, which determines the substrate specificity of MMP (reviewed by Johansson *et al.*, 2000). According to their structure and substrate specificity the large family of MMPs is sub-divided into groups. Collagenases degrade fibrillar collagens, gelatinases mostly degrade basement membrane collagen (gelatin) and stromeolysins degrade proteoglycans and glycoproteins. In addition, there are membrane type MMPs (MT-MMPs) and others MMPs.

The regulation of MMP activation occurs at different cellular levels. Expression of most MMPs is induced at the transcriptional level by AP-1 transcription factor complexes (dimers composed of Jun and Fos family members) (Karin *et al.*, 1997). There are also some transcription factors which address particular MMP genes. For example NF-kB activates MMP-9 expression (Bond *et al.*, 1998).

Post-translational regulation of MMP activity includes activation of latent MMPs and inhibition of MMP activities. Most MMPs are secreted as latent precursors (zymogens), which are proteolytically activated in the extracellular space. Latent MMPs are retained in the pro-form by a "cysteine switch" formed by covalent interaction of the conserved cysteine in the propeptide with the catalytic zinc (Wart *et al.*, 1990). The pro-peptide of most MMPs can be cleaved by various compounds, like organomercurials and a number of other extracellular proteinases, e.g. plasmin and other MMPs including MT-MMPs (Kahari *et al*, 1999)

The activity of MMPs in the pericellular space is strictly controlled by non-specific inhibitors, e.g. α2-macroglobulin and by the specific tissue inhibitors metalloproteases (TIMPs). All four members of the TIMP family are able to bind to the zinc-binding catalytic site of the MMPs, thereby preventing their catalytic activity.

A considerable body of evidence has accumulated implicating MMPs in cancer spread. A number of studies demonstrated a positive correlation between MMP expression and the invasive potential of malignant tumours. For example, overexpression of MMP-1 correlates with the poor prognosis in colorectal cancer (Murray *et al.*, 1998a). MMP-2 and MMP-3 are closely related to lymph node metastasis and vascular invasion in squamous cell carcinoma of the oesophagus (Shima *et al.*, 1992). MMP-13 and MMP-11 are associated with a high metastatic capacity in head and neck squamous cell carcinoma (Muller *et al.*, 1993).

It is interesting that in malignant tumours, most MMPs are produced by non-malignant stromal cells rather than tumour cells (Basset *et al.*, 1997). Tumour cells

can also secrete factors such as extracellular MMP inducer (EMMPRIN), which enhances the expression of MMP-1, -2, -3 by fibroblasts (Guo *et al.*, 1997). In addition, many growth factors and cytokines secreted by tumour-infiltrating inflammatory cells as well as by tumour or stromal cells are capable of modulating MMP expression.

Invasion of malignant tumours involves an interplay between tumour cells, stromal cells, and inflammatory cells and it is likely that all these cells express distinct, although somewhat overlapping patterns of MMP (reviewed by Kalahari *et al.*, 1999). For example, in squamous cell carcinoma, invading tumour cells, infiltrating inflammatory cells and stromal fibroblasts express distinct MMPs that may complement each other's substrate specificity and form a network of MMP cascades in which a single MMP cleaves a particular native or partially degraded ECM component and activates other latent MMPs (Parks *et al.*, 1993; Johansson *et al.*, 1999). As the substrate specificity of the MMPs present in the peritumoral environment is different, each MMP can be proposed to play a distinct role at different stages tumour growth and invasion. In this context it is interesting, that MMP-3, MMP-7, MMP-9 and MMP-12 have been shown to generate angiostatin from plasminogen, indicating that their expression may in fact inhibit tumour induced angiogenesis (Dong *et al.*, 1997)

A number of studies have demonstrated the expression of TIMPs in tumour stroma and tumour tissue, however the prognostic significance of their expression is not well established. For example, in breast cancer, TIMP-2 expression correlates with tumour recurrence (Visscher *et al.*, 1994), but in cervical carcinomas, TIMP-2 expression correlates with poor prognosis (Yoshiji *et al.*, 1996). Therefore, it is believed that it is more relevant to measure the MMP/TIMP ratio rather than the levels of MMP or TIMP expression alone. In general, overexpression of TIMPs by cancer cells or by the host reduces the invasive and metastatic capacity of tumour cells (Airola *et al.*, 1998; Sutinen *et al.*, 1998).

Inhibition of cancer cell invasion has also been achieved *in vitro* and *in vivo* by recombinant TIMPs or by overexpression of TIMPs using a variety of gene delivery vehicles. Unfortunately, TIMPs seem to be unsuitable for therapeutic use due to their short half-lives, and therefore a number of synthetic MMP inhibitors (MMPIs) have been designed. Those MMPIs that are in use or in the last stages of clinical trials can be sub-divided into three classes: 1) low molecular weight MMPIs (Batimastatt and

most recently Marimastat) that structurally imitate collagen and are able to chelate the zinc ion of the active centre of MMPs; 2) bryostatin compounds that are able to down-regulate PKC, thereby suppressing the transcriptional activation of AP-1-responsive MMPs; 3) tetracyclins that in addition to their well-established anti-microbial activities also possess MMP inhibitory properties (reviewed by Curran *et al.*, 2000). Since all of the aforementioned MMPIs have drawbacks such as broad specificity and side effects the search for MMPIs suitable for use in anticancer therapies continues. Advances in our understanding of tumour specificity of different MMPs may enable the rational development of selective anti-MMP drugs in the future.

1.4.1.4.2 The plasminogen activation system in invasion and metastasis

An important role for plasminogen activation in tumour invasion and metastasis is now well established. It is well known that plasminogen activators released from cancer cells catalyse the proteolytic conversion of the inactive zymogen plasminogen into the active proteinase plasmin, which in turn catalyses degradation of ECM and basement membrane proteins and thus facilitates cancer cell invasion into the surrounding tissues (reviewed by Werb, 1997).

There are two types of plasminogen activators, the urokinase-type (uPA) and tissue-type (tPA). The former is generally agreed to be the enzyme of most relevance to tumour biology, while the primary role of tPA is generation of plasmin for fibrinolysis in blood vessels. The serine proteinase uPA consists of two disulfide–bridged polypeptide chains generated by cleavage of the zymogen form of uPA, pro-uPA. The activity of pro-uPA is 250-fold less than that of two-chain uPA (Petersen *et al.*, 1988). The uPA receptor (uPAR) is a cell membrane-anchored uPA-binding protein, concentrating plasminogen activation at the cell surfaces. uPAR is known to compete with integrins for the binding of vitronectin (one of the ECM compounds). Activation of pro-uPA bound to uPAR at cell surfaces leads to generation of active uPA which in turn catalyses plasminogen activation. The most obvious consequence of pericellular plasmin generation is degradation of fibrin and other ECM proteins (reviewed by Andreasen et al., 1997). It was also shown that plasmin is able to catalyse activation of latent transforming growth factor-β (TGFβ) and to release basic fibroblast growth factor from its ECM-binding sites (reviewed by Mignatti and Rifkin, 1993). In addition, as was mentioned above, plasmin may contribute to activation of zymogen forms of MMPs. Taking into account all these

functions of plasmin, by logical inference high levels of uPA and uPAR in tumours must positively correlate with their metastatic abilities. Indeed, as it was shown in a variety of malignancies, patients with high tumour uPA levels have a shorter disease-free interval and a shorter overall survival than the patients with a low level of uPA (reviewed by Schmitt *et al.*, 1997). High levels of uPAR are also associated with poor prognosis. Further evidence suggests that the system also has plasmin-independent functions, in which intracellular signal transduction cascades are initiated by binding of uPA to uPAR, leading to tyrosine phosphorylation of Hck, focal adhesion kinase, paxillin and mitogen associated kinases and activation of Stat1 DNA-binding ability (Tang *et al.*, 1998).

There are two main plasminogen activator inhibitors, PAI-1 and PAI-2, which belong to the serpin (serine proteinase inhibitor) family. Both inhibitors are able to interact with the active site of the proteinase, resulting in a stable inactive complex (Gils *et al.*, 1998). PAI-2 was consistently found to inhibit invasion. However the finding that high tumour levels of PAI-1 predict a poor prognosis came as a surprise, and it has been an important motivation for searching for function of PAI-1 other than uPA inhibition. Currently, there is no unifying hypothesis about the role of PAI-1 in metastasis. Some observations suggest that differential expression of PAI-1 by different cell types and in different tissue areas may contribute to cancer cell-directed tissue remodelling by allowing deposition of new ECM (e.g. during angiogenesis) in tissue areas with a high uPA activity. Others support the hypothesis that PAI-1 may co-interact migration and invasion by inhibiting uPA and thereby protecting ECM proteins necessary for cell traction.

The components of the uPA system are potential targets for anti-invasive and anti-metastatic therapy. In this field there are several potential targets for anti-invasion therapies: 1) enzyme activity of uPA; 2) binding of uPA to uPAR; 3) reaction of PAI-1 with uPA; 4) biosynthesis of the components of the uPA system (for a review see Andreasen *et al.*, 2000). Presently, work in all four directions is being carried out and may form the basis for development of specific drugs against invasion and metastasis.

1.4.2 TUMOUR CELL MIGRATION, INVASION AND METASTATIC DISSEMINATION

The acquisition of migratory and invasive properties is a key event in the process of tumour progression towards metastasis. During this process the oncogenic deregulation induces a switch in epithelial cells from a stable adherent phenotype to a motile and invasive one. Normally, epithelial cells establish stable contacts between neighbouring cells and the extracellular matrix, thus maintaining a fully polarised state. Most malignant tumours are epithelial in origin (carcinomas), and transition from a normal to an invasive phenotype requires drastic reprogramming at both the genetic and physiological level, known as the epithelial-to-mesenchymal transition (EMT) (Birchmeier, 1995). The results of this transition are (i) ablation and transient activation of adhesive abilities of the cell; (ii) remodelling of the cellular cytoskeleton; (iii) recognition of chemotactic and haptotactic cues; and (iv) proteolytic processing and secretion of ECM proteins along the trajectory of movement (Schmitz *et al.*, 2000). EMT can occur either during normal embryonic development or in carcinoma cell invasion where the cells invade as cohesive clusters, showing almost the same mode of movement as mesodermal cells during heart formation (Wiens, 1996).

1.4.2.1 Cell movement across two dimensional substrate

The current concept of cell migration is based on the observations made in studies on migration of fibroblasts across planar substrates (Abercrombie *et al.*, 1970; Stossel, 1993; Huttenlocher *et al.*, 1996). In this so-called metazoan or haptokinetic model of cell movement, a single locomotion event can be observed as a continuous dynamic interplay of attachment at the cell front and de-adhesion at the rear cell edge, combined with the cell traction machinery that pulls the whole cell body in the direction of movement.

As shown in **FIGURE 1.2**, cell migration across two-dimensional (2-D) surfaces consists of at least four distinct processes. During cell movement all these steps of migration form a cycle without an obvious starting point. The initiation of migration is dependent on the binding of adhesion molecules (mainly integrins) to the ECM substrate. After ECM attachment, integrin-dependent signalling initiates multiple events resulting in focal contacts and stress fibre formation that culminate in

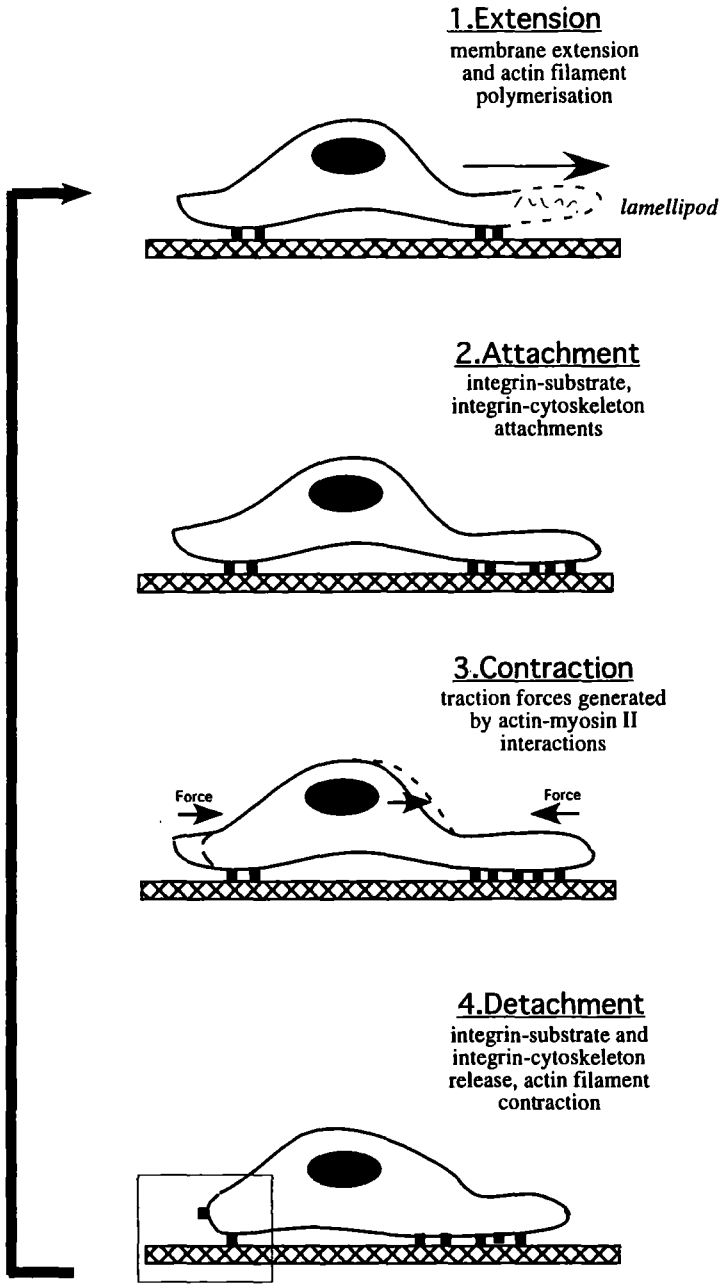


FIGURE 1.2: Schematic view of cell movement across two-dimensional surfaces.

The diagram shows four steps in cell migration: (1) Extension. Assembly of actin filaments follows membrane protrusion and lamellipod formation. (2) Attachment. Integrins which are normally free of cytoskeletal binding proteins, link to actin filaments as a consequence of substrate induced clustering. (3) Contraction. Cells contract inward from the edges towards the nucleus. (4) Detachment. Integrins are released from the cytoskeleton by either mechanical release caused by cell contraction, or by integrin dephosphorylation. Forward displacement of the cell occurs by means of the continuous interplay between these steps.

changes in cell polarity (Lauffenburger *et al.*, 1996). For cell detachment at the trailing edge and translocation of the cell mass, integrin-ligand interactions are thought to be released via integrin dephosphorylation for the recycling of receptor-membrane complexes (Bretcher *et al.*, 1996) and/or deposited at sites of cell detachment (Friedl *et al.*, 1998).

This model of adhesion-dependent cell migration has been established for the migration of fibroblasts in two-dimensional tissue models and was confirmed for tumour cells (Akiyama *et al.*, 1995; Goebeler *et al.*, 1996). However in a 3-D tissue environment, more complex strategies for cell-to-ECM interaction and migration may be present (Noble, 1987; Mandeville *et al.*, 1997).

1.4.2.2 Tumour cell locomotion within three-dimensional extracellular matrix

While haptokinetic migration is predominantly a function of adhesion and de-adhesion events in the absence of spatial barriers for the advancing cell body, the physiology of tissues requires a set of cellular strategies to overcome matrix resistance. Matrix barriers force the cells to adapt their morphology and change shape and/or enzymatically degrade ECM components, either by contact-dependent proteolysis or by protease secretion. In 3-D ECM, in contrast to a 2-D substrate, the cell shape is mostly bipolar and the cytoskeletal organisation is less stringent, frequently lacking discrete focal contacts and stress fibres (Friedl and Brocker, 2000).

Recent data based on cell migration in 3-D tissue models have implicated additional cellular interaction strategies with ECM, resulting in cell positioning and migration. These mechanisms include (1) the interaction with ECM components via low-avidity or even non-adhesive ("biophysical") interactions, (2) the formation of constriction rings and lateral protrusions at interaction sites to push and squeeze the cell body forward independent of focal attachment points, (3) the limited reorganisation of the ECM by proteolysis or mechanical means (Friedl *et al.*, 1998b).

In addition, tumour invasion migration comprises the migration of entire cell clusters or strands that maintain stringent cell-cell adhesion and communication while migrating (Nabeshima *et al.*, 1999). Lastly, cellular interactions, enzyme and cytokine secretion, and tissue remodelling provided by reactive stromal cells (i.e. fibroblasts and macrophages) also contribute to tumour cell migration.

1.4.3 MOLECULAR MECHANISMS OF CELL MOTILITY

The molecular mechanism of cell migration in 3-D environment remains obscure. It was speculated that the locomotion of certain cells, specifically rapidly moving tumour cells, might involve cell contacts provided by diffuse, highly labile contact regions of unknown molecular composition (Lee *et al.*, 1993).

A number of different molecules have been found to play an important role in migration of tumour cells across 2-D-surfaces (i.e. in Boyden chamber-type assays or scattering assays). All of the molecules involved in tumour cells migration can be sub-divided into chemoattractants (such as peptide growth factors and cytokines), cell surface molecules (like integrins), cytoskeletal proteins (like actin and vinculin) and finally signalling molecules (including PI-3 kinase, FAK, Rho GTPases). Due to space limitations only some of them will be discussed.

1.4.3.1 Motogenic factors in cell migration

The motogenic factors that stimulate the motility of tumour cells comprise a large group of tissue and serum proteins, ECM proteins and their degradation products, motility factors and growth factors (Goldberg, 1990).

The role of ECM in the induction and regulation of tumour cell motility can be considered from two different aspects. Firstly, ECM proteins and proteoglycans themselves can function as motility-inducing signals and also as directional guidance for tumour cells. Secondly, ECM, especially proteoglycans, can be a reservoir of motogenic growth factors and cytokines (Zetter and Brightman, 1990). ECM proteins may serve to protect these motility factors from proteases such that upon degradation of ECM, the motogenic factors are released and induce or enhance migration of tumour cells. Motility stimulating growth factors that bind to ECM constituents such as heparin or heparan sulfate include fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α), and hepatocyte growth factor HGF/SF (Ruoslahti and Yamaguchi, 1991; Mizuno *et al.*, 1994).

ECM proteins, including type I and IV collagens, fibronectin, laminin, vitronectin, elastin and thrombospondin, and their degradation products have chemotactic (when soluble) or haptotactic (when insoluble or substratum–bound) effects on tumour cells in *in vitro* assays (Levine *et al.*, 1995; Nabeshima *et al.*, 1997).

Among those ECM proteins listed above, fibronectin has received much attention in the context of tumour invasion. Increased deposition of this ECM protein in various cancer tissues has been reported by several groups (Hauptman *et al.*, 1995; Kaczmarek *et al.*, 1994). It has also been demonstrated that carcinoma cells stimulate fibroblasts to produce more fibronectin via TGF-β and other soluble factors (Inoue *et al.*, 1998). Migrating tumour cells possibly interact with fibronectin via α5β1 integrin, which is up-regulated in many invading cells (Baneres *et al.*, 1998).

Another group of motogens, cytokines, are chemoattractants expressed either by tumour or tumour-infiltrating cells. The cytokine and cell attachment protein osteopontin (OPN) has attracted much interest in recent studies on tumour cell migration. OPN has been shown to support various functions including macrophage chemotaxis, cell attachment and cell migration (Denhardt and Guo, 1993). The localisation of OPN in neoplastic tissues, predominantly at the leading edge of invading tumour cells suggests its possible role in invasion of the metastatic cells (Brown *et al.*, 1994). There is some direct experimental evidence now available linking osteopontin expression and metastasis. It has been shown, for example, that Ras-transformed NIH 3T3 fibroblasts that are able to produce lung metastasis, unlike non-transfected NIH 3T3, expressed elevated levels of OPN. The use of antisense OPN sequences in order to reduce the amount of OPN in these cells led to the reduction of tumorigenicity of Ras-NIH3T3 transfectants as well as their ability to colonise lungs in spontaneous metastasis assay (Gardner *et al.*, 1994). In another experiment, overexpression of OPN in the benign tumour-producing cell line Rama37 resulted in the development of metastases in half of the injected animals (Oates *et al.*, 1997). It is believed that secretion of OPN by tumour cells or tumour-associated macrophages promotes tumour cell migration and metastasis in an autocrine (or paracrine) manner, acting via cell surface receptors such as αvβ3 integrin, which induces a signalling cascade leading to the expression of genes responsible for the migratory phenotype.

1.4.3.2 Integrins in cell motility

Among the adhesion receptors, the integrin family plays a principal role in the migration of many cells, acting to "integrate" ligand interaction through ECM and cytoskeletal signals. Integrins contribute in multiple ways to the process of cell migration. First, integrin affinity for ECM proteins can be modulated by intracellular

signals in a process termed "inside-out" signalling. Second, activated integrins mobilise a diverse complex of molecules at the cell-substratum interface to generate a variety of intracellular signals in a process known as "outside-in" signalling. Third, the bridge that integrins form between the intracellular actin cytoskeleton and the ECM allows cells to exert force on their environment (Horwitz *et al.,* 1999). The transmembrane junctions between ECM and the cytoskeleton observed in many *in vitro* migration experiments have been termed focal adhesion complexes. These complexes consist of small clasters of integrins that have associated with the actin cytoskeleton to provide a structural framework for signalling proteins like PI3-kinase, focal adhesion kinase (FAK), members of the Rho family of small GTPases, RhoA, Rac and Cdc42. Cytoskeletal proteins like talin, vinculin and α-actinin are also found in focal adhesion complexes, and are thought to be essential for their function (Kumar, 1998). However *in vivo* focal adhesions are rarely observed even in vigorously migrating tumour cells (Yamada and Geiger, 1997). The reason for the discrepancy between this and the frequent development of focal adhesions *in vitro* is unknown (Myiamoto *et al.,* 1995a).

A variety of different integrin molecules have been found to mediate migration of both tumour and normal epithelial cells. However, some integrins such as α6β4 are involved in cell migration only in a neoplastic environment. Under normal physiological conditions the α6β4 integrin is a laminin receptor involved in maintaining proper epithelial architecture, but upon cellular transformation, α6β4 signalling is required for chemotaxis and invasion of carcinomas (Shaw *et al.,* 1997; Rabinovitz *et al.*, 1997).

Morphologically large spindle-shaped cells (i.e. fibroblasts, endothelial cells, and many tumour cells) with high integrin expression and strong cytoskeletal contractility utilise integrin-dependent migration strategies that are coupled to the capacity to reorganise ECM. In contrast, a more dynamic amoeboid type of migration is employed by smaller cells expressing low levels of integrins (i.e., T lymphocytes, dendritic cells, some tumour cells). This is characterised by largely integrin-independent interaction strategies and flexible morphological adaptation to pre-formed fibre strands, without structurally changing the matrix architecture.

1.4.3.3 Signalling in metastatic cell invasion

During the process of migration transmembrane proteins transmit the effects of bound ligands through their cytoplasmic tails to a variety of signalling pathways. The complex scenario of molecular interactions during this process is currently under extensive investigation and still needs to be elucidated. Here there will be discussed only some recently discovered signalling pathways in order to give an introduction into the molecular mechanism by which tumour cells acquire their motile and invasive phenotypes.

Many integrins mediate cell adhesion, and subsequent signalling after this adhesion culminates in cell motility. However only a few of them have been shown to activate specific signalling pathways. Adhesion to fibronectin via specific integrins such as $\alpha5\beta1$ has been shown to activate the Rho family GTPases: Cdc42 and Rac (Price *et al.*, 1998). Cross-linking of another integrin receptor $\alpha6\beta4$ causes the additional activation of Rho A (O'Connor *et al.*, 1998). Some pathways downstream of the Rho family of GTPases that influence subsequent cell adhesion and migration have recently been described. For example, Cdc-42 or Rac-activated PAK (p-21-activated kinase) or Rho activated Rho kinase phosphorylate and activate LIM kinase. The activated LIM kinase phosphorylates and inactivates cofilin, an actin filament depolymerising protein, thus promoting formation of stress fibres and focal complexes (Edwards *et al.*, 1999; Ohashi *et al.*, 2000). Another recently described mechanism of actin polymerisation involves activation of WASP (Wiskott-Alsrich syndrome protein) by small Rho GTPases, which subsequently activates the Arp2/3 complex (Moreau *et al.*, 2000). Arp 2/3 complex is frequently found localised to the leading edge of moving cells, seeding or "nucleating" actin filaments. By capping the slow growing "pointed" end of the elongating actin dimer, Arp 2/3 is able to initiate polymerisation of actin filaments which occurs in their fast growing "barbed" ends (Blanchoin *et al.*, 2000). These consecutive "outside-in" signalling pathways finally lead to cell spreading and formation of actin-rich lamellipodia (**Fig. 1.2** (1,2)).

Once the connection between the cell and the substrate is established the cell can effectively use that site as a point of traction for motility. This process is mediated by RhoA-activated Rho kinase, which inactivates myosin phosphatase, allowing myosin light chain (MLC) to remain phosphorylated and trigger force generation via association with actin filaments (Kimura *et al.*, 1996). In addition, many integrins in

collaboration with growth factors signal through the classical Ras/Raf/Mek/MAPK pathway which also leads to a continued phosphorylation state of MLC, stimulating cell migration (Buensuceso *et al.*, 2000).

In the following process of cell retraction (de-adhesion of trailing edge) turnover of focal adhesion complexes is believed to be driven by focal adhesion kinase (FAK). This conclusion came from the observation that fibroblasts from FAK deficient mice contain high numbers of focal adhesions and show reduced motility (Ilic *et al.*, 1995).

It is evident from the studies described above that numerous signalling molecules play an important role in adhesion, contractility and retraction of the migrating cell. Many of the pathways have not been mentioned here and many still are to be discovered. Identification of the whole spectrum of events participating in the materialisation of a fully invasive phenotype will probably allow us to develop efficient drugs against the major cause of death of cancer patients - metastasis.

1.4.4 CANCER INVASION IS A DEREGULATED FORM OF PHYSIOLOGICAL INVASION

It is a long-standing concept that tumour cells can recapitulate patterns of gene expression usually observed in invasive and motile non-transformed cells normal cells (Nicolson 1987; Sher *et al.*, 1988). Experimental evidence that the same genetic program that is used in normal cells for migration can govern metastatic progression has been recently reported by many scientific groups.

In the multicellular organism, there are obvious candidates that could form the blueprints for a part of the metastatic behaviour. For example, lymphocytes are the prime example of migratory behaviour. Tumour cell migration and metastasis share many similarities with leukocytes trafficking, which is critically regulated by chemokines and their receptors, suggesting that tumour cells may use chemokine-mediated mechanisms during the process of metastasis. Supporting these idea, Müller and co-workers recently found that the functionally active chemokine receptors CXCR4 and CCR7 are over-expressed in invading human breast cancer cells (Müller *et al.*, 2001). Furthermore, their respective ligands CXCL12/SDF-1α and CCL21/6 cytokines exhibit peak levels of expression in organs representing the first destinations of breast cancer metastasis. Signalling through CXCR4 or CCR7 mediates actin polymerisation and pseudopodia formation in breast cancer cells, and

induces chemotactic and invasive responses. The same group was able to significantly inhibit lymph node and lung metastasis of breast cancer cells *in vivo* by neutralisation of the interactions between CXCL12 and CXCR4.

CD44 is another molecule expressed in both lymphocytes and invading cells. Specifically, its variant isoforms v3, v6 and v9 have been shown to be up-regulated in T cells upon their activation (Forster-Horvath *et al.*, 2001; Seiter *et al.*, 2000). Expression of these isoforms has also been found to correlate with the metastatic potential of many tumour types (Aaltomaa *et al.*, 2001; Chun *et al.*, 2000). Thus, the shared requirement of CD44 variants for lymphocytes activation and tumour metastasis formation suggests that during tumour progression metastasising tumour cells recruit a lymphocyte specific genetic program.

It is very possible that both neoplastic and physiological invasion of tissues by migrating cells use identical components and mechanisms. For example autocrine motility factor (AMF) has been shown to be secreted by a variety of transformed cells. Binding of AMF to its receptor induces signal transduction, similar to chemotactic stimulation of neutrophil mobility, as well as the internalisation and transport of its receptor to the leading edge of migrating cell, thereby stimulating pseudopodial protrusion and cell motility (reviewed by Liotta *et al.*, 1986; Nabi, 1992). While AMF production and secretion is restricted to tumour cells exclusively, it has also been shown to stimulate motility of untransformed cells such as fibroblasts (Nabi *et al.*, 1990). Thus, AMF could play a paracrine role for normal cells whereas in tumour cells it can stimulate the migration in an autocrine fashion. This suggests that the similar signalling pathways stimulate invasion in both transformed and untransformed cells.

An increasing body of evidence suggests that the invasion of tumour cells results from the deregulation of signals involved in normal physiological responses. Unlike malignant invasion, physiological invasion ceases when the source of the stimulus is removed. For instance, the chemokine osteopontin shown to be important for cell migration is expressed transiently in T cells upon their activation (Patarca *et al.*, 1993). In contrast, in many invading tumours these molecules are expressed constitutively (Oates *et al.*, 1997). It may therefore be assumed that malignant tumour cell invasion represents the unrestrained expression of a pre-existing normal cell program for physiological invasion.

Very interesting results in support of the idea of usage of the same genetic program by both tumour and normal migrating cells came from early experiments with cell fusion. Fusion of certain non-metastatic tumour cell types with normal host cells may give rise to cells with a highly increased metastatic potential (Roos *et al.*, 1985). For instance B cell hybridomas derived from non-metastatic myeloma cells and normal B-lymphocytes upon fusion are able to form metastasis in spleen and liver (De Baetselier *et al.*, 1981; Kerbel *et al.*, 1983). In this experiment as well as in many others the fusion partner was found to be either lymphocyte or macrophage, suggesting that specific properties of these cell types (such as homing to certain tissues or inherent invasive potential), when introduced into a tumour cell, may give rise to metastatic behaviour.

Another example where normal cells need to migrate is during embryogenesis, where many different cell migration events occur. One of the most recently described proteins whose function is implicated in both embryonic neurite outgrowth and metastatic cell invasion is the heparin-binding protein amphoterin, that can act as a nucleating site of the protein-degrading complex plasmin (Parkkinen *et al.*, 1993). Amphoterin together with its receptor RAGE (for receptor for advanced glycation ends products) usually localise at the leading edge of advancing neurites during embryonic development (Hori *et al.*, 1995). The role of amphoterin in invasion of C6 glioma brain tumour cells was recently discovered by Taguchi and co-workers (2000) who showed that neutralisation of RAGE/amphoterin interactions significantly inhibits the growth, motility and local invasion of glioma brain tumour cells.

Cancer invasion can be viewed as tissue remodelling gone out of control. For instance, there are intriguing similarities between the cellular expression pattern of components of protease systems (uPA, uPAR, PAI-1 and MMPs) seen in cancer invasion and in certain types of non-neoplastic tissue remodelling which occurs, for example, during wound healing or angiogenesis (Dano *et al.*, 1999). Many integrins such as $\alpha 2\beta 1$ and $\alpha 3\beta 1$ have been found to play an important role in cell migration and tissue remodelling during wound healing (Decline *et al.*, 2001; Faull *et al.*, 2001). The same integrins are frequently found to be activated in metastatic cells (as discussed above in this chapter).

Summarising this part of the thesis, one can conclude that currently there is a large body of evidence supporting the theory that cancer cells may acquire the mechanism of metastatic activation by recapitulating processes occurring in non-

neoplastic cells capable of migration during normal physiological processes such as inflammation, embryogenesis, angiogenesis and wound healing. Thus, approaches based on the search of tumour related genes among those already found to be up-regulated in embryogenesis or inflammatory cells may lead to the discovery of novel invasion-related genes and thereby new targets for anti-metastatic therapies.

PART TWO

1.5 Immediate early genes in cancer and metastasis

A variety of extracellular signals transduced via specific receptors mediate a large diversity of intracellular signalling pathways that trigger cellular events, such as mitosis, cytoskeletal rearrangement, differentiation, and thereby orchestrate physiological processes, such as development, wound repair, and oncogenesis. The activation of receptors on quiescent cells by proliferation-inducing stimuli rapidly induces the expression of a set of specific genes whose transcription does not require novel protein synthesis, and thus involves latent transcriptional activators already present in cells. These primary response genes are termed immediate early genes (IEGs) (Rollins and Stiles, 1989). The IEG induction has a greater magnitude and longer duration when cycloheximide (an inhibitor of protein expression) is present along with the inducing agent. This so-called superinduction is thought to occur because of the abolition of expression of either IEG repressors or their degradative proteins or both.

To date about 100 IEGs have been identified that are induced in different cell types by a variety of agents like serum, PDGF, TPA, EGF, NGF, IGF TNF, UV and retinoic acid (Cochran *et al.*, 1983; Sukhatme *et al.*, 1987; Irving *et al.*, 1989; Fambrough *et al.*, 1999). The proteins encoded by IEGs include transcriptional modulators, structural proteins, cytokines, and proteins of as yet unknown function (reviewed by Herschman *et al.*, 1991).

Many IEGs were found by differential screening methods as genes rapidly activated in cells treated with growth factors or tumour promoters (Herschman *et al.*, 1978, Cochran *et al.*, 1983). These agents play key roles in embryogenesis, wound healing and tumorigenesis. It therefore seems likely that genes whose products are involved in cell transformation, proliferation or cell movement should be expected to be represented among IEGs. Indeed, one of the most extensively studied IEG encodes the Fos protein which was initially identified as mitogen-inducible gene, up-regulated immediately after stimulation of quiescent 3T3 cells with serum or growth factors leading to re-entry into the cell cycle (Riddle *et al.*, 1979). It is now a well established

that members of Fos and Jun (which is also a growth factor-induced IEG) families composes activator protein-1 (AP-1) transcription factor. The target genes of AP-1 shown to play an important role in invasion of tumour cells and include extracellular proteases (e.g. MMPs and cathepsins), cytoskeletal regulatory proteins (e.g. ezrin, vimentin, Arp 2/3) and cell-to-cell and cell-to-ECM adhesion molecules (e.g. CD44, calpactin and oncomodulin) (Ozanne *et al.*, 2000).

In addition to transcription factors, another group of genes whose products participate in the process of cell movement was found to be IEGs. In their early studies Ryseck and co-workers (1989) identified actin, fibronectin, fibronectin receptor and tropomyosin among their collection of serum-inducible IEGs. The finding that cytoskeletal and ECM proteins are IEGs was unexpected because such genes are normally induced as part of the secondary response, for which protein synthesis is required. Later it was shown that although the induction of their expression does not require *de novo* protein synthesis, mRNA levels for these four genes remain elevated for a substantial period after induction unlike RNA messages for IEG-transcription factors.

Cytokines are another class of immediate early genes that play an important role in tumour invasion and metastasis. Originally isolated as PDGF/serum inducible IEGs *KC* and *JE* genes were later found to encode cytokines (Cochran *et al.*, 1983). *KC* gene codes for MGSA (for melanoma growth stimulating activity) protein secreted by human melanoma cells, but not by normal melanocytes (Richmond *et al.*, 1988). Overexpression of MGSA in melanocytes leads to their transformation. Another immediate-early gene JE encodes monocyte chemotactic factor MCP-1, a CC chemokine that attracts monocytes, basophils and T lymphocytes to the site of inflammation. Besides its major function in recruitment of monocytes to inflammatory sites, MCP-1/JE also facilitates lymphoma lung invasion and metastasis (Wakabayashi *et al.*, 1995).

Similar to MCP-1, another immediate early gene *pip92* was found to be up-regulated in lymphocytes upon their activation (Coleclough *et al.*, 1990). The function of this protein remains to be established. Since the data presented in this thesis implicate Pip92 in tumour progression and metastasis, the literature concerning this IEG is summarised in the next section.

1.6 Immediate early gene Pip92: status at the start of this thesis

Surprisingly, it has been long time since the cDNA clone for the *pip92* (i.e. proline-reach induced protein) gene was isolated, but the functional role of Pip92 still remains obscure. Pip92 is an immediate early gene that was cloned originally by two independent scientific groups. Charles and co-workers (1990) isolated *pip92* full-length cDNA from serum-stimulated BALB/c 3T3 fibroblasts as a gene that is expressed during G0/G1 transition. Simultaneously, the group of Lefkovits isolated the same gene from activated mouse T lymphocytes treated with cycloheximide (referred to there as CHX-1, Coleclough *et al.*, 1990). Later, Pip92 appeared as an IEG in many differential display screenings. It has been reported to be induced during the differentiation of rat hippocampal H19-7 cell line (Chung *et al.*, 1998), upon stimulation of PC12 with vasoactive intestinal peptide and nerve growth factor (Eschelbach *et al.*, 1998) and after PDGF treatment in NIH3T3 cells (Fambrough *et al.*, 1999), etc.

The human homologue of *Pip92*, *ETR101* (for early TPA-responsive) cDNA was cloned from the myeloid leukaemia cell line HL-60, where it is rapidly and transiently induced by stimulation with serum, growth factors and the tumour promoter TPA.

1.6.1 Gene

A comparison between apparent size of *Pip92* and *ETR101* mRNA on agarose gels, which is about 2 kilobases, and the mRNA sequence of these genes, whose length is about 1.5 and 1.8 kb, respectively, revealed that they may lack any introns as there seems to be no space for them within the sequence. Complete absence of introns has been speculated to be necessary for a rapid response to inducers where it could greatly speed up the generation of the functional mRNA from primary transcripts (Coleclough *et al.*, 1990).

1.6.2 Promoter

The *pip92* promoter has been cloned and shown to respond to induction by serum in mouse 3T3 fibroblasts via its SRE (serum response element) (Latinkic *et al.*, 1994). The SRE, found in some other mitogen-responsive immediate early genes (i.e.

egr-1, nur 77), has been studied most extensively in the c-*fos* promoter. The SRE consists of a CArG box that has a consensus sequence $CC(A/T)_6GG$ that binds to the serum response factor (SRF) (Treisman *et al.*, 1987). When SRF is bound to the *c-fos* SRE, it recruits a ternary complex factor (TCF) to an up-stream Ets-like binding site (Dalton *et al.*, 1992; Hipskind *et al.*, 1991).

In the *pip92* promoter, the serum response element consists of at least one Ets protein binding site and a CArG site that binds SRF. In gel shift analyses, Latinkic and co-workers (1994) demonstrated that the *pip92* Ets sites bind to Elk1/TCF, a ternary complex factor that is a member of Ets family of transcription factors (reviewed by Macleod *et al.*, 1992).

In order to be active as a transcription factor Elk-1 needs to be phosphorylated. Three members of the MAPK family, the classical MAPKs (ERK1 and ERK2), JNKs, and p38 are all capable of phosphorylating Elk1 (Gille *et al.*, 1995; Jankneght *et al.*, 1993). In several sequential studies performed by Chung *et al* (1998; 2000a,b; 2001), it has been shown that in the signal transduction pathways leading to *pip92* transcriptional activation, Elk-1 is phosphorylated primarily by p38 and JNK kinases, but not by ERK1 and ERK2. The Src kinase-dependent signalling pathway stimulated by an increase of intracellular calcium levels is also capable of Pip92 activation in H19-7 hippocampal cells (Chung *et al.*, 2001)

1.6.3 TRANSCRIPTION

As for many mRNAs encoding regulatory proteins, *pip92* mRNA has a short half-life of about 10-20 minutes. By analogy with *fos*, rapid degradation of *pip92* mRNA was initially considered to be due to the presence of a degradation signal AUUUA at the 3' untranslated region (Lau *et al.*, 1987). However, sequencing of this region showed that although it is AU rich, it lacks any known specific degradation signals. Like other IEGs, Pip92 mRNA can be stabilised by the protein inhibitor cycloheximide. As for the human ETR101 mRNA, its 3' untranslated region contains GUUUG degradation signal that has also proposed to mark mRNA for degradation (Shimizu *et al.*, 1991).

Tissue distribution analysis of *pip92* mRNA in mouse tissues showed that the *pip92* message is quite ubiquitous and present in many tissues, but it is most abundant in testis, ovary, uterus, lung and intestine (Charles *et al.*, 1990). All the tissues where

pip92 is over-expressed are subject to rapid renewal and therefore are highly proliferative, a fact which may point to a role for Pip92 in proliferation.

Expression of Pip92 has been shown to be induced by a variety of ligands in a wide range of biological contexts. In some cases it is activated upon stimulation of quiescent fibroblasts with serum or growth factors leading to the induction of proliferation of these cells (Charles *et al.*, 1990). In other studies Pip92 was induced in neuronal cell death after Anisomycin or NMDA administration (Chung *et al.*, 2000a,b). In PC12 cells, the Pip92 message appears immediately after NGF treatment leading to their differentiation into neurons (Charles *et al.*, 1990). Such a diversity of reports is not surprising for an IEG. Transcriptional activation of the common IEGs in response to a variety of ligand-induced phenotypic changes has been reported multiple times and is now considered to be a hallmark of immediate early genes.

1.6.4 PROTEIN

Pip92 is a poorly investigated protein and therefore most of the data available on it to date are present in a form of predictions rather than solid experimental results. The Pip92 mRNA encodes a protein of 221 amino acids. The human protein ETR101 is two amino acids longer. Mouse Pip92 and its human homologue ETR101 are highly related to each other (see **Fig. 1.3**). The polypeptide chains of human and mouse proteins are 77% identical and 92% homologous (when conserved amino acids changes are taken into account) to each other. It should be noticed that the similarity between mouse and human protein increases towards the N-terminus, so that the first 52 amino acids are absolutely identical in both proteins. This strongly suggests that this part of the protein may represent a functional domain. Recently, another Pip92/ETR101 family member has been described. It is a 308 amino acid-long slow kinetic immediate early protein called Ier5 (Williams *et al.*, 1999). The amino terminal 49 amino acids of Ier5 are 57% identical and 90% similar to Pip92/ETR101 (see **Fig. 1.4A**). This region was predicted to form two α helices separated by a short intervening sequence (see **Fig. 1.4B**). This prediction, together with conservation of these three protein sequences, suggests that these proteins may participate in similar protein-protein or protein-nucleic acid interactions.

The predicted amino acid sequence for Pip92/ETR101 contains two nuclear localisation signals that are conserved between human and mouse proteins (**Fig. 1.3**). However, despite this prediction, Charles and co-workers (1990) showed that Pip92 is

ETR101*(human)*: MEVQKEAQRIMTLSVWKMYHSRMQRGGLRLHRSLQLSLVMRSAR
Pip92 *(mouse)*: MEVQKEAQRIMTLSVWKMYHSRMQRGGLRLHRSLQLSLVMRSAR

ELYLSAKVEALEPEVS *LPAALPSDPRLHPPREAESTAETATPDGEHPFPEPMDTQEAP
ELYLSAKVEAHQPEFPPSRRAL** DPRLHPPREAEVAVEVASPEAVQP*PEPMDTQEEV

T* AEETSACCAPRPAKVSRKRRSSS*LSDGGDVGLVPSKKARLEEKEEEEGASSEVADRL
LRVQETPALCDPPPA RVSRKRRSSSDLSDSS DAGLVPSKKARLEEVEGE* *ATSEVPDRL
! !!!

QPPPGQAEGAFPNLARVLQRRFSGLLNCSPAA*PPTAPPACEAKPACRPADSMLNVLVR
QLPPAQS EGAFPNLARVLQRRFSSLLNCGPAVPPPT*PPTCEAKPACRPADNMLNVLVR
! ✱

AVVAF
AVVAF

FIGURE 1.3: Sequence alignment of mouse Pip92 and its human homologue ETR101.

Sequence alignment of mouse and human proteins was performed manually. Identical amino acids are shown in red, conserved changes are marked in green. Stars represent gaps. The two conservative nuclear localisation signals are enclosed in boxes. The potential phosphorylation sites for PKC and PKA kinases are marked by ! . The predicted N glycosilation site is marked by an asterisk.

A.

ETR101:MEVQKEAQRIMTLSVWKMYHSRMQRGGLRLHRSLQLSLVMRSARELYLSAKVE
Pip92: MEVQKEAQRIMTLSVWKMYHSRMQRGGLRLHRSLQLSLVMRSARELYLSAKVE
Ier5: MEFKLEAH RIVSI S LG K I YN SRV QRGGIKLHKNLLVSLVLRSARQVYLS

B.

Pip92: MEVQKEAQRIMTLSVWKMYHSRMQRGGLRLHRSLQLSLVMRSARELYLSAKVE
α helices:

FIGURE 1.4: (A) Comparison of the amino termini of Pip92, ETR101 and Ier5.

Vertical bars indicate identity, plus signs indicate conservative substitutions.

(B) Predicted secondary structure of Pip92 amino terminus.

The first line is the amino acid sequence, and the second line shows position of the α helices predicted by use of SIMPA96 computer program (Levin *et al.*, 1996).

localised in the cytoplasmic fraction in 3T3 and PC12 cells by use of nuclear/cytoplasmic fractionation experiments. In the proximity of the nuclearlocalisation signals there are several putative protein kinase A (RXS and RRXS), calmodulin kinase (RXXS), and protein kinase C (RXXS or SXXR) phosphorylation sites (**Fig. 1.3**). However, Charles *et al.*, (1990) in their ^{32}P cell-labelling experiments showed that Pip92 is not phosphorylated upon its expression in activated Balb/c 3T3 cells. A single potential glycosylation site was predicted close to the carboxy-terminus of both proteins (Shimizu *et al.*, 1991).

The presence of multiple PEST sequences (amino acid-stretches rich in proline, glutamate, and serine) considered to earmark short-lived proteins suggests that Pip92/ETR101 protein is also the subject of rapid degradation. Indeed, Charles and co-workers (1990) in pulse-chase experiments estimated an extremely short half-life of about 5-10 minutes for the Pip92 protein. Rapid degradation of Pip92 mRNA and protein is consistent with its potential regulatory role in mediating the cellular response to mitogenic signals.

The molecular weight of Pip92 protein is predicted to be about 24.5 kDa. However, its apparent molecular mass observed on SDS-PAGE is about 33-35 kDa. According to amino acid sequence hydrophobicity analysis, Pip92 is a highly hydrophilic protein and therefore its poor binding to the detergent probably accounts for its slow electrophoretic mobility in the presence of SDS.

As can be seen from the reviewed reports, the Pip92 promoter has been extensively studied during the last ten years. At the same time protein studies have received much less attention. The current knowledge about Pip92 suggests that it may play an important role as a regulator of genes expressed later during mitogenic response or other induction responses. The specific functional role of the Pip92 protein awaits elucidation.

1.7 Project outline and specific aims

Despite the immense importance of tumour cell metastasis in the clinical context, the global molecular events that drives this process remains largely unknown. One of the explanations for this fact is that the metastasis is extremely complex process and the acquisition of the metastatic phenotype by the primary tumour cells requires changes of many cellular properties. This process of acquiring of the new properties by tumour cells is accomplished by alteration in the expression of a multitude of different genes. To derive a comprehensive overview of the global changes in gene expression that accompanies the transformation into a metastatic phenotype, the technique of suppression subtractive hybridisation (SSH) has been used in the Intitute für Genetik (von Stein *et al.*, 1997; Nestl *et al.*, 2001). This 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 (Daigneault *et al.*, 1995; Diatchenko *et al.*, 1996).

By use of this method two genetically-related Bsp73 rat pancreatic adenocarcinoma cell lines which differ significantly in their metastatic potential were compared (von Stein *et al.*, 1997, Nestl *et al.*, 2001). Specifically, transcripts of the highly metastatic Bsp73-ASML cell line were subtracted from those of its non-metastatic counterpart Bsp73-1AS. The Bsp73 rat pancreatic adenocarcinoma tumour progression model is comprised of several clones that have been derived from a common primary tumour and are different in their metastatic potential *in vivo* (Matzku *et al.*, 1983). The 1AS cell line form solid tumours when inoculated subcutaneously, but do not form metastases in distant organs. In contrast, the ASML cell line upon inoculation rapidly start to colonise the local lymph nodes, and spreads through the lymphatic system to finally form numerous metastases in the lung (Knierim *et al.*, 1986, Paweletz *et al.*, 1986).

After the SSH subtraction, a panel of 119 clones was found to be differentially expressed (*i.e.* up-regulated in the metastatic cell line). Sequencing of these differentially expressed clones revealed that 43 of them represent the known genes, many of which have been already described to be associated with metastasis. The

remaining 76 clones are either novel genes or genes that share homology with known ESTs (Nestl *et al.*, 2001).

An intrinsic problem with the SSH approach is the identification of genes whose expression may be functionally unrelated to metastasis. This background is due to the high genomic instability of tumour cells. The principle aim of my work was **to select and characterise those genes from the Bsp73-AS subtracted library that have strongest relevance to metastasis using secondary screening methods.**

One secondary screening approach was to look for genes that are up-regulated in metastasising cells from several tumour models. The cellular properties required for tumour cell dissemination (such as ability to detach from the primary tumour mass and migrate, resist shear stress or attach to the vascular endothelium in order to extravasate) are common for many different tumour types. The acquisition of these common features by different tumour cells is likely to be reflected by altered expression of common genes. Thus, similar patterns of metastasis-related gene expression might expected to be in different tumour types and those genes playing an important functional role in the mechanisms of many different types of tumour should be commonly expressed.

One of the essential features of metastatic cells is their ability to invade surrounding tissues. This property is also essential for many other cells capable of migration in non-pathological conditions. Processes such as embryogenesis, wound healing or inflammation all are accompanied by cell migration. One possibility is that similar molecular mechanisms underlie both the process of metastatic transformation and the activation of the migratory phenotype in normal cells. Therefore, in another secondary screen I set out to look at the expression of the subtracted genes in inflammatory cells (mobilised macrophages and activated lymphocytes) capable of migration under normal physiological situations (Janeway *et al.*, 1996). The aim was to compare the expression of these genes in macrophages and lymphocytes, which had or had not been exposed to inflammatory stimuli and therefore differed in their migratory potential.

Monocytes, which become macrophages upon maturation, are an example of cells that are able to initiate the migration program upon their activation. The migration of peripheral blood monocytes into extravascular tissues to become macrophages involves the same process that invading tumour cells use during

metastasis (*i.e.* adherence to the endothelium, traversal of the endothelial cell layer, and subsequent migration through subendothelial structures). Having arrived in their target organ, monocytes differentiate into macrophages. These so-called resident macrophages or tissue macrophages represent completely differentiated sessile cells, residing in different tissues and no longer exhibiting a migratory phenotype (Male *et al.*, 1996).

An acute inflammatory reaction is characterised by an efflux of monocytes from the circulation into inflammatory exudates. The cells occurring in inflammatory sites represent another type of macrophages, specifically called mobilised or activated macrophages. Mobilised macrophages represent young immature macrophages recruited from the blood stream into the site of inflammation. The principle difference between mobilised and resident macrophages is their migratory ability. Activated macrophages are still very mobile and even able to proliferate, unlike the resident macrophages. They are also called "responsive macrophages" because they readily respond to many inductive agents such as IFNg or LPS (Adams *et al.*, 1994).

T lymphocytes are another example of migrating cells used for the secondary screenings in my work. Both activated and non-activated (virgin) T cells are able to migrate under normal physiological conditions. The main difference between activated and non-activated lymphocytes is their migration pathways. Non-activated resting T cells migrate from thymus and bone marrow across HEV (high endothelial venules) into the secondary lymphoid tissues during the process of homing (Faveeuw *et al.*, 2000; Berg *et al.*, 1989). After activation by an antigen, activated lymphocytes move to sites of inflammation. The latter resembles migration of metastatic cells and it is very possible that the molecular equipment of both metastatic and activated T cells is similar. The activation of T lymphocytes as well as transformation of the tumour cells into invading cells is accompanied by changes in gene expression patterns (Tatham *et al.*, 1983). Identification of common genes up-regulated in these processes was the aim of this secondary screen.

The specific aims of the project were as follows:

1. Isolation and subsequent identification of genes (derived from the panel of metastasis-related subtracted genes from the SSH screen) whose expression strongly correlates with cell migration in activated lymphocytes and macrophages, and also with metastasis in multiple tumour progression models;

2. Full length cloning of selected genes, potentially involved in metastasis as defined by the three previous secondary screening procedures;

3. Functional characterisation of the full-length clones

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 CHEMICALS

All general chemicals were, unless otherwise stated, purchased from *Carl Roth GmbH + Co, Karlsruhe, Merck KgaA, Darmstadt,* or *Sigma Chemie GmbH, Deisenhofen* and were of the highest quality. All radiochemicals were supplied by *Amersham Pharmacia Biotech, Freiburg.*

2.1.2 OLIGONUCLEOTIDES

All the oligonucleotides were made by *MWG Biotech GmbH* and were all of an HPLC-purified grade (see **TABLE 2.1** on the next page for the list of the specific oligos used).

2.1.3 PLASMID CONSTRUCTS

2.1.3.1 rPip92/V5/pCDNA3.1, rPip92/HA/pCDNA3.1

The human and mouse ETR101/Pip92 sequences have been already described (Coleclough *et al.,* 1990; Charles *et al.,* 1990) and are available in public databases. The alignment analysis of these two sequences (see **Fig. 1.3**) showed that the Pip92 gene is highly conserved. Thus, one would expect that the homology between rodent genes (i.e. rat and mouse species) should be even higher than between mouse and human genes. Therefore, the PCR primers complementary to the murine Pip92 coding region were designed (CHX/pT2-5'BH, CHXGFP/Hind). Using these oligos the full-length rat Pip92 (rPip92) cDNA was amplified by PCR from the commercially available rat spleen marathon cDNA (*Clontech*). The PCR High Fidelity kit (*Clontech*) was used for the amplification exactly following the instructions provided by the company. This kit make use of the Pfu DNA Polymerase that works better that the traditionally used Taq Polymerase because of the added advantage of having a proof-reading system which eliminates the presence of possible mistakes during the amplification process. Once the PCR reaction was complete, an additional 10-min

Name of the oligo	Purpose	Sequence	Tm*	Restr. site	Position in mRNA	PCR product size
F: **CHX/pT2-5'BH**	*For cloning the coding region of rPip92*	5'-gc-ggatcc-tatggaagtacagaaagaagcgcagcgcatcatgactgtc-3'**	68°C	BamHI	The first codon***	660 bp
R: **CHXGFP/Hind**	*in frame with V5 tag in pCDNA3.1 vector*	5'-cgc-aagctt-gaaggccaccacagctcgcacc-3'	68°C	HindIII	Penultim. codon	
F: **CHXpGEX-5'**	*For generation of ΔPip92-GST fusion protein:cloning into pGEX-1 vector*	5'-gag-ggatcc-gcgaggtggcttgcg-3'	65°C	BamHI	Codon 25	350 bp
R: **CHXpGEX-3'**		5'-gag-gaattcc-gggccttcttgcttg-3'	65°C	EcoRI	Codon 142	
F: **Nov1-PCRT7s**	*For cloning of ΔNov1 in frame with His-tag PCRT7/NT vector*	5'-gctgcgaccctcggggcagat-3'	68°C	-	Codon 84	432 bp
R: **Nov1-PCRT7as**		5'-tcatgatggcggtttcggatttttc-3'	68°C	-	Stop-codon	
F: **ETR101F.**	*For cloning of the coding region of ETR101 in pCDNA3.1*	5'-gacc-ggatcc-gatggaagtgcagaaagaggcacagc-3'	68°C	BamHI	The first codon	670 bp
R: **ETR101R**		5'-gcg-ggatcc-tcagaaggccaccacggcccg-3'	68°C	BamHI	Stop-codon	
F: **ETRMIDseqF**	*For sequencing of ETR101*	texas red 5'-gaccctcgcctgcacccgc-3'	55°C	-	Bp. 303-322	-
R: **ETRMIDseqR**		texas red 5'-tggaggacgcgggcgaggtt-3'	55°C	-	Bp 619-638	
F: **NOV1START**	*For sequencing of NV1*	texas red 5'-gctcgcaccctcggggcagat-3'	55°C	-	Codon 87	-
R: **NOV1STOP**		texas red 5' tcatgatggcggtttcggatttttc-3'	55°C	-	Stop-codon	

TABLE 2.1: List of oligos

* Tm-melting temperature

** The restriction enzyme recognition site is separated by - -

*** The corresponding codones are underlined in the sequences.

incubation at 72°C in the presence of Taq Polymerase was performed for the generation of the A-overhangs, which were subsequently used for the cloning of the amplified cDNA into the pCDNA3.1/V5/TOPO vector (*Invitrogen*). The PCR primers for this amplification reaction were designed in a way that the amplified product would be in frame with Poly-His, V5 sequences of the vector (i.e. C-terminus of rPip92 was fused to the His-V5 sequence. Another feature of the CHX/pT2-5'BH, CHXGFP/Hind primers is the introduction of the BamH1, HindIII restriction nucleases sites, which flanked the amplified cDNA after the PCR reaction. These sites were used for the cloning of rPip92 sequence into the pCDNA3.1-HA = pT2 vector (a kind gift of Dr. Schweikerght). By latter cloning the rPip92 C-terminus was fused to a hemagglutinin (HA) tag. The authenticity of the insert was verified by restriction enzyme analysis of the recombinant plasmid DNA and the thorough sequencing in both directions.

2.1.3.2 ETR101/pCDNA3.1

The human homologue of the Pip92 (ETR101) was amplified from the genomic DNA of 293 cells using ETR101F and ETR101R primers by Taq Polymerase (Gibco) in the commercial buffer supplemented by glycerol (10%). The amplified full-length fragment was flanked by the restriction endonuclease BamH1 sites introduced by the PCR reaction (as indicates in the **TABLE 2.1**). An amplified ETR101 gene was subjected to BamH1 restriction enzyme digest and subsequently cloned into the BamH1 site of the pCDNA3.1 vector (*Invitrogen*). The restriction digest followed after the cloning and the sequencing proved the correct "sense" direction and faultlessness of the sequence.

2.1.3.3 NV1/pCMV

The NV1/pCMV was isolated from the ASML cDNA library (kindly provided by Dr. König) and contains the full length NV1 protein cloned in the sense direction under the CMV promoter. Two clones (namely number 2 and 4) were used for *in vitro* transcription/translation reaction (see Results) and were intended to use in the further overexpression studies.

1.1.1.4 ΔNV1-His and ΔPip92-GST

The creation of these expression constructs will be described in Methods. The authenticity of the inserts was verified by the restriction digest and sequencing as well as by the correct size of the protein they express (see Results).

1.1.4 ANTIBODIES

Antibodies were purchased from the following companies:
DAKO Diagnostika GmbH: all secondary antibodies (HRP, biotin or FITC/TRITC-conjugated); *Invitrogen:* anti-V5 mouse monoclonal antibodies; the hybridoma supernatant containing the 12CA5 anti-HA antibodies was kindly provided by Dr. Englert.

1.1.5 BACTERIA

E. coli strains used:

1. **DH 5α:** F⁻supE44Δ*lac*U169(φ80*lacZ*ΔM15)*hsdR*17*recA1 endA1 gyrA96 thi*-1 *relA*1

2. **BL21-CodonPlus (DE3)-RIL** F⁻*Omp T hsdS*($r_B^-m_B^-$)*dcm*$^+$ Tet^r galλ (DE3) *endA1* Hte (*argU ileY leuW* Cam^r)

3. **TOP10** F⁻mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Δ*lac*X74(φ80*lacZ*ΔM15) *recA1 endA1 deoR araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *nupG*

1.1.6 CELL LINES AND MEDIA

Cell lines and corresponding media used in the current study are listed in **TABLE 2.2** Tissue culture media for mammalian cells were, unless otherwise stated, purchased from *Life Technologies GmbH, Karlsruhe.* All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (*Life Technologies GmbH*). Trypsin was purchased from *Difco Laboratories, Detroit, USA*, and was diluted to 0.25% in 15 mM sodium citrate, 134 mM potassium chloride prior to use.

Cell Line	Origin	Refferences	Growth Medium
PC3	Human androgen receptor independent highly metastatic prostate carcinoma cells	Nacamoto *et al.*, 1992	RPMI, 10% FCS
LNCaP	Human androgen receptor positive prostate adenocarcinoma cells	Nacamoto *et al.*, 1992	RPMI, 10% FCS; PolyL plates
DU145	Androgen receptor positive, *c-met* expressing human prostate tumour cells	Nacamoto *et al.*, 1992	DMEM, 10% FCS
1AS	Rat Bsp73 pancreatic adenocarcinoma primary tumour cells	Matzku *et al.*, 1993; Nestl *et al.*, 2001	RPMI, 10% FCS
ASML	Rat Bsp73 pancreatic adenocarcinoma cells	Matzku *et al.*, 1993; Nestl *et al.*, 2001	RPMI, 10% FCS
MTPa	Rat 13762 NF mammary adenocarcinoma (parental tumour derived) cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	DMEM, 10% FCS
MTC	Rat 13762 NF mammary adenocarcinoma (parental tumour derived) cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	DMEM, 10% FCS
MTLy	Lymph node metastasis of the 13762 NF rat mammary adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	DMEM, 10% FCS
MTLn2	Lung metastasis of the 13762 NF rat mammary adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	DMEM, 10% FCS
MTLn3	Lung metastasis of the 13762 NF rat mammary adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	DMEM, 10% FCS
AT1 (G1)	anaplastic Dunning R-3327 rat prostate adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	RPMI, 10% FCS
AT3	a variant of the Dunning R-3327 rat prostate adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	RPMI, 10% FCS
MatLyLu	Metastasising variant of the Dunning R-3327 rat prostate adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	RPMI, 10% FCS
MatLu	Metastasising variant of the Dunning R-3327 rat prostate adenocarcinoma cells	Nicolson *et al.*, 1988; Nestl *et al.*, 2001	RPMI, 10% FCS
Cos 7	African monkey Kidney SV40 transformed cells	Barbosa, 1987	DMEM, 10% FCS
SP2/0 Ag-14	Mouse myeloma cells	Moudgil, 1986	RPMI, 10% FCS
NIH3T3	Mouse 3T3 fibroblasts	Tagliaferry, 1987	DMEM, 10% NCS

TABLE 2.2 Cell lines and corresponding media used in the experiments

1.1.7 CELL CULTURE

All cells were maintained at 37°C in an incubator (*Forma Scientific, Labortect 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 trypsinisation and re-seeded at a lower density. Trypsin treatment of cells was performed by removal of the culture medium from the cells, followed by addition of 1.5-2.0 ml of 0.25% trypsin (amount depending on the Petri dish size). The cells were 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 re-plated at the desired density in new Petri dishes.

To prepare cells for storage, logarithmically growing cells were trypsinised and re-suspended in 10ml medium. Cells were then centrifuged at 300 x g. The medium was removed and the cells re-suspended in 1 ml of freezing medium 90% FCS and 10% DMSO (Fluka Chemie AG. Switzerland) and placed in a cryovial. After incubation at –20°C for 1-6 hours, the cells were transferred to –80°C for several days, then finally to liquid nitrogen. To re-propagate cells, the vials were removed from liquid nitrogen and 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, to pellet them before being replated on Petri dishes in fresh medium.

1.2 Methods

A number of protocols and recipes for commonly used buffers used in this project were taken from the laboratory manual of Sambrook et al. (1989) and Current Protocols in Molecular Biology (Ausubel et al 1989) unless otherwise stated.

1.2.1 NUCLEIC ACID METHODS: DNA

1.2.1.1 Isolation/Purification of plasmid DNA from bacteria

1.2.1.1.1 Small scale preparation

A modification of the alkaline lysis method (Birnboim and Doly, 1979) was used as described by Maniatis *et al.* (1982). The principle of the method is as follows. Bacteria are lysed under alkaline conditions. (SDS present in the lysis mix solubilises phospholipid and protein components of the cell membrane while the alkaline conditions promote denaturing the chromosomal and plasmid DNAs as well as proteins in one step.) The lysates are neutralised and adjusted to high salt conditions by adding concentrated potassium acetate solution. (Under such high salt conditions, denatured proteins, chromosomal DNA, cellular debris, and SDS precipitate whereas the plasmid DNA renatures correctly and stays in solution.) 2×YT (2 ml) containing appropriate antibiotic was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking. 1.5 ml of cells were transferred into Eppendorf tubes and harvested by centrifugation at 7000 x *g* for 1 min. The cell pellet was re-suspended in 150 µl of an ice-cold solution of GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) by vigorous vortexing. After incubation of the sample for 5 min. on ice, 200 µl freshly prepared lysis mix (0.2 M NaOH, 1% SDS) was added and mixed until a clear solution resulted. After incubation on ice for 5 min 150 µl ice-cold potassium acetate, pH 4.8 was added. After mixing by slow, gentle inversion of the tube (vigorous treatment during the lysis procedure would shear the bacterial chromosome leaving free chromosomal DNA fragments in the supernatant), the samples were centrifuged for 10 min in a microfuge. The aqueous layer was carefully removed and transferred into a fresh tube. To precipitate DNA, 0.1 volume of 3M sodium acetate and 2 volumes of ice-cold 96% (v/v) ethanol were added. Tubes

were finally centrifuged in a microfuge for 10 min. To remove possible salt contaminations, the DNA pellet was washed once in 70% (v/v) ethanol and re-suspended in 30-50 µl of RNAse containing TE (0.1 M Tris-HCl, 0.25 M EDTA, pH 8.0) and subsequently stored at –20°C.

1.2.1.1.2 Large scale preparation

Usually, a volume of 200 ml of 2xYT medium (1.6% Tryptone, 0.5% Yeast extract, 0.5% NaCl) supplemented with an appropriate 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 × *g* for 10 min. Plasmid DNA was then isolated using Qiagen-tip 500 maxi-prep Kit (*Qiagen Inc.*) using the manufacturer's buffers and according to the supplied protocol with the slight modification that the volume of the first three solutions (I, II, III) was doubled. The purified DNA was precipitated using 0.8 volumes of isopropanol and washed in 70% ethanol before being re-suspended to a final concentration of 1 mg/ml in bi-distilled water and subsequently stored in –20°C.

1.2.1.2 Isolation/Purification of genomic DNA from eukaryotic cells or tissues

In order to isolate DNA, fresh tissue (approximately 1g) was lysed by overnight incubation in 0.6 ml of Lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.2% SDS, 500 µg/ml Proteinase K) at 55°C with shaking. Tissue culture cells were grown in a 6-well plate (*Greiner*) until sub-confluency, washed once in PBS and lysed by incubation with 0.9 ml of Lysis buffer at 37°C overnight. Lysates were then transferred into a fresh vial (*Eppendorf*) and after addition of 1/3 total volume of saturated NaCl (approximately 6-7 M NaCl) the tubes were shaken for another 15 min. The cell debris were removed by mild centrifugation at 5000 rpm for 10 min. The DNA-containing supernatant was transferred into a fresh vial and DNA was precipitated with 2 volumes of ethanol and centrifugation at 14000 rpm for 10 min at room temperature. The DNA pellet was washed with 70% ethanol and re-suspended in TE containing RNAse and stored at –20°C until used. Usually 1µg of genomic DNA was used for a 30-50 µl PCR reaction.

1.2.1.3 DNA fragment separation by agarose gel electrophoresis

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments. Gels of 0.7-1% (w/v) agarose were made in TAE (0.04 M Tris pH 7.2, 0.02 M Sodium acetate, 1 mM EDTA) buffer. To visualise DNA, ethidium bromide was added to gels to a final concentration of 0.5 μg/ml. Gels were electrophoresed horizontally in a 10 cm × 8 cm gel apparatus in a small gel tank at 80 V for 2 hours in 1×TAE buffer. Samples were mixed with loading buffer (0.01 g bromophenol blue, 0.5 M EDTA, 50 % Glycerol) prior to loading onto the gel. The DNA fragments were observed by illumination with UV light and photographs were taken by using an Eagle Eye (*Stratagene*) photocamera system.

1.2.1.4 DNA isolation from agarose gel

Isolation of DNA fragments from agarose gels was performed using the DNAeasy kit (*Biozyme*) according to the supplier's recommendations.

1.2.1.5 Determination of nucleic acid concentration

Nucleic acid concentration in aqueous solutions was determined spectrophotometrically. Readings were taken at a wavelength of 260 nm (deuterium lamp) against a blank containing a re-suspension buffer in a spectrophotometer (Biophotometer, Eppendorff) . The concentration of nucleic acid in a sample was determined on the assumption that an absorbance at 260 nm of 1.0 is equivalent to 50 μg/ml of DNA and 40 μg/ml of RNA. In order not to confuse nucleic acids concentration with that of protein, another measurement was carried out at wavelength of 280 nm. If the ratio A_{260}/A_{280} was 1.8-2.0 the DNA was of a reasonable purity. If there was contamination with protein or phenol, this ratio could be significantly less.

1.2.1.6 Restriction endonuclease digest of DNA

DNAs were digested routinely using restriction endonucleases. The 20 μl digest mixture contained 1 μg plasmid DNA, 1 × appropriate restriction enzyme buffer and 1 unit of a desired restriction enzyme. The reaction was incubated at an appropriate temperature for 1-2 hours. The time and temperature of incubation was dependent on the individual enzyme activity.

1.2.1.7 DNA ligation

The required fragments of DNA were excised from an agarose gel, purified by use of DNAeasy kit and re-suspended in 25 µl of water. Aliquots of DNA solutions were mixed together at 3:1 insert:vector molar ratio. The tubes containing the DNA mix were preheated to 65°C for 10 min and after chilling on ice the appropriate fresh ligation buffer and 1-2 units of T4 DNA ligase were added to a final volume of 20 µl. The sample was then incubated at 15°C either for 12-16 hours (in case of blunt termini ligation) or at 22°C for 2-3 hours (in case of cohesive termini ligation). In the case of the cloning into the TOPO cloning vectors (*Invitrogen*), after purification the PCR fragment was directly cloned via the T/A overhangs according to the supplier's protocol and recommendations.

1.2.1.8 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a number of commercially available Thermal Cycler machines (e.g. Perkin Elmer 9600/2400). Usually the PCR reaction in a total volume 25-100 µl was contained 10 ng of plasmid or 100 ng of genomic template DNA, 250 µM dNTP, 1-2 pmol of primers, 0.25 U to 1 U of Taq Polymerase and 1× supplier's buffer supplemented with 1.5-2 mM $MgCl_2$. Addition of 10% Glycerol into the reaction buffer helped to amplify ETR101 from the genomic DNA. Depending on the application specific cycling parameters were used for each individual PCR reaction. Usually the 25 cycles of 94°C-30 sec, 68°C-30 sec, 72°C-45 sec were carried out. The annealing temperature was a subject of change in case of the failure in obtaining the PCR product. For some PCR reaction the High Fidelity PCR Advantage Kit (*Clontech, Inc*) was used. In this case the reaction was performed according to the supplier's protocol and kit's comments. In case of TOPO (T/A) cloning an additional cycle of 72°C for 10 minutes was performed to ensure the generation of A-overhangs by Taq Polymerase in DNA templates for cloning.

1.2.1.9 Radioactive labelling of DNA/cDNA probes

For the radioactive labelling of nucleic acids the Prime It Kit (*Stratagene*, Inc) was used. Routinely, 60-100 ng of the gel-purified plasmid DNA fragment or 25 ng of freshly synthesised cDNA in a volume of 25 µl was mixed with 10 µl 5× Random Primers nanonucleotide mixture and heat-denatured in a boiling water bath for 5 min.

After rapid chilling on ice 10 µl of 5× dCTP reaction buffer, 5 µl [α-^{32}P]-dCTP (Amersham Buchler GmbH, Braunschweig, 370 MBq/ml, 10 mCi/ml), 1 µl of Klenow Large Fragment of DNA Polymerase (final concentration of 100 U/ml) were added to the reaction tube and incubated for 30 minutes at 37°C. After the labelling was completed the reaction was terminated and the volume of the solution was doubled by addition of 50 µl of TE buffer.

Unincorporated nucleotides were removed from the labelled DNA by use of Sepharose ready columns (Chroma Spin STE-100, *Clontech, Inc.*) according to the manufacturer's guidelines. Finally, the labelled DNA was eluted in a volume of 100 µl and either stored at –20°C for no longer than one week or directly used for hybridisation. The labelled probe was heat-denatured at 100°C for 5 minutes immediately before use.

1.2.1.10 Sequencing of double-stranded template DNA

1.2.1.10.1 Radioactive method

In order to sequence cloned DNA templates, the Thermo Sequenase radio-labelled terminator cycle sequencing kit (*Amersham*) was used which allows for efficient incorporation of four [α-^{33}P]-ddNTP terminators into the DNA products of PCR reaction by use of Thermo Sequenase DNA Polymerase. Sequencing of desired templates was carried out according to the supplier's protocol and kit's comments. Usually 1 µg of highly purified (maxi-prepared or phenol-chloroform purified mini-prepared) plasmid DNA was taken together with 1 pmol of an appropriate primer and 8 U of the Thermo Sequenase polymerase in a total volume 20 µl. From this mixture 4.5 µl was aliquoted into each 4 "termination" vials ("G", "A", "T", "C) each of them containing one of the [α-^{33}P]-ddNTP (*Amersham* Buchler GmbH, Braunschweig, 370 MBq/ml, 10 mCi/ml). The PCR reaction was carried out using following cycling conditions: 95°C, 30 sec; 55°C, 30 sec; 72°C, 60 sec for a total of 35 cycles. Upon termination 3 µl of each PCR reaction products was mixed with the loading buffer (TBE, 30% glycerol, 1% bromophenol-blue) and loaded into separate lanes of a 6% polyacrylamide 6M urea TBE gel. Once the run had run the desired length, the gel was removed, dried on Whatmann 3MM paper at 80°C for 2 hrs on a vacuum gel

dryer and subjected to autoradiography. Usually 18-36 hours of exposure were sufficient to give useable results.

1.2.1.10.2 Non-radioactive method using 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 5' Texas Red labelled primers. All reagents were taken from and used according to the supplier's protocol. The thermocycling conditions for the sequencing reactions were the following: 94°C, 60 sec – 1cycle; then 25 cycles of 94°C, 30 sec; 50°C, 15 sec; 72°C, 30 sec. After the PCR reaction was complete 3 µl of loading dye was added to each reaction vial. The samples were vacuum dried until the desired volume of 3-4 µl had been reached. After the gel had been pre-run for 15-45 minutes, 3 µl of each reaction sample was loaded onto the gel and allowed to run overnight. Sequences were base called from the gel using the provided software.

1.2.1.11 Screening of the ASML cDNA library

In order to isolate full-length cDNA of the NV1 gene, a cDNA library of ASML cells (a kind gift of Dr. H. König) was screened by use of a conventional bacterial library screening method (Sambrook *et al.,* 1989).

For transformation, 0.1 ng of cDNA library was electroporated into *E. coli* and 0.2 ml of cell suspension was carefully distributed onto the top of LB agar square plates (24cm x 24cm, *Nunc*). Bacteria were grown for 12-14 hrs at 37°C and transferred to nitrocellulose filters (*B&B*). The bacteria were lysed by placing the replica filter onto a puddle of 10% SDS for 3 min. The filter was then transferred onto another puddle of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min, followed by a neutralising puddle (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 5 min. Finally the membrane was placed into 2x SSC solution for another 5 minutes. For drying the filters were placed between two layers of 3 MM paper. The resulting 'sandwich' was pounded hard with the hand in order to get rid of colony debris left on the membrane surface. The DNA was cross-linked onto filters by use of a UV Stratalinker, type 2400, *Stratagene*, La Jolla, USA for one cycle. The filters were stored dry at room temperature until hybridisation.

1.2.2 NUCLEIC ACID METHODS: RNA

1.2.2.1 Isolation of total RNA from tissue or cultured cells

Animal tissue materials aimed to be used for RNA isolation were snap-frozen in liquid nitrogen immediately after upon their collection and stored at –80°C until needed. Approximately 200-500 mg of frozen tissue material was placed in a pre-chilled stone pestle containing liquid nitrogen and ground to a fine powder. The resulting powder was placed into a 50 ml Falcon tube to which 5 ml of TRI Reagent (Sigma) was added. The entire contents was then homogenised at 2000 rpm using an ultra-Turrax T25 (*IKA-Labortechnik*) for 5-10 min.

Cultured cells were grown to sub-confluency in 15 cm petri dishes (*Greiner*) and washed once in cold PBS. After removing the washing solution the cells were lysed by addition of 1 ml of the TRI Reagent (*Sigma*). The cell lysate was homogenised by passing it several times through the pipette tip and scraped off by use of a "Rubber policeman". Upon completion of homogenisation the lysates were incubated for 5-10 min at room temperature in order to allow nucleic acids to dissolve into the solution. After addition of chloroform (0.2 ml per 1 ml of TRI reagent used) and vigorous vortexing the samples were allowed to stand at room temperature for another 5-10 min and centrifuged at 13000 rpm for 15 min at 4°C. Centrifugation resulted in phase separation. The top aqueous RNA-containing layer was carefully decanted and transferred to a fresh tube. In order to precipitate the RNA, an equal volume of isopropanol was added followed by centrifugation for 10 min at 13000 rpm at 4°C. The RNA pellet was washed once with 75% ethanol and dissolved in bi-distilled water to a final concentration of approximately 10 mg/ml. The integrity of the isolated RNA was monitored by resolving it over a 1% formaldehyde/MOPS agarose gel: 10-30 μg of each sample was used per lane.

1.2.2.2 Isolation of messenger (Poly (A)+) RNA

Usually three to five 15-cm petri dishes (*Greiner*) of sub-confluent cells were used for one round of mRNA isolation. After a brief wash in cold PBS, the cells were lysed in 20 ml of STE-SDS (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) containing freshly-added 300 mg/ml proteinase K (Sigma, Muenchen). In order to shear high molecular weight DNA the cell lysates were homogenised by use

of an Ultra-Turrax T25 (IKA-Labortechnik) for 5-10 min. The DNA-associated nuclear proteins were then degraded by the action of proteinase K during the incubation of samples at 45°C for 30 min. After addition of 100 mg of pre-swollen oligo dT cellulose (type VII, Pharmacia Biotech or NEB) the samples were incubated at 45°C for another 30 minu. The final concentration of NaCl was adjusted to 0.5 M by addition of 2 ml of 5M NaCl and the resulting mixture was rotated overnight in 50-ml polypropylene tubes (Falcon) to allow the poly A^+ RNA to bind the oligo dT cellulose.

In order to get rid of the unbound material, the oligo dT cellulose was transferred to 15 ml polypropylene tubes (Falcon) and washed three times by resuspension in 10-15 ml of washing solution (100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA and 0.2% SDS) followed by centrifugation at 2000 rpm for 5 min. Finally, in order to remove the traces of any salts the cellulose was washed in 0.4 ml of cold bi-distilled water. After completion of all the washes the RNA was eluted from the oligo dT cellulose by addition of 3.5 ml of ice-cold bi-dest water and centrifuged as above at 4°C. The RNA-containing supernatant was carefully decanted from the precipitated cellulose and centrifuged once more at 4000 rpm at 4°C for 10 min in order to spin down the traces of the cellulose beads.

After transfer of the purified RNA-containing solution into a fresh tube, 0.2 ml of the eluate was withdrawn for the estimation of the RNA concentration and the remainder precipitated by adding 1 µg of yeast t-RNA as a carrier, 300 µl of 3.4 M sodium acetate and 2.5 volumes of ice-cold ethanol followed by centrifugation at 12000 × g at 4°C for 30 min. The RNA pellet was washed in 70% ethanol before being air-dried for 10 min under a fume hood. Finally, RNA was re-suspended in bi-distilled water at a final concentration of 1 mg/ml and stored at –80°C. In order to check the quality of the purified RNA, 3-5 µg of mRNA was used per lane of a formaldehyde/MOPS agarose gel.

1.2.2.3 Separation of RNA by agarose gel electrophoresis

The agarose gel electrophoresis was used for size fractionation of RNA. To prepare the gel, 1 g of Pure Agarose (Peqlab) was re-suspended in 100 ml of MOPS buffer (0.02M 4-morpholinepropanesulfonic acid, 5mM Sodium Acetate, 1mM Na_2EDTA, pH 7.0) and autoclaved. Just before pouring the gel the agarose/MOPS

mixture was melted in a microwave. After brief cooling, 3.5 ml of 37% formaldehyde and 25 µg of Ethidium bromide were added. Gels were electrophoresed horizontally in a 11 cm × 14 cm gel apparatus in a special RNAse free gel tank at 75 V for 2 hrs in 1× MOPS buffer. Prior to loading RNA samples were denatured for 15 minutes at 55°C in a freshly prepared denaturing buffer (1× MOPS, 22.6% formaldehyde, 64.5% formamide). Once denaturation was complete the samples were mixed with loading buffer (0.04 M Tris, pH 7.2, 0.02 M Sodium Acetate, 1mM EDTA, 0.01 g bromophenol-blue) and loaded onto the gel. The integrity of the mRNA was visualised by illumination with UV light and photographs were taken by using an Eagle Eye (Stratagene) photocamera system.

1.2.2.4 Northern blot hybridisation

The RNA samples were size fractionated on a 1% formaldehyde/MOPS agarose gel as described above and visualised under a UV light source. Providing the RNA was of sufficient quality and that no signs of degradation were seen, the RNA was blotted from the gel by capillary transfer to a Hybond-N nylon membrane (Amersham) using 20× SSC buffer. The blot was constructed as described in Maniatis et al. (1982). To ensure efficient transfer of the RNA, blotting was carried out overnight. The dehydrated gel was then discarded and the wet membrane was dried on a sheet of 3MM Whatmann paper at room temperature. Subsequently the blot was transferred to another sheet of Whatmann paper (RNA side up), and the RNA was covalently cross-linked to the active OH groups in the membrane using a UV Stratalinker, type 2400 (*Stratagene*, La Jolla, USA) for one cycle. The blot was stored at –20°C until needed.

1.2.2.5 *In situ* hybridisation

1.2.2.5.1 Probe labelling

The cDNA of the gene to be analysed was cloned between T7 and Sp6 promoters so that the T7 and Sp6 RNA polymerases could be used to generate ^{35}S-labelled "sense" and "antisense" RNA probes during an *in vitro* transcription reaction. Briefly, 4 µl of 5× Transcription Buffer (Gibco, BRL), 2 µl 200mM DTT, 1µl Rnasin, 4µl 2.5 mM rNTP (without UTP), 200 ng of linearised template plasmid DNA, 1 µl

Polymerase, 2 µl DEPC water were mixed together in an RNase-free reaction vial before addition of 5µl of [α-^{35}S]-UTP (Amersham) and incubation for 2 hrs at 37°C. Once the reaction had been complete, the DNA template was removed by incubation with 3 µl DNase I (RNAse free, Roche) in the presence of 3 µl of yeast t-RNA (used as a carrier) for 15 min at 37°C. To get rid of the enzyme and unincorporated material, the phenol/chloroform extraction was performed. For this purpose, the volume of the reaction was made up to 100 µl with DEPC water and 50 µl of phenol and the same volume of chloroform/isoamyl alcohol (24:1) were added. After mixing by vortexing, phase separation was achieved by centrifugation at 13000 rpm for 5 min. The top RNA-containing layer was carefully decanted, transferred to a fresh tube and the RNA was precipitated by the addition of 33 µl of 7.5 M ammonium acetate and three volumes of ice-cold ethanol. After vortexing the tubes were placed in a –80°C freezer for 10 minutes to allow RNA precipitation. Once complete the RNA was pelleted by centrifugation at 13000 rpm for 10 min and the pellet was re-precipitated once more. After removal of the ethanol the RNA pellet was vacuum dried and re-suspended in 50 µl of 0.1 M DTT. From this volume 3 µl was withdrawn for the examination of the quality of the probe and the rest was mixed with 17 µl 7.5 M ammonium acetate and 200 µl ethanol and stored at –80°C until needed.

In order to examine the quality of the probe, 2 µl of the heat-denatured RNA probe were loaded onto a 6% polyacrylamide/8 M urea gel and the appearance of a single RNA band was assured by autoradiography. The successful incorporation of [^{35}S]-UTP into the RNA probe was confirmed by measurement the cpm of 1 µl sample mixed with 3 ml of scintillation solution (SAFE-emulsifier, PACKARD Instruments) by use of a liquid scintillation counter (*Kontron*).

1.2.2.5.2 Preparation of tissue sections

Formaldehyde-fixed and paraffin-embedded material was cut into 6 µm sections. To prevent detachment of tissue during the hybridisation procedure, the tissue sections were mounted directly onto Superfrost Plus slides (*Menzel Glaeser, Breda*) and dried at room temperature overnight.

1.2.2.5.3 Pre-treatment of slides

Prior to hybridisation, the sections were de-waxed by 10 min incubation in fresh xylene. This was done twice. In order to re-hydrate the tissues, the slides were submerged stepwise in 100%, 95%, 90%, 80%, 70%, 50%, 30% ethanol for 2 min each. After 5 min incubation in 0.86% NaCl the slides were washed in PBS for 5 minutes and the RNA in the tissues was post fixed by incubation with freshly prepared 4% paraformaldehyde (PFA)/PBS for 20 min.

Traces of the fixative were removed by two washes with PBS and nucleic acids-associated proteins were digested by incubation with Proteinase K (0.2 mg/ml in PBS) for 7 min at room temperature. After washing in PBS the tissues were fixed again in 4% PFA/PBS for 5 min followed by another PBS wash. In order to reduce unspecific probe binding, the sections were acetylated in 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride for 10 min. The slides were washed in PBS prior to immersion into 0.86% NaCl for 5 min, then dehydrated in a graded series of ethanol solutions (30%-100%). Finally, the slides were air-dried and stored under RNase-free conditions until needed.

1.2.2.5.4 Hybridisation

On the day of hybridisation [^{35}S] labelled RNA probes were removed from –80°C, re-precipitated and re-suspended in Hybridisation buffer (0.02% polyvinylpyrrolidone, 0.02% ficoll, 0.038% NaH_2PO_4, pH 6.8, 5 mM EDTA, 0.3 M NaCl, 0.1 M Tris, pH 8.0, 50% formamide, 10% dextransulfate, 0.1M DTT, 1 mg/ml yeast t-RNA) containing freshly-added 0.01 M DTT. The final re-suspension volume of the RNA probes was calculated according to the cpm (counts per minutes) by use of the formula: V = 47×cpm/150000. Empirically, this volume corresponds to 1-2x10^6 cpm per slide (Sambrook *et al.*, 1989).

The probe was denatured immediately before applying to the tissue slides by heating to 90°C for 3 min. 40 µl of Hybridisation solution was used per slide. In the case where both "antisense" and "sense" probes were applied to the same slide, sections on the slide were encircled with a silicone pen (*DAKO A/S, Glostrup, Denmark*) to prevent mixing of the probes, and only half of the hybridisation solution was used per each section. Silanised coverslips were carefully applied on top of the hybridisation solution to prevent air bubbles and the sections were incubated in a

water bath at 58°C for 16-18 hrs. To prevent evaporation of the hybridisation solution the slides were placed into the plastic box lined with three 3MM Whatmann paper sheets moistened with M-solution (50% Formamide, 2× SSC).

Next morning after hybridisation was complete the unhybridised probe was washed out by incubation of the slides in M-solution containing 20 mM β-mercaptoethanol at 37°C for 15 min. The solution was then changed and the slides were subsequently incubated at 65°C for 30 min followed by transfer to 37°C and incubation for another 2 hrs. The next wash was performed in NTE solution (0.5 M NaCl, 10 mM Tris, pH 8.0, 0.5 mM EDTA) at 37°C for 15 min. To a fresh portion of the NTE solution RNAse was added (20 µg/ml), followed by incubation at 37°C for 15 min. Finally, RNAse was washed out by another incubation in NTE at 37°C for 15 min. The slides were washed once more in M-solution containing 20 mM β-mercaptoethanol at 65°C for 30 min after which all the washes were performed at room temperature. Two following washes (2× SSC – 15 min, 0.1 ×SSC – 15 min) were used to remove the salts. To de-hydrate the tissues, the slides were incubated for 2 minutes in each of the following ethanol dilutions: 30%/0.25 M ammonium acetate; 50%/0.25 M ammonium acetate; 70%/0.25 M ammonium acetate; 80%, 90%, 95%, 100%. Following de-hydration the sections were air-dried and fixed with sellotape in a film cassette and exposed to X-ray film for 16-18 hrs at room temperature.

1.2.2.5.5 Detection

In order to detect the specific hybridisation signal by autoradiography the slides were covered with photoemulsion (LM-1, *Amersham*) by dipping them twice into freshly melted half-diluted in water solution. Once covered the slides were air-dried in the dark for 2-3 hrs and stored light protected at 4°C for 3-30 days of exposure. The time of exposure was roughly estimated by analysis of the overnight exposed X-ray film (see above). To develop the signal, the slides were submerged in developing solution (D19, *Kodak*) for 3 min, washed in 1% Acetic Acid for 1 min followed by submerging into freshly-made 30% sodium thiosulphate solution for another three minutes. Traces of the developing solutions were washed out by tap water and the slides were counter-stained with hematoxylin. After mounting coverslips the signal was visualised microscopically.

1.2.3 BACTERIAL METHODS

1.2.3.1 Preparation of competent bacteria

1.2.3.1.1 Chemically competent E. coli

A single colony of *E. coli* DH5α was taken to inoculate 5 ml of LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) and allowed to grow overnight at 37°C with shaking (220 rpm). 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 min. The bacteria were sedimented by centrifugation without a brake at 3600x g for 7 min at 4°C. The pellet was carefully re-suspended in 20 ml of ice cold 0.1 M $CaCl_2$ and allowed to stand on ice for a further 10-15 min. The cells were centrifuged again under the same conditions and re-suspended 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.

1.2.3.1.2 Electrocompetent *E. coli*

As above, a single colony of *E. coli* DH5α was taken to inoculate 1 ml of YENB (7.5 g/L Bacto yeast extract, 8.0 g/L Bacto Nutrient broth) medium and the culture grown overnight at 37°C with shaking. 500 ml of fresh YENB medium was inoculated with the 1 ml overnight culture which were grown at 37°C with shaking (bacteria were harvested when the medium had an OD_{600} between 0.5 to 0.9). To harvest the bacteria, a flask was chilled on ice and spun at 4000 x *g* for 10 min at 4°C. Medium was discarded and the pellet was washed in 100 ml of cold water twice and centrifuged as previously described. Supernatant was discarded and cells resuspended in 10 ml of cold 10% glycerol and centrifuged and supernatant discarded. Bacteria were re-suspended in a final volume of 2 ml of cold 10% glycerol. The cell number in the suspension should be 1.5-3 x 10^{10} cells/ml. These competent cells can be used fresh or be frozen for future use. To freeze competent cells, cells were aliquoted into reaction tubes (40 µl/tube and placed on dry ice until frozen. Electrocompetent

bacteria were stored at –80°C and thawed on ice before use. These competent cells were good for 1-2 years.

1.2.3.2 Transformation of *E. coli*

1.2.3.2.1 Chemically

Depending on the application, 5 ng of super coiled plasmid 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 min. Following this, the cells were heat-shocked at 42°C for 90 sec before rapidly returning the tube to ice for a few min. After the addition of 1 ml 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 min 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 appropriate antibiotic. The plates were incubated for 18-24 hours at 37°C to allow bacteria to grow.

1.2.3.2.2 Electroporation

1-5 µl of DNA or 1/10 of a ligation were added to a single 40 µl aliquot of competent cells and mixed well then placed on ice for 1 min. (Salt can be removed from ligation mix by phenol/chloroform extraction and then ethanol precipitation). The mixture was transferred to a cold 0.2-cm electroporation cuvette (Bio-Rad) and electroporated in a Bio-Rad Gene Pulser according to the manufacturer's instructions (1.8 kV). The cuvette was removed from the chamber and immediately 1 ml of YENB medium was added to the cuvette. Bacteria were re-suspended, transferred to a polypropylene tube (17 mm x 100 mm) and incubated with or without shaking at 37°C for 1-3 hrs. Cells were plated on selective medium plates.

1.2.4 PROTEIN METHODS

1.2.4.1 Preparation of cell lysates

Cells of 80-90% confluency grown in 3.5 cm petri dish were washed once with PBS before 0.2 ml of lysis buffer (0.125 M Tris, pH 6.8, 2% SDS, 5% Glycerol) was added. The slurry was the homogenated by passing through a pipette tip several times and transferred to a reaction vial (*Eppendorf*). The genomic DNA was then sheared by passing through a 27G needle. After this the samples were either stored at –20°C or used directly. Before loading the samples were mixed with freshly prepared Loading Buffer (50% β-mercaptoethanol, 0.001 g bromophenol blue) to a final concentration of 8% and the proteins were denatured by heating for 3-5 minutes at 95°C. Usually 50-100 µl of lysate were used per lane in SDS-PAGE.

1.2.4.2 Determining protein concentration

Protein concentration was determined using Bio-Rad DC protein assay solutions. For this purpose 3-5 dilutions of protein standard (purified bovine serum albumin, fraction V) containing 0.2 mg/ml to 1.5 mg/ml protein were prepared. Each time the assay was performed a standard curve was plotted. To 20 µl of standards and protein samples 100 µl Bio-Rad reagent A or A' was added. After solutions had been mixed completely 0.8 ml Bio-Rad reagent B was added to each tube. After 15 min of incubation absorbencies were read at 750 nm.

1.2.4.3 Separation of nuclear/cytoplasmic fraction

In order to perform nuclear/cytoplasmic fractionation, cells were grown on 10 cm Petri dishes (*Greiner*) until sub-confluency and harvested by scraping with a Rubber Policeman in 1ml PBS. After brief centrifugation for 15 sec. the supernatant was removed and the cells were gently resuspended in Hypotonic Homogenisation buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and protease inhibitors) and incubated on ice for 15 min. Once lysis was complete, 25 µl of 10% NP-40 solution was added and the tube was vortexed vigorously for 10 sec. The cell debris along with intact nuclei were centrifuged at 13000 rpm for 1 min. The supernatant containing cytoplasmic proteins was decanted and stored at –20°C until used.

In order to extract nuclear soluble proteins, 60 µl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 25%glycerol, 0.4M NaCl, 1mM EDTA supplemented with 2.5 mM DTT, 5 µg/ml Leupeptin and 1.2mM PMSF immediately before use) was added to the pellet and the samples were placed on an Eppendorf-shaker for 30 min at 4°C. The cell debris were then removed by centrifugation at 13000 rpm for 5 min at 4°C and 50 µl of supernatant consisting of an extract of nuclear soluble proteins was carefully decanted, transferred in a fresh tube and stored at –80°C until used.

1.2.4.4 Separation of proteins by SDS polyacrylamide gel electrophoresis

SDS-Polyacrylamide protein gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (1970). The composition of 12% and 15% SDS-PAGE is described in the table below.

Components	14 cm × 14 cm gel	
	15%	12%
Acrylamide/*bis*-acrylamide (30:0.8, Carl Roth GmbH & Co, Karlsruhe)	10 ml	8 ml
3 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml
Distilled H_2O	7.5 ml	9.5 ml
10% (w/v) SDS	200 µl	200 µl
10% APS	250 µl	250 µl
TEMED	20 µl	20 µl

TABLE 2.3 Resolving gel composition.

Usually 12% or 15% polyacrylamide gels were used. In order to cast a gel, two clean glass plates, separated by spacers, were clamped together and inserted into a groove filled with 0.1% (w/v) agarose. After the agarose layer had set, the mould for polyacrylamide gel was ready. The resolving portion of the gel was prepared as shown in **TABLE 2.3** and poured into the gel mould to a level 1 cm below the well former. Then, to ensure production of a straight boundary between the resolving gel and the stacking one, the former was overlaid with 50% (v/v) butan-2-ol. After polymerisation butan-2-ol was washed off with deionised water and the gel was subsequently blotted dry. The stacking portion of the gel was prepared as follows: 1.7 ml acrylamide/ bis-acrylamide, 2.5 ml 0.5 M Tris-HCl, pH 6.8, 0.1 ml 10% SDS and 5.65 ml bi-distilled water were mixed together and the reaction was initiated by the addition of 0.1 ml 10% APS and 7.5 µl TEMED. The stacking gel was poured into the gel mould above the resolving one. The well former was then inserted and the gel was left to polymerise. After polymerisation the well former was withdrawn and gel was fixed within the gel tank. Running buffer (25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS) was used to wash off any unpolymerised acrylamide.

1.2.4.5 Coomassie Blue staining of resolved proteins

Immediately after electrophoresis gels were placed into a staining solution of 45% (v/v) methanol, 10% (v/v) glacial acetic acid containing 0.5 g/l Coomassie Blue R-250 and gently shaken at room temperature for an hour followed by destaining in a solution of 20% (v/v) methanol, 10% (v/v) glacial acetic acid until the background was clear.

1.2.4.6 Immunoblot analysis of proteins (Western blotting)

After electrophoresis, the gel was soaked in transfer buffer (25 mM Tris-HCl pH 8.3, 250 mM glycine, 20% methanol) for 10-15 min. In the meantime, a PVDF membrane (Millipore) was rinsed in 100% methanol and stored in transfer buffer. The gel was placed between a sheet of PVDF membrane and two sheets of Whatmann filter paper and assembled into a Trans/Blot Cell transfer chamber (Bio-Rad). The transfer of the protein from the gel onto the membrane was carried out for 4-16 hrs at 350 mA in transfer buffer at 4°C. To visualise the proteins and check the quality of

the transfer the PVDF membrane was washed in deionised water for 5 min. and stained with Ponceau S (*Sigma,* 0.1% in acetic acid). The membrane was destained at room temperature with deionised water for 5 min. After final rinsing the membrane was either air-dried and stored at –20°C or used directly for antibody detection.

To reduce non-specific binding of antibodies to the membrane, the membrane was incubated for 30 min in Blocking buffer (20% low-fat milk in TBS with 0.5% Tween 20) prior to detection. Once complete, the membrane was probed with the appropriate primary antibodies diluted in Blocking buffer for 1-4 hrs at room temperature. After extensive washing in TBS/0.5% Tween 20 the membrane was incubated with the appropriate peroxidase-coupled secondary antibodies diluted in Blocking buffer for 30 min at room temperature. The membrane was then washed three times in TBS/0.5% Tween 20 prior to detection of specific binding by enhanced chemifluorescence using Amersham ECL western blotting detection reagents following the manufacturer's recommended guidelines.

1.2.4.7 Coupled transcription/translation in vitro

The TNT (*Promega*) coupled rabbit reticulocyte lysate system was used to determine the authenticity of the cloned full length of NV-1. All reagents used were supplied in the kit and the assay was performed according to the accompanying outlines provided. Briefly, 1 µg of highly purified plasmid DNA was added to a reaction vial containing 25 µl of reticulocyte lysate, 2 µl reaction buffer, 1µl of amino acid mix (without methionine), 1µl RNAsin (40 U/µl), 4 µl of [^{35}S]-methionine, 1µl T7 or Sp6 RNA Polymerase (10 U/µl) in a total volume of 50 µl. The reaction was incubated at 30°C for 1h before the product was heat denatured and loaded on a 12 % SDS-PAGE. Once the run was complete, the gel was dried and exposed to autoradiography.

1.2.4.8 Enzyme-linked-immunoabsorbent assay (ELISA)

ELISA assays were used for the detection of antibody-producing cells as well as for the examination of the presence of antibodies in the serum of immunised animals. First, 96-well polycarbonate ELISA plates (*Falcon*) were coated with the antigen. For this aim, 50 µl of antigen solution containing 2-10 µg of purified antigen per ml of 100mM NaHCO3 was added into each single well of the 96 well plate followed by

incubation at room temperature for 2 hrs or at 4°C overnight. The antigen-coated plates were then washed three times in PBS by submerging the whole plate in PBS. After the last wash the unspecific background was blocked by the addition of Blocking solution (PBS, 10% FCS) and incubation at room temperature for 30 min. After draining off of the Blocking solution and another wash in PBS the plates were dried and stored at –80°C until needed. 50 µl of antibody-containing supernatant or serum diluted in the blocking solution (different dilutions were used starting from 1:50) were added to the freshly thawed antigen coated plates and incubated for 1 hr at room temperature. After draining off the unbound material the plates were washed three times in PBS as described above. The appropriate secondary antibody coupled to horseradish peroxidase (*DACO*) were applied (usually, the same volume as the antigen in a dilution 1:500 in Blocking solution) followed by incubation for 30 min at room temperature. The bound antibodies were then detected chromatographically using ABTS [2,2'azino-di-(3-ethyl-benzthiazolin-sulphonate), *Roche*] substrate as the chromogen. Once the colour development was completed (usually after 5-10 minutes) the reaction was terminated by addition of 1% SDS solution and the plates were analysed either visually or by use of spectrophotometer.

1.2.5 ANTIBODY PRODUCTION AND PURIFICATION

1.1.1.1 Purification of the ΔPip92-GST-fusion protein

In order to generate a Pip92 glutathione-S-transferase (GST) fusion protein, a fragment of the Pip92 cDNA (encoding aa 25 to 142) was cloned downstream of and in frame with the GST gene in the pGEX-1 expression vector. Once sequenced and proven to be correct, the construct was transformed into the expression *E.coli* host strain BL-21 (*Stratagen*). A single colony of bacteria was grown overnight in 50 ml LB (10 g Trypton, 5 g Yeast extract, 10 g NaCl/1L and 1 pellet of NaOH) medium supplemented with 100 µg/ml ampicillin (to select for plasmid containing bacteria) and 34 µg/ml chloramphenicol (to select for BL21). Next morning the culture was diluted 1:10 with fresh LB medium and grown for an additional 2 hrs until the bacterial growth reached stationary phase. Expression of the GST-fused protein was induced by addition of IPTG to a final concentration of 0.1 mM and the culture was grown for another 4 hrs at 37°C. The bacterial pellet was resuspended in Lysis Buffer

(0.05 M HEPES, pH 7.9, 0.015 M KCl, 0.005 M MgCl, 0.01 M EDTA; 1 ml/100 ml culture) to which 1 ml of 1 mM DTT, 1 mg/ml of lysozyme and protease inhibitor (Complete Mini, *Roche*) were added immediately before resuspension. To improve the breakage of the bacteria cell walls, cell lysates were subjected to three freeze-thaw cycles: 5 min in liquid nitrogen; 10 min at 37°C.

Once complete, the lysates were sonified (Branson cell disruptor B15, output 6) to reduce viscosity and the cell debris were removed after centrifugation at 11000 rpm for 30 min. The supernatant containing soluble proteins was incubated with glutathione-agarose (*Sigma*) for 1 hr at 4°C with rotation. In order to remove unbound material, agarose was washed 3 times in cold PBS and the specific GST fusion protein was eluted in 1 ml aliquots using 50 mM $NaHCO_3$ pH 10.8 in 10 fractions. The pH was neutralised by addition of 87 µl of 1 M Sodium Citrate pH 5.0 to each fraction. The amount and purity of the isolated protein was estimated by PAGE and subsequent Coomassie staining. Usually 10 µl of each fraction were loaded per lane of the mini-gel along with different amounts of protein standards.

1.1.1.2 Purification of His-tagged NV-1 protein (NV1-His)

To create an expression construct for NV-1, the sequence encoding the carboxy-terminus of the NV-1 protein (aa 60-229) was cloned in frame with the polyhistidine tag in PCRT7/NT vector (*Invitrogen*). NV1-His protein was purified under denaturing conditions using Ni-NTA resin (*Qiagen*) as follows. BL21 cells transformed with the expression construct were grown in 10 ml LB medium supplemented with 100 µg/ml ampicillin and 34 µg/ml of chloramphenicol overnight. Next morning the culture was 20 times diluted with fresh medium and grown at 37°C with vigorous shaking for another 30-60 min until an OD_{600} of 0.6 was reached. The expression of the NV1-His protein was induced by the addition of IPTG to a final concentration of 1 mM after which the culture was incubated for an additional 4-5 hrs and the bacteria were harvested by centrifugation at 4000 × *g* for 20 min. The cell pellet was stored overnight at –20°C. Next morning the cell pellet was thawed by incubation on ice for 15 min and the bacteria were lysed by re-suspension in 4 ml lysis buffer (100 mM NaH_2PO_4, 8 M Urea, 10 mM Tris, pH 8.0). After addition of 1ml of the 50% Ni-NTA slurry to the lysate the His-tagged protein was allowed to bind the resin by rotation for 1hr at room temperature. Once the binding was complete the resin was washed twice

in Washing buffer (100 mM NaH_2PO_4, 8 M Urea, 10 mM, Tris pH 6.3). NV1-His protein was eluted off the Ni-NTA resin with elution buffer (100 mM NaH_2PO_4, 8 M Urea, 10 mM Tris, pH 4.5). Finally, the fractions containing the most of the protein were pooled together and dialysed against PBS at 4°C overnight. After dialysis the most of the protein precipitated (about 5 mg) but some of it remained in solution (3 mg). The soluble protein was used for immunisation.

1.1.1.3 Purification of polyclonal antibodies from the whole serum

1.1.1.3.1 GST-column preparation and removal of anti-GST antibodies

In order to purify serum containing polyclonal antibodies generated against GST-fusion proteins, GST affinity columns were prepared. Usually 300 mg of CNBr-Sepharose (*Pharmacia*) was resuspended in 200 ml of bi-dest water containing 19 µl HCl. The gel was allowed to hydrate for 15-30 minutes after which the slurry was poured into a plastic column (*Econopack, Biorad*) and the excess of the HCl solution was drained off. 3mg of GST protein in 5 ml Binding Buffer (100 mM $NaHCO_3$, pH 8.6, 0.5 M NaCl) was bound to the column by rotation for 2 hrs at room temperature. Once complete 1/10 volume of 1 M ammonium chloride was added, followed by another hour of incubation. The Binding Buffer was then drained off and the gel was washed with 10 ml 100 mM sodium acetate, pH 4.0 followed by 10 ml Tris, pH 8.0. These washes were repeated twice and finally 10 ml of RIPA buffer followed by 10 ml PBS was used in order to get rid of any unbound material. After all washes the GST columns were either used directly or stored at 4°C in PBS.

To remove anti-GST antibodies, 5 ml of each serum were adjusted to B-buffer (1× TBS, 10mM EDTA, 0.1% NP-40) and all the insoluble serum proteins were removed by centrifugation at 10000 rpm for 10 min at room temperature. The supernatant was bound to 2.5 ml of GST-agarose by rotation for 2 hrs at room temperature. The flow-through consisting of purified serum was collected and stored at 4°C until used.

1.1.1.3.2 Affinity purification

For further purification of the Pip92 polyclonal antibodies and their separation from other serum proteins, affinity purification was performed. The serum previously purified against a GST column was applied to a Pip92-GST column prepared as

described above. After 2 hrs rotation at room temperature the flow-through was removed and the column was washed with 20 column volumes of Washing Buffer (0.1 M $NaHCO_3$, pH 8.0, 0.5 M NaCl) followed by 10 column volumes 10mM sodium phosphate pH 6,8. Purified anti-Pip92 antibodies were eluted with 100mM Glycine, pH 2,5 in 1 ml aliquots. In order to neutralise the elution solution each fraction was mixed with 100 µl of 1 M sodium phosphate, pH 8,0. Finally fractions containing significant amounts of antibodies (OD_{280}>0.1) were pooled together and dialysed against PBS at 4°C overnight. After addition of 0.01% sodium azide purified antibodies were stored at 4°C.

1.1.1.4 Monoclonal antibody production

1.1.1.4.1 Animal immunisation

Production of monoclonal antibodies was carried out essentially as described by J.E. Celis (Cell Biology: Laboratory handbook, 1998). Three Balb/C mice were immunised in parallel. The first injection consisting of 25 µg antigen suspended in 0.2 ml PBS mixed with an equal volume of complete Freund's adjuvant was given 17 days before the fusion was scheduled. About 50 µl of this mixture was injected subcutaneously in both hind legs of each Balb/C mice. Three days later the first booster injection was performed using antigen emulsified in incomplete Freund's adjuvant. For the subsequent booster injections on days 10, 7, 3 and 1, the antigen was dissolved in PBS only.

1.1.1.4.2 Cell fusion

The day after the last booster injection the mice were sacrificed by cervical dislocation. The popliteal lymph nodes were dissected out of the knee hollows under the sterile hood using sterile techniques. After removing all the muscle and connective tissues and washing in RPMI medium (*Gibco*), the lymph node capsule was disrupted by use of a sterile sieve and a 5ml-syringe plunger. The released lymphocytes were collected in 10 ml of RPMI medium. After centrifugation and re-suspension in another 10 ml of RPMI the cell debris were allowed to settle down for 2-3 min and the cell-containing supernatant was transferred into a fresh tube.

Exponentially growing fusion partner cells (mouse myeloma SP2/0 cells) were harvested out of 100-ml culture by centrifugation. FCS was washed out by two

washes in RPMI medium. Finally, both lymphocytes and myeloma cells were re-suspended in 10 ml RPMI and combined in one 50-ml tube (Falcon). After centrifugation at 1300 rpm for 5 min at room temperature the cells were re-suspended in 0.4 ml of medium by gentle tapping of the bottom of the tube and making a thin single layer of the cells on the bottom of the vessel. Fusion medium consisting of 0.6 ml of pre-warmed 50% PEG (Roche) was then added to give the final concentration of 30% PEG and the cells were allowed to fuse by mixing for a minute by gentle swirling. After centrifugation (1300 rpm for 5 min), the excess PEG (about 0.4 ml) was removed and 1 ml of RPMI medium was added slowly (within 30") to the cells with gentle re-suspension by shaking. Once complete, another 5 ml of RPMI medium was added within 30 sec as before. Finally, 10 ml of full medium (RPMI supplemented with 20% FCS; OPI (oxaloacetate, pyruvate, insulin 50× from *Sigma*); ECGS (endothelial cell growth supplement 15mg, *Sigma*); AH (azaserine-hypoxanthine 50× from *Sigma*); 1% Penicillin-Streptomicin, *Gibco*) was added to stop the reaction. After another centrifugation the cells were resuspended in 100 ml of full medium and plated on top of feeder cells (mouse peritoneal macrophages isolated a couple of days before the fusion as describe below) in 96 well plates (*Falcon*) by adding 100 µl per well. The hybridoma colonies appeared in about 10 days of growing under selective conditions.

1.1.1.4.3 Preparation of feeder cells

Murine peritoneal washout cells served as feeder cells for the growing hybridomas. These cells were normally harvested two days before the fusion to allow for detection of possible contamination and re-isolation of a new portion of feeder cells. The peritoneal cells isolated from 6 mice yielded enough cells for one fusion. Preparation of feeder cells was carried out in the sterile conditions. Mice were sacrificed just before the harvesting. The cells were gathered through 21G Gouge needle in sterile 0.34M sucrouse solution. 7ml of the solution was injected into the lower abdomen. After a thorough washing of the inner organs, the solution containing peritoneal cells was collected through the same needle. Each two wash-outs were pooled together in 15 ml Falcon tube. After centrifugation (1300 rpm for 5 min at RT) and single wash in PBS the cells were resuspended in RPMI full medium to final cell density of $25x10^4$ cells/ml. The cells were distributed in 10 96-well plates (*Falcon*) 100 µl/well and grown for two days before the addition of hybridoma cells.

1.1.6 CELL METHODS

1.1.6.1 Stable and transient transfection by GenePORTER

Cells used for transfection were split and re-seeded 24 hrs before transfection commenced. Usually, stable transfections were performed in 6-well petri dishes containing 3-4x10^5 cells, at a confluency of 60-80%. Transfection was performed using the liposomal transfection reagent GenePORTER (*Gene Therapy Systems*) exactly as described in the protocol provided, using 2 µg of maxi-prep plasmid DNA and 10 µl of the transfection reagent per 4h transfection. The cells were re-seeded 48 hours post-transfection and plated under appropriate antibiotic selection to generate 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 numbers had been reached. Clones were then expanded further in 10-cm dishes.

1.1.6.2 Immunofluorescence of fixed cells

Adherent cells were grown on coverslips placed on the bottom of a 6-well cell culture dish (*Greiner*) for 24-48 hrs. Once the cells reached the desired density the medium was removed and the cells washed in PBS. For fixation the cells were incubated with freshly thawed 4% paraformaldehyde/PBS solution for 30 min at room temperature. Permeabilisation of the cells was achieved by incubation with 5% NP-40/PBS solution for 5 min at room temperature. Alternatively, fixation and permeabilisation was achieved in one step by incubation with methanol for 2 min at room temperature. After the permeabilisation solution was washed out by PBS, Blocking solution (10% FCS/PBS or 4% BSA/PBS) was added to prevent unspecific background and the cells were incubated for at least 30 min at room temperature. Once blocking was complete the cells were stained with the appropriate primary antibodies diluted in Blocking buffer (approx. 1: 100) for 1-4 hrs at room temperature. After extensive washing in PBS the cells were incubated with the appropriate FITC-coupled secondary antibodies diluted in Blocking buffer (approx. 1:250) for 1 hr at room temperature. The cells were washed three times in PBS prior to mounting coverslips. When the nuclear staining was necessary the DNA was counterstained with 4µg/ml Hoechst dye for 1-2 minutes and washed in PBS before embedding. The samples were viewed by fluorescence microscopy with the appropriate set of filters.

1.1.6.3 Flow cytometry

A confluent medium-sized flask of cells was washed once with PBS (Mg^{2+} and Ca^{2+} free PBS was used throughout the procedure). The cells were then detached by incubation with trypsin for 1 min at 37°C. Afterwards the cells were centrifuged at 1500 rpm for 5 min and then re-suspended at $2x10^6$ cells per 500 µl PBS. To fix the cells, 500 µl 4% formaldehyde was added and the cells were incubated for 20 min at room temperature. The cells were washed with and re-suspended in 100 µl PBS supplemented with 5% FCS and 0.5% saponin at 10^7 cells/ml. The permeabilisation of the cells was accomplished by 10-min incubation in PBS supplemented with 5% FCS and 0.5% saponin. Primary antibodies were added to the cells at a 1:100 dilution. After two washes with PBS supplemented with 5% FCS and 0.5% saponin, the cells were re-suspended in 100 µl PBS supplemented with 5% FCS and 0.5% saponin. FITC- or PE-conjugated secondary antibodies were then added to the cells at a 1:200-1:500 dilution followed by 30-min incubation on ice. After two washes with PBS supplemented with 5% FCS and 0.5% saponin, the cells were finally re-suspended in 100 µl PBS supplemented with 5% FCS and analysed with the aid of a FACStar flow cytometer (*Becton Dickinson*).

1.1.6.4 *In vitro* cell migration assay

Usually the day before the assay matrigel basement membrane matrix (*Becton Dickinson*) was left on ice overnight for thawing and the cells were split to give them 70% confluency on the next day. To cover the filters of the upper well of 24 Transwell migration chambers (8 µm pore, *Costar*), matrigel was mixed with pre-chilled medium to a final concentration of 10 µg/ml, and 400 µl of the matrigel /medium mix was loaded into the upper compartment of each well. Matrigel was allowed to polymerase for a minimum of 1 hr at 37°C. After polymerisation remaining medium was removed and the wells were washed in PBS by placing them into a PBS bath for 2-3 min. The lower compartment of the chamber was then filled with 200 µl of medium containing 10% FCS and the Transwell was set up. Cells (10^5) were seeded in 250µl of serum-free medium into the upper compartment of the Transwell and the cells were allowed to migrate for 4 hrs at 37°C. The medium was then carefully aspirated from the upper chamber and the upper Transwell was placed into the cold 75% ethanol bath for fixation at 4°C for 20 min. After fixation the

matrigel with non-migrated cells was wiped off from the upper side of the filter and the filters were dried in the fume hood for 15 min. The migrated cells remaining on the lower part of the filter were visualised by staining with Crystal Violet solution (1 mg/ml in water) for 25 min at room temperature. After staining the filters were dried again. Each filter was analysed microscopically and the migrated cells were counted.

3 RESULTS

PART ONE

3.1 Selection of metastasis-associated genes from 1AS/ASML SSH library

In order to find genes that are involved in general mechanisms of invasion and to reduce the large initial number of genes isolated in the SSH screen to a smaller number of potentially more valuable genes, the expression of the SSH genes in non-transformed migratory cells was analysed. During the process of inflammation cell migration occurs under normal physiological conditions. Different cells have been shown to participate in the inflammatory reaction. Many of these cells such as T cells, neutrophils or macrophages need to be recruited from distant organs to the site of inflammation (reviewed by Jutila *et al.*, 1989). Many of the migratory properties these cells are recapitulated in metastatic tumour cells. I therefore looked at the expression of the genes isolated in the SSH screen in migratory inflammatory cells. The expression of these genes was compared with that in corresponding non-activated cells, which had not been exposed to inflammatory stimuli and therefore did not exhibit a migratory phenotype so that genes involved in the migratory process could be identified.

3.1.1 IDENTIFICATION OF CLONES WHOSE EXPRESSION IS UP-REGULATED IN MOBILISED MACROPHAGES BUT NOT IN RESIDENT MACROPHAGES

The identification of those clones out of the Bsp73 ASML/1AS SSH subtractive library which are expressed in mobilised macrophages was performed by use of the reverse Northern Screening technique. For this purpose, mRNA isolated from either mobilised or resident macrophages was converted to cDNA by use of Reverse Transcriptase. The synthesised cDNA was subsequently used as a probe for hybridisation with the differentially expressed clones from the Bsp73-1AS/ASML SSH library. To do this the library clones were PCR amplified, electrophoretically resolved and blotted onto membranes. The membranes were then hybridised with the ^{33}P-radiolabelled probes. Eight genes out of the Bsp73-1AS/ASML library were found

to be up-regulated in the mobilised macrophages but not in the resident ones (**Fig. 3.1**). These data suggest that the 8 genes that were differentially expressed in ASML and mobilised macrophages might belong to the basic set of transcripts functionally important for the invasive migratory phenotype.

3.1.2 DETECTION OF CLONES WHOSE EXPRESSION IS UP-REGULATED IN THE ACTIVATED LYMPHOCYTES BUT NOT IN THE NON-ACTIVATED ONES

The next cells that were used for the selection of genes associated with metastasis were activated and non-activated T lymphocytes. Unlike non-stimulated T lymphocytes, antigen-activated T cells are able to traverse almost all types of tissues on their way to the inflammatory site (reviewed by Campbell *et al.*, 2000). The migratory phenotype of the activated lymphocytes highly resembles the metastatic one and thus one can suggest that changes in gene expression occurred during the T cells activation might be recapitulated by tumour cells during their transformation into invading metastatic cells.

In my experiments, lymphocytes were isolated from the murine spleen and lymph nodes. Unlike macrophages, T cells were activated *in vitro* by use of the phytolectin Concanavalin A that is known to induce a T cell response similar to that initiated by cytokines during an inflammatory reaction (Harding *et al.*, 1983). Expression of genes in the SSH library in activated lymphocytes was compared to that in unstimulated "naïve" T cells. To do this, mRNA obtained from the lymphocytes was used for cDNA production which in turn was used as a probe for hybridisation with the differentially expressed SSH library cDNAs blotted onto membranes as described previously.

In these experiments, (see **Fig. 3.2**) 9 clones out of the 1AS/ASML subtracted library were found to be differentially expressed in activated T cells. Contrary to my expectation, no clones were up-regulated in both mobilised macrophages and activated lymphocytes. Nevertheless, elevated expression of these genes during both transition from primary tumour to metastasis (as defined by SSH) and activation of the migratory phenotype in inflammatory response cells (*i.e.* in activated or mobilised macrophages) suggests that their gene products might be functionally involved in the general mechanisms of cell migration and invasion. Therefore, all the 17 genes (8 up-

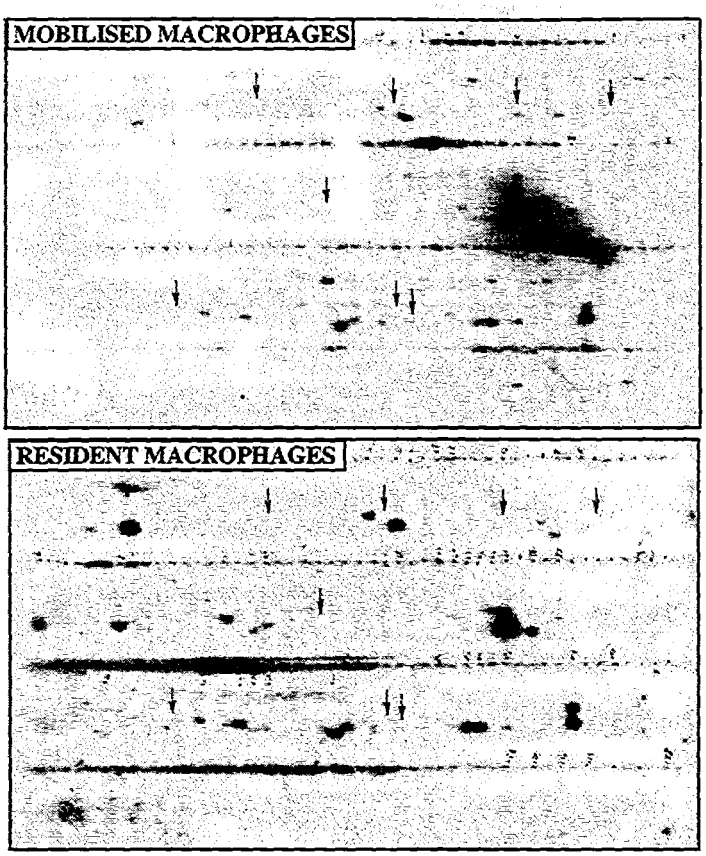


FIGURE 3.1: Hybridisation screening for genes up-regulated in mobilised macrophages but not in resident macrophages.
Duplicate filters containing amplified inserts ofclones derived from 1AS/ASML cDNA library were hybridised with double-stranded ^{32}P-labelled cDNA produced either from mobilised macrophages (**upper panel**) or resident macrophages (**bottom**). The arrows indicate those clones that show expression only in mobilised macrophages.

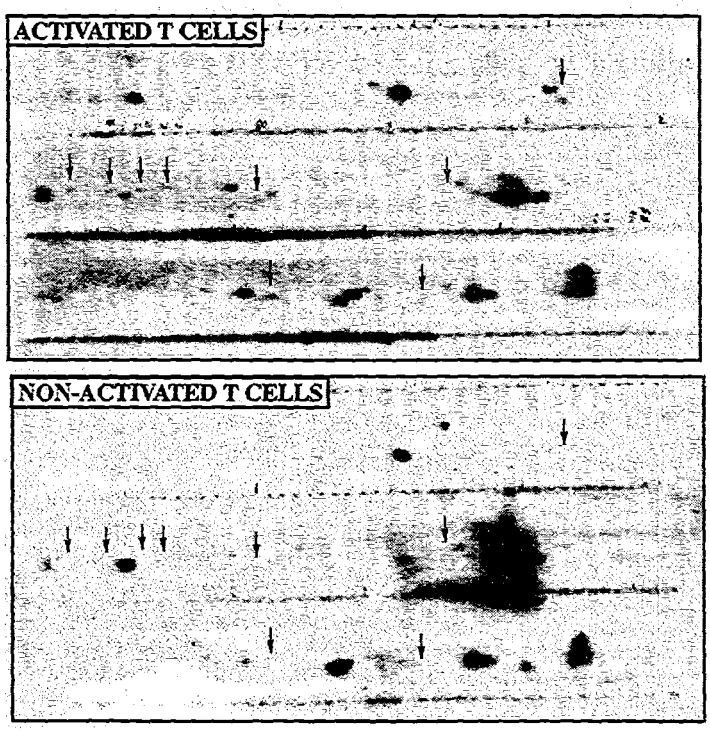


FIGURE 3.2: Hybridisation screening for genes up-regulated in activated lymphocytes but not in non-activated lymphocytes.
Duplicate filters containing amplified inserts ofclones derived from 1AS/ASML cDNA library were hybridised with double-stranded ^{32}P-labelled cDNA produced either from activated T cells (**upper panel**) or non-activated T cells (**bottom**). The arrows indicate those clones that show expression only in activated lymphocytes.

regulated in mobilised macrophages and 9 activated during T cells stimulation) were used for further secondary screening approaches.

3.1.3 THE EXPRESSION OF SOME OF THE IDENTIFIED CLONES CORRELATES WITH METASTATIC POTENTIAL IN MULTIPLE TUMOUR PROGRESSION MODELS

To determine which of the 17 "migration-related" clones selected in previous screens belong to a general pool of gene products shared by different tumour types, and thus to find those which are more likely to be functionally important for metastasis, their expression in several tumour progression models was analysed. Two rat tumour progression models in addition to the Bsp73 pancreatic carcinoma model were chosen for this analysis: mammary and prostate rat adenocarcinomas. The prostatic cell system (Isaacs *et al.*, 1986) comprises of five tumorigenic cell lines of differing *in vivo* metastatic potencies. The G line produces no metastases upon inoculation into Fisher F344 rats, whereas AT.1, AT.3 sublines are moderately metastatic. MatLu and MatLyLu both metastasise preferentially to the lymph nodes and lung/lymph nodes respectively. Concerning rat mammary adenocarcinoma cell system 13762NF (Nicolson *et al.*, 1988b; Nicolson *et al.*, 1988c), the parental line MTPa was reported not to metastasise *in vivo*. MTC was described as being poorly metastatic. The lines MTLN-2, MTLN-3 were demonstrated to produce metastases to varying degrees (Nicolson *et al.*, 1988b). The described phenotypes of all these tumour cell lines were verified in our Institute as described by Nestl *et al.*, 2001. Additionally, human prostate carcinoma cells with differing metastatic potential in immuno-compromised (non-metastasising LNCap, low-metastatic Du-145 and highly-metastatic PC3 cell lines, Nacamoto *et al.*, 1992) were included in the analysis.

Northern blots containing mRNA isolated from different tumour cell lines were hybridised with PCR products derived from the cDNAs of the 17 selected clones. Following this screening, two clones whose expression correlates with metastasis in most of the tumour models analysed were selected for further characterisation. One of these clones, the clone #147 encodes a novel previously undescribed cDNA. It was found to be up-regulated in mobilised macrophages in my initial screening. As can be seen in **Fig. 3.3**, clone #147 exhibits more pronounced expression in cell lines with high metastatic ability in comparison to non-metastatic ones. In all three tumour

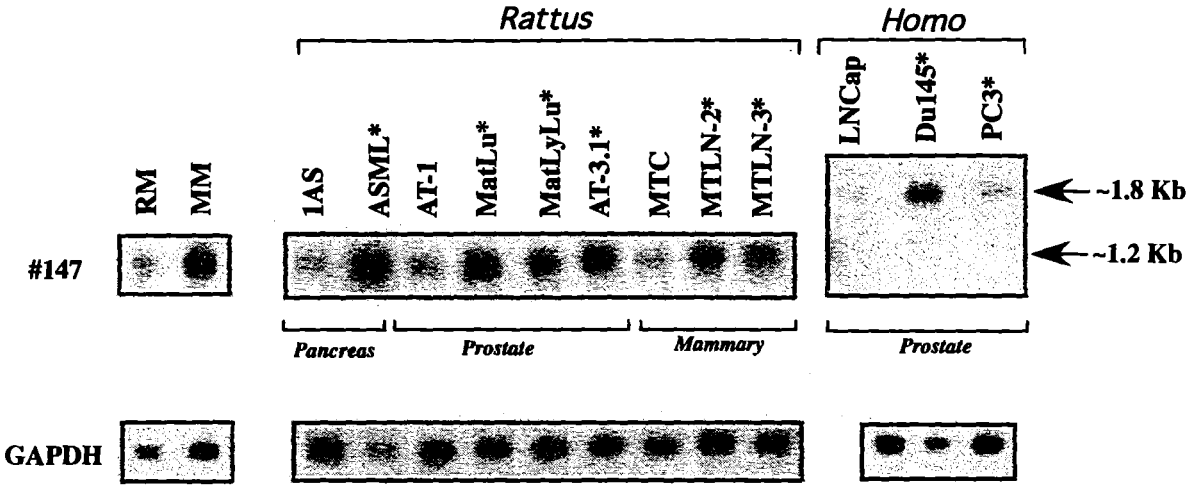


FIGURE 3.3: Expression of the clone #147 is up-regulated in mobilised macrophages as compared to resident macrophages and correlates with metastatic potential in several tumour progression models.

A PCR amplified insert of the clone #147 was used as a probe for hybridisation of Northern blots containing mRNA isolated from mobilised (**MM**) and resident (**RM**) macrophages and several tumour cell lines of pancreatic (1AS; ASML), prostate (AT1, AT3.1, MatLu, MatLyLu; LNCap, Du145, PC3) and mammary(MTC, MTLN-2, MTLN-3) tumour progression models of rat and human origin. Tumour cell lines with high metastatic potential are marked by asterisks. Hybridisation with a GAPDH probe served as a control for equal loading.

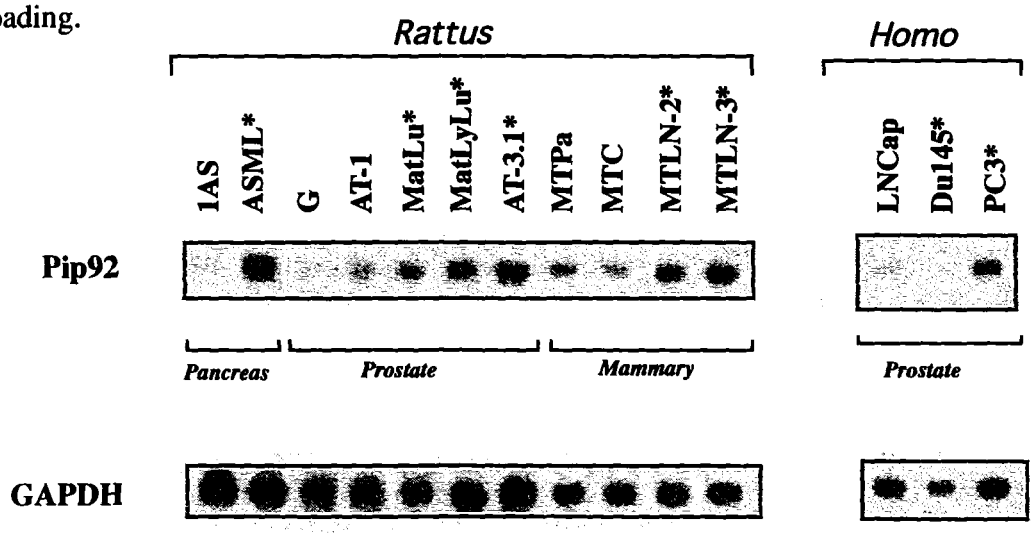


FIGURE 3.4: Expression of Pip92 correlates with metastatic potential in several tumour progression models.

Pip92 cDNA was used as a probe for hybridisation to Northern blots containing mRNA isolated from tumour cell lines of pancreatic (1AS, ASML), prostate(G, AT-1, MatLu, MatLyLu, AT3.1; LNCap, Du145, PC3) and mammary(MTPa, MTLN2, MTLN3) tumour progression models of rat and human origin. Tumour cell lines with high metastatic potential are marked by asterisks. Hybridisation with a GAPDH probe served as a control for equal loading.

progression cell systems analysed, correlation of the expression of this gene and the metastatic ability of the cell lines is obvious.

The second clone which was found to be highly expressed in activated T cells but not in non-activated ones and whose expression is correlated with metastasis in all tumour progression models studied is a previously described gene called *pip92* (**Fig. 3.4**). Pip92 encodes a relatively recently discovered protein whose expression has not been associated with metastasis before. Its cDNA has been cloned from murine and human sources, but not from rat. The finding of its possible involvement in cell invasion encouraged further studies on this gene.

As the Pip92 and NVM-1 proteins represent two different lines of my project of studying of metastasis-related genes, they will be considered here separately. Part Two will be devoted to the work that has been done on the characterisation of Pip92 protein and its role in cell motility and tumour progression. Part Three will concern the characterisation of the NVM-1 protein.

PART TWO

3.2 Characterisation of Pip92 protein and its role in tumour progression, metastasis and cell motility

3.2.1 ISOLATION OF THE FULL-LENGTH OF *PIP92*

To facilitate functional studies, it was first necessary to obtain the full length rat Pip92 cDNA sequence as the clones derived from the subtracted library were only short sequences about 120-140 bp long.

3.2.1.1 Cloning of the coding region of rat Pip92 (rPip92)

Only murine (Pip92) and human (ETR101) homologues of the rat Pip92 cDNA fragment isolated in the SSH screen have been published. The high similarity of the isolated partial rat cDNA sequence to the murine Pip92 gene permitted PCR primers to be designed which should amplify the full length rat Pip92 cDNA (see list of oligos in Chapter 2 of this thesis). Spleen rat cDNA (*Clontech*) was used for the PCR reaction for the amplification of the full-length rat Pip92 cDNA. After sequencing and alignment analysis of the coding region of rat Pip92 it was found that the murine and rat sequences are highly conserved (**Fig. 3.5**). The predicted amino acid sequence of rPip92 is 100% identical to the murine Pip92 protein. Such a strong conservation between rat and mouse sequences is highly suggestive of an important critical function for Pip92 in cellular metabolism.

In early studies of the Pip92, Coleclough *et al.* (1990) suggested that the *pip92* gene may lack introns as the molecular size of the signals obtained in genomic Southern blot and Northern blot analyses were very similar. In order to obtain an expression construct of ETR101, the human homologue of Pip92, human genomic DNA isolated from 293 human embryonic kidney cell line was used for a PCR reaction with ETR101F and ETR101R primers (see Chapter Two). Subsequent sequencing directly confirmed the lack of any introns within the ETR101 gene, demonstrating for the first time that ETR101 is an intronless gene.

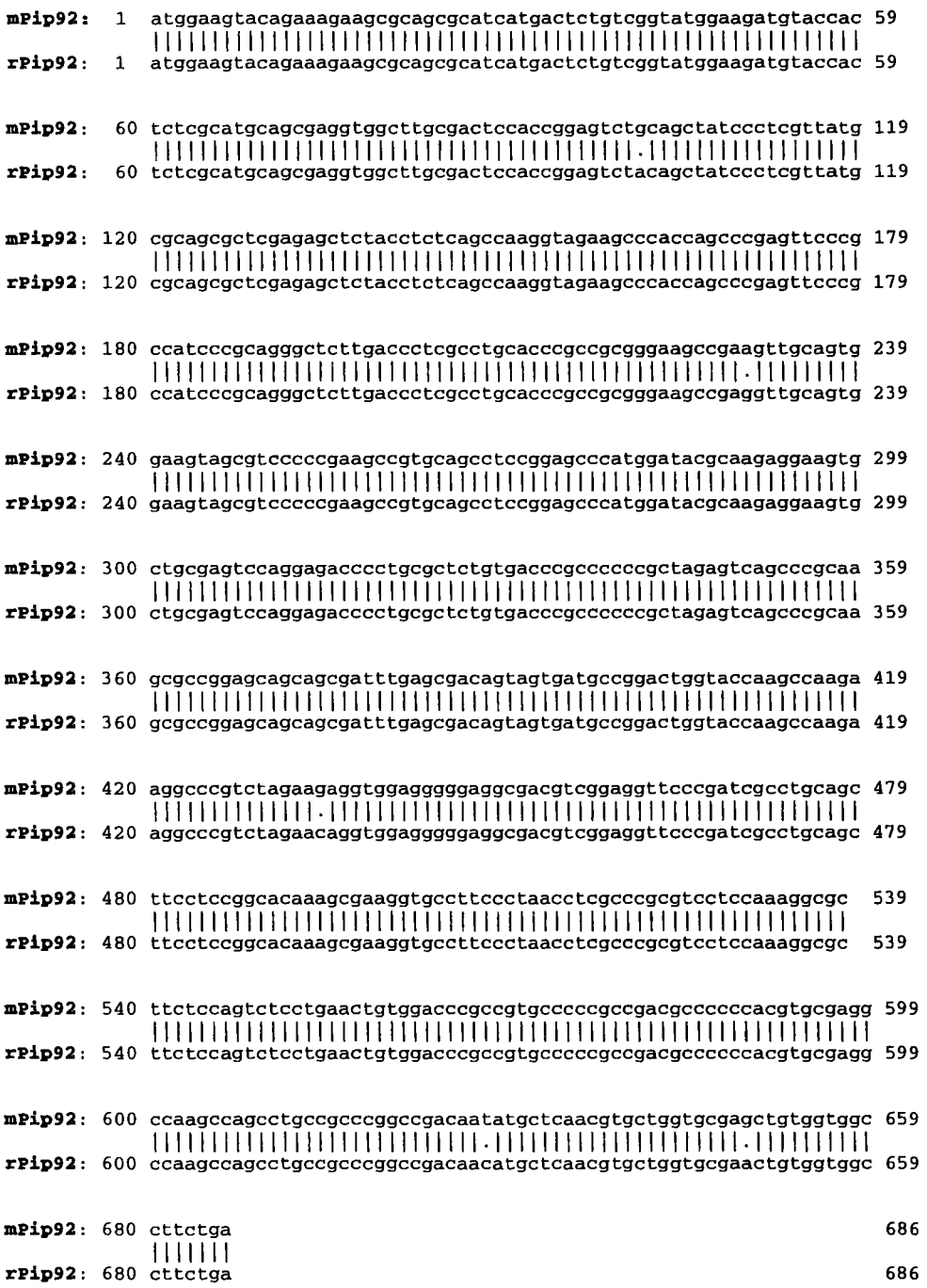


FIGURE 3.5: Alignment of mouse and rat Pip92 ORF nucleotide sequences. Analysis of the nucleotide sequence alignment of the mouse and rat Pip92 cDNAs (mPip92 and rPip92 respectively showed very high identity of two proteins. The first string in each lane represents the murine Pip92 sequence and the second string corresponds to the rPip92. Identity is depicted by vertical bars.

3.2.1.2 Creation of the Pip92/V5/pCDNA3.1, Pip92/HA/pCDNA3.1 and ETR101/pCDNA 3.1 expression constructs

In order to perform functional studies using rPip92 and ETR101, their cDNAs were used to create expression constructs by cloning their coding regions downstream of the CMV promoter in the pCDNA3.1 mammalian expression vector (*Invitrogen*). In addition, C-terminus of rPip92 was fused to polyhistidine and a V5 protein tag. The His tag permits purification of the protein over nickel columns, while the V5 protein tag is a 14 amino acid epitope derived from the P and V proteins of the paramyxovirus SV5 (Southern *et al.*, 1991). This "epitope tagging" allows for the detection of an expressed protein against the background of the endogenous protein within eucaryotic cells by using the antibody that specifically recognise the tag. Another construct with rPip92 was created where its cDNA 3' terminus was fused to a human hemagglutinin protein tag (HA-tag) derived from human influenza virus (Gill *et al.*, 1996). In order to investigate the role of Pip92 in human cell systems, the ETR101 coding region was inserted into the same pCDNA3.1 vector under the CMV promoter. The expression constructs created in this way were used further for functional studies.

3.2.2 DETERMINATION OF THE INTRACELLULAR LOCALISATION OF PIP92 PROTEIN

The Pip92 protein contains the two nuclear localisation signals within the protein sequence and has homologies to nuclear proteins such as JunD, but the protein has been published as being cytoplasmically located (Charles *et al.*, 1990). I therefore set out to see if I could substantiate the cytoplasmic location of Pip92.

3.2.2.1 COS7 cells overexpressing HA-tagged Pip92 protein showed nuclear localisation of the Pip92 protein

COS7 cells were transiently transfected with HA-Pip92 protein and the intracellular localisation of the overexpressed protein was determined by use of the anti-HA 12CA5 monoclonal antibodies. 95% of positive (transfected) cells showed nuclear staining of the protein (**Fig 3.6 A**). The other 5% of the transfectants showed either a slight cytoplasmic staining or the cytoplasmic staining in the presence of destroyed nuclei. Pip92 polyclonal antibodies showed similar predominantly nuclear staining of the protein.

To exclude the possibility that the HA-tag was responsible for the nuclear localisation of the Pip92 protein, another expression construct of V5-tagged Pip92 protein was stably transfected into the COS7 cell line. Probing of a Western blot containing nuclear and cytoplasmic fractions of the obtained cell lines with anti-V5 antibodies showed the localisation of Pip92 to the nuclear fraction (**Fig 3.6B**). These results are in agreement to those obtained in the previous immunofluorescence assay (**Fig. 3.6A**), convincingly demonstrating the nuclear localisation of Pip92 in these cells.

To check the validity of the fractionation procedure, the same Western blot was re-probed with anti-PCNA (proliferating cell nuclear antigen) antibodies which should be found only in the nuclear soluble fraction; with anti-lamin antibodies whose antigen should appear only in the nuclear insoluble fraction and with anti-Paxillin antibodies whose antigen is a cytoskeleton-associated protein and therefore stains exclusively the cytoplasmic fraction. As can be observed on **Figure 3.6B**, all the control antibodies behaved as predicted, indicating that the fractionation procedure was valid.

This data are the first demonstration that Pip92 can be found in nuclei.

3.2.2.2 1AS cells overexpressing V5-tagged Pip92 protein showed both nuclear and cytoplasmic staining of the Pip92 protein

To further investigate the intracellular localisation of Pip92 protein in cells where the endogenous protein is known to be expressed and therefore possible interacting molecules should be present, 1AS cells were stably transfected with the V5-rPip92 expression construct. The immunofluorescent assessment of the transfected cells by use of anti-V5 antibodies revealed overall staining of the cells, with the most pronounced signal coming from the cytoplasm (**Fig. 3.6C**). Nuclear/cytoplasmic fractionation experiments confirmed the observations obtained by immunofluorescence (**Fig. 3.6D**), namely that in 1AS cells the majority of Pip92 is found in the cytoplasm and only a fraction is in the nucleus.

From these experiments I conclude that the Pip92 can be localised to the nuclei as well as to the cytoplasm. The possible localisation of Pip92 to the nuclei significantly broadens its putative cellular functions. On the other hand, one cannot rule out the possibility that the exogenous overexpression of Pip92 leads to its nuclear location but its natural occurrence is cytoplasmic. An important question is, what are

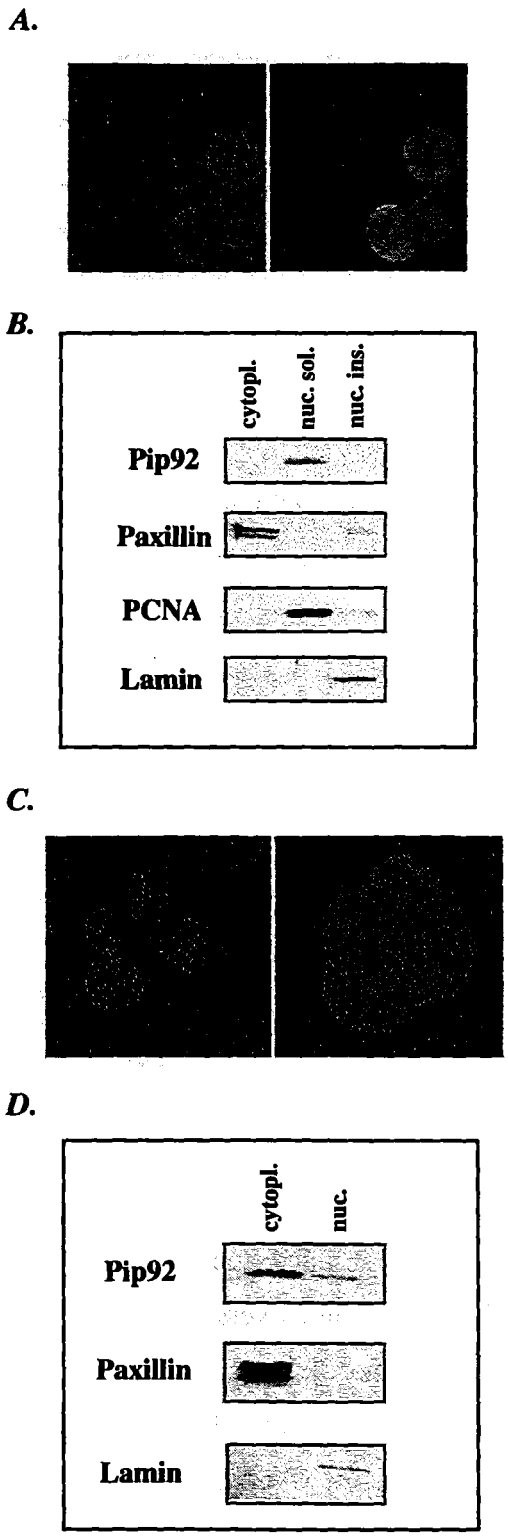


FIGURE 3.6: Pip92 protein can be localised to the nucleus and to the cytoplasm. Immunofluorescence of COS7 (**A**, right panel) and 1AS cells (**C**, right panel) expressing Pip92 protein. The nuclei are visualised by Hoechst staining (**A,C** left panel). Either COS7 (**B**) or 1AS (**D**) cells were fractionated on cytoplasmic and nuclear fractions which were resolved on the SDS PAGE, transferred to the membrane that was immunoblotted with anti-Pip92, anti-Paxillin, anti-PCNA or anti-Lamin antibodies. See text for details.

the factors regulating Pip92 protein localisation in the cell? Can, for example, the nuclear localisation of Pip92 be cell cycle dependent? To answer these questions, one would need to study the endogenous protein. I therefore set out to make anti-Pip92 antibodies an important tool for the further studies of Pip92.

3.2.3 GENERATION OF PIP92 POLYCLONAL ANTIBODIES

Recombinant Pip92 fusion protein was prepared and used to make polyclonal serum, which was then purified and characterised.

3.2.3.1 Fusion protein preparation

To make an antigen with which to generate antibodies against the Pip92 protein, a cDNA fragment encoding amino acids 25 to 142 was fused up-stream of the Glutathione-S-transferase gene (GST) in the pGEX-1 vector. This construct directs the inducible synthesis of a Pip92-GST fusion protein. This ΔPip92-GST expression construct was transformed into the LB21 Codon Plus *E.coli* strain (*Invitrogen*). Following 4 hours of induction of the protein synthesis the fusion protein was purified using Glutathione-coupled agarose (*Sigma*) (**Fig. 3.7**). The purified product was sent to Sigma-Genosis in order to immunise New Zealand White Rabbits. Serum containing anti-Pip92 antibodies was further used for purification and analysis.

1.1.1.2 Purification of the Pip92 antibody

1.1.1.2.1 Purification of the serum of antibodies generated against GST

Collected serum containing immunoglobulins against Pip92 was purified using CyanogenBromide agarose beads (*Pharmacia*) coupled with GST protein in order to get rid of those antibodies raised against the GST fusion protein partner during the immune response. ELISA assays following serum purification showed that antibodies in the serum directed against GST protein decreased significantly in comparison to that before purification (data not shown). Western blot analysis of 10ng purified GST and the same amount ΔPip92-GST protein loaded into PAGE and blotted onto the PVDF membrane showed that the purified antibodies cross-react only with Pip92 protein but not with GST alone. A single band of the expected molecular weight of about 36 kDa appeared only in the lane where Pip92 protein was loaded. No signal

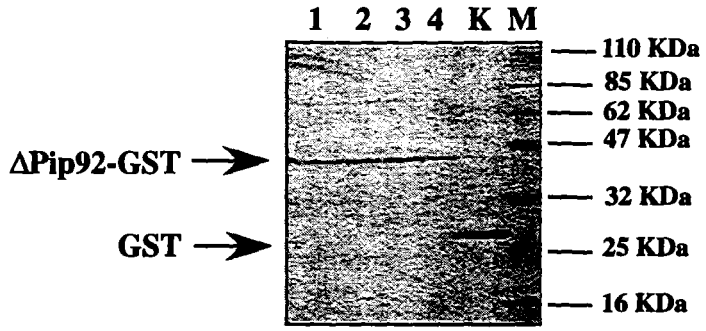


FIGURE 3.7: Purification of the ΔPip92-GST fusion protein.
The central part of the Pip92 protein (amino acids 25 to 142) was fused to the GST protein to direct the inducible synthesis of ΔPip92-GST fusion protein. The fusion protein was purified by use of glutathione-coupled agarose. An aliquot representing 0.5% of each purified fraction was loaded into separate lanes of SDS PAGE. The gel was subsequently stained with Coomassie Blue. Lanes **1-4** represent fractions 3-6 of the purified protein; lane **K** is the GST protein alone used as a control for the purification procedure; lane **M**: protein molecular weight marker.

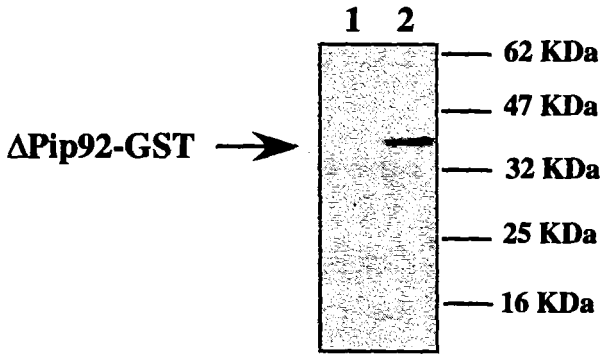


FIGURE 3.8: Anti-Pip92 polyclonal antibodies cross-react with the ΔPip92-GST protein but not with the GST protein alone in western blot analysis.
10 ng of the purified GST protein (**lane 1**) and ΔPip92-GST (**lane 2**) fusion protein were loaded into separate lanes of SDS PAGE. The gel was subjected to Western blot analysis with anti-Pip92 polyclonal antibodies.

was observed in the lane with pure GST protein (**Fig. 3.8**). This assay also demonstrates the sensitivity of the anti-Pip92 polyclonal antibody. Specifically, this experiment showed that as little as 10ng of the purified Pip92 is sufficient to be detected by Western blot immunostaining.

Thus, ELISA assays as well as Western blot analysis both proved that after purification over a GST column there were no antibodies capable of cross-reacting with GST detectable in the anti-Pip92 antibody-containing serum.

1.1.1.1.2 Affinity purification by use of antigen-coupled CyanogenBromide agarose

Further purification of anti-Pip92 polyclonal antibodies was accomplished by use of immunoaffinity purification. For this purpose, purified ΔPip92-GST fusion protein was bound to CyanogenBromide agarose beads (*Pharmacia*). The efficiency of cross-linking was monitored by checking the flow-through solution on a Coomassie stained SDS PAGE. GST-purified serum was passed over this column and after appropriate incubation and washes (as described in Materials and Methods), the anti-Pip92 antibodies were eluted under the low pH conditions with 100mM Glycine pH 2,5 in 1 ml aliquots. In order to neutralize the elution solution each fraction was mixed with 100 µl of 1M sodium phosphate pH 8,0. Finally 4 fractions containing significant amounts of antibodies (OD_{280}>0.1) were pooled together and dialyzed against PBS at 4°C overnight. The resulting solution containing purified antibodies was used for subsequent experiment.

1.1.1.3 Characterisation of the purified serum

An ELISA assay performed with the purified serum showed that Pip92 polyclonal antibodies could still bind to the immobilised ΔPip92-GST protein at a dilution of 1:7000 (data not shown). To show the applicability of anti-Pip92 polyclonal antibodies in immunoprecipitation assays, they were used for the precipitation of the *in vitro* produced HA-tagged Pip92 protein. The antiserum raised against Pip92 but not the preimmune serum immunoprecipitated the Pip92 protein translated *in vitro* (**Fig 3.9**, lane 3 and 2). The same protein of molecular weight about 33 kDa was precipitated by anti-HA 12CA5 mouse monoclonal antibodies which were used as a positive control (**Fig 3.9**, lane 1).

Pip92 polyclonal antibodies were further investigated using Western blot analysis of lysates obtained from NIH3T3 cells transfected with either HA-tagged Pip92 protein or empty vector. In this experiment Pip92 polyclonal antibodies cross-react with the ectopically expressed Pip92 protein, showing a signal of the expected molecular weight. The identity of the HA-tagged Pip92 protein was proved by use of the anti-HA 12CA5 monoclonal antibody, which was used as a positive control (**Fig. 3.10**).

To check the potential of Pip92 polyclonal antibodies in immunofluorescence assays, immunostaining of NIH3T3 cells transfected with an HA-tagged Pip92 expression vector was performed. In this experiment, Pip92 polyclonal antibodies showed a similar staining pattern as the control anti-HA 12CA5 monoclonal antibodies (**Fig. 3.11**). In immunofluorescent assessment, anti-Pip92 polyclonal antibodies were also able to cross-react with the human homologue of Pip92/ETR101 when it is overexpressed in COS7 cells (data not shown). The cross-reactivity of the anti-Pip92 antibodies generated against the rat Pip92 sequence with the human protein might be explained by the high conservation of this molecule between the two species.

From these data, I conclude that Pip92 polyclonal antibodies can be successfully used for immunoprecipitation of the corresponding antigen, in Western blot analysis as well as in immunohistochemistry assays.

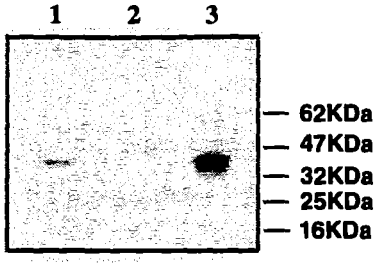


FIGURE 3.9: Pip92 polyclonal antibodies precipitate Pip92 protein. Pip92 polyclonal antibodies were used for immunoprecipitation of *in vitro* transcribed/translated hemagglutinin (HA)-tagged ^{35}S-labelled Pip92 protein (**lane 3**). Anti-HA 12CA5 monoclonal antibody precipitated the same protein (**lane 1**). No immunoprecipitation was observed when the prebleed was used (**lane 2**).

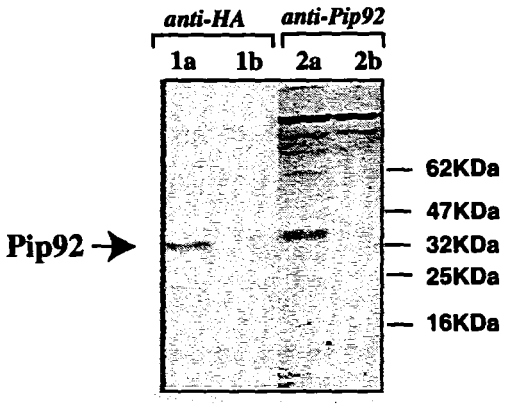


FIGURE 3.10: Western blot with Pip92 polyclonal antibodies. Pip92 polyclonal antibodies were used for western blott analysis of lysates of NIH3T3 cells overexpressing either hemagglutinin (HA)-tagged Pip92 protein (**lane 2a**) or empty vector (**lane 2b**). Anti HA 12CA5 monoclonal antibody were used as positive control (**lanes 1a,b**).

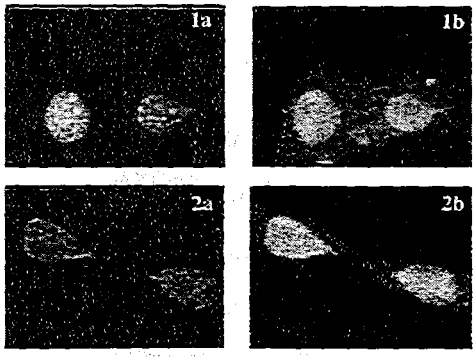


FIGURE 3.11: Immunofluorescence with Pip92 antibodies. Pip92 polyclonal antibodies were used for immunostaining of hemagglutinin (HA)-tagged Pip92 protein over-expressed in NIH3T3 cells (**1b**). Anti HA 12CA5 monoclonal antibody were used as positive control (**2b**). Cell nuclei were visualised by Hoechst staining (**1a, 2a**).

1.1.4 SUB-CELLULAR LOCALISATION OF THE ENDOGENOUS PIP92 PROTEIN IN NIH3T3 CELLS

Growth of fibroblasts to confluency and serum deprivation is known to synchronise their cell cycle and bring the cells to the quiescent state (G_0). Stimulation of the quiescent fibroblasts with serum induces DNA replication and their transition to the G_1 phase (Adolph *et al.*, 1993). The *pip92* gene belongs to the immediate early gene family. Its expression has been shown to be rapidly induced upon serum stimulation in Balb/c 3T3 fibroblasts (Charles *et al.*, 1990). Fast induction of the Pip92 expression in fibroblasts during the transition from the quiescent to the proliferative state might suggest its critical role in this process. Thus, in order to get an idea about the possible function of the Pip92 protein in the G_0/G_1 transition, I set out to investigate the intracellular localisation of the endogenous Pip92 protein in serum-stimulated and non-stimulated murine NIH3T3 fibroblasts.

To check the kinetics of Pip92 induction in NIH3T3 cells, Northern hybridisation analysis of total RNA isolated from cells harvested at different stages of cell maintenance (*i.e* subconfluency, confluency, serum starvation, serum stimulation at different time points) was performed. As can be seen in **Figure 3.12**, *pip92* expression is hardly detectable in unstimulated cells (*i.e.* subconfluent stage). No expression of the Pip92 was observed in resting NIH3T3 cells (**Fig. 3.12**, the second and third lanes).

Transcription of *pip92* is activated immediately after serum stimulation, reaches a peak level by 30 minutes and is attenuated to low levels 2 hours after serum addition. This experiment defined the time points at which the localisation of the Pip92 protein would be investigated.

The immunofluorescent assessment of the endogenous Pip92 in the NIH3T3 cells growing to sub-confluency and immunostained with anti-Pip92 polyclonal antibodies revealed a weak but exclusively cytoplasmic localisation of the protein (see **Fig. 3.13A**). According to the Northern hybridisation analysis described above, transcription of *pip92* is minimal at this stage explaining the weakness of the staining obtained in immunofluorescence. On the other hand, the cells induced with serum for 30 minutes showed a strong staining all over the cells with the most fluorescent signal coming from the nuclei (see **Fig. 3.13B**). The predominantly nuclear localisation of the Pip92 in the cells overexpressing the protein observed in this experiment is in

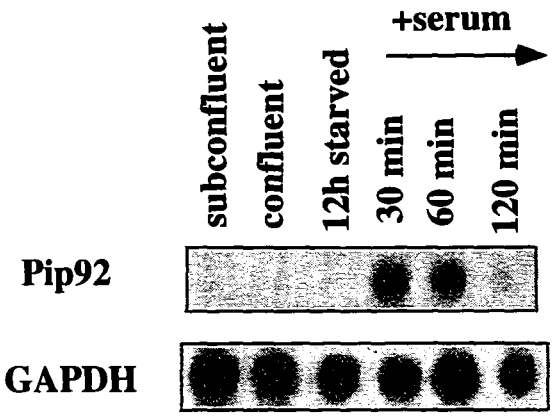


FIGURE 3.12: Induction of the expression of Pip92 upon serum stimulation of NIH3T3 cells.
Total RNA was isolated from either uninduced (first three lines) or serum induced NIH 3T3 cells at the different time points of the stimulation. The resulted Northern blot was hybridised with the radiolabelled Pip92 DNA fragment. The product of the expected size (about 2Kb) was observed. The bottom line showed the loading control signal obtained after GAPDH hybridisation.

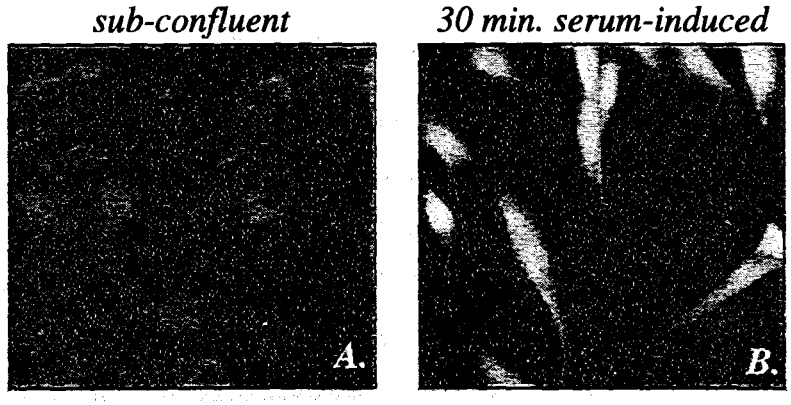


FIGURE 3.13: The Pip92 is localised to nuclei and cytoplasm of the serum induced NIH3T3 cells.
Either uninduced (**left**) or serum induced for 30 min (after 12 hours of serum starvation) (**right**) NIH3T3 cells were immunostained with the Pip92 polyclonal antibodies followed by FITC-conjugated anti-rabbit secondary antibodies. The stained samples were viewed by fluorescence-detective microscopy. A very weak cytoplasmic staining was observed in unstimulated NIH3T3 cells (**left**). After 30 min of serum stimulation and thus during the maximal induction of Pip92 expression (see Fig. above), the Pip92 was localised to the nuclei and the cytoplasm of the cells (**right**).

agreement with the analysis of ectopically expressed Pip92 in NIH3T3 cells (see **Fig. 3.11**). However, in the case of the Pip92 transfected cells the nuclear staining was significantly more pronounced, possibly reflecting the amount of protein expressed.

Thus, these experiments showed that not only exogenous but also naturally expressed Pip92 can be localised to the nucleus. The change of the Pip92 protein localisation during G_0/G_1 transition from cytoplasmic to the nuclear might indicate it has a nuclear function, for example transcription of secondary message genes which drive cells through cell cycle progression.

1.1.5 FUNCTIONAL ANALYSIS OF PIP92 PROTEIN

The induction of Pip92 expression upon the onset of the migration program either in activated lymphocytes or metastatic cell lines strongly suggest a possible role for this protein in cell migration. To address the question of the involvement of the Pip92 protein in cell motility, the migration ability of cells overexpressing Pip92 protein was investigated.

1.1.5.1 Generation of stable transfectants over-expressing Pip92 protein

Based on the fact that Pip92 is highly differentially expressed in the Bsp73 rat pancreatic adenocarcinoma cell system, and based on the hypothesis that Pip92 is a possible mediator of cell motility, the tentative assumption was drawn that the overexpression of Pip92 in the non-metastatic Bsp73-1AS cell line (which has a low endogenous levels of Pip92) could have functional consequences concerning the invasive ability of 1AS cells. I reasoned that overexpression of Pip92 in 1AS cells might lead to the induction of a putative signalling pathway leading to a motogenic-invasive phenotype in the non-metastatic cell line. Stable transfectants of 1AS cells were therefore generated by the transfection of the Pip92 expression construct into 1AS 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 anti-V5 antibodies (**Fig. 3.14**). Two positive clones of 1AS cells as well as two independent clones of vector-transfected cells were used for further assays.

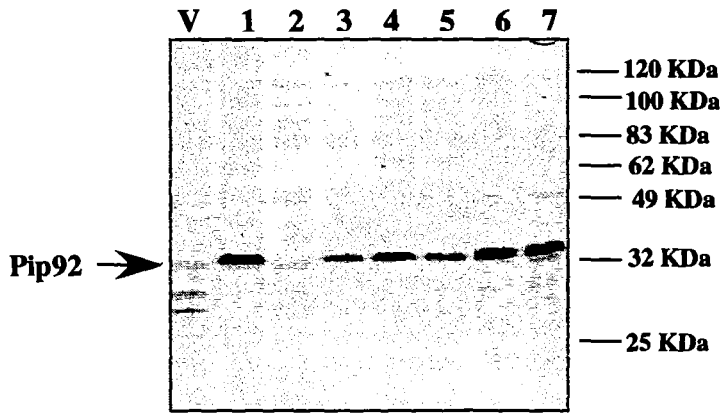


FIGURE 3.14: Identification of 1AS clones stably overexpressing Pip92 protein. Cell lysates obtained from the individual antibiotic-resistant clones of 1AS cells transfected either with Pip92/V5/pCDNA3.1 construct (**lanes 1-7**) or pCDNA3.1 vector (**lane V**) were loaded into separate lanes of the SDS PAGE. The gel was blotted to the membrane and the resulting membrane was probed with anti-V5 antibodies. In the experiment shown, only one clone (**lane 2**) appeared to be negative out of 7 clones being tested.

1.1.5.2 Over-expression of Pip92 in 1AS cells facilitates migration of these cells in vitro

To get an insight into the functional role of Pip92 in the context of cell migration, the 1AS rat pancreatic tumour cells stably overexpressing Pip92 protein were analysed for their ability to migrate through matrigel in classical *in vitro* cell migration assays (Pratt *et al.*, 1984). The porous polycarbonate filters of the transwell migration chamber (*Costar*) were coated with matrigel. Matrigel is a soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) tumour that solidifies at room temperature to form a genuine reconstitute of the basement membrane (Kleinman *et al.*, 1986). Triplicate samples of 10^5 cells of two Pip92 over-expressing clones and two vector controls were seeded in serum-free medium on top of matrigel-coated filters of the upper compartment of the transwell migration chamber. The cells were allowed to migrate towards medium supplemented with 10% FCS located in the lower chamber of the transwell. After four hours the assay was completed by removing the matrigel together with non-migrated cells. Cells which had migrated adhered to the bottom side of the filter. These were fixed and stained with Crystal Violet (as described in Materials and Methods). The migrated cells were analysed microscopically and counted. As one can see in **Fig. 3.15** the number of Pip92 over-expressing cells which migrated was 3-4-fold higher than that of the vector control.

From this experiment I conclude that Pip92 indeed plays a role in migration of 1AS cells *in vitro* by conferring invasive motile properties onto recipient tumour cells.

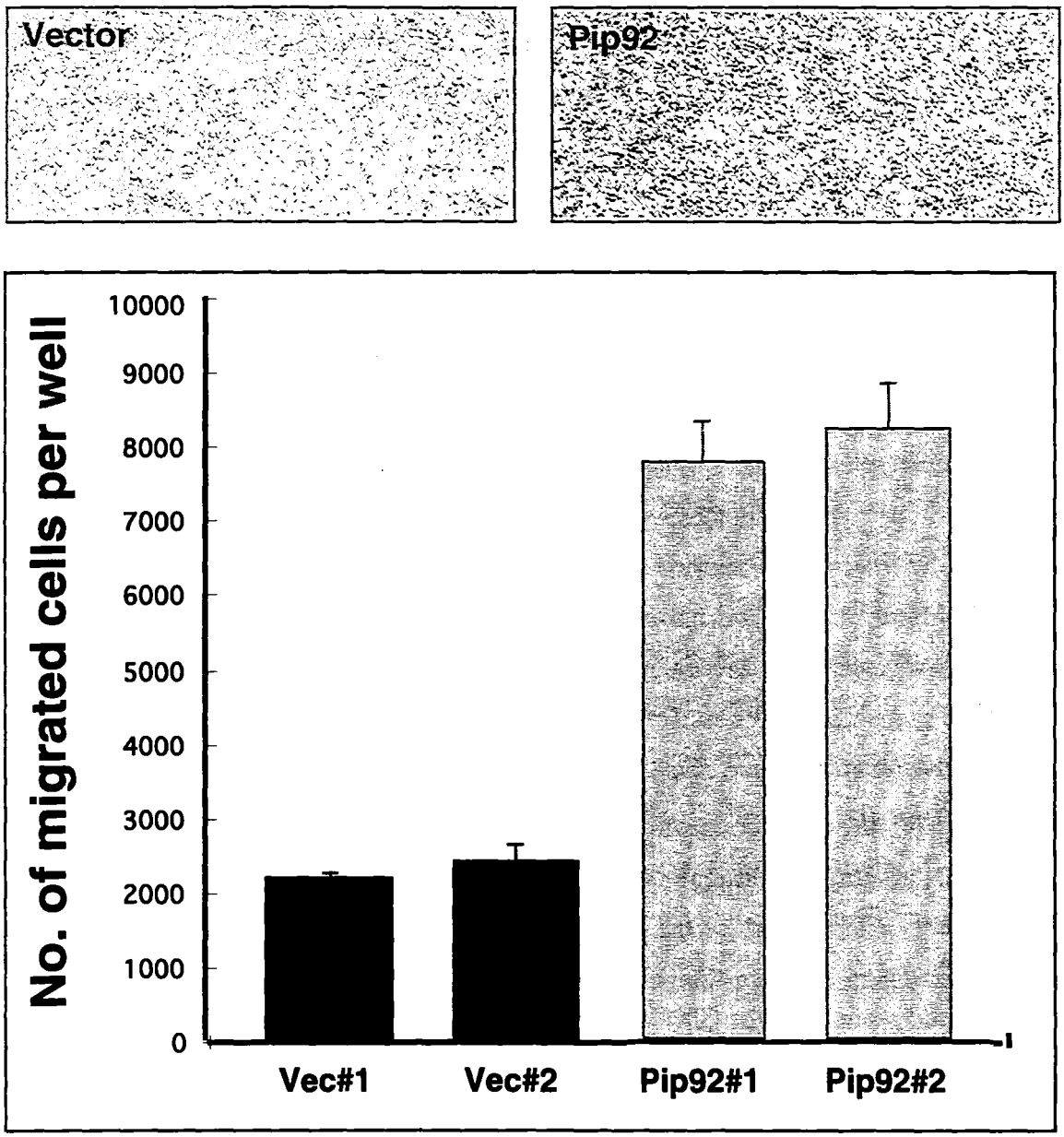


FIGURE 3.15: Elevated expression of Pip92 protein enhances the migration of transfected 1AS cells *in vitro*. 1AS cells transfected with either Pip92 or empty vector were seeded on top of matrigel-coated filters of transwell migration chambers and allowed to migrate towards FCS- containing medium placed in the lower chamber. Photos of the migrated cells that adhered to the bottom side of the filters and stained with crystal violet are shown in the upper panel. Cells were subsequently counted and a diagram reflecting the number of cells of two independent clones transfected either with empty vector (**dark blue bars**) or the Pip92 over-expressing construct (**light blue bars**) is shown on the bottom. The data represent the mean value of two independent experiments performed in triplicate.

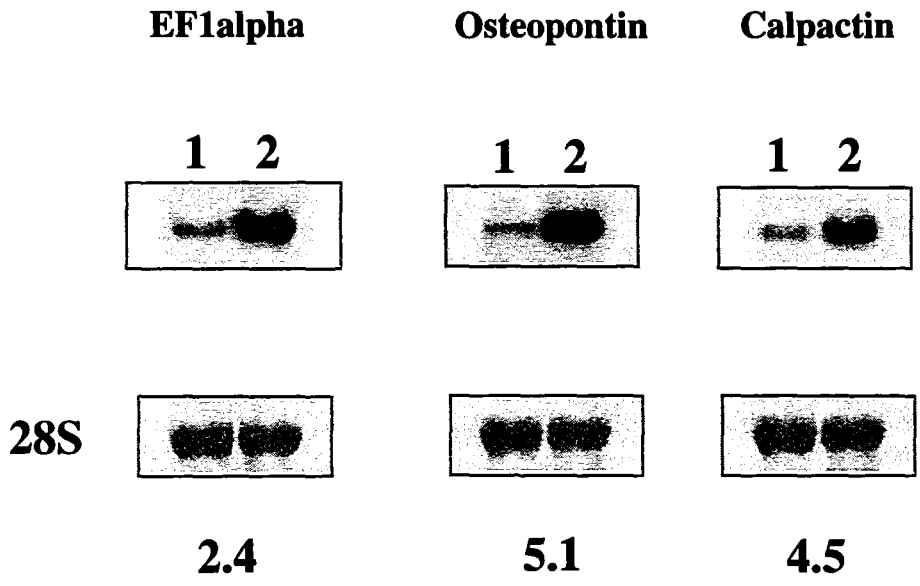
1.1.6 INVESTIGATION OF THE MOLECULAR MECHANISM OF PIP92 ACTION

The enhanced motility of 1AS cells transfected with Pip92 speaks for the activation of a program of genes involved in cell motility by the Pip92 protein. Furthermore, the regulated nuclear localisation of the Pip92 protein as well as the similarity to the HIV integrase DNA binding domain and the basic region of the JunD protein (Coleclough *et al.*, 1990) suggest that Pip92 may be able to regulate transcription. The following assay was therefore performed to investigate this possibility.

1.1.6.1 Reverse Northern Screening identifies Pip92 target genes

If Pip92 protein is capable of modulating transcription, its over-expression in cells should lead to transcriptional changes and thus differential expression of some genes. To check this hypothesis a search for Pip92 regulated genes was performed. Reverse Northern hybridisation screening of cDNA arrays of a rat spleen cDNA library (obtained from the resource center of the German human genome project (RZPD)) was used for the detection of genes that are up- or down-regulated in response to Pip92 as follows. Either a Pip92 expression vector or empty vector was transiently transfected into Bsp73-1AS cells. The mRNA obtained from these cells was used for the production of ^{33}P radiolabelled cDNA probes for hybridisation. Reverse Northern hybridisations were performed with both probes sequentially on the same library filter to reduce the possibility of false positives coming from unequal DNA spotting on different filters. In this experiment the signal intensity of each clone which hybridised with the cDNA from the Pip92 transfectants was compared to the hybridisation signal intensity of the same DNA spot hybridised with the vector control probe, by use of the computational analysis (AIDA Array Compare software).

Following this screening, 40 clones out of about 28000 individually spotted clones on the array filter were found to be differentially expressed (namely up-regulated) in Pip92-transfected 1AS cells (**Table 3.1**). No genes were found to be down-regulated by Pip92 overexpression in this cell system. The up-regulation of the expression of the 40 genes was further confirmed by the Southern blot analysis. In this experiment the inserts were excised out of the vectors of the rat spleen library and electrophoretically resolved on an agarose gel. The resulting gel was blotted onto a membrane. The membrane was



FICURE 3.16: Pip92 induces the expression of certain genes.
Equal amounts of total RNA isolated from 1AS cell line either transfected with an empty vector (**lane 1**) or Pip92 expression construct (**lane 2**) were loaded into the separate lanes of agarose gel which was subsequently subjected to Northern blot. The membrane was probed with ^{32}P-radiolabelled EF1 alpha, osteopontin or calpactin probes (**upper panel**). Equal loading was ensured by staining of the blotted membrane with Methylene Blue and subsequent photographic documentation of the 28S ribosomal RNA band (**bottom**). Fold of the signal enhancement in Pip92-expressing cells (indicated under each blot) was quantified using AIDA computer software.

then hybridised with ^{32}P-radiolabelled cDNA derived from either Pip92-overexpressing or empty vector transfected cells. This Southern analysis substantiated that 80% of the selected clones indeed represent genes up-regulated in Pip92-transfected 1AS cells (data not shown). These clones were then sequenced. Due to redundancy of the clones identified after sequencing only 17 individual genes were identified. The identity of these clones is shown in the table below.

NN	Name of the gene	Fold of induction
1	Vimentin	1.4
2	Elongation factor 1 alpha	2.4
3	Novel 1	d.c
4	Cytolplasmic gamma isoform of actin	1.6
5	Rat sialoprotein –osteopontin (Spp1)	5.1
6	Novel 2	d.c
7	Synapsin (Syn1)	1.4
8	Peptidilpropyl isomerase (cyclophelin A)	d.c
9	Phosphoglycerate kinase	1.4
10	Stromal cell derived factor 2 (Sdf2)	d.c
11	Novel 3	d.c
12	Rat alpha tubulin	1.6
13	Novel 4	d.c
14	Tubulin A	1.6
15	Novel 5	d.c
16	Spraque-Dawley transketolase	d.c
17	Calpactin 1 (Anxa)	2.6

TABLE 3.1 List of the genes up-regulated in Pip92 over-expressing 1AS cells. Fold of induction as determined by Northern analysis of gene expression in cells either overexpressing Pip92 or transfected with an empty vector; d.c. indicates that the up-regulation of the clone have not been verified by Northern hybridisation.

1.1.6.2 Direct Northern Analysis proves differential expression of the target genes

The Pip92-regulated genes were identified by Reverse Northern or Southern screenings. It was therefore important to determine their differential expression directly at the mRNA level as the extent of differential expression is hard to determine accurately in Reverse Northern analysis. Direct Northern analysis is more sensitive and provides additional information (e.g. transcript size). For this purpose a selection

of the 17 clones from the rat spleen library found to be up-regulated by Pip92 in the previous screens were used as probes for hybridising Northern blots of RNA isolated from mass cultures of the 1AS cell line transfected with either Pip92 protein or empty vector. Nine genes out of 17 found were analysed in this way. As can be seen in **Fig. 3.16,** the differential expression status of three genes analysed in this way is clearly visible (only clones where the degree of differential expression was more than two fold are shown). Six other genes checked by direct Northern analysis also showed differential expression, but extent of the change in gene expression was less pronounced (see **Table 3.1**).

This experiment confirmed that the presence of Pip92 in cells indeed changes the expression of some genes, strongly suggesting a transcriptional regulatory role for Pip92 in cells. Whether the Pip92 protein is directly involved in the activation of these genes acting as a transcription factor or participates in signalling pathways leading to the transcriptional activation of these proteins still needs to be elucidated. Whether the proteins activated by Pip92 (**Fig 3. 16**) are involved in the migratory phenotype of the Pip92-overexpressing cells is also remains unclear and therefore could represent a basis for further investigations. However it should be noted that osteopontin, the gene most highly up-regulated by Pip92 expression, has previously been shown in many studies to be involved in promoting cell migration (Oates *et al.*, 1997).

1.1.7 SEARCH FOR A CORRELATION OF THE PIP92 EXPRESSION WITH THE METASTATIC POTENTIAL IN HUMAN TUMOURS

The ultimate aim of my study as well as that of many other studies on metastasis-related genes is to prove the relevance of findings made in rodent or other tumour systems to the human *in vivo* situation. One way of doing this is by immunohistological analysis of tumour tissues dissected from cancer patients.

1.1.7.1 *In situ* hybridisation of human tumour sections with radiolabelled Pip92 mRNA showed that the expression of Pip92/ETR101 is up-regulated in tumour cells

To test if the expression of the *pip92* gene correlates with tumour metastasis not only in *in vitro* tumour progression models (see **Fig. 3.4**) but also *in vivo*, *in situ* hybridisation on human tumour material was performed. For this aim, ^{35}S-radiolabelled anti-sense RNA of ETR101 (the human homologue of Pip92) was used as a probe for *in situ* hybridisation to tissue sections of invasive mammary ductal carcinoma tumours dissected from three patients. Strong expression of Pip92 mRNA was observed in the invasive tumour cells but not in the surrounding tissues (**Fig. 3.17 A**, **B**, and **C**). Hybridisation with the sense RNA of the same gene was used as a negative control and showed no signal on autoradiography (**Fig. 3.17 A***, **B***, **C***).

Thus, Pip92/ETR101 whose expression was identified as being metastasis associated in several tumour progression cell line systems also shows an enhanced expression in human mammary tumours. In order to confirm these results at the protein level, anti-Pip92 polyclonal antibodies were used to immunostain the aforementioned tumour sections.

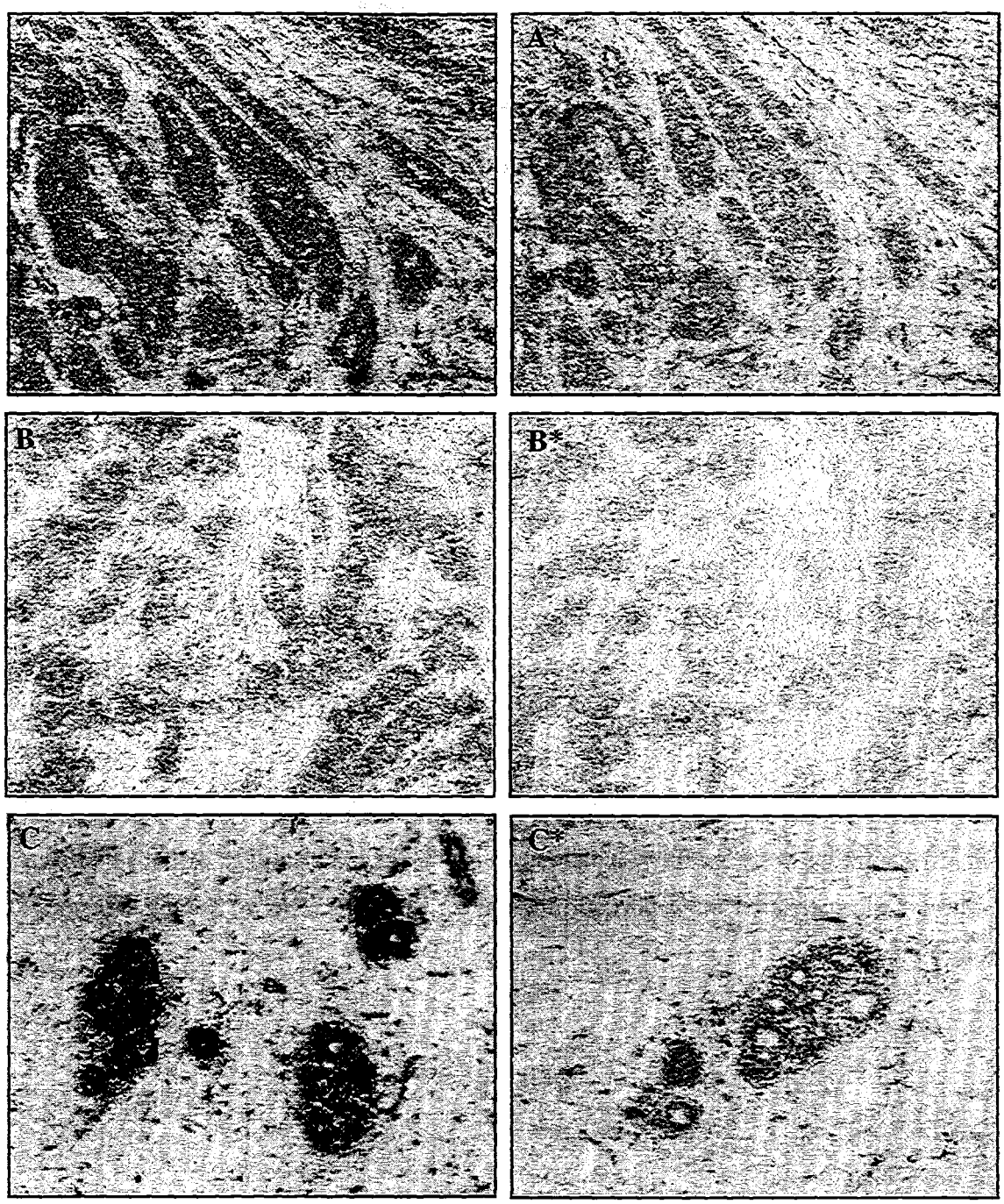


FIGURE 3.17: Tumour cells of invasive ductal carcinomas express Pip92.
^{35}S radiolabelled anti-sense RNA of ETR101 (a human homologue of Pip92) was used as a probe for *in situ* hybridisation to tissue sections of invasive mammary ductal carcinoma tumours dissected from three patients (**A, B, C**). Hybridisation with the sense RNA of the same gene was used as a negative control (**A*, B*, C***).

1.1.7.2 Immunostaining of the human tumour sections with anti-Pip92 polyclonal antibodies shows that Pip92/ETR101 is expressed in tumour cells but not in normal mammary duct epithelium

Sections of tumours dissected from patients with invasive ductal adenocarcinoma were immunostained with anti-Pip92 polyclonal antibodies. Positive staining was observed in the invading tumour cells but not in normal tissue of all three patients examined (**Fig. 3.18 A, B, C**). No staining was detected in the healthy ducts of the mammary gland, suggesting that the Pip92/ETR101 protein is not expressed in normal ductal epithelia (**Fig. 3.19 left panel**). More detailed examination of the immunostained tumour material revealed that tumour-infiltrating lymphocytes were also positively stained. This is consistent with the data described by Lau *et al.*, as well as with our own observations indicating that the expression of Pip92 is up-regulated in activated lymphocytes. No staining was observed when pre-immune serum or only secondary antibodies were used (**Fig. 3.18A*, B*, C*; 3.19 right panel**).

These data confirm the results obtained in *in situ* hybridisations. As the most of the mammary tumours arising in humans are of ductal epithelia origin, the absence of positive staining in the healthy ducts coupled with the high amount of the protein in tumour cells speaks for an important function for Pip92/ETR101 in human mammary tumours.

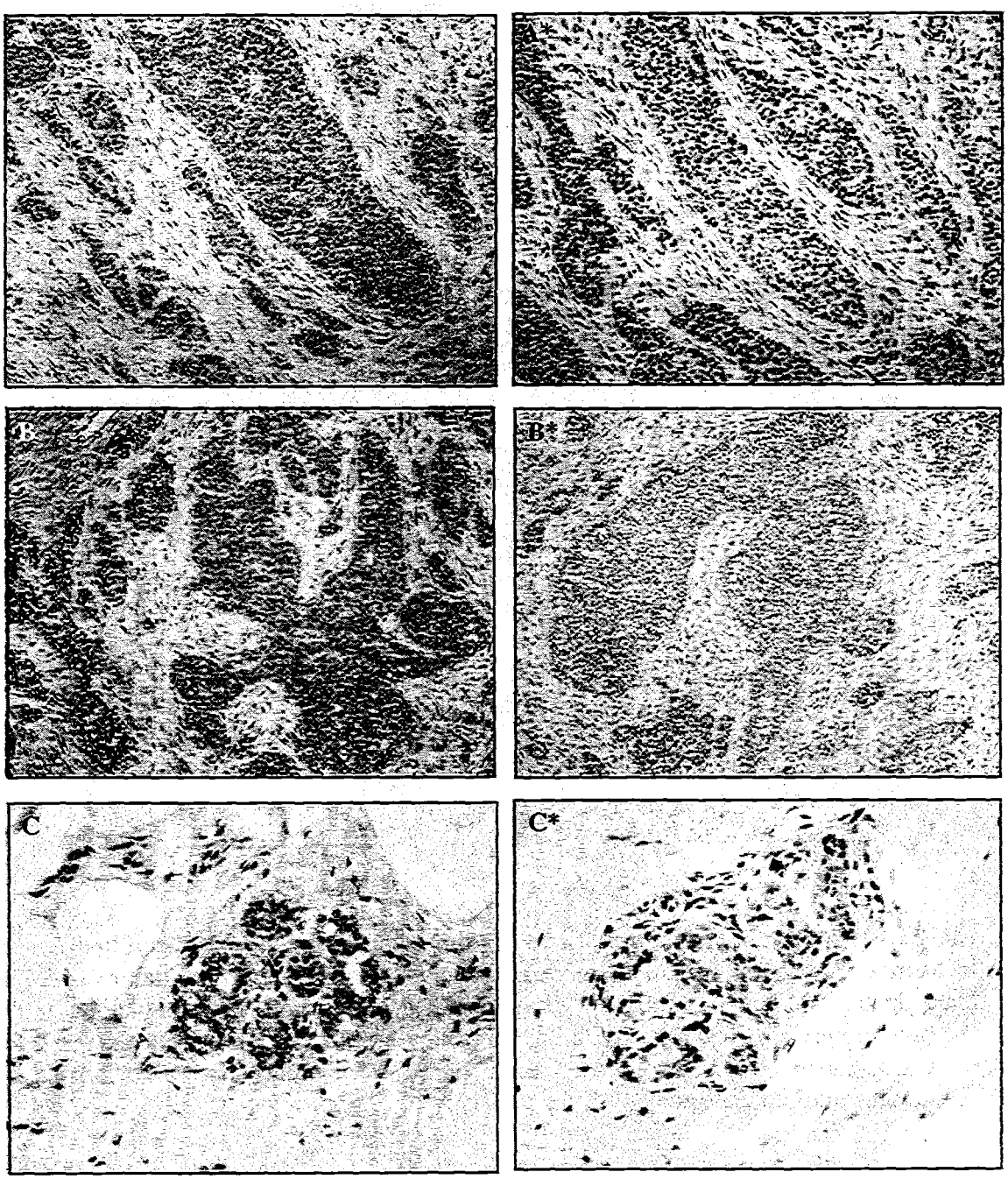


FIGURE 3.18: Anti-Pip92 antibodies immunostain tumour cells in sections of human tumours.

Tissue sections of the invasive mammary ductal carcinoma tumours dissected from three patients (**A, B, C**) were immunostained with anti-Pip92 polyclonal antibodies. Positive cells are stained in red (AEC staining). No staining was observed when preimmune serum was used (**A*, B*, C***).

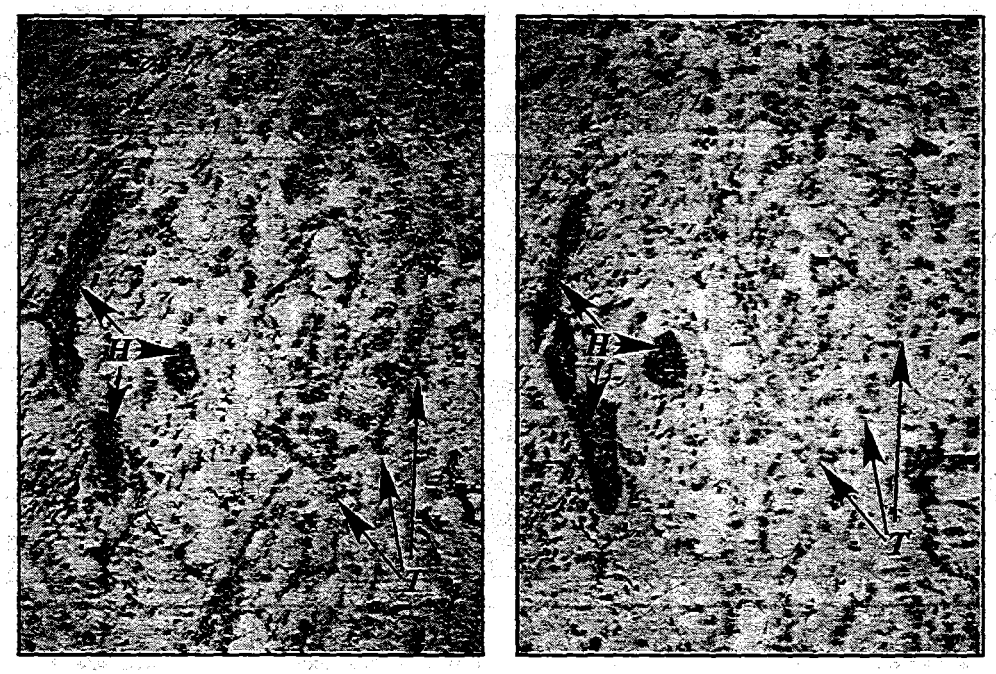


FIGURE 3.19: Pip92 antibodies immunostain only tumour cells but not healthy ducts in human mammary ductal carcinoma tumours. Tissue sections of the invasive mammary ductal carcinoma tumours were immunostained with anti-Pip92 polyclonal antibodies (**left panel**). Positive DAB staining (in yellow-brown) was observed only in tumour cells (**T**), but not in non-neoplastic healthy ducts (**H**). No staining was observed when preimmune antibodies were used (**right panel**).

PART THREE

1.3 Initial characterisation of NVM-1 protein, a novel metastasis-associated gene

1.3.1 ISOLATION OF THE FULL-LENGTH cDNA OF THE #147 CLONE AND IDENTIFICATION OF ITS TRANSLATION PRODUCT

The full-length cDNA of the #147 clone was isolated from an ASML cDNA library. Several independent clones selected during the ASML library screen were found to contain identical sequences. The length of the isolated cDNA is 1025 bp, in concordance with the estimated mRNA length of about 1.2 kb (see **Fig. 3.3**), assuming a contribution of 100-200 nucleotides in poly(A) (**Fig. 3.20**). The cDNA fragment of 151 bp length initially isolated from the 1AS/ASML SSH library spans the region from 424 bp until 575 bp and is situated in the middle of the full-length cDNA sequence.

Data base searches using this sequence performed against the nucleic acid and protein databases (BLAST, Altschul *et al.*, 1997) showed no significant similarities between the cDNA sequence and any other known genes. However, analysis of the ESTs (Expressed Sequenced Tags) database revealed numerous hits in rodent tissues such as embryo, thymus, lymph nodes and brain, strongly supporting the likelihood that this sequence is a part of a transcription unit. The gene was named *nvm-1* for the first novel metastasis-associated gene out of the Bsp73-1AS/ASML SSH library to be further studied. According to the BLAST search made against human ESTs, a similar sequence of a very high identity of 87% is expressed in human embryos as well as in a number of tumour tissues such as hepatocellular carcinoma, retinoblastoma and prostate adenocarcinoma.

In order to identify the open reading frame (ORF) of the *nvm-1* gene, the ORF Finder program (NCBI) was used. A single long open reading frame of 686 bases was identified encoding a 229 amino acids polypeptide (**Fig 3.20**). Upon completion of the human genome project, I discovered that the human homologue of the rat *nvm-1* gene (GeneBank accession number NM 024558) is located on chromosome 14. This finding allow me to investigate the promoter and the structure of the human *nvm-1* gene (*hnvm-1*).

```
1 GAAAGGCCTG TGGCGGGCAG GAGCTGGAAT ATCGTCCGCC GAGGAGCGGGA

  52 atgccgtccgtcgtggagccggaggtggaggatccgctctggagc
     M  P  S  V  V  E  P  E  V  E  D  P  L  W  S     15
  97 tttgtgcgggttctggagaagcgagatgggaccgcgcttcgactg
     F  V  R  V  L  E  K  R  D  G  T  A  L  R  L     30
 142 cagcagtacggctccggaggcgtgggttgcgttgtgtgggacgcc
     Q  Q  Y  G  S  G  G  V  G  C  V  V  W  D  A     45
 187 gccatcgtccttgccaaatacctggagacgccggggttttctggc
     A  I  V  L  A  K  Y  L  E  T  P  G  F  S  G     60
 232 gatggggcccacgctctgagtcggcgctcggtgctggaactgggt
     D  G  A  H  A  L  S  R  R  S  V  L  E  L  G     75
 277 tccggcacgggggccgtggggctcatggctgcgaccctcggggca
     S  G  T  G  A  V  G  L  M  A  A  T  L  G  A     90
 322 gatgttatagtcactgatcttgaggagctacaagacttgctgaaa
     D  V  I  V  T  D  L  E  E  L  Q  D  L  L  K    105
 367 atgaatattaatatgaacaagcatcttgtgactggttctgttcaa
     M  N  I  N  M  N  K  H  L  V  T  G  S  V  Q    120
 412 gccaaggtactgaaatggggtgatgacatagaagacttgatgtca
     A  K  V  L  K  W  G  D  D  I  E  D  L  M  S    135
 457 ccagactacatattaatggcggactgcatatactatgaggagtcc
     P  D  Y  I  L  M  A  D  C  I  Y  Y  E  E  S    150
 502 ttggaaccattgctgaaaacactaaaagatctcagcggatctgaa
     L  E  P  L  L  K  T  L  K  D  L  S  G  S  E    165
 547 acttctattatatgttgttacgaacaacgtacaatggggaaaaat
     T  S  I  I  C  C  Y  E  Q  R  T  M  G  K  N    180
 592 ccagaaattgagaagaaatattttgagctcctgcagctagacttt
     P  E  I  E  K  K  Y  F  E  L  L  Q  L  D  F    195
 637 gactttgaaaaaatccctttggacaaacatgatgaagaataccga
     D  F  E  K  I  P  L  D  K  H  D  E  E  Y  R    210
 682 agtgaagacattcatattctacacatcaggaagaaaaatccgaaa
     S  E  D  I  H  I  L  H  I  R  K  K  N  P  K    225
 727 ccgccatcatga 738
     P  P  S  *                                     229
739  AGCCCTTTTC CA
751  TTACCCGTGC CCCCAGCATC CTGGGTAAGG TAAAGCTGTG ACCACGCATG
801  CACACCGCAT GGAAGACTGC CTTACAGAGT TCTCCAGCAT GTCTTAGAGA
851  TCTGGCTCCT GGATGACCAC TGCCTTGGAC TGCCCACAGT GTGAAATGCC
901  TGCCTGCCCG TGGGCCTGAA ATCTAACTTA GGTTTACTTA GCTCAGCATC
951  AAGTTCTGTT TAAAAGAAAC ATTGACAATC ACTCAAATAA AGATTAATTT
1001 TGAGGACAAA AAAAAAAAAA AAAAA
```

FIGURE 3.20: Nucleotide and predicted protein sequence of the rat NVM-1. The full-length cDNA of the #147 clone was isolated from the cDNA library. Four out of six isolated clones were sequenced and showed the identical sequence presented above. The length of the cDNA sequence is 1025 bp, in concordance with the estimated mRNA length being about 1.2 kb assuming a contribution 100-200 amino acids in poly(A). The major open reading frame predicts a protein of 229 amino acids. The predicted amino acid sequence is shown bellow the open reading frame. Potential phosphorylation sites predicted by use of NetPhos program are marked by red-serine residues, blue tyrosine residues and green-threonine residues.

1.3.2 COMPUTER ANALYSIS OF THE PREDICTED *NVM-1* PROMOTER SEQUENCE REVEALED MULTIPLE BINDING SITES FOR DIFFERENT TRANSCRIPTION FACTORS

In order to investigate the promoter of the *nvm-1* gene, the genomic sequence of approximately 1.5 kb upstream of the initiating AUG of the *hnvm-1* gene was analysed. As the transcription start is hard to determine solely from the DNA sequence, a putative trancription initiation site was designated ca. 30 bp downstream of the perfect TATA box consensus found 270 bp up-stream of the initiating codon (see **Fig. 3.21**). However, the PromoterInspector computer program (Heinemyer *et al.*, 1998) for the prediction of promoter regions in mammalian genomic sequences identified the putative promoter sequence comprising 260 bp-DNA sequence immediately up-stream of the AUG (*i.e.* between potential TATA box and AUG). Therefore, this sequence was included into the initially chosen sequence up-stream of the initiator codon during the analysis for the identification of the putative transcription factor binding sites within the *hnvm-1* promoter sequence. For this prediction the TRANSFAC computer software (Kolchanov *et al.*, 2000) was used. As can be seen in **Figure 3.21** multiple putative transcription factor binding sites were identified in the *hnvm-1* promoter DNA sequence. It should be noted that only perfect matches *i.e.* the exact patterns of transcription factor binding sites found in *hnvm-1* promoter sequence have been included in this analysis. Besides multiple predicted SP-1 binding sites in the GC-rich sequence immediately upstream of the AUG, two NF-1 and an ATF binding sites consensi were found in the same region. Four glucocorticoid receptor elements (GREs) were predicted to be located within the analysed *hnvm-1* putative promoter, suggesting the possible involvement of steroid hormones in the regulation of *hnvm-1* gene expression. Whether these and other predicted transcription factor-binding sites are functional will be a subject of future investigations.

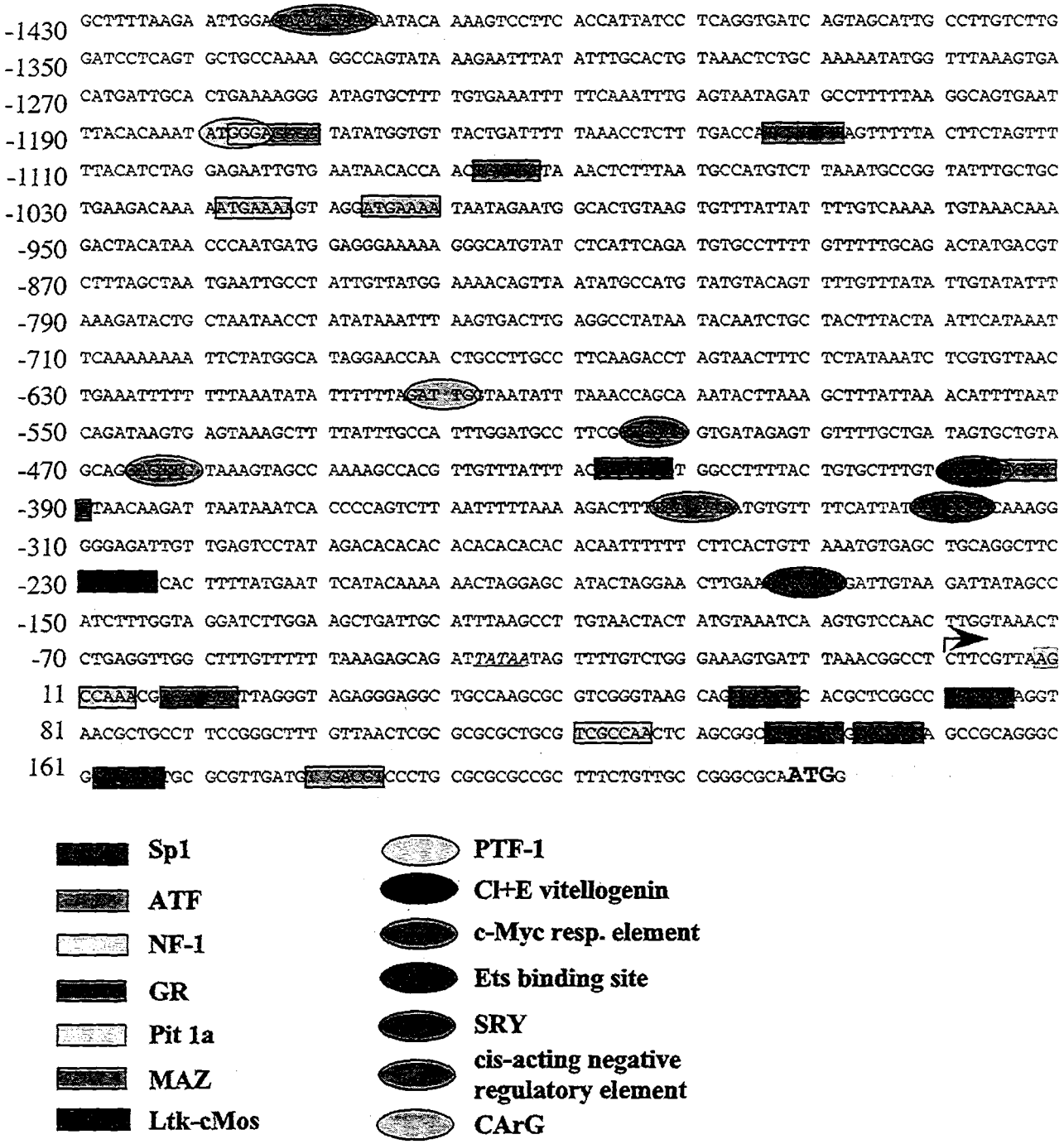


FIGURE 3.21 : Nucleotide sequence of the human NVM-1 promoter upstream of the initiating methionine codon (ATG). The TATA box consensus is shown in underlined italics. The putative transcription start site is indicated by an arrow. Putative transcription binding sites were identified using the TRANSFAC program. These sites are marked symbolically on the sequence with a key at the bottom to explain the symbols.

1.3.3 THE *NVM-1* GENE CONSISTS OF 6 EXONS, WHICH ARE ALTERNATIVELY SPLICED

The open reading frame of *hnvm-1* predicted from the genomic sequence encodes a putative protein of 144 amino acids (GenBank accession number XM017401). This sequence would be encoded by six exons (see **Fig. 3.22, 3.24**). The predicted size of the hNVM-1 protein is considerably shorter than the predicted amino acid sequence of the rat cDNA. Furthermore, alignment of the human and rat amino acid sequences showed that they diverge considerably at their C-termini (**Fig. 3.23**).

```
Rnvm1   1  MPSVVEPEVEDPLWSFVRVLEKRDGTALRLQQYGSGGVGCVVWDAAIVLAKYLETPGF 60
           |   +| +|||| |||||||||||| |||||| |||||||||||||||+|||||| |
Hnvm1   1  MADTLESSLEDPLRSFVRVLEKRDGTVLRLQQYSSGGVGCVVWDAAIVLSKYLETPEF 60

Rnvm1  61  SGDGAHALSRRSVLELGSGTGAVGLMAATLGADVIVTDLEELQDLLKMNINMNKHLVT 120
           |||||||||||||||||||||||||||||||||+|||||||||||||||||||||||||
Hnvm1   61  SGDGAHALSRRSVLELGSGTGAVGLMAATLGADVVVTDLEELQDLLKMNINMNKHLVT 120

Rnvm1  121 GSVQAKVLKWGDDIEDLMSPDYILMADCIYYEESLEPLLKTLKDLSGSETSIICCYEQ 180
           ||||||  +           +
Hnvm1  121 GSVQAKGGRNRRLSFSTRLHTDGRLHIL****************************** 144

Rnvm1  126 RTMGKNPEIEKKYFELLQLDFDFEKIPLDKHDEEYRSEDIHILHIRKKNPKPP     229

Hnvm1  126 **************************************************
```

FIGURE 3.23: Alignment of rat and human (NM024558) NVM-1 protein sequences. Sequence alignment of the rat (Rnvm1) and human (Hnvm1) NVM-1 proteins. The first string in each lane represents the rat NVM-1 sequence and the second string corresponds to the human NVM-1 protein sequence (GenBank accession number NM024558). Vertical bars designate identity, plus signs indicate conservative amino acid substitutions, stars were used to fill the space in the absence of amino acids.

Sequence alignment of rat and human cDNAs revealed the insertion of a short intervening DNA stretch (11 bp) into the rat sequence that was absent in the deduced human cDNA. The inclusion of this short intervening DNA stretch into the sequence leads to the shift of the reading frame and thus to a dramatic change in the protein size. A thorough analysis of *hnvm-1* gene led to the surprising finding of the "lost" sequence at the 5'-terminus of intron 2 of the *hnvm-1* gene. Thus, this 11-nucleotide sequence was included during mRNA splicing in rat ASML cells, whereas in the predicted human sequence this region seems to be skipped by the splicing machinery.

Close inspection of the alternatively spliced sequence suggests that alternative splice donor sites might be used (see **Fig. 3.22**). An important question to answer next was whether alternative splice donor sites are used or whether splicing is aberrant in ASML cells or the predicted human ORF is incorrect. The search of the public data

bases (BLAST) for the presence of different splice variants among ESTs allowed this question to be answered. It revealed the existence of both mRNA species in human tissues. Thus, the alternative splice donor sites really are used, and results in the production of mRNA species encoding proteins which differ dramatically in their length and C-terminal sequences (**Fig. 3.25**). Furthermore, according to the EST search the inclusion of the 11-nt. DNA stretch seems to be a characteristic feature of mRNAs derived from embryonic tissues, perhaps suggesting a requirement for the "long form" of the hNVM-1 protein in embryogenesis.

Further analysis of the *hnvm-1* gene revealed that the long form of the protein is encoded by 6 exons. The alternative splice donor sites are found at the 3'end of exon2 **(Fig 3.22, 3.25)**

Exon 1

.....ATGGCGGATACGCTGGAGTCCTCGCTGGAGGACCCACTGCGGAGCTT
M A D T L E S S L E D P L R S F
TGTGCGAGTTTTGGAGAAGCGGGATGGTACAGTGCTACGACTACAGCAGT
V R V L E K R D G T V L R L Q Q Y
ATAGCTCCGGTGGCGTGGGTTGCGTTGTGTGGGACGCTGCCATTGTCCTTT
S S G G V G C V V W D A A I V L S
CTAAATACCTGGAAACGCCCGAGTTTTCTGGCGACGGGGCCCACGCGCTG
K Y L E T P E F S G D G A H A L
AGCCGGCGGTCGGTGCTGGAGCTGGGTTCGGGCACCGGGGCCGTGGGGCT
S R R S V L E L G S G T G A V G L
CATGGCTGCTACCCTCGGgtaagagctggcgggcgggcgggatgtgggggcgcc.....
M A A T L G

SPLICE-VARIANT 1:

Exon 2

.....tcagtcgacgttattttatagGGCTGATGTTGTAGTCACCGATCTTGAGGAATTGC
A D V V V T D L E E L Q
AAGACTTGCTGAAGATGAATATTAATATGAACAAGCATCTTGTCACTGGT
D L L K M N I N M N K H L V T G
TCTGTTCAAGCCAAGgtactgaaatggtttgtatggcctttcag.....
S V Q A K

Exon 3

.....tgaaatatttgtttggcatttttaagGGGGGAAGAAATAGAAGGCTTTCCTTCTCCACC
G G R N R R L S F S T
CGACTTCATACTGATGGCCGACTGCATATACTATGAAGAGgtaagtatccagtgagc
R L H T D G R L H I L *
gaagtctcttcacttagaagatgaaccttattccatgaatt.....

SPLICE-VARIANT 2:

Exon 2

.....tcagtcgacgttattttatagGGCTGATGTTGTAGTCACCGATCTTGAGGAATTGC
A D V V V T D L E E L Q
AAGACTTGCTGAAGATGAATATTAATATGAACAAGCATCTTGTCACTGGT
D L L K M N I N M N K H L V T G
TCTGTTCAAGCCAAG<u>GTACTGAAATG</u>gtttgtatggcctttcag.....
S V Q A K V L K W

Exon 3

.....tgaaatatttgtttggcatttttaagGGGGGAAGAAATAGAAGGCTTTCCTTCTCCACC
G E E I E G F P S P P
CGACTTCATACTGATGGCCGACTGCATATACTATGAAGAGgtaagtatccagtgagc
D F I L M A D C I Y Y E E
gaagtctcttcacttagaagatgaaccttattccatgaatt.....

Exon 4

.....caaaaaatatattctttttaaatagTCTTTGGAGCCATTGCTGAAAACTCTAAAAGA
S L E P L L K T L K D
TATCAGCGGATTTGAAACTTGTATTATATGTTGTTATGAACAACGAACAAT
I S G F E T C I I C C Y E Q R T M
GGGGAAAAATCCAGAAATTGAGAAAAAATATTTTGAGgtaagtattaagtactctattat
G K N P E I E K K Y F E
ctttgtagtga.....

Exon 5

.....aatttgaagtatgtctcttttaaatttatagCTCCTTCAGCTAGATTTTGACTTTGAAAAAA
L L Q L D F D F E K I
TTCCTTTGGAAAAACATGATGAAGAGTATCGAAGTGAAGATATTCATATT
P L E K H D E E Y R S E D I H I
ATATACATCAGAAAGAAAAAATCGgtaaataacattctcagttcttcattctccaagg.....
I Y I R K K K S

Exon 6

.....aatcaatcttttaagtttccttttttttgttatagAAATTTCCATCGTGA.....
K F P S *

FIGURE 3.22: Exon sequences and flanking intron regions of the 6 exons of NVM-1 gene. Underlined is a stretch of 11 bp downstream of exon 2, which was found to be alternatively spliced. The alternative splice donor sites are marked in red. The predicted protein sequence is shown below the each exon.

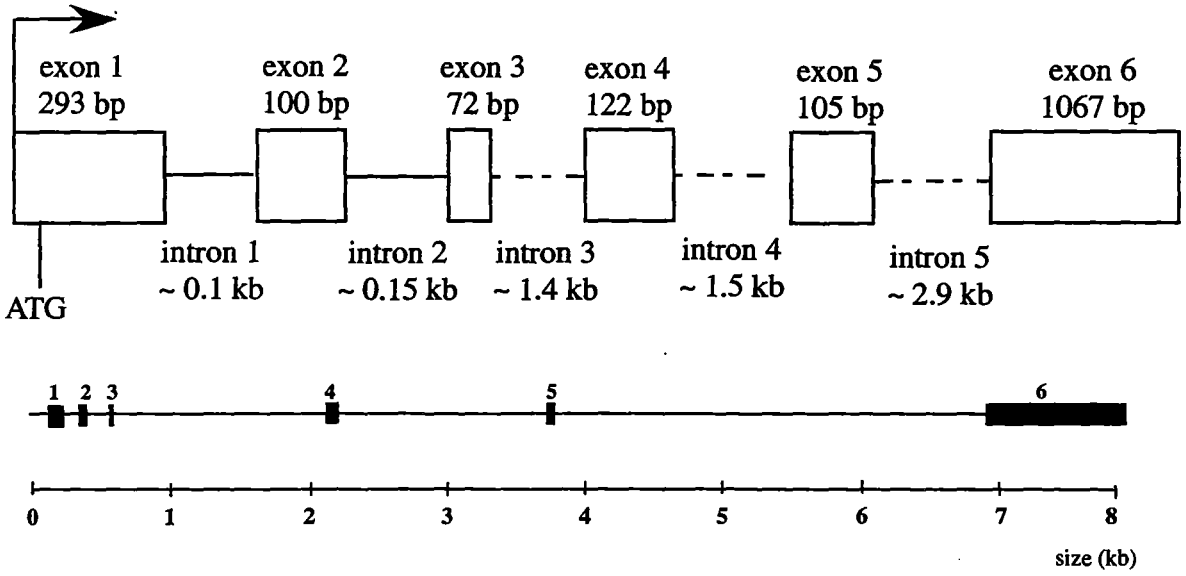


FIGURE 3.24: Schematic drawing of the exon organisation of the complete hNVM-1 gene. The entire hNVM-1 gene spans over 8 kb of genomic DNA (q14) and consists of 6 exons. Bottom panel represents the scheme of the gene drawn to scale. Exons are designated by black boxes and introns are shown as lines. The upper panel shows the more detail scheme of the hNVM-1 gene showing the size of each element.

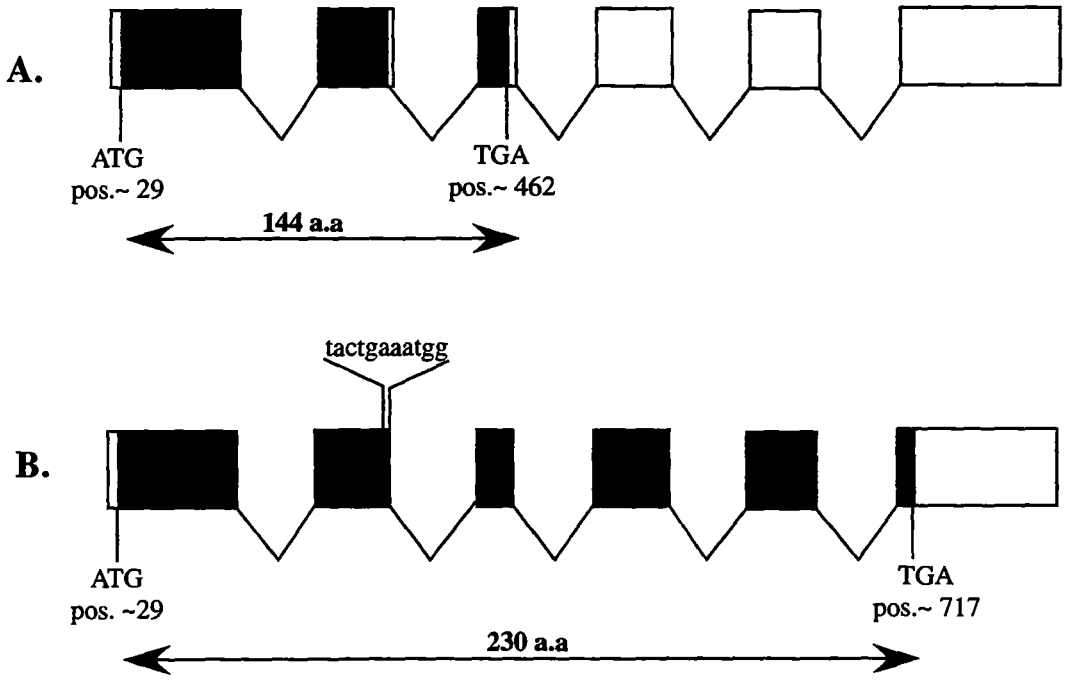


FIGURE 3.25: A schematic drawing of the alternative splicing of the hNVM-1 gene. 5' and 3'-untranslated regions of the hNVM-1 mRNA are shown as open boxes, the translated regions are designated as red boxes, the alternatively spliced intervening DNA stretch is represented as a pink box and the corresponding DNA sequence of this region is shown above the box.

1.3.4 DESCRIPTION OF THE PREDICTED RAT NVM-1 PROTEIN

The predicted protein has no significant homology to any other molecule. According to the amino acid sequence the predicted molecular weight of the long form is 26 kDa. The protein is rich in leucine, lysine glutamate and aspartate residues. A search of the database using ProDom, PRINTS and Pfam computational analysis programs did not identify any previously described motifs in the NVM-1 protein. The only program that recognised some protein signatures within the NVM-1 protein sequence was BLOCKS (Henikoff *et al.,* 1994). Several motifs including an adenylate cyclase class I motif, spanning the region 47-62 a.a of the NVM-1protein, and eukaryotic thiol (cysteine) proteases active sites (a.a. 77-87) motif were found by the BLOCKS database search. The significance of this prediction is hard to interpret as the position scores of these predictions are rather low. According to the PSORT analysis program (Nakai and Kanehisa, 1992) the protein is predicted to be cytoplasmic. Kyte and Doolittle analysis of the hydrophobic profile of the protein predict that NVM-1 is likely to be a soluble protein as most of its amino acid are hydrophilic.

1.3.5 TRANSLATION OF THE NVM-1 PROTEIN *IN VITRO*

To confirm that the predicted ORF observed in the full length of NVM-1 cDNA from the ASML library can be translated into protein, *in vitro* transcription and translation of NVM-1 protein was performed. Two independent cDNAs for the NVM-1 gene isolated from ASML cDNA library were used as templates for *in vitro* transcription and translation. The translated protein was ^{35}S radiolabelled and subjected to SDS PAGE. A protein of an expected molecular weight of about 25 kDa was observed (**Fig. 3.26**). No product was observed when the antisense promoter was used for transcription (see **Fig. 3.26** Sp6 lanes), confirming the correct choice of the cDNA strand encoding the NVM-1 protein.

1.3.6 THE EXPRESSION PROFILE OF NVM-1 IN RAT TISSUES

According to the results obtained from the computer search for human ESTs encoding hNVM-1, the gene is expressed in a variety of tissues (see § 3.10). To characterise the expression of rat *nvm-1* gene (*rnvm-1*), the tissue distribution of rNVM-1 RNA in different rat tissues was analysed by Northern hybridisation (see

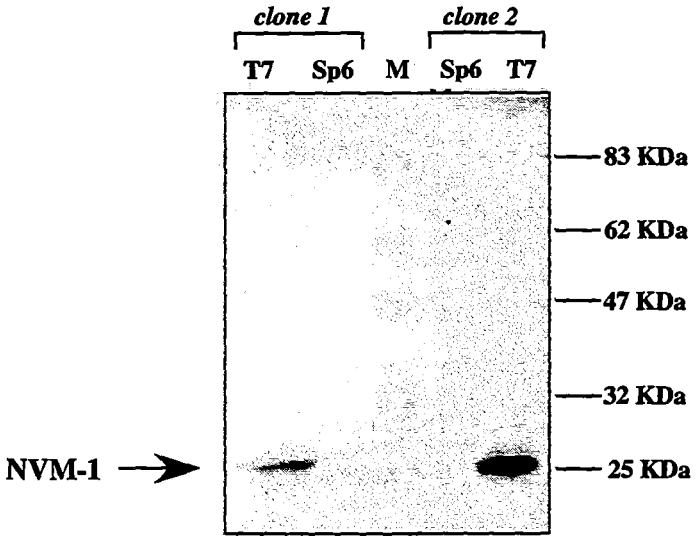


FIGURE 3.26: The *in vitro* transcription and translation of the NVM-1 protein. Two independent cDNAs for the *nvm-1* gene (**clone 1** and **clone 2**) isolated from the ASML cDNA library were used as templates for *in vitro* transcription/translation. the translated protein was ^{35}S-radiolabelled and subjected to SDS PAGE. A protein of the molecular weight of about 25 KDa was observed after radiography(the first and last lanes). No protein was produced when the reverse promoter (Sp6) was used for transcription (the second and fourth lanes): M-indicates the protein molecular weight marker.

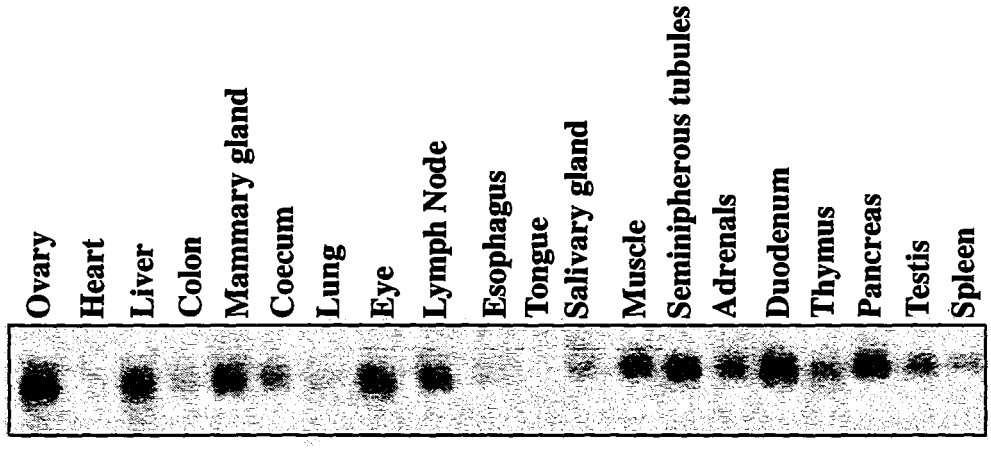


FIGURE 3.27: Tissue distribution of the NVM-1 mRNA in rat tissues. 10 µg of the total RNA obtained from different rat tissues was loaded into each lane of the agarose-formaldehyde gel. After electrophoresis the gel was subjected to Northern blotting to the membrane. The membrane was hybridised with the ^{32}P-radiolabelled NVM-1 cDNA.

Fig. 3.27). To this end, total RNAs isolated from various adult BD10 rat tissues were equally loaded into separate lanes of a formaldehyde/agarose gel. Each lane of the gel was loaded with 10µg of RNA. After electrophoretic resolving, the RNA was transferred to the filter by Northern blot transfer. The resulting membrane was hybridised with the ^{32}P-radiolabelled full-length NVM-1 cDNA. As can be seen in **Figure 3.27** NVM-1 is rather ubiquitously expressed. However, the level of *nvm-1* gene expression seems differ from tissue to tissue. Its mRNA is most abundant in ovary, seminiferous tubules, duodenum and pancreas whereas in heart, lung, tongue and spleen the *nvm-1* message was hardly detectable. Knowledge of NVM-1 expression pattern in different tissues might help in future predictions of the NVM-1 protein function.

1.3.7 THE NVM-1 EXPRESSION IS UP-REGULATED IN HUMAN TUMOURS IN COMPARISON TO NORMAL TISSUES

To address the question of whether NVM-1 expression correlates with human tumour progression *in vivo*, Southern hybridisation analysis was used. A cDNA array containing samples from different tumour types as well as corresponding normal tissues dissected from patients was used for hybridisation with a ^{33}P radiolabelled fragment of NVM-1 cDNA. The expression level of the NVM-1 gene was found to be elevated in tumours of stomach and uterus origin (**Fig. 3.28**). The fold of the signal enhancement was evaluated by use of the AIDA computer program. Depending on the patient, the level of NVM-1 expression in tumours was shown to be increased up to 7.8 fold in comparison to normal tissues, perhaps suggesting a functional role of the NVM-1 in the development of certain tumours. However, this type of analysis does not clarify which cells (tumour or the host stromal cells) up-regulate NVM-1 expression. On the other hand, it remains unclear if the observed up-regulation of the expression of the gene leads to NVM-1 protein synthesis or whether post-transcriptional events leave the protein level unaltered.

To answer these questions one would require an anti-NVM-1 antibody for use in immunohistochemical analysis of human tumour tissue. Moreover, such an antibody would of course be a valuable tool for further investigation of the NVM-1 protein function. I therefore generated anti-NVM-1 monoclonal antibodies as described below.

1.3.8 CREATION OF THE ANTI-NVM-1 MONOCLONAL ANTIBODIES

In order to make an antigen for use in generating anti-NVM-1 monoclonal antibodies, recombinant NVM-1 protein was generated using an *E. coli* BL21-CodonPlus-RIL bacterial cell system (*Stratagene*) that allows high amounts of heterologous protein to be generated. After purification, the NVM-1-His recombinant protein was used immunisation of mice. The lymphocytes of the immunised animals were subsequently fused to the murine myeloma cells to generate hybridoma clones, some of which were capable of production of specific anti-NVM-1 antibodies. Detailed description of all the steps in the generation of the anti-NVM-1 monoclonal antibodies is given below.

1.1.1.1 Purification of His-ΔNov1 protein

In order to direct the inducible synthesis of NVM-1 protein in *E. coli*, the carboxy-terminus of the NVM-1 protein (which due to its structure should be the most antigenic according to GCG program, Womble *et al.*, 2000) was cloned in frame with the polyhistidine tag under the T7 promoter in the PCRT7/NT vector (*Invitrogen*). Four hours after induction of protein synthesis, the ΔNVM-1-His protein was purified under denaturing conditions as described in Materials and Methods. As one can observe in **Fig. 3.29**, this method of purification yielded very high amounts of protein (0.5mg/ml in the case of the ΔNVM-1-His). To neutralise the protein solution and remove the contaminating salts fractions containing the ΔNVM-1-His protein were dialysed against PBS. This treatment caused the precipitation of most of the purified protein, but the supernatant still contained sufficient soluble protein for use in immunisations.

1.1.1.2 Animal immunisation, cell fusion and ELISA screen of hybridoma supernatants for anti-NVM-1 antibody production

Although a large variety of methods concerning monoclonal antibody production have been described, I chose one that has several advantages (initially described by Brodsky *et al.*, 1985). It uses a rapid immunisation protocol, requires comparatively little antigen and proved to be very efficient in my hands.

The immunisation of Balb/c mice and subsequent hybridoma formation was performed exactly as described in Materials and Methods. Once the individually

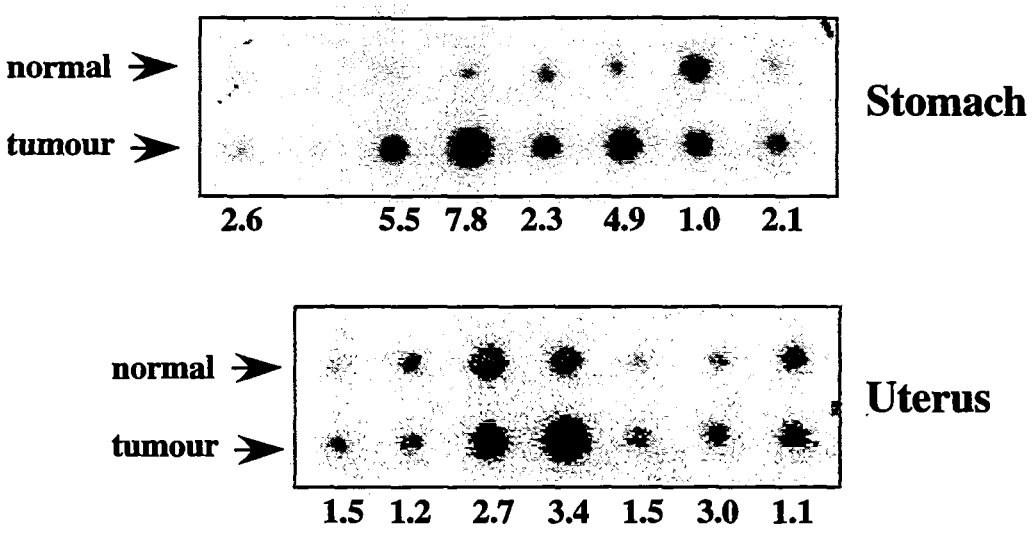


FIGURE 3.28: The NVM-1 expression is up-regulated in human tumours in comparison to normal tissues. A cDNA array containing samples from different types of tumours as well as corresponding normal tissues dissected from patients was used for hybridisation with a ^{33}P-radiolabelled fragment of the NVM-1 cDNA. Two portions of the resulting autoradiographed filter are shown. The fold of the signal enhancement in tumour tissues (the lower spots in each panels) in comparison to the normal tissues (the upper spots) are shown below the each evaluated dot.

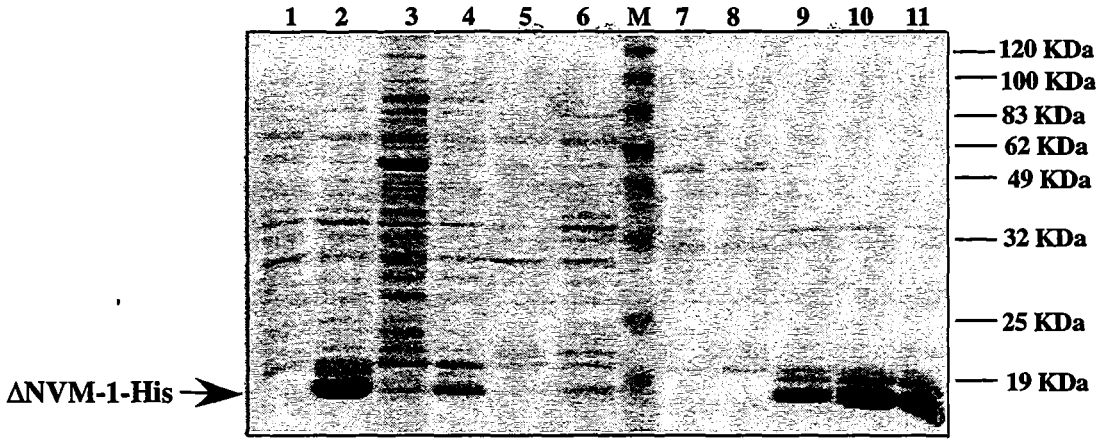


FIGURE 3.29: Purification of the Δ NVM-1-His protein. In order to direct the inducible expression of the NVM-1 antigen the C-terminus of the NVM-1 protein (a.a. 60-204) was cloned in frame with the polyhistidine tag under the T7 promoter. The ΔNVM1-His protein was purified under denaturing conditions by use of Ni-coupled agarose beads. Aliquots of the bacterial lysates or purified material representing each step of the purification procedure was loaded into separate lanes of SDS PAGE. To visualise the proteins the gel was stained in Coomassie Blue. Lanes **1,2**-represent bacterial lysates uninduced and induced for the NVM-1-His protein synthesis correspondingly; lane **3**-soluble proteins; lane 4-membrane bacterial fraction; lanes **5-8**-the unbound material and washes; lanes **9-11**: fractions of the purified protein, **M**-indicates the protein molecular weight marker.

growing hybridoma clones became sub-confluent, their supernatants were transferred onto antigen-coated ELISA plates to perform a primary screening of the growing clones. After this analysis, 70% (about 1400 single clones) were positive (data not shown). Those clones that gave the strongest signal on ELISA were rechecked on ELISA again and finally 200 positive clones, *i.e.* hybridomas producing anti-NVM-1 antibodies in high amounts, were selected for further screening of their specificity on Western blot analysis.

1.1.1.2.1 Screening of the anti-NVM-1 antibodies specificity on Western blot

The ELISA method was used predominantly for quantitative determination of the anti-NVM-1 antibody content in the tested hybridoma supernatants, and those antibodies which had the highest affinity to the antigen were also selected by this method. However, not all of these antibodies would necessarily be expected to work in other immunoassays such as Western blot analysis. Therefore, the reactivity of the antibodies in other immunoassays was checked further.

Supernatants that gave promising results in ELISA derived from 200 selected clones were further tested in Western blots. For this purpose, lysates obtained from ASML cells which produce high amount of endogenous NVM-1 (see **Fig 3.3**) were separated on SDS PAGE without slots. The resulted gel containing protein bands over the total width of the gel was subjected to Western blotting. The obtained membrane was cut into strips. Supernatants were applied separately to individual strips so that many samples were compared simultaneously (see Fig. **3.30**). This technique is often called a "strip-blot" method. In total 31 clones out of 200 tested showed a single specific signal of an appropriate size in this assay.

Since in this quick Western blot analysis it is impossible to include a negative control into each screen, further analysis of those supernatants showing a single specific signal on Strip-blots was required. An example of this secondary Western blot screen is shown on **Fig. 3.31**. In this type of experiment a conventional SDS PAGE was run where lysates of 1AS cells producing low amounts of the NVM-1 protein and ASML cells expressing high amounts of the antigen were loaded into adjacent slots. To ensure equal loading, the protein contents of both lysates were measured and the same amounts of both ASML and 1AS derived proteins were loaded. After Western blot transfer the membrane was immunostained with anti-

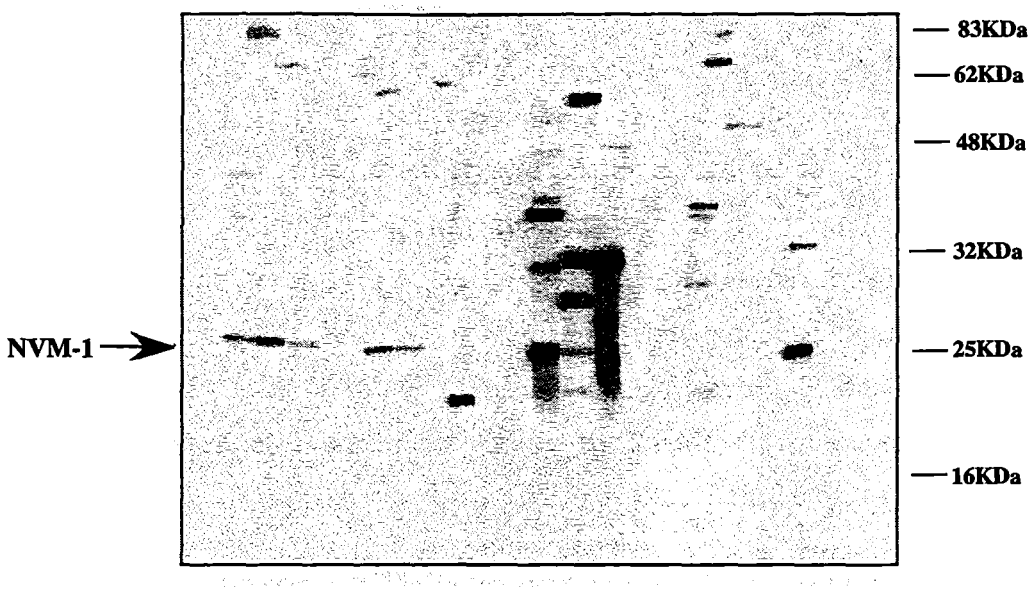


FIGURE 3.30: Screen for the specificity of anti-NVM-1 monoclonal antibodies in Western blot analysis. Anti-NVM-1 monoclonal antibodies derived from different hybridoma clones were used for Western blot analysis of lysates obtained from ASML cells expressing high amounts of the NVM-1 protein. The blot was cut into strips and each strip was probed with an individual supernatant. Most of the hybridoma supernatants on the left side of the shown blot contain specific antibodies against NVM-1, giving a single band of the right size (about 25 kDa) in Western blot analysis.

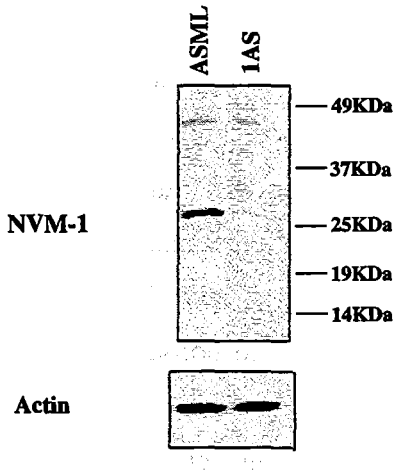


FIGURE 3.31: Anti-NVM-1 monoclonal antibodies detect the NVM-1 protein in Western blot analysis. Anti-NVM-1 monoclonal antibodies were used for Western blot analysis of lysates of ASML and 1AS cell lines expressing high and low levels of NVM-1 protein respectively (**upper panel**). The blot was striped and re-probed with anti-actin polyclonal antibodies to ensure equal loading (**bottom**).

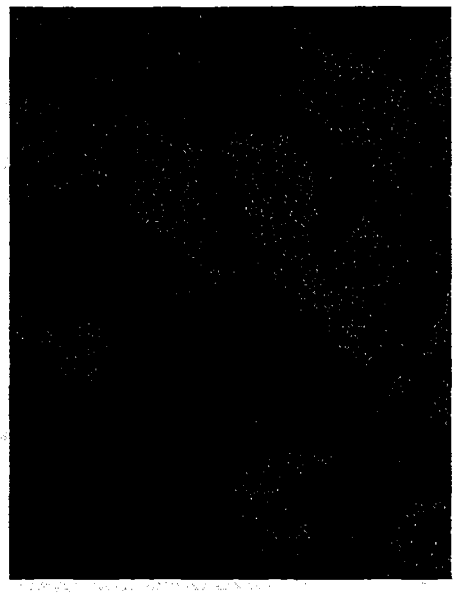
NVM-1 antibodies already shown to give a single specific band in the previous strip-blot assay. As can be seen on the **Fig. 3.31** these antibodies show a weak signal with 1AS cells and a strong signal in ASML cell lysate-containing lane. To confirm the equal transfer to the membrane the same membrane that was used for the immunostaining with anti-NVM-1 antibodies was probed with anti-actin antibodies, a conventional normalisation marker protein equally expressed in many tissues. Acting staining demonstrated equal loading for both cell lines. These data confirm the differential expression of NVM-1 observed in Northern blot analysis (see **Fig 3.3**) and also the validity of the SSH.

1.1.9 INTRACELLULAR LOCALISATION OF NVM-1 PROTEIN

NVM-1 is an absolutely novel protein. My experiments suggest it might have a role in tumour progression, metastasis and cell motility. Thus, the knowledge of its specific functions in the normal and especially tumour cells would be of a great interest and may be therapeutically relevant. As a starting point in this direction, I set out to determine the intracellular localisation of the NVM-1 protein.

To analyse the NVM-1 sub-cellular localisation, MTLN-3 cells expressing high levels of the NVM-1 protein (as judged from the mRNA expression experiments, see **Fig. 3.3**) were investigated. This cell line rather than the ASML that also expresses high amount of the NVM-1 protein was chosen for these experiments because of its adhesive abilities. The MTLN-3 cells, unlike the ASML cells used for our previous experiments with anti-NVM-1 antibodies, are able to adhere and spread. It was therefore much easier to study microscopically the intracellular location of the NVM-1 protein in these cells in comparison to non-adhesive non-spreading cells. As the NVM-1 protein has been already predicted to be cytoplasmic according to its polypeptide sequence, a permeabilisation step was used before the application of the anti-NVM-1 antibodies. The cells were immunostained with the selected anti-NVM-1 monoclonal antibody (#185) exactly as described in Materials and Methods. The sub-cellular localisation of the NVM-1 protein was visualised by use of fluorescently-labelled secondary anti-mouse antibodies and subsequent microscopical assessment. As is shown in **Fig. 3.32A**, the NVM-1 protein was localised to the cytoplasm of the investigated cells, confirming the earlier predictions.

A.



B.

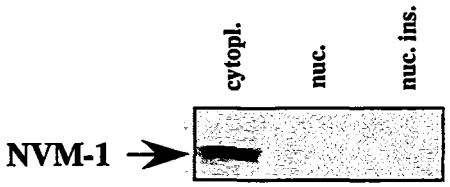


FIGURE 3.32: NVM-1 is localised to the cytoplasm. MTLN-3 cells were immunostained with the anti-NVM-1 monoclonal antibodies. The subsequent visualisation of the positive cells by use of fluorescently labelled anti-mouse antibodies revealed the cytoplasmic localisation of the NVM-1 protein (**A**). Similar results were obtained upon fractionation of ASML cells into cytoplasmic (**cytopl**), nuclear (**nuc**) and nuclear insoluble (**nuc ins**) fractions. An aliquot of each fractions was loaded into separate lanes of SDS PAGE, electrophoretically resolved and transferred to a membrane. The subsequent probing of the membrane with anti-NVM-1 monoclonal antibodies showed a protein band of the expected size (about 25 kDa) exclusively in the cytoplasmic fraction (**B**).

The cytoplasmic localisation of the protein was also shown by cellular fractionation assays. In these experiments lysed ASML cells were subdivided into three fractions (see **Fig 3.32B**): cytoplasmic (containing soluble cytoplasmic proteins), nuclear (comprising nuclear soluble proteins) and nuclear insoluble (containing nuclear insoluble fraction). An aliquot of each fraction was loaded into separate lanes of SDS PAGE. The gel was subjected to Western blotting and the membrane was immunostained with anti-NVM-1 monoclonal antibodies. As can be seen on the **Fig. 3.32B** the NVM-1 protein was observed exclusively in the cytoplasmic fraction. These results are in concordance with the immunofluorescence staining (**Fig 3.32A**) proving the cytoplasmic localisation of the NVM-1.

1.1.10 ANTI-NVM-1 ANTIBODIES IMMUNOSTAIN THE RAT MTLN-3 TUMOURS

One of the most important reasons for the generation of anti-NVM-1 monoclonal antibodies was their utilisation in immunostaining of tumour tissues in order to identify the significance of the differential status of the NVM-1 protein in tumour progression and metastasis. To define the conditions for use of the anti-NVM-1 monoclonal antibodies for the immunohistological analysis of tumour tissues, one would need a tumour type where the NVM-1 protein is expected to be overexpressed. For my experiments the rat tumours produced by subcutaneous injections of MTLN-3 cell line were therefore chosen. These cells have elevated expression of the NVM-1 mRNA (see **Fig. 3.3, 3.32**) and therefore high levels of the NVM-1 protein is anticipated.

Sections of formaldehyde-fixed and paraffin-embedded MTLN-3 tumours were immunostained with anti-NVM-1 monoclonal antibodies. As can be seen in **Fig. 3.33** positive signal was observed in tumour cells but not in non-neoplastic tissues. The cytoplasm of the tumour cells was stained, again confirming the cytoplasmic location of the protein. It should be noted that the staining was observed mostly at the tumour periphery where most of the living tumour cells are localised. The presence of high amounts of the NVM-1 protein in the margins of tumours perhaps speaks for a function for this protein in cell invasion and metastasis.

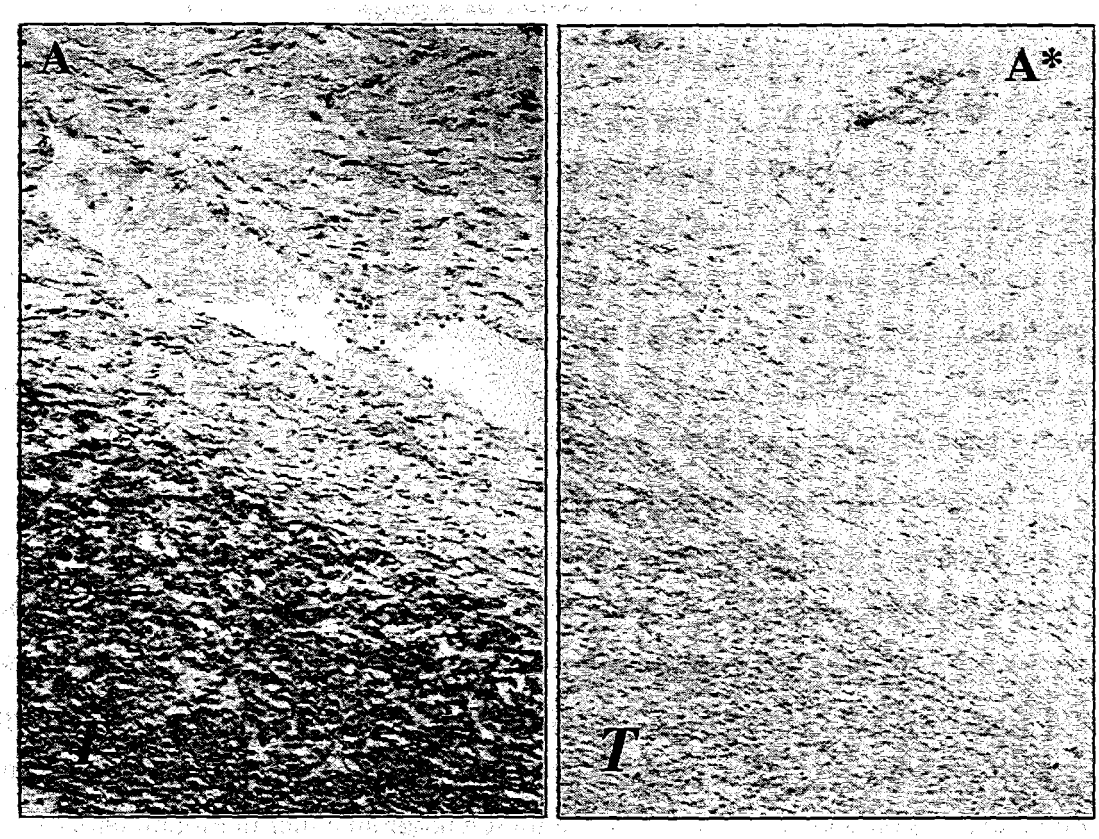


FIGURE 3.33: Anti-NVM-1 monoclonal antibodies immunostain rat MTLN3 tumours. MTLN3 tumours were generated by subcutaneous injections of MTLN-3 rat mammary adenocarcinoma cells into syngeneic animals. Sections of formaldehyde-fixed and paraffin-embedded tumours were immunostained with anti-NVM-1(#185) monoclonal antibodies. Positive staining (in yellow-red, NovaRed) was observed in tumour cells (**T**) but not in normal tissues (**A**) . No staining was detected when only secondary antibodies were used (**A***).

4 DISCUSSION

The ability to analyse the expression of a large number of genes has been revolutionising many areas of biology and medicine. Novel technologies such as SAGE, SSH or the variety of microarray-based approaches will certainly have a major impact on cancer research, where it may potentially lead to the identification of the new therapeutic targets. These approaches often identify tens or hundreds of genes which are differentially expressed. However, only a few follow-up studies have been published since the era of differential expression screenings began, which have attempted to investigate the functional significance of such differential expression. This "functional genomics" remains a major challenge for modern biology.

My work is an example of such a follow-up study, in which the large quantity of genes initially identified by SSH has been narrowed down by further correlative studies and finally led to the selection of two genes (namely NVM-1 and Pip92) that are candidates for having a functional involvement in metastasis. Both NVM-1 and Pip92 genes have been selected for further characterisation because of the up-regulation of their expression during cell migration. The up-regulation of NVM-1 and Pip92 expression upon the onset of the migration program either in inflammatory cells or metastatic cell lines strongly suggested their possible participation in a common migration program utilised by motile cells.

Experimental evidence for the direct involvement of Pip92 in the increase of the invasive abilities of 1AS cells was obtained upon over-expression of the Pip92 protein in these cells, which resulted in the enhanced invasion of these cells *in vitro* (**Fig. 3.15**). From these experiments, one could hypothesise that quantitative change of the Pip92 in 1AS cells might trigger a certain cellular mechanism leading to the acquisition of invasive properties by tumour cells.

How might Pip92 influence tumour metastasis and motility? Since the time of Pip92 isolation, because of its rapid induction and rapid degradation coupled with the potential of rapid regulation by phosphorylation, the protein has always been considered to play a regulatory role in the cell. When Coleclough *et al.,* (1990) first isolated the murine Pip92 they were able to demonstrate patches of similarity between Pip92 and JunD cDNAs (**Fig. 4.1A**). The DNA region of JunD homologous to Pip92 comprises a part of its basic region responsible for JunD DNA binding. In addition,

while performing search for known protein motifs within the Pip92 protein sequence, I found some patches of homology between Pip92 and the HIV integrase DNA-binding domain. Both findings may hint at the potential ability of Pip92 to bind DNA as a part of its proposed regulatory function. The ProDom computer program did not identify any known DNA-binding motifs in the Pip92 protein. However, the amino terminus of the protein, which is absolutely identical in both human, mouse and rat proteins according to predictions may form helix-turn-helix structure (see **Fig. 1.4B**). A similar protein conformation has been found to be responsible for the DNA-binding properties of many transcription factors (reviewed by Suzuki, 1994). Thus, one can hypothesise that Pip92 may function as a transcription factor.

A.

```
Pip92
#57                                                                    #122
PESPPSRRALDPRL  HPPREAEVAVE        VASPEAQPP  EPMDTQEEVLRVQETPALCDPPPARVSRKR
|  ||+  | | |    || ||  | +  |      | |   | |   | | | | |     |   |     | ++|||
PFPPPPG ALGPPPPPHPPR LAALKDEPQTVPDVPSFGDSPPLSPIDMDTQERIKA  ERKRLRNRLAANATRKR
#212                                                                   #270
JunD
```

B.

```
Pip92
#114                                  #143
PARVSR KRRSSSDLS  DSSDAGLVPSKKARL
| | ++   | +   +     |+||   +||  +| |++
PAKLLWKGEGAVVIQDNSDIKVVPRRKAKI
#20                                   #49
HIV-1 integrase
```

FIGURE 4.1: Protein sequence similarities between Pip92 and two DNA-binding protens (JunD and HIV integrase).
The amino acid sequence of the Pip92 (upper line) compared to the JunD (**A**) or HIV integrase (**B**) polypeptide sequences (bottom line in each case). Vertical bars indicate identity, plus signs indicate conservative substitutions.

Contrary to the putative role of Pip92 as a transcription factor, Charles *et al.* (1990) in their study on intracellular localisation of the Pip92 protein found it to be localised exclusively to the cytoplasm of both Balb/c 3T3 and PC12 cells. The obvious discrepancy between the latter finding and the presence of two nuclear localisation signals within the Pip92 sequence (**Fig. 1.3**) coupled with the intriguing suggestion of possible Pip92 DNA-binding ability compelled me to start my own investigations on Pip92 intracellular localisation. To my surprise, ectopically expressed Pip92 protein was located exclusively in the nuclei of the transfected COS7

cells (**Fig. 3.6A**). Similar experiments with NIH3T3 cells confirmed these results, showing predominantly nuclear location of the protein (**Fig. 3.11**). Moreover, the endogenous Pip92 protein whose expression was induced in NIH3T3 cells by serum stimulation showed nuclear and cytoplasmic localisation, with most of the signal coming from the nuclei (**Fig. 3.13**). Interestingly, in the unstimulated NIH3T3 cells in which the expression of the Pip92 is low, the Pip92 protein was found only in cytoplasm of the cells immunostained with anti-Pip92 polyclonal antibodies. This may speak for the possible nuclear efflux of the Pip92 protein upon serum stimulation. The possibility that the protein may shuttle between different cellular compartments might indicate functional polymorphism; *i.e.* its ability to play distinct roles in the nucleus and in the cytoplasm. As follows from the experiments with NIH3T3 cells, the nuclear localisation of the Pip92 is observed only upon overexpression of the protein. This might suggest the presence of a cytoplasmic Pip92-anchoring protein that sequesters Pip92 in the cytoplasm. When Pip92 is ectopically expressed, the amount of the anchoring protein becomes insufficient to retain the excess of Pip92 in the cytoplasm, which leads to the translocation of the Pip92 protein to the nucleus. The presence of two nuclear localisation signals (NLS) within the Pip92 sequence argues for active translocation of Pip92 to the nucleus. Such a notion is supported by the fact that in COS7 cells, Pip92 was found to be located exclusively in the nucleus. NLS are known targets for the nuclear transport proteins (such as importin family receptors) that upon binding to their import substrates mediate their nuclear transport (Görlich, 1998). Pip92 protein mutants with modified nuclear localisation signals would clarify the mechanism of Pip92 translocation to the nucleus.

What is the spatial status of the Pip92 protein in cancer cells? The analysis of Pip92 protein in human mammary ductal carcinoma tumours showed that the protein is localised to the cytoplasm of the tumour cells (see **Fig. 3.18, 3.19**). However, only a limited number of samples have been analysed and the histories of the patients were not available, furthermore haematoxylin co-staining would tend to obscure meak nuclear signals in patient material. Thus, based on the current data no conclusions on the involvement of the particular nuclear function of Pip92 in the metastasis could be drawn. It will be interesting to look at how the nuclear localisation of Pip92 correlates with the patients history (*i.e.* with the metastatic status of the analysed tumours). An interesting experiment to be performed is the investigation of the sub-cellular

localisation of the Pip92 protein in tumour cell lines differing in their metastatic abilities. Will the nuclear localisation of the protein correlate with the metastatic potential of these cells?

Some of my experiments, however, speak against the nuclear function of Pip92 in the initiation of the migration program. For example, in 1AS cells where the over-expression of Pip92 leads to the enhanced motility of the transfected cells (see **Fig. 3.15**) only a small portion of the protein is located to the nucleus, and the majority of the ectopically expressed protein is localised to the cytoplasm (see **Fig. 3.13C,D**). On the other hand, by analogy with transcription factors, even a small amount of nuclear protein may be sufficient to induce changes in gene expression.

To check for the ability of Pip92 to modulate the transcription of other proteins, analysis of transcriptomes of cells overexpressing either Pip92 or the empty vector was performed. This analysis yielded a number of differentially expressed genes. Besides 1AS cells, I also compared the differential gene expression in 293 cells transfected with either human homologue of Pip92 (ETR101) or the empty vector. This experiment identified a number of genes other than those found in 1AS cell system whose expression is up-regulated only in ETR101 transfectants (data not shown) although the differential expression of these genes has not yet been verified by Northern hybridisation analysis. It is highly likely that the list of genes regulated by Pip92 is perhaps incomplete and might be broadened by use of other libraries or approaches, for example by SSH.

Another question concerns the cellular level at which Pip92 exerts its action. From the patches of similarity between Pip92 and DNA-binding domains of the HIV integrase and the JunD proteins, one can imagine the possibility for Pip92 to directly bind DNA. Band shift analysis with the promoters of the Pip92 regulated genes may partially answer this question. However, my preliminary experiments for searching the specific DNA binding sites for Pip92 were not successful, possibly indicating on the absence of direct Pip92-DNA interaction or the requirement for additional cofactor. Alternatively, Pip92 may indirectly regulate gene transcription, perhaps by modulating the activity of DNA-binding transcription factors.

It is interesting to investigate the Pip92 downstream effectors found in this screen. The most interesting candidate for a further analysis is the osteopontin. This protein is believed to be directly involved in cell migration (Oates *et al.*, 1997). Could it be that the osteopontin, whose expression is induced by Pip92, is responsible for the

enhanced migration of 1AS-Pip92 transfectants? Future attempts to interfere with 1AS cell migration by use of blocking antibodies against osteopontin, for example, should help to answer this question.

Overexpression of Pip92 in 1AS cells enhances their invasive ability. Will this change lead to the acquisition of the metastatic phenotype of the transfected 1AS cells *in vivo*? I am currently evaluating this question. However, my preliminary data suggest that the enhanced invasive ability of 1AS cells is not sufficient to induce complete metastatic transformation of 1AS cells. It is to be expected: the migratory ability is only a single property among many others that tumour cells need to acquire in order to metastasise (reviewed by Sleeman, 2000). Opposite experiments with interference of the metastatic phenotype by targeting a component of the metastatic program (migration ability) might destroy the whole system and prevent the metastasis. Therefore, the ablation of the Pip92 action by antisense or dominant —negative approaches in highly metastatic ASML cells has more chances to affect their metastatic spread *in vivo*. Such approaches would be the subject of future investigations.

A simplified current model for Pip92 action is shown in **Fig. 4.2** and can be expressed in one sentence: Pip92 is able to induce the expression of genes, whose products lead to the acquisition of the migratory phenotype by tumour cells.

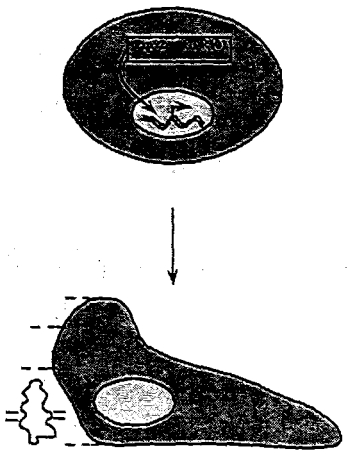


FIGURE 4.2: A model for the functional role of Pip92 in the cell.
Upon overexpression the Pip92 protein translocates to the nucleus and regulates the expression of genes whose products are responsible for the changes in cellular motility properties.

The second line of my project concerned the isolation and characterisation of the novel metastasis-associated gene, which was named *nvm-1*. The full-length DNA sequence of the *nvm-1* was isolated from an ASML cDNA library. Correlation of the *nvm-1* expression with the metastatic potential of tumour cells of different tumour models, together with elevated expression in mobilised macrophages in comparison to resident macrophages made it an interesting candidate for further elucidation of the molecular events in the metastatic transformation of tumour cells.

Upon the completion of the human genome sequencing project it became apparent that the human *hnvm-1* gene is localised to the chromosome 14. The DNA sequence analysis of the *hnvm-1* gene allowed for characterisation of the gene structure and description of the putative *nvm-1* promoter. As it is difficult to determine the transcription start without experimental evidence, the transcription initiation site was deduced empirically and located 20-30 bp downstream of the found TATA box consensus. To identify the exact transcription start point one would need to perform primer extension experiments.

In my screenings, the *nvm-1* showed differential expression in metastatic and non-metastatic cells as well as in mobilised and resident macrophages (**Fig. 3.3**). Moreover, the expression of human *nvm-1* was found to be up-regulated in tissue samples from several tumours in comparison to normal tissues (**Fig. 3.28**). As an initial attempt to investigate the molecular basis of the up-regulated expression of NVM-1 in cancer tissues and metastatic cell lines, I performed an analysis of the putative *nvm-1* promoter. The DNA sequence of 1.5 kb up-stream of the initiating codon was designated as a putative promoter sequence. A computer search for potential transcription factor-binding sites within the selected 5' flanking region was performed by use of the TRANSFAC program that revealed several probable promoter elements. Putative regulatory sequences, which include several SP-1 sites, four GR binding sites, two NF-1 sites and an ATF-1 site were found in the region. Thus, these transcription factors may play a role in the up-regulation of NVM-1 expression observed in the highly metastatic cell lines. Understanding of the mechanisms which regulate the expression of *nvm-1* may therefore provide insight into the biologic consequences of the augmented expression of this gene during metastasis. Future experiments using nested deletion mutants of the putative *nvm-1*

promoter fused to reporter genes will help to identify which region of the analysed 5'-flanking genomic DNA has the functional promoter activity.

A 600 bp difference between human and rat NVM-1 mRNA sequences estimated by Northern blot analysis (**Fig. 3.3**) is caused by the differences in their 3'-UTR lengths (data not shown). This could be a possible consequence of the use of alternative sites of pre-mRNA cleavage/polyadenylation in different species. The importance of an additional 600 bp in the human 3'-flanking region is unclear. The presence of several AU-rich motifs in hNVM-1 mRNA may influence the stability of hNVM-1 mRNA.

Comparison of the human genomic sequence with the predicted hNVM-1 cDNA sequence identified 6 exons and 5 introns spanning a region longer than 8 kb of the DNA sequence of chromosome 14. The sizes of the exons range from 72 bp (exon 3) to >1000 (exon 6, which also contains the 3'-UTR). The sequence of the splice donor and acceptor sites follows the GT/AG rule (Mount *et al.*, 1982). A thorough analysis of the hNVM-1 exon2/intron2 junction revealed two splice donor sites separated by 11 nucleotides. According to published ESTs, both sites are alternatively utilised by the splice machinery during the rNVM-1 and hNVM-1 mRNA processing. The difference between the two transcripts derived from alternative splice-site usage is hard to identify by common Northern blot analysis. Therefore, it is difficult to judge which particular form is produced in the human prostate adenocarcinoma cell lines and rat cell lines used for the Northern hybridisation analysis shown on **Fig. 3.3**. However, because the alternative splice-site usage results in proteins of different sizes (229 amino acids as opposed to 144 amino acids), the different rNVM-1 and hNVM-1 isoforms would be perfectly detectable by Western blot analysis. In the experiment shown in **Fig. 3.31**, it appears that ASML cells express only the long form of rNVM-1.

Future experiments aimed to determine the size of the NVM-1 protein in different tissues will show the possible correlation of the involvement of one or another splice variant in specific cellular processes. Identification of tumour-associated specific isoforms of several proteins such as CD44, Mox 1 or FGFR2 has been reported during the last decade (Arch *et al.*, 1992; Xo *et al.*, 1999; Carstens *et al.*, 2000). As NVM-1 is quite ubiquitously expressed in normal tissues (see **Fig. 3.27**), one might predict that the different isoforms could be utilised by normal and tumour cells. Isolation of the long form of the protein from the highly metastatic

ASML cell line might speak for the requirement of this form in tumour progression and metastasis. Another hint of the possible preference of the long form of hNVM-1 protein in tumour cells may be extrapolated from the analysis of published ESTs. The presence of the intervening 11bp stretch in all ESTs derived from embryonic tissues speaks for the possible need of the long form of NVM-1 during embryogenesis. In the context of cell proliferation and cell migration, embryogenesis is often compared to tumorigenesis and metastasis and the presence of shared molecules acting in a similar way during tumour progression and embryogenesis supports the validity of this comparison (see Introduction).

On the basis of my current data, I cannot draw any firm conclusions about the functional role of the NVM-1. It is still too early to envisage any models on the NVM-1 protein function. No known protein motifs or domains have been found within the NVM-1 polypeptide by use of various molecular biology search engines. Based on predicted protein solubility, cytoplasmic localisation and because of the presence of the potential phosphorylation sites for PKC, cAMP and casein kinases, one could speculate that it might be a cytoskeleton-associated regulatory protein functioning in migration, perhaps mediating cytoskeleton rearrangement in response to specific signalling, for example. However, this is a purely speculative prediction and I believe that the data obtained during my work will provide the critical framework for a more precise delineation of a role of NVM-1 in cancer.

The primary aim of this work to find the novel genes associated with metastasis has been achieved. As Pip92 has never been associated with metastasis and NVM-1 is a totally novel gene, the data presented in this thesis for the first time provide experimental evidence on possible involvement of their gene products in tumour cell migration and metastasis. Both of the genes which have been characterised in this work could also prove to serve as new tumour markers or targets for therapeutic intervention and thus be potentially used in the diagnosis and therapy of human cancer.

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