THE UNIVERSITY OF MANITOBA

GENETIC AND EPIGENETIC REGULATION OF THE METASTATIC PHENOTYPE

by

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To Dermot Egan and Mavis Egan, for everything.

OPTIMISM

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"Its only Rock and Roll but I like it!"

ABSTRACT

The metastatic phenotype is a complex set of properties which results in tumor dissemination throughout a host organism. Experimental systems have been set up to analyze genetic and epigenetic regulation of this phenotype in both 10T1/2 and NIH-3T3 murine fibroblast cell lines. Transfection of an activated *ras*-oncogene into 10T1/2 cells can result in metastatic transformation. Analysis of *ras* expression in these cells as well as in progression variants of a non-metastatic cell line revealed a correction between oncogene expression and metastatic potential. Pre-induction of p21 synthesis in NIH-3T3 line 433, which contains v-H-*ras* under transcriptional control of the glucocorticoid sensitive MMTV-LTR, significantly increased metastatic efficiency. In addition, all *ras* oncogenes studied which could transform NIH-3T3 cells could induce metastatic behavior. This work suggests that the *ras* oncogene product p21 can directly regulate the metastatic phenotype in 10T1/2 and NIH-3T3 cells. Oncogenes encoding serine/threonine or tyrosine kinases were also introduced into NIH-3T3 cells and tested for tumorigenic and metastatic properties in nude mice. These genes could potently induce metastatic behavior. In an attempt to analyze individual events in metastatic transformation, 10T1/2 cells were transfected or infected with *myc* and *ras* oncogenes in dialyzed calf serum. These cells were tumorigenic but non-metastatic revealing that oncogene cooperation is insufficient for complete metastatic transformation. An additional stochastic event which can be induced by a factor present in fetal bovine serum

is also required. Finally, dramatic regulation of metastatic potential of either 10T1/2 or NIH-3T3 transformants can be achieved through exposure to specific polypeptide growth factors.

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INTRODUCTION

The Metastatic Process

i) General Features

Metastasis is a complex process which results in the growth of secondary tumors at sites discontiguous from the primary neoplasm. It is this metastatic spread of cancer that is responsible for the majority of cancer deaths since conventional therapy is often unsuccessful in the treatment of widely disseminated disease. It is, therefore important to understand the characteristics of malignant cells that distinguish them from benign tumor cells. This has been an area of intensive research for many years (Nicolson,1982) but has failed to yield universal answers.

Successfully metastatic cells must dissociate from the primary tumor, intravasate into the vasculature or lymphatics, survive potential immune attack and hydrodynamic pressure drops in the vasculature, bind to endothelium, extravasate into the surrounding tissue and finally grow into an autonomous tumor (Figure 1,Poste and Fidler,1980). This challenge necessarily dictates a complex phenotype likely involving the combined action of a large number of gene products. Since this process is so complex and because it does occur frequently as a result of tumor progression, it is likely that these properties are not acquired or controlled independently, but may be regulated through the concerted action of a small number of key gene

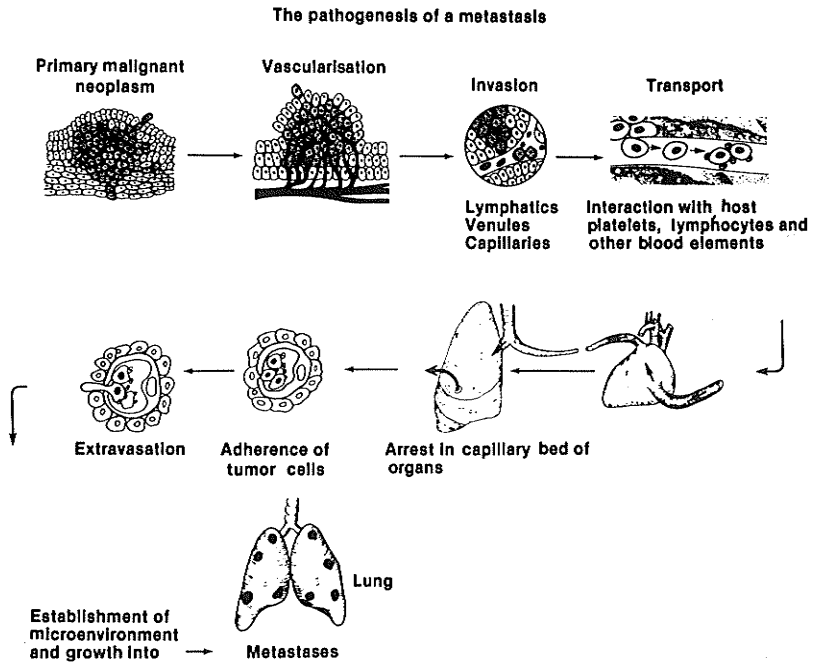

Figure 1: The Metastatic Process (from Poste and Fidler,1980)

products. With this reductionist approach specific hypothesis can be formulated and tested in an effort to learn more about the enemy; the metastatic cell.

ii) Routes of Dissemination

In some tumors, metastatic spread occurs along mechanical barriers such as nerve sheaths and facial planes. In addition, neoplasms growing in major body cavities may shed cells which upon reattachment to cavity walls may establish secondary lesions. However, these two forms of tumor spread are not characteristic of highly malignant behaviour. Aggressive tumors often invade tissues and spread with little regard for anatomical or physical boundaries.

The establishment of secondary lesions often occurs through specific pathways. Human colon carcinomas normally spread to the liver, whereas malignant melanoma preferentially metastasizes to the lung (Nicolson,1982). The "Seed and Soil" hypothesis may provide an explanation for this phenomena (Paget,1889). It states that tumor cell properties and microenvironment in which it is present are both important determinants of successful establishment for potential metastatic cells. Fidler has obtained a series of B16 melanoma cell lines with increasing metastatic potential through sequential selection of lung tumors in ten cycles of intravenous injection (Fidler,1973). The line obtained

after the tenth cycle (F10) was stably more metastatic than the cell line obtained after one cycle (F1). Serial selection of brain lesions following intravenous injection of B16 melanoma cells resulted in the isolation of a cell line which metastasized preferentially to the brain (Brunson,1978). Therefore, both the metastatic potential and the anatomical route of metastasis can be tumor cell determinants. Alternatively, the immune status of the host as well as properties of specific tissue endothelium, extracellular matrix and local production of growth promoting factors are all believed to be potent regulators of both the extent and routes of malignant dissemination (Nicolson,1982 & Roos,1984).

iii) Required Characteristics;Invasion,Extravasation and Immune Escape

Tumor dissemination through the vasculature requires specific enzymatic activities and physical properties. Tissue compartments are bordered by basement membranes; metastatic cells therefore must initially traverse the basement membrane in order to access the bloodstream and once again during implantation. Since many of the structural components of basement membranes are conserved throughout the body it is likely that invasion into blood vessels and extravasation out of the blood stream are very similar processes. These tissue boundaries are composed of

a type IV collagen matrix, glycoproteins such as laminin and entactin as well as heparin sulfate proteoglycans. Lance Liotta has described a three step model of tumor invasion (Figure 2,Liotta,1986). This model involves attachment, dissolution of matrix components and motility. In recent times Liotta and others have made great progress in understanding the molecular mechanisms responsible for these three steps.

Basement membrane specific attachment is often mediated through the attachment of cells to laminin. Highly malignant cells often express greater numbers of the 67,000 dalton laminin receptor on their surface. In addition, laminin can increase the metastatic potential of cells. This may be partially mediated through the ability of laminin to induce type IV collagenase activity (Turpeeniemi-Hujanen,1986). Recently the pentapeptide YIGSR, which represents the cell binding domain of laminin, was shown to interfere with the interaction of laminin with its receptor. Iwamoto et. al. have taken advantage of this property to show that injection of, or pretreatment of, cells with the YIGSR pentapeptide could also dramatically inhibit the formation of metastatic lung nodules by B16 melanoma cells injected intravenously (Iwamoto et.al.,1987). Recently, an additional type of laminin receptor has been described which is related to the integrin family of cell surface receptors (Gehlsen et.al.,1988). Therefore, the interaction of cells

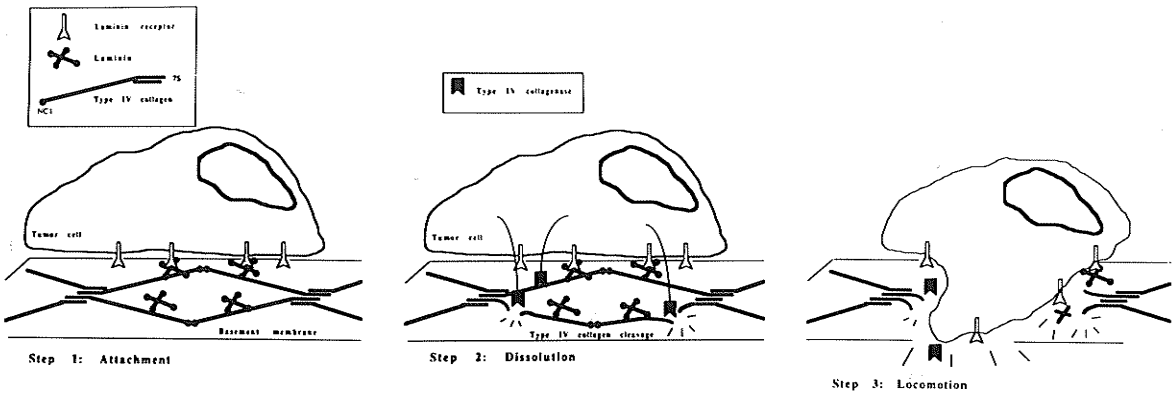

Chart 1. Three-step hypothesis of tumor cell invasion of extracellular matrix. Schematic diagram (not to scale) of tumor cell invasion of the basement membrane. Step 1 is tumor cell attachment to the matrix. This process may be mediated by specific attachment factors such as laminin, which form a bridge between the cell surface laminin receptor and type IV collagen. Step 2 is local degradation of the matrix by tumor cell-associated proteases. Such proteases may degrade both the attachment proteins as well as the structural collagenous proteins of the matrix. Type IV collagenase makes a single cleavage 25% of the distance from the amino terminus of type IV collagen. Proteolysis may be localized at the tumor cell surface where the amount of active enzyme outbalances the natural protease inhibitors present in the matrix. Step 3 is tumor cell locomotion into the region of the matrix modified by proteolysis. The direction of locomotion may be influenced by chemotatic factors. Continued invasion of the extracellular matrix may take place by cyclic repetition of these three steps.

Figure 2: Three Step Model of Invasion and Metastasis (from Liotta,1986)

with laminin would likely yield receptor specific consequences.

In 1983,Kalebic et. al. described the destruction of basement membrane specific collagen by highly metastatic tumors and endothelial cells (Kalebic et.al.,1983). Type IV collagenase is secreted in latent form which upon activation is 62-65KD molecular weight. This metalloproteinase is itself activated through proteolytic cleavage. It is likely that this activity is controlled both through production of latent type IV collagenase and through production of the enzymes which activate it. The tissue destructive activities of tumor cells and those displayed by many normal cells during wound repair or tissue remodelling are believed to be controlled through complex cascades of limited proteolysis which activates and deactivates specific enzymes. This is the scenario by which many of the activities in the blood coagulation process are controlled (Furie and Furie,1988 & Mignatti et.al.,1986). In fact, many of the important regulators of blood coagulation are believed to be involved in regulating the metastatic spread of tumors. Recently the gene coding for type IV collagenase has been cloned (Liotta,1988a). Sequence analysis indicates that the latent protein is activated through cleavage of the N-terminal 80 amino acids. This occurs at a sequence homologous to the sequence in type I collagenase which is the target of its activation by cleavage. It is believed

that plasminogen activators can activate these collagenases through a proteolytic cascade involving the intermediate activation of plasmin. The importance of plasminogen activators in invasion and metastasis is well established (Dano et.al.,1985). Most recently, transfection of recombinant plasminogen activator genes into poorly metastatic clones of ras-transformed NIH-3T3 cells resulted in a dramatic increase in their metastatic potential (Axelrod et.al.,1988).

The third step in Liotta's model of basement membrane invasion is the motility of tumor cells. Several labs have been successful in isolating motility factors which may be responsible for the movement and migration of malignant cells. In addition, many of the well characterized growth factors which are secreted by tumor cells are capable of stimulating not only the growth of the cells secreting them but their motility and communication with non-tumorous stroma. Recently Liotta and co-workers have cloned the gene for Autocrine Motility Factor (AMF) and therefore the mechanisms and importance of AMF deregulation in malignant transformation should become clear soon. Liotta's lab have also made major advances in understanding signal transduction following the interaction of AMF with its receptor (Stracke et.al.,1987).

Although the processes of invasion and extravasation in and out of the vasculature described above is essential for

haematological dissemination, metastasis will not occur if the immune system can eliminate circulating tumor cells. Transfection of major histocompatibility antigens into metastatic cells can result in their elimination through T-cell dependant immune rejection (Wallich et.al,1985). Consistent with a role for T-cell mediated immunity in tumor cell surveillance, much evidence has been accumulated on the role of MHC class I antigen expression in the regulation of tumor growth and spread (Reviewed in Tanaka et.al.,1988). Histocompatibility independent mechanisms of tumor cell surveillance are also believed to be important regulators of tumor spread (Talmadge et.al.,1980;Karre et.al.,1980;Warner and Dennert,1982 & Greenberg et.al.,1987a). Recent evidence from this lab has demonstrated that Natural Killer cells are effective at reducing the metastatic spread of ras-transformed fibroblasts in vivo (Greenberg et.al.,1987a).

Finally, if a tumor cell has traversed tissue boundaries and survived immune surveillance it must also be capable of forming an autonomous tumor at the new site. Growth in this new environment requires the ability to grow in the presence of normal cells. The presence of normal cells is often strongly inhibitory for growth of tumor cells both in vitro and in vivo (Land et.al.,1986 & Dotto et.al.,1988). Nicolson et. al. have reported that inhibition of gap junctional communication correlated with the metastatic potential of rat 13762NF mammary

adenocarcinoma cells (Nicolson,1988). The successful metastatic cell will not only be resistant to the inhibitory signals which may be present in the new environment but will also be able to either produce its own positive signals and/or be capable of responding to growth promoting activities which may be present.

iv) Generation of Metastatic Variants; Models and Evidence

Several models have been suggested to explain how the complex metastatic phenotype can arise. These models are concerned with the cell-autonomous characteristics which result in metastatic spread and not with the host-cell interactions which depend on abnormal host responses. Two models have been suggested to explain the origin of metastatic cells within a tumor population. The model first proposed by Fidler states that metastasis results from a non-random selective process, whereby metastatic variants possessing a defined phenotype emerge from the tumor population with characteristics which permit their dissemination and growth at secondary sites (Fidler and Kripke,1977). Alternatively, Harris et. al. (1982) and Weiss (1983) also believe that the metastatic phenotype is selected for, but that it is an unstable phenotype which reverts in the absence of selective pressure. These two models can simplistically be viewed as involving selection for a phenotype which is either stable or unstable.

In Fidler's F1/F10 experiment described above (Fidler,1973), he clearly demonstrated the selection of a stably more metastatic cell line (F10) from the poorly metastatic B16-F1 line. This work indicated that selection for cells with stable alterations, which imparted on them a greater ability to successfully disseminate, could occur in vivo. Weiss views the generation of metastatic cells within a tumor as a random process (Weiss,1983). He states that although variants may arise which can alter the rate at which metastasis occurs, these variants do not determine whether or not metastatic spread takes place. This view is largely based on the heterogeneous nature of tumor cell populations, and on the demonstration that individual clones from a metastatic population are often non-metastatic. Weiss has named his model the "Transient Metastatic Compartment". Harris et. al., in an eloquent series of experiments, demonstrated that the vast majority of clones within both the F1 and F10 populations were non-metastatic but that the F10 line had a greater rate of generation of metastatic variants (Hill et.al.,1984). The model proposed by this group to explain the generation of metastatic variants within a population is termed "Dynamic Heterogeneity" (Hill et.al.,1984). They suggest that highly metastatic cells result from the selection of a phenotype within which the rate of variant generation is enhanced. This group therefore believes that the heterogenous nature

of malignant populations is the critical determinant for induction of malignant dissemination. Much effort has also been directed at understanding the molecular mechanisms responsible for generation of this complex phenotype.

Both genetic and epigenetic models have been proposed to explain the generation of metastatic variants within a tumor cell population. The genetic model suggests that metastatic cells arise through the accumulation of mutations effecting primary nucleotide sequences of the genome. This model may include point mutations, amplification of genes, or deletion of genetic material. In contrast, the epigenetic model involves altered regulation of gene expression, which can arise through alterations in chromatin structure. Generation of metastatic variants through epigenetic mechanisms may not always be caused by nuclear events but may also theoretically involve self sustaining cytoplasmic or membrane determinants such as ion concentrations. There is considerable evidence to support both of these models. In 1985, three labs reported that the ras oncogene could transform NIH-3T3 cells to the metastatic phenotype and in doing so proved that genetic mechanisms could at least partially account for the generation of metastatic variants within tumors (Thorgeirsson et.al.,1985;Bernstein and Weinberg,1985/1988 & Greig et.al.,1985). Definitive evidence for epigenetic regulation of metastatic behaviour was obtained by Raz and Ben-Ze'ev in

1983 when they showed that cell shape could have a profound effect on the metastatic capability of B16-F1 melanoma cells (Raz and Ben-Ze'ev,1983). This effect was fully reversible, demonstrating that genetic mechanisms were not responsible. Additional evidence for both models is abundant.

It would seem likely that all of the above models are correct to some degree and need to be incorporated into a more complete picture of the metastatic phenotype and its determinants. Definitive evidence for selection of stable alterations in metastatic potential, as well as selection for cells which transiently enter the metastatic compartment, have been obtained. It therefore seems likely that selection for stable alterations will occur when those alterations arise within the tumor cell population. If however, no stable alterations arise in variants within the tumor population, then there will be a transient selection for dissemination of cells which express the biochemical properties necessary for metastatic spread at that time. This selection could be for cells which are in a given compartment of the cell cycle. Once a secondary lesion has been established, the new tumor population would loose its synchronous nature and return to a population much like the primary tumor. This example is not meant as a thesis but an illustration of the types of determinant which could explain the data accumulated. The "stable" or non-random model of Fidler is an example of tumor progression which is dependent

on variant generation. The transient models arise from experiments in which tumor progression is not allowed to occur, and as a result the phenotype selected for, is unstable.

The genetic and epigenetic models proposed to explain generation of metastatic variants are also both well proven and therefore need to be incorporated into a larger theory. It is now clear that alterations in gene expression can occur through changes in either the DNA or chromatin structure of that DNA. The mechanisms responsible for tumor progression to the metastatic phenotype will depend on many things including the type of carcinogens or tumor promoters involved in transformation. The resulting phenotype will always have both genetic and epigenetic determinants. The simplest illustration of this comes from the abundant work done on transgenic mice which result in tissue/oncogene/and promoter specific phenotypes. This work clearly shows that chromatin structure (epigenetic determinant/cell type specific expression) and the transgene (genetic determinant) are both important regulators of tumorigenic and even metastatic phenotypes (Palmiter and Brinster,1986 & Muller et.al.,1988).

v) Biochemical Properties; Regulation by External Signals

In section (iii) above some of the essential biochemical activities required for metastatic dissemination

were reviewed. Many of these properties involve specific interaction with basement membrane components as discussed in Lance Liotta's three step model (Liotta,1986). The interaction of metastatic tumor cells with basement membranes is often facilitated through expression of increased numbers of available laminin receptors on their cell surface (Wewer et.al.,1986 & Wewer et.al.,1987). The molecular basis of this phenomena is unknown. Availability of molecular probes to investigate the control mechanisms of laminin receptor expression and affinity should facilitate detailed analysis of normal and aberrant receptor function. Preferential association of cells with certain types of extracellular matrix including basement membrane components can be induced through transformation by specific oncogenes (Bober et.al.,1988). Once a tumor cell has interacted with basement membrane constituents like laminin, other integrins, or even organ specific molecules, then it must degrade the matrix in order to establish a path of invasion. As discussed previously the enzymatic destruction of matrix likely involves a proteolytic cascade (Mignatti et.al.,1986). In many cases the key enzymes associated with invasion and metastasis have been identified and studied. Three enzymes which have been suggested as playing a key role in malignant spread are the plasminogen activators, type iv collagenase and MEP or cathepsin L (Dano et.al.,1985;Liotta,1986 & Denhardt et.al.,1987). These

proteases can be regulated through many of the pathways normally associated with control of growth and differentiation. Plasminogen Activators can be induced through treatment of cells with tumor promoting phorbol esters (Wigler and Weinstein,1976). Similarly type iv collagenase can be regulated through treatment of cells with phorbol esters like TPA or through the direct effects of the activated ras oncogene (Liotta et.al.,1988a). In addition, cathepsin L can be induced by growth factors such as Platelet Derived Growth Factor (PDGF), TPA, or ras transformation (Rabin et.al.,1986). The motility of cells can also be stimulated by growth factors or oncogenes (Martinet et.al.,1986 & Varani et.al.,1986). Finally, it is clear that oncogenes can regulate the immunogenicity of tumor cells. This regulation can involve T-cell mediated immunity or non MHC restricted mechanisms such as through the function of natural killer cells (Bernards et.al.,1986;Trimble et.al.,1986 & Greenberg et.al.,1987b). The effects of oncogenes on the immunogenicity of cells is oncogene and cell type specific and can play a profound role in the metastatic dissemination of tumors (Bernards et.al.,1986). In summary, many of the properties that are essential for metastasis, including adhesion dissolution and motility through basement membranes as well as escape from immune surveillance, are regulated by growth factors and/or oncogenes. This fact strongly suggests that the same

mechanisms and pathways that control the growth and differentiation of cells, and ultimately are responsible for tumorigenic transformation, are also important regulators of the metastatic phenotype.

Oncogenes and Tumorigenic Transformation

i) Dominant Oncogenes; Viral and Cellular

The discovery of dominant acting **oncogenes** can be traced back almost eighty years to the work of Peyton Rous on what later became known as Rous Sarcoma Virus (RSV)(Rous,1911). The significance of RNA tumor viruses on human cancer was not recognized for many years but gradually in the 1960's work on retroviruses began to take centre stage in cancer research. With the newly available molecular techniques in the 1970's, research focused on dissection of the retroviral genome. This work culminated with the discovery by Stehelin, Varmus, Bishop and Vogt that RSV contained a non-essential sequence of cellular origin which was responsible for transformation (Stehelin et.al.,1976). It was clear that the viral *src* (for sarcoma) oncogene had a normal cellular counterpart which became known as the *src* proto-oncogene. Moreover, this discovery proved that the cellular genome contained at least one gene which could be activated to participate in transformation. The study of RNA tumor viruses has resulted in the isolation

and description of several dozen viral oncogenes which also have cellular origins (Bishop,1985).

Erikson and co-workers revealed that the protein product of v-src (pp60src) was a protein kinase which phosphorylated its targets on tyrosine residues rather than the serine/threonine residues which are most commonly the targets of phosphorylation (for review see Bishop,1985)). The viral pp60src protein was constitutively elevated for its tyrosine kinase activity providing the first biochemical description of an alteration in a cellular function which could promote transformation. The first genetic manipulation of a cellular gene which proved to result in oncogenic activation was achieved by George Vande Woude and colleagues. This group fused the mouse cellular mos proto-oncogene (c-mos) to a strong viral promoter. The result was a potent chimeric oncogene which achieved transformation through increased or inappropriate expression of a normal cellular protein (Blair et.al.,1981). A separate avenue of research beginning in the late 1970's has resulted in the isolation of cell associated oncogenes which have become activated through non-viral carcinogenesis. This work was initiated through the discovery by Chialo Shih in Robert Weinberg's lab that DNA from chemically transformed cells could be used to transfer the neoplastic phenotype into non-transformed recipient cells (Shih et.al.,1979). This work resulted in the cloning of a human bladder carcinoma

oncogene simultaneously in three labs (Goldfarb et.al.,1982;Pulciani et.al.,1982 & Shih and Weinberg,1982). It was then found that oncogenes isolated from RNA tumor viruses and cellular oncogenes isolated through transfection studies were related. The cellular oncogene isolated from colon and lung carcinoma was homologous to the Kirsten-ras oncogene (Ki- or K-ras) from Kirsten Murine Sarcoma Virus (Der et.al.,1982) and the bladder carcinoma oncogene described above was homologous to the Harvey-ras oncogene (Ha- or H-ras) from Harvey Murine Sarcoma Virus (Parada et.al.,1982 & Santos et.al.,1982). The next important step was to characterize the alteration responsible for activation of transforming potential. This was achieved quickly by the Weinberg and Scolnick labs in a collaborative effort and independently by Mariano Barbacid's group (Tabin et.al.,1982 & Reddy et.al.,1982). The same conclusion was reached soon after by two other groups (Taparowsky et.al.,1982 & Capon et.al.,1983). Activation of the bladder carcinoma derived T24/EJ H-ras was achieved through a single nucleotide change which resulted in the replacement of glycine at codon 12 by valine. Recently Levinson has demonstrated that an additional mutation is present in the T24/EJ allele, and that this mutation results in overexpression of the oncoprotein product p21 (Cohen and Levinson,1988).

Work in Edward Scolnick's lab in the late 70's was

focused on the study of viral ras genes isolated from both Harvey and Kirsten Murine Sarcoma Viruses. By the time the two fields of oncogene research merged, Scolnick's lab had found the ras-genes encoded protein products of 21 kilodaltons which were anchored in the inner plasma membrane. In addition, it was clear that p21 molecules bound guanine nucleotides (Reviewed in Barbacid,1987). These pieces of information led to the idea that ras proteins were involved in signal transduction in the same way as classical G-proteins were. In such a model, p21 which was associated with GTP was in an excited state and capable of stimulating its target second messenger system. Hydrolysis of the bound GTP molecule yielded the inactive p21-GDP complex. It was therefore very satisfying to learn that activated ras oncogenes were impaired in their intrinsic GTPase activity, which suggested that chronic stimulation of the ras target would occur (Finkel et.al.,1984;Gibbs et.al.,1984;Manne et.al.,1985;McGrath et.al.,1984 & Sweet et.al.,1984)(Reviewed in Barbacid,1987).

Although the discovery and isolation of oncogenes can be traced back through two separable and well defined experimental pathways, the mechanisms responsible for the activation of cellular oncogenes are diverse. There are numerous distinct mechanisms by which oncogenic activation can occur. One example is fusion of proto-oncogene sequences to strong promoters or enhancers as illustrated by

Vande Woude and described above (Blair et.al.,1981). This process can also occur through retroviral integration with subsequent overexpression of an adjacent cellular proto-oncogene such as the myc gene in avain leukosis virus induced T-cell lymphomas (Hayward,1981).

A second mechanism of oncogene activation is achieved through overexpression as a result of gene amplification. Once again the myc oncogene was found to be altered, this time through 50-fold amplification in the human HL60 leukaemia cell line (Collins and Groudine,1982 & Della-Favera,1982).

Thirdly, inappropriate expression of proto-oncogenes can occur as a result of chromosomal translocation. This is the case for activation of the myc gene in many human Burkitt's lymphomas. It is believed that juxtaposition of myc and immunoglobulin enhancer sequences results in overexpression. Recent work has demonstrated that mutations at the exon1/intron1 border of c-myc may occur as a result of the translocation. These mutations can lead to a loss of coding potential for the larger of the two myc proteins (Hann et.al.,1988) as well as loss of the binding site for a transcriptional repressor which is believed to block the elongation of myc transcripts when terminal differentiation occurs (Cesarman et.al.,1987).

Additional mechanisms include structural alteration which can be achieved through point mutation as described

above for ras. The list of alterations which are capable of activating the oncogenic potential of cellular proto-oncogenes will likely include alleviation of all possible types of control. The work described above by Arthur Levinson demonstrates that the T24/EJ H-ras allele is partially activated through a mutation in the last intron which affects the normal splicing profile of this gene (Cohen and Levinson,1988 & Doniger,1988). Recently the lck proto-oncogene was demonstrated to be altered through translational activation as a result of retroviral insertion near the gene (Marth et.al.,1988). In addition, it is now believed that the cellular p53 gene is activated through mutations which deactivate its function, yet allow it to complex with the normal product from the unaltered p53 allele (Munroe and Benchimol,1988 submitted). This activation process can be described as a dominant negative mutation.

In recent years the function of several oncogenes has been elucidated. However, the majority of oncogene products are still not described to this level. Structure/function analysis has succeeded in grouping the oncogenes or proto-oncogenes into distinct classes which are based initially on subcellular localization and further on enzymatic properties and sequence homologies (Weinberg,1985, Table 1). The oncogene products are believed to be involved in signal transduction from the external environment to the nucleus.

Table 1
Oncogene and Proto-Oncogene Families

Growth Factors

PDGF-A chain
PDGF-B chain (sis)

int-1

int-2
bFGF
hst/K-FGF

TGF-α
EGF

IL2
IL3

M-CSF
GM-CSF

Growth Factor Receptor

ros

EGF-R/erb-b
Neu/erb-b2

kit
M-CSF-R/fms

trk

met

ret

tcr-B

Thy-1

Neuronal-type Receptors

Angiotensin receptor/mas

Cytoplasmic tyrosine kinases

abl
arg

src
yes
lck
hck
fyn
lyn
tkl
tco

fes/fps

fgr

pim

G-Proteins

H-ras
K-ras
N-ras

Kinase Regulators

crk

Ion Channels

H+pump

Serine/Threonine Kinases

mos

raf/mil
A-raf

Protein Kinase C

Transcription Factors

jun
jun-B

fos
fra-1
fos-r

Zinc Finger Protein Evi-1/Fim-3

Nuclear Oncogenes of Unkown Function

c-myc
N-myc
L-myc

myb

ets-1
ets-2
erg

ski

p53

Hormone Receptors

T3 Thyroid Hormone Receptor/erb-A

Unknown Function or Location

rel-cytoplasmic or nuclear

dbl

orf-F

bcl-2

When activated, these proteins likely send their normal signal in the absence of an input signal or to a level not consistent with the input signal intensity (see Weinberg,1985 & Bishop,1987 for review). Inappropriate growth promoting signals can be induced by growth factors such as PDGF B-chain otherwise known as the c-sis oncogene (Waterfield et.al.,1983;Doolittle et.al.,1983;Chiu et.al.,1984;Huang et.al.,1984 & Gazit et.al.,1984), or through inappropriate expression of hemopoietic growth factors such as IL2 or IL3 as a result of retroviral insertion (Chen et.al.,1985 & Ymer et.al.,1985). Oncogenic activation can also occur through aberrant growth factor receptor function such as through truncation of the Epidermal Growth Factor Receptor in the v-erb-b oncogene (Downward et.al.,1984), or in the Macrophage Colony Stimulating Factor Receptor derived oncogene v-fms (Sherr et.al.,1985). More recently these same two receptors have been shown to be activated through overexpression which will increase the sensitivity of the cell to the growth factor in question (Velu et.al.,1987;Paolo Di Fiore et.al.,1987;Roussel et.al.,1987 & Gisselbrecht et.al.,1987). The next level in signal transduction is mediated by membrane anchored or cytoplasmic kinases as well as the G-protein ras molecules. Through still unknown mechanisms these molecules send signals into the nucleus. Activation of cytoplasmic tyrosine kinases such as the src family gene

products, or kinases related to the abl, fgr, or fes/fps gene families is normally achieved through structural alteration which results in the loss of controlling domains, thus yielding constitutively activated or derepressed enzymes. Alternatively, it is very likely that these kinases promote transformation when expressed at higher than normal levels, since once again this would increase sensitivity and an inappropriately high signal would be transmitted. Indeed partial transformation has been achieved through overexpression of the normal c-src gene product (Johnson et.al.,1985). The ras family of signal transducers can also be activated through structural alterations and overexpression (Barbacid,1987) which both result in overstimulation of the ras target GAP protein (Trahey and McCormick,1987 & Vogel et.al.,1988). The cytoplasmic serine/threonine kinase oncogenes can be activated through overexpression as has been demonstrated with the mos proto-oncogene (Blair et.al.,1981) or through structural alteration as shown for the raf gene (Ishikawa et.al.,1987). The same situation prevails for some nuclear oncogenes. The c-myc gene is normally activated through overexpression or a block to repression of expression resulting in inappropriate expression (Cesarman et.al.,1987). It is clear that mutations within the coding sequence of c-myc can also contribute to its transforming potential (Frykberg et.al.,1987). The transcriptional

regulators fos and jun are also likely activated through both changes in expression and in structure, such that they do not need their normal activation in order to alter transcription from AP1 enhancer sequences (Reviewed in Verma and Graham,1987 & Vogt and Tjian,1988). The above discussion is not meant as a review of oncogenes but merely as a guide to the types of gene products which when altered can promote transformation. In addition it is now clear that oncogenic activation can occur through overexpression and/or structural alterations in most types of oncogenes. These mechanisms result in increased sensitivity of the growth promoting cascade or alleviation of negative control over such signals.

ii) Multistep Nature of Cancer; Oncogene Complementation

Cancer is a multistep disease, and it is believed that tumor progression occurs through the accumulation of alterations in independent characteristics (Nowell,1976). Experimental work with the mouse skin initiation/promotion system has reproduced the multistep nature of transformation. A single treatment of mouse skin by a carcinogen results in initiation. Tumor formation will occur only if the initiated area is treated with repeated exposure to tumor promoters such as phorbol esters. The tumor promoter causes outgrowth of the initiated cell, resulting in the formation of a benign papilloma. Extensive

promoter treatment can result in progression of the papilloma into an invasive carcinoma as a consequence, it is believed, from the acquisition of additional mutations within the expanding papilloma population (Quintanilla et.al.,1986).

In vitro studies with polyoma virus by Rassoulzadegan, Cuzin and colleagues has revealed that the different protein product of this virus contribute different biological activities (Rassoulzadegan,1982). The sum total of these activities is the ability of the virus to transform non-established cells in culture. This DNA virus therefore, transformed cells through the cooperation of distinct biological activities. A dramatic illustration of complementation involving cellular oncogenes was described in 1983 (Reviewed in Land et.al.,1983b). Earl Ruley transformed Baby Rat Kidney cells to a highly tumorigenic phenotype with the E1A oncogene of human Adenovirus together with T24-H-ras (Ruley,1983). Neither gene alone was active in this assay. Furthermore, Land, Parada and Weinberg transformed primary Rat Embryo Fibroblasts through complementation between the EJ-H-ras oncogene and an activated v-myc construct (Land et.al.,1983a) which represents cooperation between two cell derived sequences. Ruley later advanced this work through the demonstration that in vitro establishment was not sufficient for transformation by an activated ras gene (Franza

et.al.,1986). In fact, expression of the ras oncogene at levels above one fifth of the endogenous ras expression seemed to be antiproliferative and perhaps lethal (Franza et.al.,1986). This work demonstrated that the oncogene complementation story was not as simple as originally perceived.

iii) Recessive Oncogenes

Despite convincing evidence that transformation can be achieved through the acquisition of dominant acting mutation in oncogene sequences, the transformed phenotype is usually recessive (For Review see Klein,1987). Fusion of normal and cancer cells reveals that the normal phenotype is dominant. This dominance of the non-transformed phenotype has been associated with the acquisition of specific chromosomes (Weissman et.al.,1987). In addition, karyotypic analysis of cancer cells has linked the loss of genetic material to neoplastic transformation and tumor progression. Finally, genetic linkage analysis of familial cancers has associated deletions with tumors such as Retinoblastoma.

Transformation of primary hamster cells in vitro by the combined action of myc and ras is associated with the loss of one copy of chromosome 15 (Oshimura et.al.,1985). This chromosome loss is believed to involve the deletion of an anti-oncogene. Recently the first anti-oncogene has been cloned through the isolation of human chromosome sequences

deleted in Retinoblastoma tissue (Dryja et.al.,1986 & Reviewed in Weinberg,1988). A cDNA corresponding to the Rb gene was then cloned using the genomic fragment encompassing the deleted sequences (Friend et.al.,1986). It has been speculated that numerous additional candidate tumor-suppressor genes exist on the basis of association of specific deletions with specific tumor types. In addition, analysis of informative polymorphic loci reveals that in some cases the transformed phenotype is associated with the loss of one specific allele (a) and duplication of the other (b). This fact suggests that, a normal copy of a closely linked tumor-suppressor gene has been lost and the chromosome or chromosome region specifying a mutant copy of the same anti-oncogene has been duplicated. This would result in the duplication of one allele of a linked polymorphic loci (b) and the deletion of the allele which resides close to the normal tumor-suppressor gene (a) (for Review see Hansen and Cavenee,1988 & Ponder,1988). It is also possible that many anti-oncogenes have already been cloned for reasons other than their potential tumor-suppressing abilities. In Friend virus induced erythroleukaemia the p53 gene is very frequently deactivated through rearrangement and retroviral insertion, suggesting that its loss plays an important role in transformation (Mowat et.al.,1985). In addition, the deletion of sequences on chromosome 3 which is associated with lung cancer in

humans is now believed to involve the loss of one type of Thyroid hormone receptor. This could result in the inability of the target cell to respond to a differentiation inducing signal involving this receptor (Leduc et.al.,1988 & Houle et.al.,1988). The importance of growth inhibitory pathways in transformation has been recently highlighted through the demonstration that the E1A oncogene product of Adenovirus binds the product of the Rb gene (Whyte et.al.,1988). This suggests that transformation is at least partially achieved through the titration of tumor-suppressor activities. Subsequently it has been shown that the Large T antigen of the unrelated virus SV40 also binds the product of Rb (DeCaprio,1988). Finally, the essential involvement of recessive oncogenes in transformation has been demonstrated by Ruley and co-workers in the REF 52 system. As discussed above, Ruley has shown that in vitro establishment is not sufficient to render a cell susceptible to ras-mediated transformation (Franza et.al.,1986). Transformation by ras requires the cooperating activity of E1A or Large T (Franza et.al.,1986 & Hirakawa and Ruley,1988). Consistent with the fact that the two DNA virus oncogenes are capable of titrating out the product of the Rb gene, Ruley has found that transformation by ras can occur if the cells undergo a deactivation of endogenous Rb. In some cases this deactivation is a result of deletions within the Rb gene (Ragozzino et.al.,1988). The fields of

Dominant and Recessive Cancer Genetics are now inseparable. It is clear that both types of mutations are involved in tumorigenic transformation. This is not surprising since Cancer must involve, not only the acquisition of inappropriate positive signals but the lack of response to the negative signals which maintain homeostasis.

iv) Cocarcinogens and Chromatin Structure; Epigenetic Defects

In 1980, Guernsey and co-workers demonstrated that transformation of C3H/10T1/2 cells by X-rays was dependent on the presence of Thyroid Hormone T3 in the growth media (Guernsey et.al.,1980). It has also been found that EGF can stimulate the transformation of rat granulosa cells by Kirsten Murine Sarcoma Virus (Harrison and Auersperg,1981). In addition, Weinstein's group has found that ras-induced transformation of 10T1/2 can be dramatically increased by either tumor promoters or by a factor present in fetal bovine serum (Hsiao et.al.,1984 & Hsiao et.al.,1987). The effect of tumor promoters such as TPA and the factor in fetal bovine serum are synergistic and therefore, at least partially, work through distinct pathways (Hsiao et.al.,1987). In this work, Hsiao has excluded the possibility of an increase in transfection frequency as a result of TPA or FBS treatment and therefore these factors stimulate transformation as the response to oncogene

activation in cells that are capable of resisting the transformation. These cocarcinogens promote transformation through the induction of contributory cellular events, suggesting that epigenetic defects are necessary for transformation. Consistent with this notion, many embryonal carcinomas are capable of forming completely normal offspring when they are re-established in the correct embryonic environment. In these cases there are no genetic defects present. This fact indicates that a minimum of zero stable genetic defects are necessary to establish the transformed phenotype. All of the transforming defects may be epigenetic which can result in tumor formation solely as a result of altered chromatin structure. This in turn can lead to the inappropriate expression of normal genes and therefore, transformation. Cancer can therefore result from different combinations of dominant, recessive and epigenetic defects. All of these must be considered in its study.

The Molecular Basis of Metastatic Spread

i) Metastatic Transformation by ras

In 1985, the fields of oncogene research and the study of metastasis were brought together by several groups. Work in the labs of Lance Liotta, Robert Weinberg and George Poste all established that NIH-3T3 cells transformed by activated ras genes were metastatic in T-cell deficient nude

mice (Thorgeirrson et.al.,1985;Bernstein and Weinberg,1985 & Greig et.al.,1985). This idea had arisen independently in many other labs including our own. Work by Ann Chambers' group, using the embryonal chick experimental metastasis assay confirmed that ras-transformed NIH-3T3 cells were also metastatic in this system (Bondy et.al.,1985). Ruth Muschel soon established that the effects of ras were cell type specific and also that, in contrast to mutated ras genes, overexpression of the proto-oncogene was not able to induce metastasis in 3T3 cells (Muschel et.al.,1985). Next, the Khoury and Liotta labs collaborated to show that primary cells transformed by ras alone were highly metastatic but the same cells transformed by the Adenovirus serotype 2 E1A gene in cooperation with ras were non-metastatic (Pozzatti et.al.,1986). The metastatic transformation by ras was therefore, not limited to aneuploid established cell lines and this metastatic conversion could occur in the absence of dramatic cytogenetic alterations (Muschel et.al.,1986). Bradley, working in the ras-lab of Jackson Gibbs at Merk Sharp & Dohme, surveyed the effects of ras genes from yeast to man and activated through overexpression and/or structural alteration on the metastatic ability of 3T3's (Bradley et.al.,1986). This group concluded that all transforming ras genes were able to induce metastasis formation and all with equal potency. This led to their model of a ras induced qualitative switch mechanism for

access to the metastatic phenotype. In an effort to understand the influence that ras had on metastasis, we analyzed a series of 10T1/2 clones transformed by ras. A correlation between ras expression and metastatic potential was noted. In addition, preinduction of the glucocorticoid regulatable MMTV-ras gene in NIH-3T3 line 433 resulted in an increase in metastatic potential (Egan et.al.,1987a;Chapter 1). We then proposed a model in which the ras protein p21 was directly involved in regulating the metastatic phenotype in the same way that p21 can regulate the transformed phenotype (Egan et.al.,1987a). If this model was correct then 1) all transforming ras constructs should be able to induce metastatic behaviour in NIH-3T3 cells and 2) the metastatic potential of ras genes should be related to their transforming potential. Two pieces of information in the literature were inconsistent with the model. It had been reported that ras oncogenes were able to induce the metastatic phenotype, whereas overexpression of the proto-oncogene was not (Muschel et.al.,1985). If proto-ras could transform through increased stimulation of the ras target (GAP) why could it not induce metastasis formation? The second piece of information that was not consistent with our model was Bradley's conclusion that all transforming ras genes including the overexpressed proto-oncogene could induce experimental metastasis formation with equal potential (Bradley et.al.,1986). If the proto-oncogene was

much less potent in transformation assays and did not have impaired GTPase activity or any of the other activating alterations, why could it induce metastasis as efficiently as activated oncogenes? In order to resolve these issues we tested several NIH-3T3 lines transformed by either amplification of the normal gene, LTR activation of the normal gene, or reversion of activating mutations in the viral H-ras gene. We found that, consistent with our model of direct regulation and the models of ras function (involving the stimulation of GAP with subsequent generation of second messenger formation), the proto-oncogene was able to induce the full metastatic phenotype but not as effectively as the ras oncogenes (Egan et.al.,1989;Chapter 2). In addition, all transforming ras genes were able to induce metastasis including several which contained activating mutations not analyzed by Bradley. Therefore through correlative evidence, co-induction of ras and metastasis formation and structure function analysis, we have shown that ras can directly regulate the metastatic behaviour in the same way as it regulates transformation. The ability of ras to induce metastasis formation in cell lines has been extended to include several different parental cell types and metastasis formation in their respective syngeneic hosts (Egan et.al.,1987a;Collard et.al.,1987 & Waghorne et.al.,1987). Recently, Hill et.al. (1988) have found that the expression of p21 in ras-

transformed NIH-3T3 was indicative of metastatic potential of a series of independent clones and subclones. This experiment provides additional support for a direct role of ras in metastasis formation (Liotta,1988b).

ii) Metastatic Transformation by Other Oncogenes

Despite the strong evidence linking ras to metastasis in experimental systems, it is clear that not all human tumors contain activated or amplified ras genes. Malignant dissemination can therefore occur in the absence of alterations in ras. Analysis of NIH-3T3 cells transformed by receptor type tyrosine kinases, cytoplasmic tyrosine kinases and cytoplasmic serine/threonine kinases were all found to be metastatic (Egan et.al.,1987b;Chapter 3). The ability of the cytoplasmic tyrosine kinase src to induce metastatic behaviour through the direct action of pp60src kinase had been demonstrated earlier by Ann Chambers in the chick embryo experimental metastasis assay (Chambers and Wilson,1985). The ability of the cytoplasmic tyrosine kinase oncogene fes/fps to induce metastasis has been confirmed in CCL39 fibroblasts (Sadowski et.al.,1988). The wide range of structurally divergent oncogenes which are able to induce the metastatic phenotype would suggest that possibly a few limited biochemical pathways within the cell are involved. We have suggested that alterations in inositol phospholipid metabolism may be key regulators of

this process. A recent report supports this view. It has been found that the metastatic potential of B16 melanoma cells correlates with the level of protein kinase C activity, and that stimulation of protein kinase C with tumor promoters enhanced metastatic spread (Gopalakrishna and Barsky,1988). Although this pathway may be the primary defect responsible for metastatic dissemination of many or all tumor types, it is likely that many different pathways will modulate the malignant potential of tumor cells. Bernards et.al. (1986) have found that overexpression of the nuclear N-myc oncogene in Neuroblastoma cells had a dramatic effect on their metastatic behaviour. A similar conclusion has been reached with the nuclear oncogene p53 (Pohl et.al.,1988). These nuclear oncogenes are not likely to directly affect inositol phospholipid metabolism as a result of their location, but their deregulation may result in the indirect alteration of non-nuclear events such as regulation of second messenger generation.

iii) Regulation of Metastasis by Growth Factors

In the seed and soil hypothesis, interaction between the tumor cell and its environment are highlighted (Paget,1889). A significant element of this interaction is the production of and response to soluble growth factors by both stroma and tumor. In addition, numerous examples of autocrine growth factor production by tumors now exist

(Reviewed in Sporn and Roberts,1985). Can polypeptide growth factors play a significant role in regulating not only the growth of tumors but their metastatic spread? In an effort to understand the role that growth factors and growth autonomy play in malignant spread of tumor, Chadwick and Lagarde (1988) have isolated growth autonomous variants from the non-metastatic, growth factor dependent, CCL39 hamster lung fibroblasts. These cells coincidentally developed both the metastatic and growth factor independent phenotypes. This was likely due to the acquisition of activating mutations in oncogenes which is known to affect both production of, and response to, growth factors (Stern et.al.,1986) in addition to the well documented effect of oncogenes on metastasis (Liotta,1988c). In order to test this question more directly we have analyzed the effect of growth factors on the metastatic phenotype using both stable and transient assays. Notably NIH-3T3 cells transformed by bFGF were highly metastatic in nude mice, whereas treatment of ras-transformed 10T1/2 cells prior to injection into a syngeneic host dramatically inhibited their metastatic behaviour (Egan et. al.,1988b;Chapter 5). Growth factors can positively or negatively regulate the metastatic phenotype depending on the cell type, host status and/or step in the malignant process in question. Cytokines can also have a dramatic effect on metastasis formation through the augmentation of immunogenicity of tumor cells (Taniguchi

et.al.,1987).

References

Axelrod,J.H., Reich,R. and Miskin,R., 1988. Human Recombinant Plasminogen Activators Enhance the Invasive and Metastatic Behaviour of H-ras transformed NIH/3T3 Cells. 4th International Congress of Cell Biology Proceedings P1.2.44(MW9.32),48.

Barbacid,M., 1987. ras Genes. A. Rev. Biochem. 56, 779-827.

Bernards,R., Dessain,S.K. and Weinberg,R.A., 1986. N-myc Amplification Causes Down-Modulation of MHC Class 1 Antigen Expression in Neuroblastoma. Cell 47, 667-674.

Bernstein,S.C. and Weinberg,R.A., 1985/1988. Expression of the Metastatic Phenotype in Cells Transfected with Human Metastatic Tumor DNA/Correction. Proc. Natl. Acad. Sci. USA. 82, 1726-1730./ 85, 5581.

Bishop,J.M., 1985. Viral Oncogenes. Cell 42, 23-38.

Bishop,J.M., 1987. The Molecular Genetics of Cancer. Science 235, 305-311.

Bober,F.J., Birk,D.E., Shenk,T. and Raska,K. Jr., 1988. Tumorigenicity of Adenovirus-Transformed Cells: Collagen

Interaction and Cell Surface Laminin are Controlled by the Serotype Origin of the E1A and E1B Genes. Journal of Virology **62**, 580-585.

Bondy,G.P., Wilson,S. and Chambers,A.F., 1985. Experimental Metastatic Ability of H-ras-transformed NIH-3T3 Cells. Cancer Research **45**, 6005-6009.

Bradley,M.O., Kraynak,A.R., Strorer,R.D. and Gibbs,J.B., 1986. Experimental Metastasis in Nude Mice of NIH-3T3 Cells Containing Various ras Genes. Proc. Natl. Acad. Sci. USA. **83**, 5277-5281.

Brunson,K.W., Beattie,G. and Nicolson,G.L., 1978. Selection and Altered Properties of Brain-Colonising Metastatic Melanoma. Nature **272**, 543-545.

Cesarman,E., Dalla-Favera,R., Bentley,D. and Groudine,M., 1987. Mutations in the First Exon are Associated with Altered Transcription of c-myc in Burkitt Lymphoma. Science **238**, 1272-1275.

Chadwick,D.E. and Lagarde,A.E., 1988. Coincidental Aquisition of Growth Autonomy and Metastatic Potential During the Malignant Transformation of Factor-Dependent CCL39 Fibroblasts. Journal of the National Cancer Institute

80, 318-325.

Chambers,A.F. and Wilson,S., 1985. Cells Transformed with a ts Viral src Mutant are Temperature Sensitive for in vivo Growth. Mol. Cell. Biol. 5, 728-733.

Chiu,I.-M., Reddy,E.P., Givol,D., Robbins,K.C., Tronick,S.R. and Aaronson,S.A., 1984. Nucleotide Sequence Analysis Identifies the Human c-sis Proto-Oncogene as a Structural Gene for Platelet-Derived Growth Factor. Cell 37, 123-129.

Cohen,J.B. and Levinson,A.D., 1988. A Point Mutation in the Last Intron Responsible for Increased Expression and Transforming Activity of the c-Ha-ras Oncogene. Nature 334, 119-124.

Collard,J.G., Schijven,J.F. and Roos,E., 1987. Invasive and Metastatic Potential Induced by ras-Transfection into Mouse BW5147 T-Lymphoma Cells. Cancer Research 47, 754-759.

Dano,K., Andreasen,P.A., Grondahl-Hansen,J., Kristensen,P., Nielsen,L.S. and Skriver,L., 1985. Plasminogen Activators, Tissue Degradation and Cancer. Adv. Cancer Res. 44, 146-239.

DeCaprio,J.A., Ludlow,J.W., Figge,J., Huang,C.-M., Lee,W.-H., Marsilio,E., Paucha,E. and Livingston,D.M., 1988. SV40

Large Tumor Antigen Forms a Specific Complex with the Product of the Retinoblastoma Susceptibility Gene. Cell 54, 275-283.

Denhardt,D.T., Greenberg,A.H., Egan,S.E., Hamilton,R.T. and Wright,J.A., 1987. Cysteine Proteinase Cathepsin L Expression Correlates Closely with the Metastatic Potential of H-ras-Transformed Murine Fibroblasts. Oncogene 2, 55-59.

Di Fiore,P.P., Pierce,J.H., Fleming,T.P., Hazan,R., Ullrich,A., King,C.R., Schlessinger,J. and Aaronson,S.A., 1987. Overexpression of the Human EGF Receptor Confers an EGF-Dependent Transformed Phenotype to NIH 3T3 Cells. Cell 51, 1063-1070.

Doniger,J., 1988. Alternative Splicing Results in a Truncated N-ras Protein. Oncogene 2, 293-294.

Doolittle,R.F., Hunkapiller,M.W., Hood,L.E., Devare,S.G., Robbins,K.C., Aaronson,S.A. and Antoniades,H.N., 1983. Simian Sarcoma Virus onc Gene, v-sis, Is Derived from the Gene (or Genes) Encoding Platelet-Derived Growth Factor. Science 221, 275-277.

Dotto,G.P., Weinberg,R.A. and Ariza,A., 1988. Malignant Transformation of Mouse Primary Keratinocytes by Harvey

Sarcoma Virus and its Modulation by Surrounding Normal Cells. Proc. Nalt. Acad. Sci. USA. 85, 6389-6393.

Egan,S.E., McClarty,G.A., Jarolim,L., Wright,J.A., Spiro,I., Hager,G. and Greenberg,A.H., 1987a. Expression of H-ras Correlates with Metastatic Potential: Evidence for Direct Regulation of the Metastatic Phenotype in 10T1/2 and NIH-3T3 Cells. Mol. Cell. Biol. 7, 830-837.

Egan,S.E., Wright,J.A., Jarolim,L., Yanagihara,K., Bassin,R.H. and Greenberg,A.H., 1987b. Transformation by Oncogenes Encoding Protein Kinases Induces the Metastatic Phenotype. Science 238, 202-205.

Egan,S.E., Broere,J.J., Jarolim,L., Wright,J.A. and Greenberg,A.H., 1989. Coregulation of Metastatic and Transforming Activity of Normal and Mutant ras Genes. International Journal of Cancer. In the press.

Egan,S.E. Wright,J.A., Jarolim,L., Rogelj,S., and Greenberg,A.H., 1988. Regulation of the Metastatic Phenotype by Growth Factors. Manuscript in preparation.

Fidler,I.J., 1973. Selection of Successive Tumour Lines for Metastasis. Nature New Biol. 242,148-149.

Fidler,I.J. and Kripke,M.L., 1977. Metastasis Results from Preexisting Variant Cells Within a Malingnant Tumor. Science 197, 893-895.

Franza,B.R.Jr., Maruyama,K., Garrels,J.I. and Ruley,E.H., 1986. In Vitro Establishment is not a Sufficient Prerequisite for Transformation by Activated Ras Oncogenes. Cell 44, 409-418.

Frykberg,L., Graf,T. and Vennstrom,B., 1987. The Transforming Activity of the Chicken c-myc Gene can be Potentiated by Mutations. Oncogene 1, 415-421.

Furie,B and Furie,F.C., 1988. The Molecular Basis of Blood Coagulation. Cell 53, 505-518.

Gazit,A., Igarashi,H., Chiu,I.-M., Srinivasan,A., Yaniv,A., Tronick,S.R., Robbins,K.C. and Aaronson,S.A., 1984. Expression of the Normal sis/PDGF-2 Coding Sequence Induces Cellular Transformation. Cell 39, 89-97.

Gehlsen,K.R., Dillner,L., Engvall,E. and Ruoslahti,E., 1988. The Human Laminin Receptor Is a Member of the Integrin Family of Cell Adhesion Receptors. Science 241, 1228-1229.

Gisselbrecht,S., Andre,C., Galibert,F. and Tambourin,P.,

1987. Frequent c-fms Activation by Proviral Insertion in Mouse Myeloblastic Leukaemias. Nature 329, 259-261.

Gopalakrishna,R. and Barsky,S.H., 1988. Tumor Promoter-Induced Membrane-Bound Protein Kinase C Regulates Hematogenous Metastasis. Proc. Natl. Acad. Sci. USA. 85, 612-616.

Greenberg,A.H., Egan,S.E., Jarolim,L., Gingras,M.-C. and Wright,J.A., 1987a. Natural Killer Cell Regulation of Implantation and Early Lung Growth of H-ras-Transformed Fibroblasts in Mice. Cancer Research 47, 4801-4805.

Greenberg,A.H., Egan,S.E., Jarolim,L. and Wright,J.A., 1987b. NK Sensitivity of H-ras Transfected Fibroblasts is Transformation-Independent. Cellular Immunology 109, 444-450.

Greig,R.G., Koestler,T.P., Trainer,D.L., Corwin,S.P., Miles,L., Kline,T., Sweet,R., Yokoyama,S. and Poste,G., 1985. Tumorigenic and Metastatic Properties of "Normal" and ras-Transfected NIH/3T3 Cells. Proc. Natl. Acad. Sci. USA. 82, 3698-3701.

Guernsey,D.L., Ong,A. and Borek,C., 1980. Thyroid Hormone Modulation of X-Ray-Induced in vitro Neoplastic

Transformation. Nature 288, 591-592.

Hann,S.R., King,M.W., Bentley,D.L., Anderson,C.W. and Eisenman,R.N., 1988. A Non-AUG Translational Initiation in c-myc Exon-1 Generates an N-terminally Distinct Protein Whose Synthesis is Disrupted in Burkitt's Lymphoma. Cell 52, 185-195.

Hansen,M.F. and Cavenee,W.K., 1988. Tumor Suppressors: Recessive Mutations that lead to Cancer. Cell 53, 172-173.

Harrison,J. and Auersperg,N., 1981. Epidermal Growth Factor Enhances Viral Transformation of Granulosa Cells. Science 213, 218-219.

Harris,J.F., Chambers,A.F., Hill,R.P. and Ling,V., 1982. Metastatic Variants are Generated Spontaneously at a high rate in Mouse KHT Tumor. Proc. Acad. Natl. Sci. USA. 79, 5547-5551.

Hill,R.P., Chambers,A.F., Ling,V., and Harris,J.F., 1984. Dynamic Heterogeneity: Rapid Generation of Metastatic Varients in Mouse B16 Melanoma Cells. Science 224, 998-1001.

Hill,S.A., Wilson,S. and Chambers,A.F., 1988. Clonal Heterogeneity, Experimental Metastatic Ability, and p21

Expression in H-ras-Transformed NIH-3T3 Cells. Journal of the National Cancer Institute **80**, 484-490.

Hirakawa,T. and Ruley,H.E.,1988. Rescue of Cells from ras-Oncogene Induced Growth Arrest by a Second, Complementing, Oncogene. Proc. Natl. Acad. Sci. USA. **85**, 1519-1523.

Houle,B., Dobrovic,A and Bradley,W.B.C., 1988. Implication of erb-A b, Encoding a Thyroid Hormone Receptor, in Small-Cell Lung Cancer (SCLC). Proceedings of the XVI International Congress of Genetics. Genome **30** Supplement 1,page 197 Abstract 32.21.12.

Hsiao,W.,-L.,W., Gattoni-Celli,S. and Weinstein,I.B., 1984. Oncogene-Induced Transformation of C3H 10T1/2 Cells is Enhanced by Tumor Promoters. Science **226**, 552-555.

Hsiao,W.,-L.,W., Lopez,C.,A., Wu,T. and Weinstein,I.B., 1987. A Factor Present in Fetal Calf Serum Enhances Oncogene-Induced Transformation of Rodent Fibroblasts. Mol. Cell. Biol. **7**, 3380-3385.

Huang,J.S., Huang,S.S., Deuel,T.F., 1984. Transforming Protein of Simian Sarcoma Virus Stimulates Autocrine Transformation of SSV-Transformed Cells through PDGF Cell-Surface Receptors. Cell **39**, 79-87.

Ishikawa,F., Takaku,F., Nagao,M. and Sugimura,T., 1987. Rat c-raf Oncogene Activation by a Rearrangement that produces a Fused Protein. Mol. Cell. Biol. 7, 1226-1232.

Iwamoto,Y., Robey,F.A., Graf,J., Sasaki,M., Kleinman,H.K., Yamada,Y., Martin,G.R., 1987. YIGSR, A Synthetic Laminin Pentapeptide, Inhibits Experimental Metastasis Formation. Science238, 1132-1134.

Johnson,P.J., Coussens,P.M., Danko,A.V. and Shalloway,D., 1985. Overexpressed pp60src can Induce Focus Formation Without Complete Transformation of NIH 3T3 Cells. Mol. Cell. Biol. 5, 1073-1083.

Karre,K., Klein,G.O., Kiessling,R., Klein,G. and Roder,J.C., 1980. Low Natural in vivo Resistance to Syngeneic Leukaemias in Natural Killer-Deficient Mice. Nature 284, 624-626.

Land,H., Parada,L.F. and Weinberg,R.A., 1983a. Tumorigenic Conversion of Primary Embryo Fibroblasts Requires at least two Cooperating Oncogenes. Nature 304, 596-602.

Land,H., Parada,L.F. and Weinberg,R.A., 1983b. Cellular Oncogenes and Multistep Carcinogenesis. Science 222, 771-778.

Land,H., Chen,A.C., Morgenstern,J.P., Parada,L.F. and Weinberg,R.A., 1986. Behavior of myc and ras Oncogenes in Transformation of Rat Embryo Fibroblasts. Mol. Cell. Biol. 6, 1917-1925.

Leduc,F., Hajj,C., Ayoub,J. and Bradley,W.E.C., 1988. Recessive Genetic Changes Near the erb-A b Locus Implicated in the Phenotype of Small-Cell Lung Cancer vs Non Small-Cell Lung Cancer. Proceedings of the XVI International Congress of Genetics. Genome 30 Supplement 1, page 197, Abstract 32.21.11.

Liotta,L.A., 1986. Tumor Invasion and Metastasis-Role of the Extracellular Matrix: Rhoads Memorial Award Lecture. Cancer Research 46, 1-7.

Liotta,L.A., Guirguis,R., Stracke,M., Kohn,E. and Schiffman,E., 1988a. Augmentation of Laminin Receptors in Cytokine Induced Psuedopodia Protrusion. 4rth International Congress of Cell Biology Symposia S38.3

Liotta,L.A., 1988b. Editorial; H-ras p21 and the Metastatic Phenotype. Journal of the National Cancer Institute 80, 468-469.

Liotta,L.A., 1988c. Editorial; Growth Autonomy: The Only Requirement for Metastasis? Journal of the National Cancer Institute 80, 300.

Marth,J.D., Overell,R.W., Meier,K.E., Krebs,E.G. and Perlmutter,R.M., 1988. Translational Activation of the lck Proto-oncogene. Nature 332, 171-173.

Martinet,Y., Bitterman,P.B., Mornex,J.-F., Grotendorst,G.R., Martin,G.R. and Crystal,R.G., 1986. Activated Human Monocytes express the c-sis Proto-Oncogene and Release a Mediator showing PDGF-like activity. Nature 319, 158-160.

Mignatti,P. Robbins,E. and Rifkin,D.B., 1986. Tumor Invasion through the Human Amniotic Membrane: Requirement for a Proteinase Cascade. Cell 47, 487-498.

Mowat,M., Cheng,A., Kimura,N., Bernstein,A. and Benchimol,S., 1985. Rearrangements of the Cellular p53 Gene in Erythroleukemic Cells Transformed by Friend Virus. Nature 314, 633-636.

Muller,W.J., Sinn,E., Pattengale,P.K., Wallace,R. and Leder,P., 1988. Single-Step Induction of Mammary Adenocarcinoma in Transgenic Mice Bearing the Activated c-neu Oncogene. Cell 54, 105-115.

Muschel,R.J., William,J.E., Lowy,D.R. and Liotta,L.A., 1985. Harvey ras Induction of Metastatic Potential Depends Upon Oncogene Activation and the Type of Recipient Cell. American Journal of Pathology **121**, 1-8.

Muschel,R.J., Nakahara,K., Chu,E., Pozzatti,R. and Liotta,L.A., 1986. Karyotypic Analysis of Diploid or Near Diploid Metastatic Harvey ras Transformed Rat Embryo Fibroblasts. Cancer Research **46**, 4101-4108.

Nicolson,G., 1982. Cancer Metastasis: Organ Colonization and the Cell-Surface Properties of Malignant Cells. Biochimica et Biophysica Acta **695**, 113-176.

Nicolson,G.L., Dulski,K.M. and Trosko,J.E., 1988. Loss of Intracellular Junctional Communication Correlates with Metastatic Potential in Mammary Adenocarcinoma Cells. Proc. Natl. Acad. Sci. USA. **85**, 473-476.

Nowell,P.C., 1976. The Clonal Evolution of Tumor Cell Populations. Science **194**, 23-28.

Oshimura,M., Gilmer,T.M., and Barret,J.C., 1985. Nonrandom Loss of Chromosome 15 in Syrian Hamster Tumours Induced by v-Ha-ras plus v-myc Oncogenes. Nature **316**, 636-639.

Paget,S., 1889. The Distribution of Secondary Growths in Cancer of the Breast. Lancet i,571-573.

Palmiter,R.D. and Brinster,R.L., 1986. Germ Line Transformation of Mice. Ann. Rev. of Genetics 20, 465-499.

Pohl,J., Goldfinger,N., Radler-Pohl,A., Rotter,V. and Schirrmacher,V., 1988. p53 Increases Experimental Metastatic Capacity of Murine Carcinoma Cells. Mol. Cell. Biol. 8, 2078-2081.

Ponder,B., 1988. Cancer; Gene Losses in Human Tumours. Nature 335, 400-402.

Poste,G and Fidler,I.J., 1980. The Pathogenesis of Cancer Metastasis. Nature 283, 139-145.

Pozzatti,R., Muschel,R., Williams,J., Padmanabhan,R., Howard,B., Liotta,L.A. and Khoury,G., 1986. Primary Rat Embryo Cells Transformed by One or Two Oncogenes Show Different Metastatic Potentials. Science 232, 223-227.

Quinttanilla,M., Brown,K., Ramsden,M. and Balmain,A., 1986. Carcinogen-Specific Mutation and Amplification of Ha-ras during Mouse Skin Carcinogenesis. Nature 322, 78-80.

Rabin,M.S., Doherty,P.J. and Gottesman,M.M., 1986. The Tumor Promoter Phorbol 12-Myristate 13-acetate Induces a Program of Altered Gene-Expression Similar to that Induced by Platelet-Derived Growth Factor and Transforming Oncogenes. Proc. Natl. Acad. Sci. USA. **83**, 357-360.

Ragozzino,M., Kuo,A., Kohl,N., O'Brien,M. and Ruley,H.E., 1988. Mechanisms of Oncogene Collaboration: Activation and Inactivation of a Dominant Repressor. Proceedings from Fourth Annual Meeting on Oncogenes; Frederick Maryland, page 300.

Rassoulzadegan,M., Cowie,A., Carr,A., Glaichenhaus,N., Kamen,R. and Cuzin,F., 1982. The Roles of Individual Polyoma Virus Proteins in Oncogenic Transformation. Nature 300, 713-718.

Raz,A. and Ben-Ze'ev,A., 1983. Modulation of the Metastatic Capability in B16 Melanoma by Cell Shape. Science 221, 1307-1310.

Roos,E., 1984. Cellular Adhesion, Invasion and Metastasis. Biochimica et Biophysica Acta 738, 263-284.

Rous,P., 1911. Transmission of a Malignant New Growth By

Means of a Cell Free Filtrate. Journal of the American Medical Association 56, 198.

Roussel,M.F., Dull,T.J., Rettenmier,C.W., Ralph,P., Ullrich,A. and Sherr,C.J., 1987. Transforming Potential of the c-fms Proto-Oncogene (CSF-1 Receptor). Nature 325, 549-552.

Ruley,E.H., 1983. Adenovirus Early Region 1A Enables Viral and Cellular Transforming Genes to Transform Primary Cells in Culture. Nature 304, 602-606.

Sadowski,I., Pawson,T. and Lagarde,A., 1988. v-fps Protein-Tyrosine Kinase Coordinately Enhances the Malignancy and Growth Factor Responsiveness of Pre-neoplastic Lung Fibroblasts. Oncogene 2, 241-248.

Sherr,C.J., Rettenmier,C.W., Sacca,R., Roussel,M.F., Look,A.T. and Stanley,E.R., 1985. The c-fms Proto-Oncogene is Related to the Receptor for the Mononuclear Phagocyte Growth Factor, CSF-1. Cell 41, 665-676.

Sporn,M.B. and Roberts,A.B., 1985. Autocrine Growth Factors and Cancer. Nature 313, 745-747.

Stern,D.F., Roberts,A.B., Roche,N.S., Sporn,M.B. and

Weinberg,R.A., 1986. Differential Responsiveness of myc- and ras-Transfected Cells to Growth Factors: Selective Stimulation of myc-Transfected Cells to Epidermal Growth Factor. Mol. Cell. Biol. 6, 870-877.

Stracke,M.L., Guirguis,R., Liotta,L.A. and Schiffmann,E., 1987. Pertussis Toxin Inhibits Stimulated Motility Independently of the Adenylate Cyclase Pathway in Human Melanoma Cells. Biochemical and Biological Research Communication 146, 339-345.

Talmadge,J.E., Meyers,K.M., Prieur,D.J. and Starkey,J.R., 1980. Role of NK Cells in Tumour Growth and Metastasis in Beige Mice. Nature 284, 622-624.

Tanaka,K., Yoshioka,T., Bieberich,C. and Jay,G., 1988. Role of the Major Histocompatibility Complex Class I Antigens in Tumor Growth and Metastasis. Ann. Rev. Immunol. 6, 359-380.

Taniguchi,K., Petersson,M., Hoglund,P., Kiessling,R., Klein,G. and Karre,K., 1987. Interferon gamma Induces Lung Colonization by Intrevenously Inoculated B16 Melanoma Cells in Parallel with Enhanced Expression of Class I Major Histocompatibility Complex Antigens. Proc. Natl. Acad. Sci. USA. 84, 3405-3409.

Thorgeirsson,U.P., Turpeenniemi-Hujanen,T., Williams,J.E., Westin,E.H., Heilman,C.A., Talmadge,J.E. and Liotta,L.A., 1985. NIH/3T3 Cells Transfected with Human Tumor DNA Containing ras Oncogenes Express the Metastatic Phenotype in Nude Mice. Mol. Cell. Biol. 5, 259-262.

Trimble,W.S., Johnson,P.W., Hozumi,N. and Roder,J.C., 1986. Inducible Cellular Transformation by a Metallothionein-ras Hybrid Oncogene Leads to Natural Killer Cell Susceptibility. Nature 321, 782-784.

Turpeeniemi-Hujanen,T., Thorgeirsson,U.P., Rao,C.N. and Liotta,L.A., 1986. Laminin Increases the Release of Type IV Collagenase from Malignant Cells. J. Biol. Chem. 261, 1883-1889.

Varani,J., Fligiel,S.E.G. and Wilson,B., 1986. Motility of H-ras Oncogene Transformed NIH-3T3 Cells. Invasion Metastasis 6, 335-346.

Velu,T.J., Beguinot,L., Vass,W.C., Willingham,M.C., Merlino,G.T., Pastan,I. and Lowy,D.R., 1987. Epidermal Growth Factor-Dependent Transformation by a Human EGF Receptor Proto-Oncogene. Science 238, 1408-1410.

Verma,I.M. and Graham,W.R., 1987. The fos Oncogene. Advances

in Cancer Research 49, 29-52.

Vogel,U.S., Dixon,R.A.F., Schaber,M.D., Diehl,R.E., Marshall,M.S., Scolnick,E.M., Sigal,I.S. and Gibbs,J.B., 1988. Cloning of Bovine GAP and its Interaction with Oncogenic ras p21. Nature 335, 90-93.

Vogt,P.K. and Tjian,R., 1988. Jun: A Transcriptional Regulator Turned Oncogenic. Oncogene 3, 3-7.

Waghorne,C., Kerbel,R.S. and Breitman,M.L., 1987. Metastatic Potential of SP1 Mouse Mammary Adenocarcinoma Cells is Differentially Induced by Activated and Normal Forms of c-H-ras. Oncogene 1, 149-155.

Wallich,R., Bulbuc,N., Hammerling,G.J., Katzav,S., Segal,S. and Feldman,M., 1985. Abrogation of Metastatic Properties of Tumor Cells by de novo Expression of H-2K Antigens following H-2 Gene Transfection. Nature 315, 301-305.

Warner,J.F. and Dennert,G., 1982. Effects of a Clonal Cell Line with NK Activity on Bone Marrow Transplants, Tumour Development and Metastasis in vivo. Nature 300, 31-34.

Waterfield,M.D., Scrace,G.T., Whittle,N., Stroobant,P., Johnsson,A., Wasteson,A., Westermark,B., Heldin,C.-H.,

Huang,J.S., Deuel,T.F., 1983. Platelet-Derived Growth Factor is Structurally Related to the Putative Transforming Protein p28sis of Simian Sarcoma Virus. Nature 304,35-39.

Weinberg,R.A., 1985. The Action of Oncogenes in the Cytoplasm and the Nucleus. Science 230, 770-776.

Weinberg,R.A., 1988. Finding the Anti-Oncogene. Scientific American September 1988, 44-51.

Weiss.L., 1983. Random and Nonrandom Processes in Metastasis, and Metastatic Inefficiency. Invasion Metastasis 3, 193-207.

Wewer,U.M., Liotta,L.A., Jaye,M., Ricca,G.A., Drohan,W.N., Claysmith,A.P., Rao,C.N., Wirth,P., Coligan,J.E., Albrechtsen,R., Mudryj,M. and Sobel,M.E., 1986. Altered levels of Laminin Receptor mRNA in various Human Carcinoma Cells that have different abilities to bind Laminin. Proc. Natl. Acad. Sci. USA. 83, 7137-7141.

Wewer,U.M., Taraboletti,G., Sobel,M.E., Albrechtsen,R. and Liotta,L.A., 1987. Role of Laminin in Tumor Cell Migration. Cancer Research 47, 5691-5698.

Whyte,P., Buchkovich,K.J.,Horowitz,J.M., Friend,S.H.,

Raybuck,M., Weinberg,R.A. and Harlow,E., 1988. Association Between an Oncogene and an Anti-Oncogene: the Adenovirus E1A Proteins bind to the Retinoblastoma Gene Product. Nature **334**, 124-129.

Wigler,M. and Weinstein,I.B., 1976. Tumor Promoter Induces Plasminogen Activator. Nature **259**, 232-233.

I EXPRESSION OF H-*ras* CORRELATES WITH METASTATIC POTENTIAL: EVIDENCE FOR DIRECT REGULATION OF THE METASTATIC PHENOTYPE IN 10T1/2 AND NIH-3T3 CELLS

This work appears in published form elsewhere;

Expression of H-<u>ras</u> Correlates with Metastatic Potential: Evidence for Direct Regulation of the Metastatic Phenotype in 10T1/2 and NIH-3T3 cells.

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ABSTRACT

Using three independent approaches, we have studied the effects of H-ras on metastasis formation. Analysis of five in vitro ras-transfected 10T1/2 clones with either flat or refractile morphologies revealed a relationship between metastatic potential, H-ras expression and anchorage-independent growth. Four metastatic variants derived from a poorly metastatic, low H-ras expressing line all expressed high levels of H-ras RNA and grew efficiently in soft agar. Activation of H-ras expression in the metastatic tumors had occurred through amplification and rearrangement of H-ras sequences. In addition, pre-induction of p21 synthesis in NIH-3T3 line 433, which contains v-H-ras under transcriptional control of the glucocorticoid sensitive MMTV LTR, significantly increased metastatic efficiency. Glucocorticoid treatment of normal or pEJ transformed NIH-3T3 cells did not affect metastatic potential. These data reveal a direct relationship between ras expression and metastasis formation and suggest that metastatic and transformed phenotypes may be coregulated in ras transformed 10T1/2 and NIH-3T3 cells.

INTRODUCTION

Tumor progression is the tendency of tumors to become more aggressive with time. The widely accepted model is that described originally by Foulds in which progression was characterized by emergence of new variants with a selective advantage for growth in the host (10). Metastatic spread is the most important form of tumor progression because it is the most life threatening aspect of the disease. Metastasis is a complex process involving invasion through host barriers into the vasculature, surviving circulating host immune defences followed by implantation, extravasation and growth at sites distant to the primary neoplasm (23, 24, 26, 33).

Much effort has been directed at understanding the metastatic cascade yet little is known about the mechanisms involved. On the other hand, the critical events in cell immortalization and transformation have been partially elucidated and attributed to mutation or disregulation of a group of genes collectively known as oncogenes. These genes are normally responsible for maintenance of control over diverse cellular functions including proliferation, differentiation, morphology, communication and motility (21, 39, 32, 2, 19). Consequently, they are good candidates for study of the metastatic process which also requires alterations of many of these functions (23, 26, 33).

Recent studies have shown that primary and established

rodent fibroblasts transformed by activated ras sequences can form metastases (11, 22, 27, 37). Exclusive selection and analysis of in vitro transformed foci however, raises the possibility that ras transformation may be a permissive event for expression of the metastatic phenotype but not directly involved. In order to resolve this question we have analyzed the relationship between ras expression and metastatic potential. ras-mediated transformation may be the initial event which allows for expression of secondary cellular characteristics important in metastatic conversion, or it may be more directly involved in maintaining and regulating the metastatic phenotype through expression and action of its gene product. Recent work has demonstrated a relationship between levels of H-ras oncogene (20,41) or proto-oncogene (28) expression and tumorigenic potential. In addition, it has been shown that morphological reversion of the ras-transformed phenotype may be achieved through microinjection of anti-p21 antibodies (7). Consequently, ras expression is critical in maintaining the transformed phenotype. In this study we provide evidence for the direct involvement of ras expression in metastatic potential, suggesting that ras-mediated tumorigenic and metastatic phenotypes may be regulated through a common mechanism in both 10T1/2 and NIH-3T3 cells.

Materials and Methods

Gene Transfer, Plasmids and In Vitro Derived Cell Lines

DNA mediated gene transfer or transfection was carried out using the calcium phosphate method as previously described (40). The plasmid pAL8A was constructed by introducing the 6.6 Kb T24 H-ras insert into the BamHl site of pSV2neo. Following transfection of pAL8A into 10T1/2 cells, three morphologically transformed cell lines were established and cloned from foci observed at confluence. These cell lines, designated CIRAS 1,2 and 3, were subsequently shown to be resistant to 400 ug/ml of G418 sulphate (Gibco, Grand Island, NY). Two other cell lines were isolated through selection in 400 ug/ml G418. These two lines, NR3 and NR4, were morphologically non-transformed. Focus formation was observed at a frequency of 2 x 10^{-4}/cell/ug DNA. In contrast, following G418 selection the frequency of resistance was 4 x 10^{-4}/cell/ug DNA which resulted in 60% flat and 40% refractile colonies. MDS.R cell lines are radiation transformed 10T1/2 cells which were selected for anchorage independent growth and tumorigenicity (29). All 10T1/2 derived cell lines were grown in either MEM or F12 media (Flow Laboratories, McLean, Va) supplemented with 10% fetal calf serum (Gibco Laboratories).

NIH-3T3 line 433, which contains v-H-ras under control of the glucocorticoid sensitive MMTV LTR (plasmid pA9)(12),

was grown in MEM (Gibco) medium and 10% FCS, with or without 2 x 10^{-6} M dexamethasone (Sigma, St. Louis, Mo) for 7 days prior to injection into BALB/c nu/nu mice. Growth with dexamethasone under these conditions results in a 20-fold increase in p21 synthesis (12). Control cell lines pEJ1 and pEJ3 were obtained through transfection of pEJ H-ras into NIH-3T3 cells and cloned by limiting dilution, from transformed foci. NIH-3T3 derived cell lines were maintained in MEM media supplemented with 10% calf serum (Colorado Serum, Denver, Co). All cells were kept in culture for a maximum of approximately two months before discarding and returning to frozen stocks to minimize drift from the original clones.

Experimental and Spontaneous Metastasis Assays

Metastatic potential was determined by the experimental metastasis assay using a 3 x 10^5 tumor cell inoculum (except with NIH-3T3 line 433 where 5x10^5 cells were used) injected in 0.2 ml volume into the tail vein of mice (8). Cells were lightly trypsinized from subconfluent cultures, washed and adjusted to the appropriate concentration in Hanks balanced salt solution. Recipient animals were sacrificed by ether anesthesia twenty-one days later and Bouin's solution [picric acid, formaldehyde, and acetic acid (15:5:1)] injected intratracheally. The stained lungs were then removed and metastatic foci counted under a dissecting microscope.

Spontaneous lung metastasis formation was assayed between 30 and 60 days after subcutaneous injection. Lung metastases were occasionally visible but normally detected as micrometastases by culturing enzymatically disaggregated lung cells in 400 ug/ml G418 (see below).

In Vivo Derived Cell Lines

In vivo derived lines were obtained by dissecting out the tumor, cutting and teasing into small fragments and enzymatically disaggregating (800 ug/ml collagenase type I, Sigma; 10 U/ml hyaluronidase type I-S, Sigma; and 0.05% trypsin-EDTA, Gibco), followed by selection of plasmid carrying cells in 400 ug/ml G418 sulphate for 3 days. NR3.1L lines were derived from 2 C3H/HeN mice with rare experimental lung metastases following intravenous injection of 10^6 NR3 cells. NR3.3 and NR3.4 were isolated from non-regressing tumors 40-45 days after subcutaneous injection of 3×10^5 NR3 into C3H/HeN mice.

Growth in Semisolid Medium

Growth in soft agar was performed basically as previously described (35) with a 0.5% Bacto-agar (Difco, Detroit, Mich.), 10% FCS, MEM base layer and a 0.33% agar, 10% FCS, MEM growth layer. Colonies were scored after 14 days.

Northern and Southern Blot Analysis

Total RNA was prepared by the quanidinium/cesium chloride method previously described (3) and 20 ug

electrophoresed on 1% formaldehyde gels (15). RNA was then transferred to nitrocellulose and hybridized at 68°C for 16 hours to a 32p-labelled (3 x 10^8 cpm/ug) nick translated (31) v-H-ras probe (Oncor Inc., Gaithersberg, MD). The filters were washed in 2 x SSC (1 x SSC is 0.15M NaCl and 0.015 M sodium citrate), 0.1% SDS (2 x 15 min. at room temperature (RT)) followed by 0.1 x SSC, 0.1% SDS (1 x 30 min.at RT, and 1 x 30 min. at 65°C). Autoradiography was carried out at -70°C using Kodak X-Omat AR film and Cronex lighting plus intensifying screens. Total RNA (20 μg) was also electrophoresed on 0.6% agarose gels in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA)(18, 36) and stained with ethidium bromide to confirm relative quantitation and ensure that degradation had not occurred.

Southern blot analysis was performed with two different protocols using BamH1 and EcoR1. When BamH1 was used, 20ug of genomic DNA was digested and separated on a 0.6% agarose gel, transferred to a nylon membrane (BioRad) with 0.4 N NaOH and hybridized for 24 hours to a ^{32}P-labelled (Klenow extension)(6)(3 x 10^8 cpm/ug) v-H-ras probe (50% formamide, 42°C) as previously described (30). Alternatively, 20 μg of genomic DNA were digested with EcoR1 and electrophoresed on a 0.45% agarose gel. DNA was then transferred to nitrocellulose (34) and hybridized at 42°C (50% formamide, 10% dextran sulfate) for 16 hours to a ^{32}P-labelled (nick translated)(31)(3 x 10^8 cpm/ug) v-H-ras probe. Following

hybridization, filters were washed in 2 x SSC, 0.1% SDS (2 x 15 min. at RT) and 0.1 x SSC, 0.1% SDS (2 x 30 min. at 65°C).

Densitometric Analysis

Relative levels of H-ras mRNA was determined by densitometry using a BioRad Model 165D Transmittance/Reflectance Scanning Desitometer. Peaks were cut, weighed and ratios determined from 3 Northern blots. Reprobing with ß-tubulin or c-myc revealed that differences in ras RNA were not a result of differential loading of mRNA (not shown)

RESULTS

METASTATIC POTENTIAL OF IN VITRO DERIVED CELL LINES

Transfection of pAL8A into 10T1/2 cells allowed us to introduce T24 H-ras linked to a selectable (neomycin resistance) marker gene. Five clones were picked and expanded for analysis. Three transformed foci gave rise to the cell lines CIRAS 1, 2, and 3, whereas cell lines NR3 and NR4 arose from morphologically flat colonies selected in G418. CIRAS-1, -2 and -3 cells were disorganized and consistently overlapped, NR3 cells were flat, contact inhibited and organized (similar to 10T1/2), whereas NR4 cells were intermediate for these characteristics (Figure 3). The isolation of cell lines with different colony morphologies ultimately enabled us to analyze the

relationship between ras expression and various phenotypic properties including tumorigenic and metastatic potentials.

Survival and tumour latency data (Table 2) indicate that CIRAS-2 and -3 are the most tumorigenic of the five lines, CIRAS-1 and NR4 show intermediate tumor latency and NR3 is poorly tumorigenic with many of the tumours regressing. Subcutaneous injection of up to 10^7 cells of control 10T1/2 into either syngeneic C3H/HeN or immunodeficient BALB/c nu/nu mice did not result in tumor formation.

Spontaneous metastases were detected in all mice injected with CIRAS-1, 2 or 3, while three of five mice injected with NR4 and none of five injected with either NR3 or parental 10T1/2 developed tumors at other sites (Table 2). In order to quantitate metastatic potential, the lung colonization or experimental metastasis model was employed. Using this assay, CIRAS-2 and -3 were highly metastatic, CIRAS-1 and NR4 were relatively low, and NR3 was virtually non-metastatic with only 3 out of 43 mice injected producing lung tumors and two of these at the highest cell inoculum (10^6). Control 10T1/2 transfected with pSV2neo produced only rare experimental metastases (one tumor each, in 2 of 6 mice injected with 3×10^5 cells each). Radiation transformed 10T1/2 cell lines (MDSR) were tumorigenic but not metastatic (Table 2).

A correlation between anchorage-independent growth and

metastatic potential has been observed in several systems (4,35). We assessed the agar cloning efficiency of all five transfected lines and the 10T1/2 control (Table 2). Correlating well with in vivo metastatic potential, CIRAS-2 and -3, the most metastatic lines grew very efficiently in agar, while CIRAS-1 and NR4 grew very poorly and were inefficient at forming lung foci. NR3 rarely produced agar colonies (not shown) or metastases. 10T1/2 never grew in soft agar.

Southern blotting revealed the presence of novel H-ras sequences in all five lines (Figure 4), however, gene copy number did not correlate with in vivo behaviour. Northern blot analysis was also carried out and 1.2 Kb messages were detected in all lines (Figure 5). In addition, CIRAS-1 also expressed high levels of a 4.7 Kb species. Densitometric analysis of three Northern blots revealed a relationship between expression of H-ras and metastatic potential in immunocompetant C3H/HeN mice (Fig. 6a) as well as in NK cell deficient C3H/HeJ bg/bg mice and their normal bg/+ littermates (Fig. 6b). Consideration of the 4.7 Kb species as a translatable message (5) yielded the relationship shown in Fig. 6a ($r=0.899$). Inclusion of only 1.2 Kb species in the calculations yielded essentially the same result ($r=0.844$).

IN VIVO SELECTION OF METASTATIC VARIANTS

We next isolated four cell lines from two mice bearing

rare NR3 derived experimental lung metastasis (NR3.1L A,B,C,D). These four lines were cultured from two distinct lung colonies in each animal and therefore represent tumors arising from four independent cells or emboli in vivo. The lines were all morphologically transformed (NR3.ILB, Fig. 3). In addition, they were highly tumorigenic, metastatic (Table 3) and grew efficiently in soft agar (not shown). Two lines were also isolated from mice bearing non-regressing tumors following subcutaneous injection of NR3 cells. Once again, these tumor lines (NR3.3 and NR3.4) were also refractile (Fig. 3), highly tumorigenic and metastatic (Table 3).

All six in vivo derived cell lines expressed high levels of H-ras RNA as compared to the poorly expressing NR3 parental line from which they arose (Fig. 7). Evidence that gene rearrangement and amplification had occurred in the lung metastases (NR3.IL) was found on Southern blotting (Fig. 8). All four metastatic lines showed complex and identical restriction patterns with novel H-ras sequences indicating that they were of clonal origin. The subcutaneous tumors NR3.3 and NR3.4 were activated through gene rearrangement resulting in only a single novel H-ras sequence. Both NR3.3 and NR3.4 also exhibited the identical restriction band, again indicating common origin. Southern blot analysis of these lines following BamH1 digestion (not shown) confirms the identity of restriction patterns between

all four metastatic lines and, independently, between the two subcutaneous tumors. INDUCTION OF v-H-RAS IN NIH-3T3 CELLS

The relationship between activated H-ras expression and metastatic potential was then confirmed in the NIH-3T3 system using line 433. Line 433 contains a plasmid (pA9) in which v-H-ras is transcriptionally regulated by the glucocorticoid sensitive MMTV LTR. It is able to enhance p21 production 20-fold in the presence of physiological amounts of glucocorticoids resulting in a "phenotype switch" (12). Following incubation in the presence or absence of dexamethasone for seven days, line 433 was injected intravenously into BALB/c nu/nu mice. Preinduction of v-H-ras expression prior to injection resulted in a 2.5- to 3.0-fold increase in metastatic effeciency. In contrast, dexamethasone pretreatment of NIH-3T3, pEJ H-ras transformed NIH-3T3 or T24 H-ras transformed 10T1/2 controls did not effect their metastatic potential (Table 4).

DISCUSSION

We have studied the effects of activated H-ras on metastatic potential in both 10T1/2 and NIH-3T3 cells and report that metastatic efficiency is closely related to the level of H-ras expression. H-ras RNA levels correlated with tumorigenicity, anchorage-independent growth, and metastatic potential of a series of T24 H-ras transfected 10T1/2 cell

lines. Metastatic potential was assessed initially by frequency of spontaneous metastases and quantitated with the experimental metastasis assay. This assay involves survival in the circulation, implantation, extravasation and growth, and has been found to correlate very well with spontaneous metastasis formation (13, 24). Although metastasis formation following intravenous injection does not require invasion through host barriers, this process may be quite similar to the extravasation process which is required for successful lung colonization.

The relationship between ras expression and metastatic efficiency was observed in immunocompetant (Fig. 6a), natural killer (NK) cell deficient bg/bg (Fig. 6b) as well as in T-cell deficient BALB/c nu/nu mice (10a). The higher frequency of tumors in the bg/bg suggests that NK cells can reduce metastatic burden but do not affect relative metastatic efficiency (10a). In vitro experiments also indicate that these cell lines are not sensitive to macrophage kill (not shown). Furthermore flow cytometric analysis revealed that expression of MHC class 1 antigens on these lines was unaffected by ras transfection (B. Elliot and A.H. Greenberg, unpublished data). Consequently, it is unlikely that immune regulation is responsible for the relative metastatic efficiencies observed. It should also be noted that tumorigenicity and anchorage-independent growth potential of these lines follow the same

relationship, suggesting that ras expression may affect all three properties directly and through a common mechanism.

Radiation transformed 10T1/2 lines (MDSR) were found to be tumorigenic but non-metastatic, indicating that tumorigenic transformation of 10T1/2 is insufficient for expression of the metastatic phenotype. In contrast pSV2neo transfected 10T1/2 controls produced occasional lung metastases indicating that ras transformation is not the only mechanism by which metastatic cells may be generated. This is not unexpected since activated ras sequences can be detected in only a
minority of human tumors (14). The mechanism by which these 10T1/2 neo cells have acquired metastatic ability is unknown. Possibly, integration of transfected plasmid and carrier DNA was responsible for activation of local genes regulating the metastatic phenotype.

The six in vivo derived NR3 lines were all highly tumorigenic, metastatic, and expressed high levels of H-ras RNA, indicating that selection of metastatic cells also resulted in selection for high H-ras expression. The existence of common restriction banding patterns on Southern blots (Fig. 8) suggests that the four NR3.1L lines likely arose from the same cell, while the two subcutaneous tumor lines were derived from a different but common variant. Activation of H-ras expression may have occurred within the in vitro NR3 population giving rise to preexisting high

H-ras expressing subpopulations that preferentially formed lung tumors on inoculation. Derivation of lung metastases and subcutaneous lines in these experiments was carried out using parallel NR3 cultures of different passage number. Consequently the selection of two distinct subpopulations (at subcutaneous and lung sites) is likely a result of the presence of different variants in these NR3 inocula. The existence of such variants in the NR3 population has been confirmed through in vitro selection of anchorage independent or focus forming metastatic subclones (M.-C. Gingras and A.H. Greenberg unpublished results). Alternatively, it is possible that selection for subcutaneous tumor growth or metastatic ability resulted in outgrowth of different phenotypic variants from an NR3 population containing both subpopulations. This explanation seems less likely since NR3.1L lines were selected for lung colonization but are tumorigenic at subcutaneous sites, and NR3 tumor lines (NR3.3 and NR3.4) were selected for subcutaneous growth yet are metastatic.

The relationship between ras expression and metastatic potential was also evident in the NIH-3T3 system using line 433 which contains v-H-ras under transcriptional control of the glucocorticoid sensitive MMTV LTR. Induction of v-H-ras p21 synthesis prior to intravenous injection resulted in a significant increase in metastatic potential. Physiological glucocorticoids presumably induced v-H-ras in vivo (12) and

could be responsible for metastases from cells not incubated in dexamethasone prior to injection. Although both lines would express v-H-ras in vivo, in vitro induction likely provided a kinetic advantage that allowed more cells to survive and form lung tumors. This result also suggests that activated ras expression is important in the early events following intravenous injection (implantation and extravasation). Control experiments revealed that dexamethasone treatment did not potentiate metastatic efficiency of lines constitutively expressing H-ras.

Greig et al have recently reported that NIH 3T3 cells are both tumorigenic and metastatic under specific conditions (11). This work suggests that transformation of 3T3 cells by activated H-ras may be enhancing a pre-existing metastatic phenotype. These results emphasize the necessity for caution when interpreting the malignant characteristics of NIH-3T3 derived transformants. Although transfection of activated ras can enhance the metastatic potential of a pre-existing malignant phenotype (38), it has been shown that ras expression in NIH-3T3 cells results in enhanced secretion of collagenase IV activity as well as imparting the ability to invade amnion basement membranes on these cells (37). These properties are not shared by NIH-3T3 cells or spontaneous transformants (37). In addition, induction of the metastatic phenotype by activated ras does not require aneuploid recipient cells such as NIH-3T3 but

has been observed in diploid fibroblasts (22, 27). Consequently, ras transformation can result in metastatic conversion in some cell systems. Previous studies have not determined whether this phenomenon is a direct result of ras expression, or if ras transformation may only be permissive for expression of secondary events which then lead to appearance of the metastatic phenotype. Our studies were undertaken to evaluate the relative importance of these two possible roles for ras in metastasis formation. In doing so, we have confirmed previous work and report that 10T1/2 cells are also susceptible to ras-mediated metastastic conversion. More relevant to the hypothesis that ras directly effects the expression of the metastatic phenotype, a correlation between ras expression and metastatic potential was demonstrated: 1) The metastatic potential, tumorigenicity and anchorage independent growth properties of five ras transfected lines all followed the same relationship and correlated with H-ras RNA expression, (2) in vivo derived metastatic variants exhibited high H-ras expression and (3) induction of the v-H-ras gene resulted in enhanced metastatic efficiency. This argues against integrational activation of "metastatic-genes" as a mechanism of ras-mediated metastatic conversion. In particular, induction of ras expression in line 433 clearly effects expression of the metastatic phenotype, presumably without karyotypic changes. Although this study involves a

limited number of ras-transfected cell lines, we feel that the three independent approaches taken together, strongly support a tight link between ras action and the metastatic phenotype. This relationship has not yet been established at the protein level (ras p21), however, the correlation between metastatic, tumorigenic and anchorage independent characteristics of these lines suggests that ras affects all three processes through a common mechanism. These results provide evidence for a direct role of activated ras in regulating and maintaining the metastatic phenotype.

In contrast to the results obtained in 10T1/2, NIH-3T3, and diploid fibroblasts, Mushel et al report that ras-transformed C127 cells are not metastatic (22). Therefore, not all lines expressing activated ras sequences are converted to the metastatic phenotype. Indeed, infection of PC12 neuronal cells by ras containing retroviruses induces them to differentiate (25). It has been suggested that ras p21 is important in signal transduction processes via secondary messangers (16), and evidence is now accumulating that p21 might normally be involved in coupling of receptors for polypeptide agonists to phospholipase C (1, 9). Consequently, phenotypic alterations induced by ras would be cell-type dependent and likely influenced by genes that are transcriptionally active or that are capable of being induced as a result of p21 function. Identification and characterization of these

"metastatic genes" or gene products which are induced or altered by ras may lead to a greater understanding of the process by which transformed cells evolve to the metastatic phenotype. Subsequent to submission of this manuscript Bradley et al. reported that various ras constructs were capable of converting NIH-3T3 to metastatic cells (2a). In addition, this group described rapid acquisition of the metastatic phenotype without in vitro selection. These results are consistent with our findings but the effect of ras expression on metastatic potential reported here argues for direct regulation of the metastatic phenotype as opposed to the qualitative induction suggested by Bradley et al. (2a).

Table 2. Tumorigenic and Metastatic Characteristics of T24-H-*ras*-Transformed 10T½ Lines in Syngeneic C3H/HeN Mice

Cell Line	Tumorigenicity[a]			Exptl Metastases[b]		Frequency of spontaneous metastases[c]	Frequency (mean ± SE) of cloning in soft agar with tumor inoculum[d]			Division Time
	Frequency	Latency (days [mean ± SE])	Survival (days [mean ± SE])	Frequency	No. (mean ± SE)		10^3	10^4	10^5	
10T½	0/12			0/12	0	0/5	0±0	0±0	0±0	14
CIRAS-1	13/13	10.0±1.0	66.5±7.0	20/27	14±5	5/5	0±0	0.6±4	14±2	14
CIRAS-2	11/11	6.5±0.7	58.2±9.5	8/8	118±6	5/5	9±2	360±11	>500	9.7
CIRAS-3	11/11	6.5±0.7	36.2±4.2	14/14	121±20	5/5	7±1	296±99	>500	12.3
NR4	10/10	10.7±1.4	58.5±12.4	12/19	2±0.5	3/5	0±0	1±1	0.2±0.2	14
NR3	6/8	49.6±2.0	106.0±7.0	1/13	0.1±0.1	0/5	0±0	0±0	0±0	15.3
MDS.R1	5/5	7.1±0.9	NT[e]	0/3	0	0/5				
MDS.R5	5/5	8.1±1.1	NT	0/6	0	0/5				
MDS.R9	5/5	8.7±1.3	NT	0/3	0	0/5				
MDS.R25	5/5	4.0±0	NT	0/4	0	0/5				

[a] Results are reported for subcutaneous injection of 3×10^5 cells. Survival data is taken from one experiment with four to eight mice per group. NR3 survival is based on three mice that developed nonregressing tumors, and latency is based on six mice with detectable tumors.

[b] Data were obtained after injection of 3×10^5 cells, except for MDS lines, which were injected as a 10^6 cell inoculum.

[c] Gross spontaneous metastases were occasionally seen; however, micrometastases detected by culturing lung cells in 400 μg of G418 per ml were detected for CIRAS-1, CIRAS-2, CIRAS-3 and NR4 tumors.

[d] Five replicate plates were used for each experiment point.

[e] NT, Not tested.

Table 3. In Vivo Selection of Progression Variants of NR3 Line

Cell Line	Tumorigenicity		Experimental Metastases	
	Latency (days [mean ± SE])	Frequency	Freqeuncy	No. (mean ± SE)
NR3	49.6±2.0	6/8	1/13	0.1±0.1
NR3.1LA	6.8±0.5	5/5	6/6	49±7
NR3.1LB	9.8±1.5	5/5	6/6	40±18
NR3.1LC	6.7±0.7	5/5	6/6	30±6
NR3.1LD	5.8±0.6	5/5	6/6	13±7
NR3.3	9.8±0.5	5/5	4/4	16±5
NR3.4	13.8±2.2	5/5	4/4	42±19

Table 4. Lung Colony Formation After p21 Induction in Line 433

Tumor Line	Transfected gene	Dexamethasone Treatment[a]	No. of lung metastases (mean ± SE[2]) (n)
433	MMTV LTR[b]/v-H-ras	-	70±12 (7)
		+	181±13[c] (9)
pEJ	pEJ H-ras	-	148±40 (7)
		+	141±40 (8)
CIRAS-1	T24 H-ras	-	3±1 (7)
		+	4±1 (9)
NIH 3T3		-	0.4±0.2 (5)
		+	0.6±0.6 (5)

[a] 2×10^{-6} M.
[b] MMTV LTR. Mouse mammary tumor virus long terminal repeat.
[c] $p < 0.001$.

FIGURE LEGENDS

Figure 3: Photomicrographs illustrating morphological differences between (A) 10T1/2, and T24 H-ras transfected cell lines (B) CIRAS-1 (C) CIRAS-2 (D) CIRAS-3 (E) NR3 (F) NR4 as well as in vivo derived metastatic variants (G) NR3.4 and (H) NR3.1LB

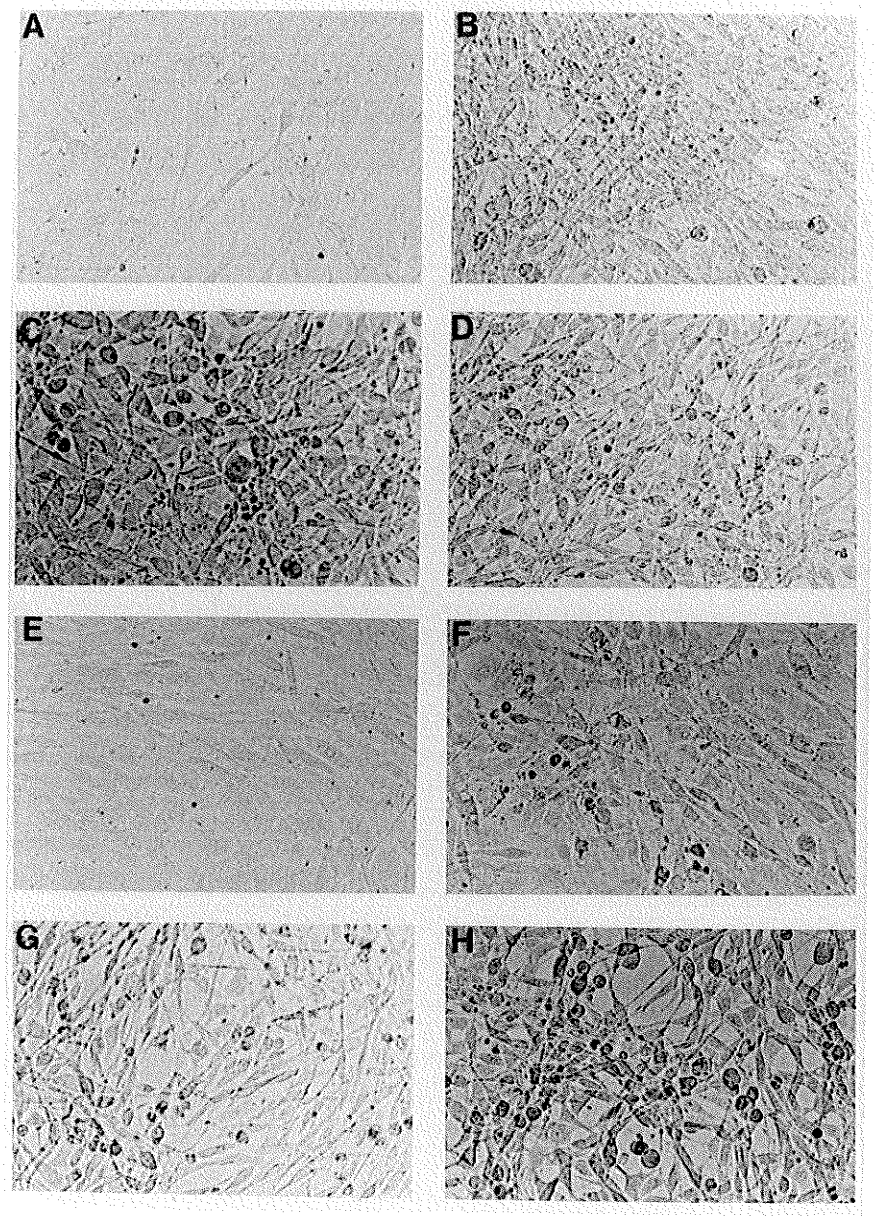

A
B
C
D
E
F
G
H

Figure 4: Southern blot analysis of 20ug of Bam H1-digested genomic DNA showing the presence of T24 H-ras sequences in transfectant lines. (C1, CIRAS-1; C2, CIRAS-2; C3, CIRAS-3; N3, NR3; N4,NR4)

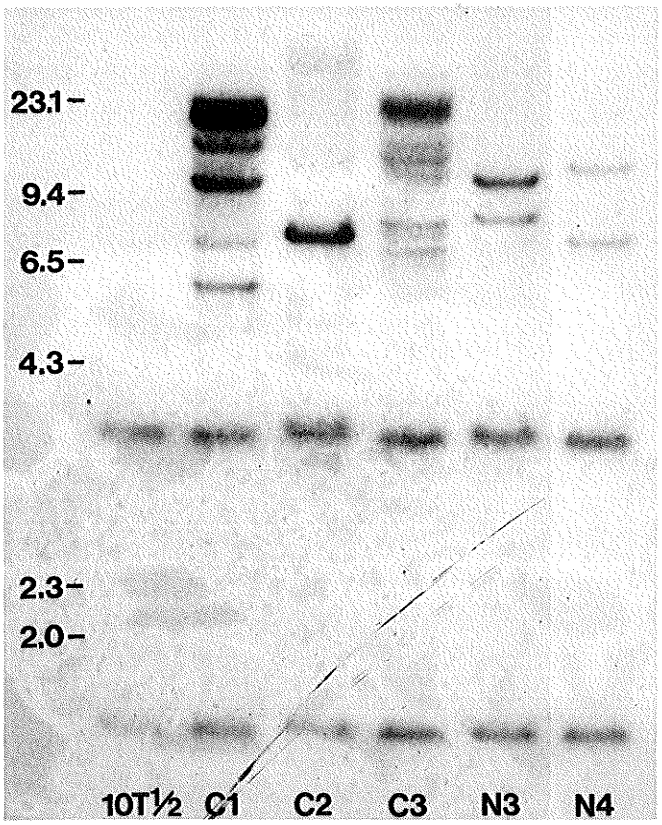

23.1
9.4
6.5
4.3
2.3
2.0
10T½
C1
C2
C3
N3
N4

Figure 5: Northern blot analysis of T24 H-ras transfected cell lines (lower panel). Transcripts of 1.2 and 4.7 Kb were detected. Top panel shows ethidium bromide stained non-denaturing gel with 20 ug of total RNA from each cell line indicating equal loading for northern blots. (C1, CIRAS-1; C2, CIRAS-2; C3, CIRAS-3; N3, NR3; N4, NR4)

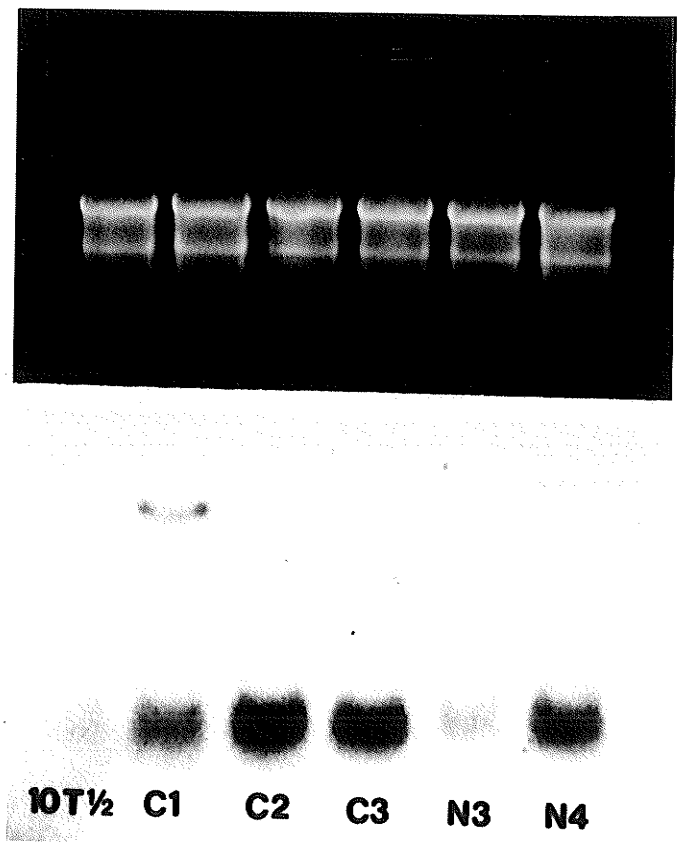

10T½
C1
C2
C3
N3
N4

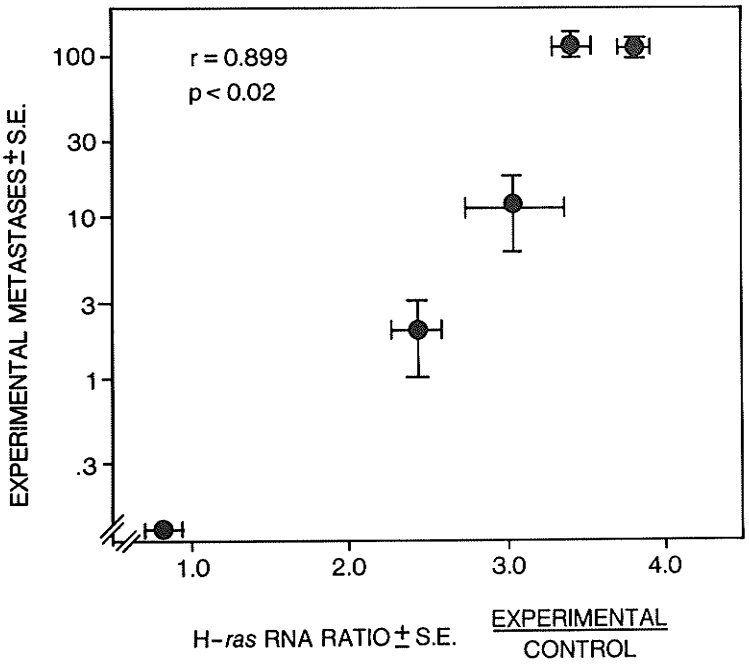
Figure 6: Relationship between metastatic potential and H-ras expression assessed in:

a) Immunocompetant syngeneic C3H/HeN mice

and in

b) Natural killer cell deficient syngeneic C3H/HeJ bg/bg mice and normal heterozygous bg/+ littermates

Rank order of H-ras RNA ratios from lowest to highest are NR3, NR4, CIRAS-1, CIRAS-3 and CIRAS-2. CIRAS-2 was not tested in bg/bg mice. All RNA ratios are calculated by comparing ras-transfected lines to 10T1/2 controls.


r = 0.899
p < 0.02
100
30
10
3
1
.3
EXPERIMENTAL METASTASES ± S.E.
1.0
2.0
3.0
4.0
H-ras RNA RATIO ± S.E.
EXPERIMENTAL
CONTROL

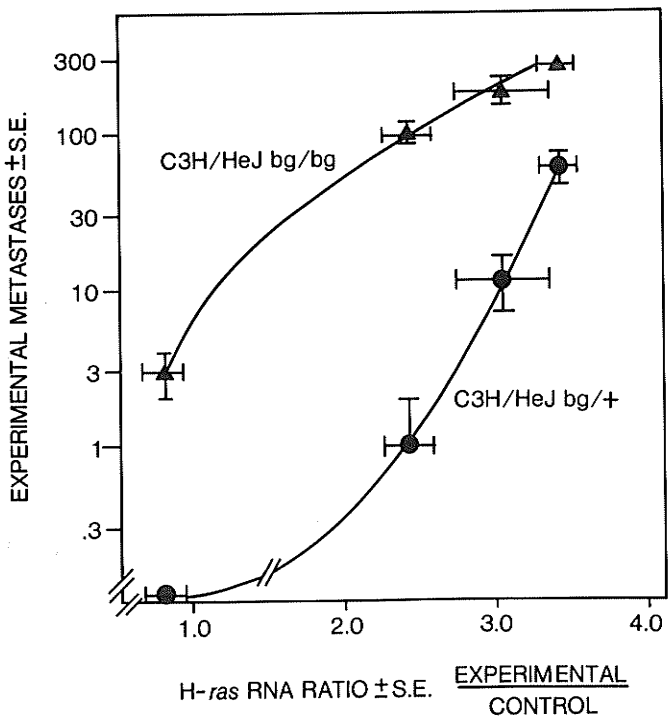

300
100
30
10
3
1
.3
EXPERIMENTAL METASTASES ± S.E.
C3H/HeJ bg/bg
C3H/HeJ bg/+
1.0
2.0
3.0
4.0
H-ras RNA RATIO ± S.E.
EXPERIMENTAL
CONTROL

Figure 7: (Lower panel) Northern blot analysis of H-*ras* RNA levels from NR3 derived subcutaneous tumor lines (3.4, NR3.4 AND 3.3, NR3.3) and NR3 derived lung metasteses lines (1LA, NR3.1LA; 1LB, NR3.1LB; 1LC, NR3.1LC; and 1LD, NR3.1LD) with NR3 (N3) and 10T1/2 controls. Top panel shows ethidium bromide stained non-denaturing gel with 20ug of total RNA from each cell line indicating equal loading for northern blots.

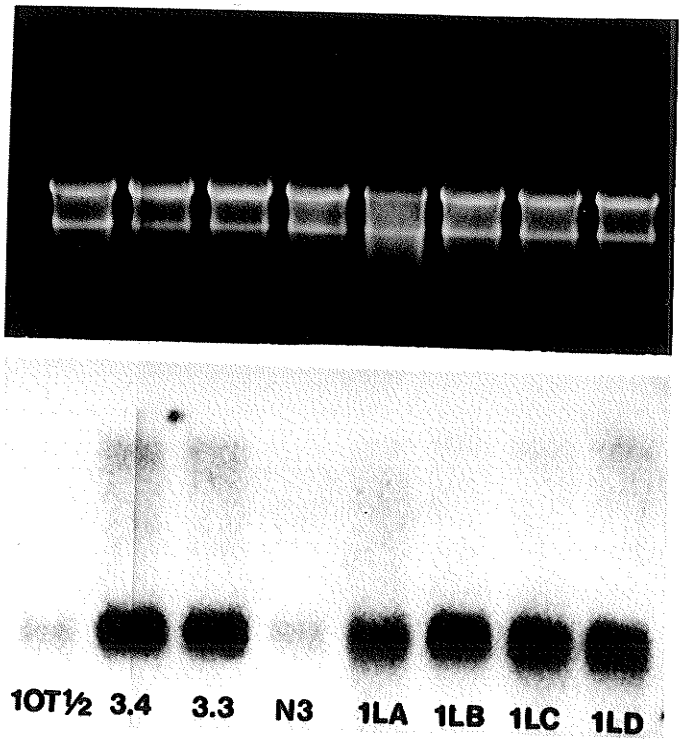
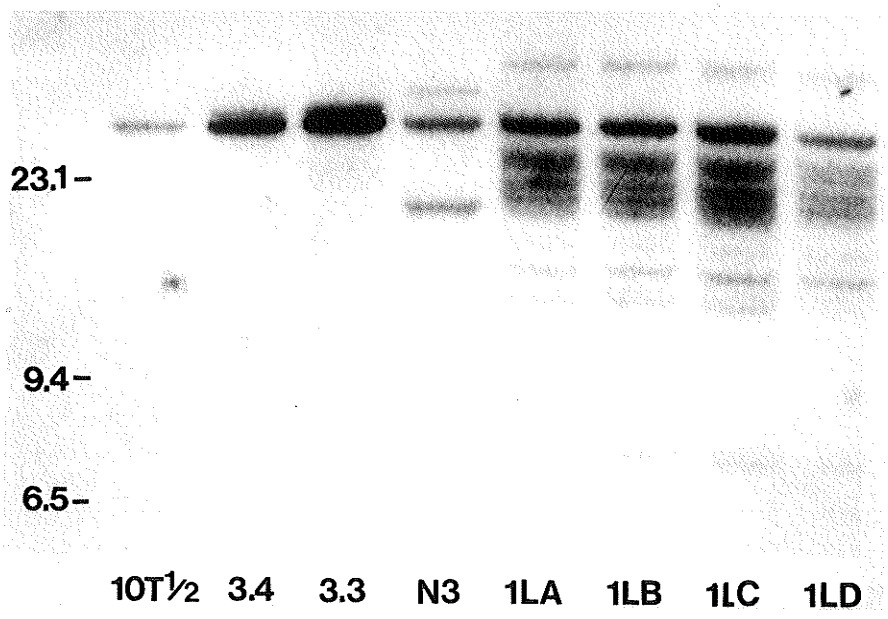

1OT½
3.4
3.3
N3
1LA
1LB
1LC
1LD

Figure 8: Southern blot analysis of 20ug of EcoR1-digested genomic DNA from NR3 (N3) derived subcutaneous tumor lines (3.4, NR3.4 and 3.3, NR3.3) and lung metastases (1LA, NR3.1LA; 1LB, NR3.1LB; 1LC, NR3.1LC and 1LD, NR3.1LD)


23.1–
9.4–
6.5–
10T½
3.4
3.3
N3
1LA
1LB
1LC
1LD

REFERENCES

1. **Bourne, H.R.** 1986. News and Views: GTP-binding proteins, one molecular machine can transduce diverse signals. Nature (London) 321:814-816.

2. **Chang, C.-C., J. E. Trosko, H.-J. Kung, D. Bonbick and F. Matsumura.** 1985. Potential role of the src gene product in inhibition of gap-junction communication in NIH-3T3 cells. Proc. Natl. Acad. Sci. USA. 82:5360-5364.

2a. **Bradley, M.O., A.R. Kraynak, R.D. Storer, and J.B. Gibbs.** 1986. Experimental metastasis in nude mice of NIH-3T3 cells containing various ras genes. Proc. Natl. Acad. Sci. USA 83:5277-5281.

3. **Chirgwin, J. M., A. E. Przbyla, R. J. McDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299.

4. **Cifone, M. A., and I. J. Fidler.** 1980. Correlation of patterns of anchorage-independent growth with in vivo behaviour of cells from a murine fibrosarcoma. Proc. Natl. Acad. Sci. USA 77:1039-1043.

5. **Ellis, R. W., D. DeFeo, M. E. Furth, and E. M. Scolnick.** 1982. Mouse cells contain two distinct ras gene mRNA species that can be translated into a p21 onc protein. Mol. Cell. Biol. 2:1339-1345.

6. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

7. **Feramisco, J. R., R. Clark, G. Wong, N. Arnheim, R. Milley and F. McCormick.** 1985. Transient reversion of ras oncogene-induced cell transformation by antibodies specific for amino acid 12 of ras protein. Nature (London) 314:639-642.

8. **Fidler, I. J., and M. L. Kripke.** 1977. Metastasis results from pre-existing variant cells within a malignant tumor. Science 197:893895.

9. **Fleischman, L. F., S. B. Chahwala, and L. Cantley.** 1986. ras-transformed cells: Altered levels of phosphatidylinositol4-5-bisphosphate and catabolites. Science 231:407-410.

10. **Foulds, L.** 1975. Neoplastic Development, Volume 2. Academic Press, Inc. (London).

10a. **Greenberg, A.H., S.E. Egan, L. Jarolim, M.-C. Gingras and J.A. Wright** 1987. NK Cell Regulation of Implantation and Early Lung Growth of H-ras-Transformed 10T1/2 Fibroblasts. Cancer Res., 47:4801-4805.

11. **Greig, R. G., T. P. Koestler, D. L. Trainer, S. P. Corwin, L. Miles, T. Kline, R. Sweet, S. Yokoyama and G. Poste.** 1985. Tumorigenic and metastatic properties of "normal" and ras-transfected NIH/3T3

cells. Proc. Natl. Acad. Sci. USA 82:3698-3701.

12. **Huang, A. L., M. C. Ostrowski, D. Berard and G. L. Hager.** 1981. Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from Mouse Mammary Tumor Virus. Cell 27:245-255.

13. **Kripke, M. L., E. Gruys, and I. J. Fidler.** 1978. Metastatic heterogeneity of cells from an ultraviolet light-induced murine fibrosarcoma of recent origin. Cancer Res. 38:2962-2967.

14. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Cellular oncogenes and multistep carcinogenesis. Science 222:771-778.

15. **Lehrach, H., D. Diamond, J. W. Wozney and H. Baldtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical re-examination. Biochemistry 16:47434751.

16. **Levinson, A. D.** 1986. Normal and activated ras oncogenes and their encoded products. Trends Genet. 15:81-85.

17. **Liotta, L. A.** 1986. Tumor invasion and metastasis - Role of the extracellular matrix: Rhoads Memorial Award Lecture. Cancer Res. 46:1-7.

18. **Maniatis, T., E. F. Fritsch, J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

19. **Martinet, Y., P. B. Bitterman, J.-F. Mornex,**

G. R. Grotendorst, G. R. Martin, and R. G. Crystal. 1986. Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF-like activity. Nature (London) 319:158-160.

20. **Monoharan, T. H., J. A. Burgess, D. Ho, C. L. Newell and W. E. Fahl.** 1985. Integration of a mutant c-Ha-ras oncogene into C3H/10T1/2 cells and its relationship to tumorigenic transformation. Carcinogenesis **6**:1295-1301.

21. **Mulcahy, L. S., M. R. Smith and D. W. Stacey.** 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH-3T3 cells. Nature (London) 313:241-243.

22. **Muschel, R. J., J. E. Williams, D. R. Lowy and L. A. Liotta.** 1985. Harvey ras induction of metastatic potential depends upon oncogene activation and the type of recipient cell. Amer. J. Pathol. 121:1-8.

23. **Nicolson, G. L.** 1982. Cancer metastasis; organ colonization and the cell-surface properties of malignant cells. Biochim. Biophys. Acta 695:113-176.

24. **Nicolson, G. L. and G. Poste.** 1983. Tumor implantation and invasion at metastatic sites. Int. Rev. Exp. Pathol. 25:77-181.

25. **Noda, M., M. Ko, A. Ogura, D. Liu, T. Amano, T. Takano, and Y. Ikawa.** 1985. Sarcoma viruses carrying ras

oncogenes induce differentiation associated properties in a neuronal cell line. Nature (London) 218:73-75.

26. **Poste, G., and I. J. Fidler.** 1980. The pathogenesis of cancer metastasis. Nature (London) 283:139-145.

27. **Pozzatti, R., R. Muschel, J. Williams, R. Padmanabhan, B. Howard, L. Liotta and G. Khoury.** 1986. Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science 232:223-227.

28. **Pulciani, S., E. Santos, L. K. Long, V. Sorrentino and M. Barbacid.** 1985. ras gene amplification and malignant transformation. Mol. Cell. Biol. 5:2836-2841.

29. **Raaphorst, G.P., J. A. Vadasz, E. I. Azzam, M. D. Sargent, J. Borsa, and M. Einspenner.** 1985. Comparison of heat and/or radiation sensitivity and membrane composition of seven x-ray-transformed C3H 10T1/2 cell lines and normal C3H 10T1/2 cells. Cancer Res. 45:54525456.

30. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. 13:7207-7221.

31. **Rigby, P. W., M. Dieckmann, C. Rhodes and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nicktranslation with DNA polymerase I. J. Mol. Biol. 113:237-251.

32. **Rohrschneider, L., and S. Reynolds.** 1985. Regulation

of cellular morphology by the rous sarcoma virus src gene: analysis of fusiform mutants. Mol. Cell. Biol. 5:3097-3107.

33. **Roos, E.** 1984. Cellular adhesion, invasion and metastasis. Biochim. Biophys. Acta. 738:263-284.

34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

35. **Stackpole, C. W., D. M. Fornabaio and A. L. Alterman.** 1985. Phenotypic interconversion of B16 melanoma clonal cell populations: relationship between metastasis and tumor growth rate. Int. J. Cancer 35:667-674.

36. **Thompson, C. B., P. B. Challoner, P. E. Neiman and M. Groudine.** 1986. Expression of the c-myb proto-oncogene during cellular proliferation. Nature (London) 319:374-380.

37. **Thorgeirsson, V. P., T. Turpeenniemi-Hujanen, J. E. Williams, E. H. Westin, C. A. Heilman, J. E. Talmadge and L. A. Liotta.** 1985. NIH/3T3 cells transfected with human tumor DNA containing activated ras oncogenes express the metastatic phenotype in nude mice. Mol. Cell Biol. 5:259-262.

38. **Vousden, K. H., S. A. Eccles, H. Purvies and C. J. Marshall.** 1986. Enhanced spontaneous metastasis of mouse carcinoma cells transfected with an activated

c-Ha-ras-1 gene. Int. J. Cancer 37:425-433.

39. **Weissman, B., and S. A. Aaronson.** 1985. Members of the src and ras oncogene families supplant the epidermal growth factor requirement of BALB/MK-2 keratinocytes and induce distinct alterations in their terminal differentiation program. Mol. Cell. Biol. 5:3386-3396.

40. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223-232.

41. **Winter, E., and M. Perucho.** 1986. Oncogene amplification during tumorigenesis of established rat fibroblasts reversibly transformed by activated human ras oncogenes. Mol. Cell. Biol. 6:2562-2570.

II COREGULATION OF METASTATIC AND TRANSFORMING ACTIVITY OF NORMAL AND MUTANT *ras* GENES

This work appears in published form elsewhere;

Coregulation of Metastatic and Transforming Activity of Normal and Mutant ras genes.

Sean E.Egan, James J.Broere, Lenka Jarolim, Jim A.Wright, and Arnold H.Greenberg. 1989.

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ABSTRACT

We have analyzed the metastatic properties of NIH-3T3 cells transformed by H-*ras* activated through overexpression and/or mutation. Our results reveal that elevated expression of the H-*ras* proto-oncogene can induce the complete metastatic phenotype. Cells transformed by the proto-oncogene have a lower metastatic potential than cells transformed by a mutated *ras* gene. *ras* oncogenes activated through alterations in codon 12 which encode p21 molecules with impaired GTPase activity, codon 59 which produce p21 molecules that release bound guanine nucleotides faster, or codon 61 which produce p21 having impaired GTPase activity and altered nucleotide release properties, are all able to induce the metastatic phenotype. Leucine 61 activated oncogenes with an additional mutation in codons 116, 117 or 119, resulting in a reduced affinity for guanine nucleotides, are also capable of inducing metastatic behavior. These data indicate that *ras* genes which are capable of transforming are also capable of inducing the full metastatic phenotype in NIH-3T3 cells. This suggests that both phenotypes are induced through an increase in p21-GTP concentration in *ras* transformed cells. This established model for *ras* mediated transformation can also explain the qualitative and quantitative regulation of metastatic behavior by *ras*.

INTRODUCTION

Metastasis is a complex process that results in tumor growth at sites distant to the primary neoplasm. Over the last few years, much effort has been directed at understanding the metastatic cascade and in particular, the genetic and epigenetic defects which are responsible for the spread of tumor cells. In 1985, several groups showed that activated ras genes could induce the metastatic phenotype in NIH-3T3 cells (Thorgeirsson et al, 1985; Bernstein and Weinberg, 1985; Greig et al, 1985). This finding has since been confirmed and the observation extended to other non-senescing fibroblast lines and non-metastatic tumors (Egan et al, 1987a; Waghorne et al, 1987; Collard et al, 1987). Recent work from our laboratory has revealed that the metastatic behavior of ras-transformed cells is directly regulated by ras (Egan et al, 1987a). Furthermore, the metastatic phenotype can be induced by many different kinase encoding oncogenes (Egan et al, 1987b). These two observations have led us to hypothesize that alterations in signal transduction are important not only in transformation but in regulating the metastatic dissemination of tumor cells.

A large body of literature now exists with respect to the study of ras genes and their products (for review see Barbacid, 1987). Consequently, studies on the regulation of metastatic behavior by ras is aided by extensive genetic and

biochemical analysis of this oncogene. The ras gene family codes for proteins of 21 kilodaltons in higher eukaryotes. These proteins, and their functionally conserved relatives in all eukaryotic organisms including yeast, are believed to be involved in signal transduction at the periphery of cells. All ras genes are homologous to the classical G-proteins which couple signalling and/or second messenger pathways. Like other G-proteins ras p21 has intrinsic GTPase activity which is responsible for regulating the coupling activity of p21. It is believed that the ras-GTP active complex is capable of stimulating the target second messenger system and therefore, the concentration of this moiety will determine the activity of the second messenger (for review, see Gibbs et al, 1985; Levinson, 1986; Barbacid, 1987). Mutation or overexpression of ras will lead to an increase in this complex, often resulting in transformation of permissive cell types. This model enables us to evaluate the qualitative and quantitative effects of ras mediated transformation on the metastatic behavior of NIH-3T3 cells.

MATERIALS AND METHODS

Cell Lines

The derivation of all cell lines has been previously described. NIH-3T3 cells transformed by the Harvey ras proto-oncogene are RS485 (Chang et al, 1982) 115-111, 115-

143 (Pulciani, et al, 1985) and Gly, Ala (Lacal et al, 1986a). These cells were supplied by Dr. Esther Chang, Dr. Mariano Barbacid and Dr. Stuart Aaronson respectively. Cell lines transformed by the Leu61 activated H-ras oncogenes were received from Dr. Geoff Cooper (Der et al, 1986b). Finally, the cell lines transformed by Abelson virus LTR regulated ras genes were obtained from Dr. Stuart Aaronson (Lacal et al, 1986a). All cell lines were grown in alpha minimal essential medium (Flow Laboratories) supplemented with 10% calf serum (Colorado).

Immunoprecipitation

Exponentially growing cells were labelled for 20 hours with 100 μCi/ml of ^{35}S methionine in methionine-free alpha minimal essential medium with 5% dialyzed fetal calf serum (Gibco) on 100 mm tissue culture plates (Lux Scientific). Monolayers were gently washed twice with phosphate buffered saline and lysed on the plate with 400 ul of lysis buffer at 4°C [150 mM NaCl, 10 mM sodium phosphate (pH 7.25), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 0.2% sodium azide, and 0.004% sodium floride]. Cell lysates were passed through a 25 gauge needle 5 to 10 times and then centrifuged at 10,000 gav in microfuge tubes for 10 minutes to remove the DNA. 5 x 10^7 TCA-precipitable counts were then incubated with anti-ras p21 immunoprecipitation reagent (Cetus, Emerville, CA) for 90 minutes. Alternatively, labelled lysates were precleared

with rabbit anti rat-Pansorbin (Sigma Chemical Co. and Calbiochem, respectively) for 15 minutes, centrifuged and the supernatants incubated with 10 μl of Y13-259 rat anti-ras antibody (Oncogene Science) for 2 hours. Finally, rabbit anti rat-Pansorbin (preabsorbed with mouse cell extract) was added for 15 minutes. Precipitates from either protocol were collected by centrifugation through 600 μl of 1 M sucrose in lysis buffer, washed twice in lysis buffer and once in TE. The precipitates were finally resuspended in 50 μl of SDS page sample buffer, boiled and electrophoresed in 12% polyacrylamide gels. Gels were dried and visualized by autoradiography. It was important for cell lysis to be carried out on the plate.

Immunoblotting

Cell lysates were prepared as described in the previous section. Protein concentrations were determined with a colorimetric assay kit (Bio Rad). Aliquots containing 40 μg of protein were mixed with electrophoresis sample buffer, boiled and electrophoresed on 12% polyacrylamide minigels. Proteins were electroblotted onto nitrocellulose as described by Towbin et al (1979). Following transfer, the dried nitrocellulose was blocked for 1/2 hour with 5% BSA in TTBS [0.5% Tween 20, 50 mM Tris-Cl (pH 7.6), 150 mM NaCl] and then incubated overnight at 4°C in 1/100 dilution of mouse pan-reactive anti-ras antibody (Cetus, Emerville, CA) in TTBS. The blots were then washed 3 times for 30 minutes

each in TTBS and then incubated in 1/1000 dilution of goat anti-mouse conjugated with alkaline phosphate (Sigma Chemical Co.) in TTBS for 2 hours. Following incubation with second antibody, blots were washed 3 times for 30 minutes each in TTBS. The antibody complexes were then detected by development of the alkaline-phosphatase reaction (Blake et al, 1984).

Experimental and Spontaneous Metastasis Assays

Metastatic potential was determined by the experimental metastasis assay. Tumor cell inoculum of either 10^5 or 3 x 10^5 were injected in a 0.2 ml volume into the tail vein of BALB/c nu/nu mice. Cells were lightly trypsinized from subconfluent cultures, washed and resuspended in Hanks balanced salt solution. Recipient animals were sacrificed 21 days later and Bouins' solution injected intratracheally. The stained lungs were then removed, and metastatic foci were counted under a dissecting microscope. Spontaneous lung metastasis formation was assayed as previously described (Egan et al, 1987a).

Growth in Semisolid Medium

Anchorage independent growth in 0.33% Agar was assayed as previously described (Stackpole et al, 1985; Egan et al, 1987a).

RESULTS AND DISCUSSION

Metastatic Potential of Cells Overexpressing Normal p21

Transformation of NIH-3T3 cells through overexpression of a normal ras gene product was originally reported by DeFeo et al (1981). This result can be interpreted in terms of an increase in p21-GTP concentration even though the active (p21-GTP) to inactive (p21-GDP) ratio may be unaltered. If this model of proto-oncogene mediated transformation is correct and the transformed and metastatic phenotypes are coregulated as we have suggested previously, (Egan et al, 1987a), then proto-oncogene transformed NIH-3T3 cells should be metastatic. In order to test this hypothesis we have analysed 3T3 cell lines transformed by normal H-ras genes which were obtained from three laboratories. Cell line RS-485 was transformed by the normal human c-H-ras-1 gene regulated by the Ha-MuSV Long Terminal Repeat (LTR) (Chang et al, 1982). The "Gly, Ala" cell line was transformed by the v-H-ras gene from BALB-MuSV (v-bas) with codon 12 changed from lysine to glycine. This gene, therefore, does not contain any activating mutations in its coding sequences and is being regulated by the Ab-MuLV LTR as described (Lacal et al, 1986a). In addition, the cell lines 115-111 and 115-143 were transformed by transfection of approximately 30 and 100 gene copies, respectively, of the normal human c-H-ras-1 gene into NIH-3T3 cells (Pulciani et al, 1985).

The metastatic potential of these cell lines was determined using the experimental metastasis assay. All four lines were metastatic following intravenous injection (Table 5). This result is in apparent contrast to the results reported by Muschel et al (1985), and in agreement with Bradley et al (1986). The reason for this discrepancy may be due to slight variations in experimental design and interpretation. Intravenous (iv) injections of 5×10^4 cells did not produce lung colonies in either of these studies. In both the study of Bradley et al (1986) and this one, the assay was performed using a higher cell inoculum and in so doing the propensity for metastatic spread became apparent. All of this data can be reconciled if the oncogene is more potent than the proto-oncogene at inducing metastatic behavior. Indeed, cell line pEJ3 which is transformed by the pEJ ras oncogene (Egan et al, 1987a) is much more metastatic than the proto-oncogene transformed lines (Table 5). This point has also been noted by Waghorne et al (1987) using the non-metastatic SP1 mammary adenocarcinoma cells. It appears then that the metastatic potential of proto-oncogene transformed NIH-3T3 cells is only detectable at high cell inoculum.

We have also isolated cell lines from two distinct lung nodules in each of two mice injected with 115-143 cells iv. and one mouse injected with 115-111 cells iv.. These six experimental metastases lines were analysed for p21

expression and for the presence of transforming sequences. As found by Bradley and coworkers (1986), these six experimental metastases derived cell lines also did not express more p21 than their parental in vitro lines (data not shown). Metastases from proto-oncogene transformed cells may arise through selection of variant cells in which mutational activation of ras has occurred. If this is the case then proto-ras transformation of NIH-3T3 cells would be insufficient to induce the metastatic phenotype. We have transfected NIH-3T3 cells with DNA isolated from each of these six cell lines in order to score for transforming ras genes. Cell line NR3.4 which contained one copy of activated ras (Egan et al, 1987a) was used as a control. All six lines were negative in this assay as were the parental 115-143 and 115-111 cells (Pulciani et al, 1985). In addition, no altered forms of p21 were detected by immunoprecipitation from these in vivo derived lines (data not shown). Together these data establish that overexpression of the normal ras gene product is sufficient to induce the metastatic phenotype in NIH-3T3 cells. Furthermore, proto-oncogene transformed cells cannot only colonize the lung following intravenous injection, but they are also fully capable of metastasizing from a subcutaneous tumor (Table 5).

The level of p21 ras expression achieved in NIH-3T3 cells must exceed a threshold in order to induce

transformation (Pulciani et al, 1985). It has recently been demonstrated that phenotypic properties of transformation can be titrated by regulating the level of a steroid-inducible N-ras proto-oncogene (McKay et al, 1986). We have tested whether the level of expression of p21 is indicative of metastatic potential (Figure 9), and detected no correlation between expression of ras in these four proto-oncogene transformed lines and their metastatic properties. This is in contrast to both our previous results, where the level of ras-RNA in ras-transformed 10T½ cells corresponded to metastatic potential (Egan et al, 1987a), as well as similar observations made by other groups using 3T3 cells or a T cell lymphoma (Radinsky et al, 1987; Collard et al, 1987). Our result may in part be due to the different cell culture history of the proto-oncogene-transformed lines. However, a lack of correlation between p21 levels and metastatic behavior has also been observed by Waghorne et al (1987) following transfection of normal or activated H-ras into a non-metastatic adenocarcinoma. Another possible explanation for our results could come from the potential influence of other oncogenes in the ras proto-oncogene transfected cells. It is well established that oncogene complementation with ras can promote transformation and tumor progression in some cell lines (Land et al, 1983; Ruley, 1983; Bernards et al, 1986). This latter possibility is difficult to establish, and will require a more direct

test of the hypothesis that oncogene complementation can enhance metastatic progression. Because of this issue we believe that the direct regulation of metastatic behavior by ras is best observed by induction of oncogene expression through an inducible promoter (Egan et al, 1987a). Although p21 proto-oncogene expression does not predict metastatic ability in our cell lines, we have observed that the tumorigenic and metastatic ability of the ras transformed cells correspond quite closely (Egan et al, 1987a).

Metastatic Potential of Cells Transformed by viral-ras Genes

Many viral oncogenes contain multiple activating mutations, each independently capable of causing transformation of suitable recipient cells. This is the case for v-H-ras derived from Harvey Murine Sarcoma Virus (Ha-MSV). This gene has minimally three oncogenic alterations. v-H-ras is regulated by a potent viral Long Terminal Repeat which has replaced normal cellular promoter and enhancer sequences. In addition, Exon -1 has been lost during transduction of c-H-ras and consequently a much higher level of ras expression is achieved (Cichutek and Duesberg, 1986). This altered regulation leads to transformation through overexpression as discussed above. The second activating mutation in v-H-ras occurs at codon 12 where glycine is replaced by arginine. This alteration causes a change in structure which results in impaired GTPase activity (Barbacid, 1987). It has recently been

shown that codon-12 activated p21 molecules do not interact with the cellular GTPase Activator of GAP protein (Trahey and McCormick, 1987). Consequently such molecules will remain in the active state (p21-GTP) and therefore will stimulate their target second messenger system constitutively. The third oncogenic mutation in v-H-ras occurs at codon 59, where alanine has been replaced by threonine. This mutation results in an autokinase activity of unknown significance (Shih et al, 1982), and an alteration in nucleotide exchange which is believed to be responsible for the transforming ability (Lacal and Aaronaon, 1986b). p21 molecules with a threonine at residue 59 release their bound guanine nucleotides three- to nine-fold faster than normal p21 molecules. In an intracellular environment where GTP is in at least tenfold molar excess over GDP, an increase in nucleotide exchange should also result in an increase in p21-GTP concentration (Lacal and Aaronsone, 1986b). In addition, the threonine 59 mutation increases the halflife of p21 and this effect will also contribute to an increase in p21-GTP active complex (Ulsh and Shih, 1984).

We have tested the metastatic properties of NIH-3T3 cells transformed by ras genes regulated by a viral LTR (Ab-MuLV) and containing combinations of normal or mutant codons 12 and 59. As reported by Bradley et al (1986), we found that ras genes with mutations in either codon 12, codon 59

or both codons 12 and 59 are all capable of inducing the metastatic phenotype. We were unable to detect any complementation between codons 12 and 59 mutations. Cells transformed by v-H-ras which contained both arginine 12 and threonine 59 mutations were not more metastatic than cells transformed by ras containing threonine 59 or a lysine 12 mutation alone (Table 6). Once again, in this limited survey we observed an increase in metastatic potential of cells transformed by viral genes containing a mutation in coding sequences as compared to the "Gly, Ala" cell line transformed by overexpression of normal p21. This result confirms the conclusion reached above that the oncogene is more potent at inducing metastatic behavior than the proto-oncogene. From our previous study demonstrating an increase in metastatic potential through induction of v-H-ras expression, and the present result illustrating an increase in metastatic potential caused by the structural mutations in v-H-ras, it is apparent that both alterations in ras complement each other in the induction of metastatic behavior.

We have analyzed the steady state levels of p21 in these four cell lines by Western blotting in order to compensate for the different half lives of these proteins (Ulsh and Shih, 1984). Determination of expression through immunoprecipitation of labelled extracts will lead to an under-estimation of the differences in steady state levels.

Although all four genes are regulated by the Abelson Murine Leukemia virus LTR, the p21 species containing threonine 59 have accumulated to a much higher level (Figure 10). The ras gene product from "Gly, Ala" was barely visible on the Western blot and is therefore not seen upon reproduction in the figure. ras is expressed to a high level in "Gly, Ala" (Figure 9) and consequently the cell line "Gly, Thr" which has a much higher steady state level of p21 may be highly metastatic not only because of the mutation effecting guanine nucleotide exchange but because of ras levels. Both mutation and over-expression of ras as a result of viral alterations lead to an increase ras-GTP active complex and increase metastatic potential when compared to cells transformed by ras genes with only alterations in expression or coding sequence (Egan et al, 1987a).

The Metastatic Phenotype is Induced by Guanine Nucleotide Binding Mutant ras Genes

ras oncogenes with an activating mutation in codon 61 have been isolated from human tumors and through chemical carcinogenesis (Barbacid, 1987). The substitution of leucine for glutamine at codon 61 has been shown to decrease GTPase activity and result in a higher rate of GDP release relative to GTP (Der et al, 1986a; Lacal and Aaronson, 1986b). Consequently, deactivation of the active ras-GTP complex would occur slower than normal through impaired phosphatase activity and when p21 is deactivated, the bound

GDP would be released at a higher rate and then rapidly be replaced by GTP. This replacement of GDP by GTP would occur because of the high intracellular GTP to GDP ratio discussed ealier (Lacal and Aaronson, 1986b; Proud, 1986). Recently, mutations in other domains of ras have been shown to induce transforming potential. These mutations occur in the region from codon 116 to 119. Analysis of homologous regions in other G-proteins and the structural model of p21 (McCormick et al, 1985) reveal that these mutations affect interaction between p21 and the nucleotide ring structure. Consequently, mutations of this region result in a lower affinity for guanine nucleotides. This lower affinity can result in the establishment of a nucleotide free pool of p21. This nucleotide free pool will end up binding GTP more often than GDP due to the concentration differences of these two species and can consequently raise the steady state active p21-GTP concentration (Sigal et al, 1986; Walter et al 1986). Mutations in this region in combination with the viral mutations (codon 12 and 59) have been shown, however, to abolish nucleotide binding completely and therefore to abolish transformation (Clanton et al, 1986).

We have analysed NIH-3T3 cells transformed by ras genes with leucine 61 activating mutation regulated by the MoMLV LTR (Der et al, 1986b). This mutation is sufficient to induce the metastatic phenotype (Bradley et al, 1986; Egan et al, 1987b). Additional mutations in the nucleotide

binding region (codons 116-119) have been introduced into the leucine 61 oncogene and the resulting oncogenes found to retain their transforming potential despite up to a 5000-fold decrease in nucleotide binding affinity (ras leucine 61/glutamine 117) (Der et al, 1986b). Once again we find that ras genes capable of inducing transformation also induce the metastatic phenotype (Table 7).

In summary, the data presented above, together with the previously reported work (Muschel et al, 1985; Bradley et al, 1986; Egan et al, 1987a) establish that ras genes activated by different mechanisms can induce the metastatic phenotype in NIH-3T3 cells. Oncogenic alterations in ras genes are believed to induce transformation through an increase in GTP-ras active complex which results in a greater level of stimulation of the target second messenger system (Barbacid, 1987). This second messenger system may involve the formation of diacylglycerol from a novel source (Lacal et al, 1987). If the metastatic and transformed phenotypes are coregulated as we have previously proposed (Egan et al, 1987a), then all transforming ras genes should induce metastatic behavior, and the quantitative differences observed in the transforming potential of various ras genes should be reflected in the metastatic potential of transformed cells. The data presented here establish both principles and therefore support our hypothesis of direct regulation of both transformed and metastatic phenotypes by

ras in *ras* transformed cells.

Table 5. Metastatic Properties of NIH-3T3 Cells Expressing Normal ras Genes or A Mutant ras Oncogene

Cell Line	Transforming[b] Oncogene	Experimental Metastasis Frequency	Metastasis No (mean±SE)	Spontaneous[a] Metastasis
pEJ3	pEJ-ras	5/5	114±31	NT[c]
RS-485	c-H-ras/Ha-MuSV LTR	8/9	33±20	2/5
Gly, Ala	(gly12)v-bas/Ab-MuLV LTR	5/5	67±35	NT
115-111	c-H-ras	9/10	15±6	3/5
115/143	c-H-ras	9/10	58±16	3/5
NIH-3T3	-	3/10	0.5±0.3	0/4

[a] Experimental metastasis were counted 21 days after the intravenous inoculation of 3×10^5 cells. Visible spontaneous metastasis were scored between 30 and 60 days following subcutaneous injection of 3×10^5 cells.

[b] Line 115 - 111 and 115 - 143 were transformed by 30 and 100 copies of the proto-oncogene, respectively.

[c] NT = Not tested.

Table 6. Metastatic Potential of Cells Transformed by v-H-*ras* Genes

Cell Line	Amino Acid Substitution	Experimental Metastases	
		Frequency	No. (mean ± SE)
"Gly, Ala"	gly^{12}, ala^{59}	3/5	0.8 ± 0.3
"Gly, Thr"	gly^{12}, thr^{59}	5/5	32 ± 13
"Lys, Ala"	lys^{12}, ala^{59}	3/6	15 ± 9
"Arg, Thr"	arg^{12}, thr^{59}	5/5	18 ± 5

[a] 10^5 cells were injected intravenously and lung colonies counted on day 21.

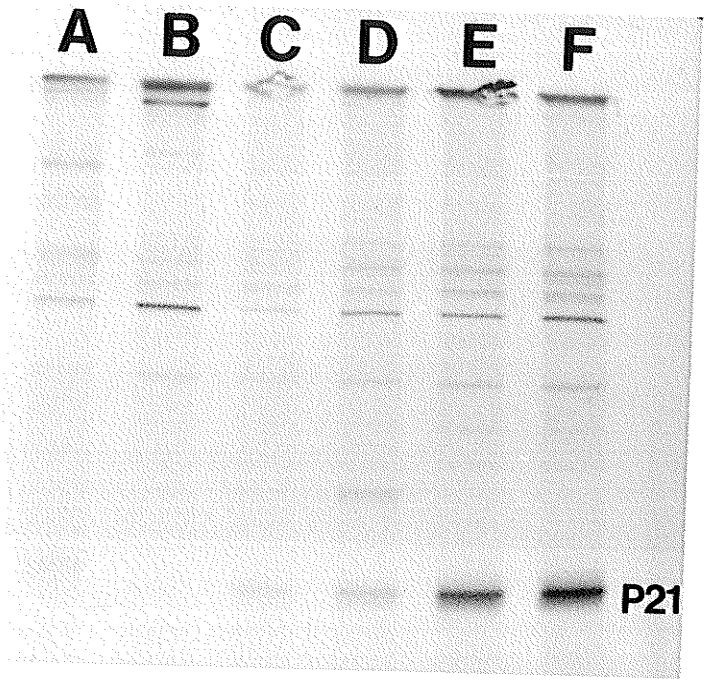
Table 7. Metastatic Properties of Cells Transformed by Nucleotide Binding Mutant *ras* Genes

Cell Line	Amino Acid Substitution	Experimental Metastasis (10^5 cell iv) Frequency	Experimental Metastasis (10^5 cell iv) No (mean ± SE)	Spontaneous Metastasis[a] Frequency	Soft Agar Cloning % ± S.E.
NZ 61.8	Leu 61	8/10	8.6 ± 2.4	2/5	15 ± 2
NZ 61L	Leu 61	6/10	33 ± 24	NT	17 ± 1
NZ 3.6	Leu 61/His 116	5/5	113 ± 24	NT	56 ± 3
NZ 7.5	Leu 61/His 119	5/5	41 ± 19	NT	79 ± 2
NZ 5.4	Leu 61/Glu 117	2/3	35 ± 33	3/5	26 ± 2
NZ 5.1	Leu 61/Glu 117	5/5	4.2 ± 1.0	NT	36 ± 2

[a] Subcutaneous injection of 3×10^5 cells.

FIGURE LEGENDS

Figure 9: Immunoprecipitation of p21 from NIH-3T3 cells expressing normal or mutant ras genes. A) pEJ3 is transformed by the pEJ oncogene, B) NIH-3T3, C) RS-485 is transformed by MoMSV LTR regulated c-H-ras gene, D) "Gly,Ala" is transformed by an Ab MuLV LTR regulated v-bas gene with codon 12 reverted to the normal glycine residue, E) 115-111 is transformed by transfection of 30 copies of c-H-ras, F) 115-143 is transformed by transfection of 100 copies of c-H-ras.


A
B
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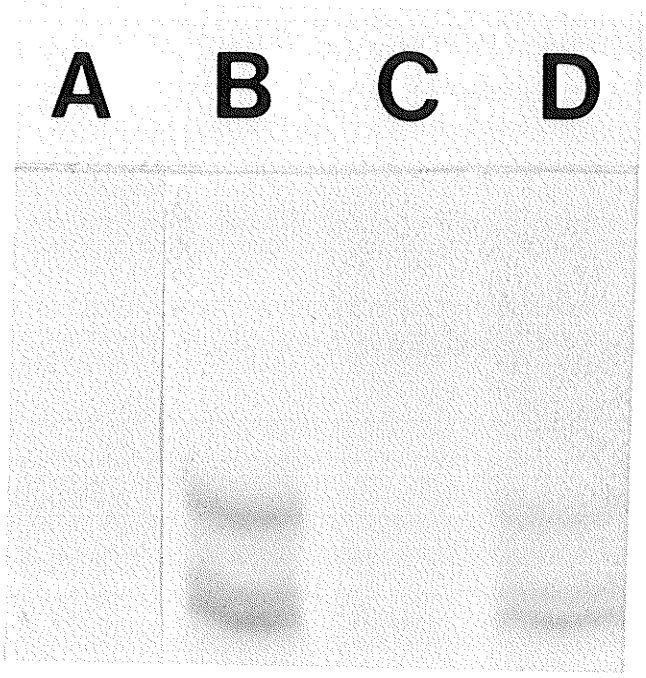
P21

Figure 10: Western blot of p21 from viral-*ras* transformed NIH-3T3. Cells are transformed with Abelson Leukemia Virus Long Terminal Repeat regulated v-H-*ras* genes containing the designated residues at codon 12 and 59. A) "Gly,Ala", B) "Gly,Thr", C) "Lys,Ala", D) "Arg,Thr".


A
B
C
D

REFERENCES

Barbacid, M. (1987). ras Genes. **Ann. Rev. Biochem., 56,** 779-827.

Bernards, R., Dessain, S.K. and Weinberg, R.A. (1986). N-myc Amplification Causes Down-Modulation of MHC Class I Antigen Expression in Neuroblastoma. **Cell, 47,** 667-674.

Bernstein, S.C. and Weinberg, R.A. (1985). Expression of the Metastatic Phenotype in Cells Transfected with Human Metastatic Tumor DNA. **Proc. Natl. Acad. Sci. USA, 82,** 1726-1730.

Blake, M.S., Johnston, K.H., Russel-Jones, G.J. and Gotschlich, E.C. (1984). A Rapid, Sensitive Method for Detection of Alkaline Phosphatase-Conjugated Anti-Antibody on Western Blots. **Anal. Biochem., 136,** 175-197.

Bradley, M.O., Kraynak, A.R., Storer, R.D. and Gibbs, J.B. (1986). Experimental Metastasis in Nude Mice of NIH-3T3 Cells Containing Various ras Genes. **Natl. Acad. Sci. USA, 83,** 5277-5281.

Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R.

(1982). Tumorigenic Transformation of Mammalian Cells Induced by a Normal Human Gene Homologous to the Oncogene of Harvey Murine Sarcoma Virus. **Nature, 297,** 479-483.

Cichutek, K. and Duesberg, P.H. (1986). Harvey ras Genes Transform Without Mutant Codons, Apparently Activated by Truncation of a 5' Exon (exon-1). **Proc. Natl. Acad. Sci. USA, 83,** 2340-2344.

Clanton, D.J., Hattori, S. and Shih, T.Y. (1986). Mutations of the ras Gene Product p21 that Abolish Guanine Nucleotide Binding. **Proc. Natl. Acad. Sci. USA, 83,** 5079-5080.

Collard, J.G., Schijven, J.F. and Roos, E. (1987). Invasive and Metastatic Potential Induced by ras-Transfection into Mouse BW5147 T-Lymphoma Cells. **Cancer Research, 47,** 754-759.

DeFeo, D., Gonda, M.A., Young, H.A. and 4 others (1981). Analysis of Two Divergent Rat Genomic Clones Homologous to the Transforming Gene of Harvey Murine Sarcoma Virus. **Proc. Natl. Acad. Sci. USA, 78,** 3328-3332.

Der, C.J., Finkel, T. and Cooper, G.M. (1986a). Biological and Biochemical Properties of Human ras^H Genes Mutated at

Codon 61. **Cell, 44,** 167-176.

Der, C.J., Pan, B.-T. and Cooper, G.M. (1986b). ras^{H} Mutants Deficient in GTP Binding. **Mol. Cell. Biol., 6,** 3291-3294.

Egan, S.E., McClarty, G.A., Jarolim, L., Wright, J.A., Spiro, I., Hager, G. and Greenberg, A.H. (1987a). Expression of H-ras Correlates with Metastatic Potential: Evidence for Direct Regulation of the Metastatic Phenotype in 10T½ and NIH-3T3 Cells. **Mol. Cell. Biol., 7,** 830-837.

Egan, S.E., Wright, J.A., Jarolim, L., Yanagihara, K., Bassin, R.H. and Greenberg, A.H. (1987b). Transformation by Oncogenes Encoding Protein Kinases Induces the Metastatic Phenotype. **Science, 238,** 202-205.

Gibbs, J.B., Sigal, I.S. and Scolnick, E.M. (1985). Biochemical Properties of Normal and Oncogenic ras p21. **Trends in Biochemical Sci., 10,** 350-353.

Grieg, R.E., Koestler, T.P., Trainer, D.L. and 6 others (1985). Tumorigenic and Metastatic Properties of "Normal" and ras-transfected NIH-3T3 Cells. **Proc. Natl. Acad. Sci. USA, 82,** 3698-3701.

Lacal, J.C., Srivastava, S.K., Anderson, P.S. and Aaronson, S.A. (1986a). Ras p21 Proteins with High or Low GTPase Activity Can Efficiently Transform NIH-3T3 Cells. **Cell,** 44, 609-617.

Lacal, J.S. and Aaronson, S.A. (1986b). Activation of ras p21 Transforming Properties Associated with an Increase in the Release Rate of Bound Guanine Nucleotide. **Molec. Cell. Biol.,** 6, 4214-4220.

Lacal, J.S., Moscot, J. and Aaronson, S.A. (1987). Novel Source of 1,2-diacylglycerol Elevated in Cells Transformed by Ha-ras Oncogene. **Nature,** 330, 269-272.

Land, H., Parada, L.F. and Weinberg, R.A. (1983). Tumorigenic Conversion of Primary Embryo Fibroblasts Requires at Least Two Cooperating Oncogenes. **Nature,** 304, 596-602.

Levinson, A.D. (1986). Normal and Activated ras Oncogenes and their Encoded Products. **Trends in Genetics,** March, 81-85.

McCormick, F., Clark, B.F.C., 1a cour, T.F.M. and 3 others (1985). A Model for the Tertiary Structure of p21, the Product of the ras Oncogene. **Science, 230,** 78-82.

McKay, I.A., Marshall, C.J., Cales, C. and Hall, A. (1986). Transformation and Stimulation of DNA Synthesis in NIH-3T3 Cells are a Titratable Function of Normal p21 N-ras Expression. **EMBO J., 5,** 2617-2621.

Muschel, R.J., Williams, J.E., Lowy, D. and Liotta, L.A. (1985). Harvey ras Induction of Metastatic Potential Depends upon Oncogene Activation and the Type of Recipient Cell. **Am. J. Pathol., 121,** 1-8.

Proud, C.G. (1986). Guanine nucleotides, Protein Phosphorylation and the Control of Translation. **Trends Biol. Sci., 11,** 73-77.

Pulciani, S., Santos, E., Long, L.K., Sorrentino, V. and Barbacid, M. (1985). ras Gene Amplification and Malignant Transformation. **Mol. Cell. Biol., 5,** 2836-2841.

Radinsky, R., Kraemer, P.M., Raines, M.S., Kung, H.-J. and Culp, L.A. (1987). Amplification and Rearrangement of the Kirsten ras Oncogene in Virus-Transformed BALB/c 3T3 Cells

During Malignant Tumor Progression. **Proc. Natl. Acad. Sci. USA., 84,** 5143-5147.

Ruley, H.E. (1983). Adenovirus Early Region 1A Enables Viral and Cellular Transforming Genes to Transform Primary Cells in Culture. **Nature, 304,** 602-606.

Shih, T.Y., Stokes, P.E., Smythers, G.W., Dhar, R. and Oroslan, S. (1982). Characterization of the Phosphorylation Sites and the Surrounding Amino Acid Sequences of the p21 Transforming Proteins Coded for by the Harvey and Kirsten Strains of Murine Sarcoma Viruses. **J. Biol. Chem., 257,** 11,767-11,773.

Sigal, I.S., Gibbs, J.B., D'Alonzo, J.S. and 4 others (1986). Mutant ras-Encoded Proteins with Altered Nucleotide Binding Exert Dominant Biological Effects. **Proc. Natl. Acad. Sci. USA, 83,** 952-956.

Stackpole, C.W., Fornabaio, D.M. and Alterman (1985). Phenotypic Interconversion of B16 Melanoma Clonal Cell Populations: Relationship Between Metastasis and Tumor Growth Rate. **Int. J. Cancer, 35,** 667-674.

Thorgeirrson, U.P., Turpeenniemi-Hujanen, T., Williams, J.E. and 4 others (1985). NIH-3T3 Cells Transfected with Human Tumor DNA Containing Activated ras Oncogenes Express the Metastatic Phenotype in Nude Mice. **Mol. Cell. Biol., 5,** 259-262.

Towbin, H., Stahelin, T. and Gordon, J. (1979). Electrophoretic Transfer of Proteins From Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. **Proc. Natl. Acad. Sci. USA, 76,** 4350-4354.

Trahey, M. and McCormick, F. (1987). A Cytoplasmic Protein Stimulates Normal N-ras p21 GTPase, but Does Not Affect Oncogenic Mutants. **Science, 238,** 542-545.

Ulsh, L.S. and Shih, T.S. (1984). Metabolic Turnover of Human c-ras^H p21 Protein of EJ Bladder Carcinoma and its Normal Cellular and Viral Homologs. **Mol. Cell. Biol., 4,** 1647-1652.

Walter, M., Clark, S.G. and Levinson, A.D. (1986). Activation of Human p21 ras by a Novel Mechanism. **Science, 233,** 649-652.

Waghorne, C., Kerbel, R.S. and Breitman, M.L. (1987).

Metastatic Potential of SP1 Mouse Mammary Adenocarcinoma Cells is Differentially Induced by Activated and Normal Forms of c-H-*ras*. **Oncogene, 1,** 149-155.

III TRANSFORMATION BY ONCOGENES ENCODING PROTEIN KINASES INDUCES THE METASTATIC PHENOTYPE

This work appears in published form elsewhere;

Transformation by Oncogenes Encoding Protein Kinases Induces the Metastatic Phenotype.

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ABSTRACT

Oncogenes encoding serine/threonine or tyrosine kinases were introduced into the established rodent fibroblast cell line NIH-3T3 and tested for tumorigenic and metastatic behavior in T-cell deficient nude mice. Transforming oncogenes of the *ras* family were capable of converting fibroblast cell lines to fully metastatic tumors. Cell lines transformed by the kinase oncogenes *mos*, *raf*, *src*, *fes* and *fms* formed experimental metastases and in some cases, these genes were more efficient at metastatic conversion than a mutant *ras* gene. In contrast, cells transformed by either of two nuclear oncogenes, *myc* and p53, were tumorigenic when injected subcutaneously but were virtually non-metastatic following intravenous injection. These data demonstrate that, in addition to *ras*, a structurally divergent group of kinase oncogenes can induce the metastatic phenotype.

Metastasis is the process by which tumor cells spread and colonize secondary sites throughout an organism. For a cell to be able to metastasize, many specialized characteristics are required, including the ability to invade through host barriers into the vasculature, survive in the circulation, extravasate and subsequently establish and grow. Recent work has firmly established that NIH-3T3 and 10T1/2 cells transformed by ras genes are capable of forming metastases (1,2), and this appears to be a direct result of ras function (2). Also, transfection of activated ras into poorly metastatic murine adenocarcinoma cells significantly enhances metastatic potential (3). However, it is unlikely that aberrant ras function is essential for all tumor dissemination since not all metastases contain activated ras sequences (4), and correlative studies have implicated amplification of non-ras oncogenes in progression of specific tumors (5,6). To directly test the hypothesis that other oncogenes may be involved in regulating or inducing metastatic activity, we have assessed the ability of NIH-3T3 clone 7 cells transformed by a wide variety of oncogenes to form experimental and spontaneous metastases. We report here that transformation by all kinase-encoding oncogenes tested results in the metastatic conversion of this fibroblast line.

Representative oncogenes of the cytoplasmic serine/threonine kinases were evaluated for their ability to

induce experimental metastases. Three lines of NIH-3T3 cells transformed by v-mos were isolated and cloned from transformed foci after Moloney murine sarcoma virus infection (7). Southern blot analysis revealed the presence of v-mos sequences in each clone (7). These freshly transformed cell lines (which were not releasing virus) were injected intravenously for assessment of lung colonizing ability. All three mos-transformed clones produced lung tumors, although frequencies varied significantly (Table 8). Two of the lines were equal in metastatic ability or more metastatic than the ras-transformed positive control. The relatively low metastatic potential of Mos 3 was likely due to a low level of mos expression observed on Northern blots, since the level of mos expression correlated with metastatic efficiency of the three clones (7). NIH-3T3 cells transformed by the raf oncogene, which also encodes a serine/threonine kinase, were next examined. v-raf and A-raf transformed cells were cloned from foci following transfection of cloned viral DNA, and have been shown to contain and express their respective transforming sequences (8,9). Both raf-transformed cell lines were also capable of forming experimental metastases in nude mice (Table 8).

We next tested oncogenes which code for tyrosine kinase products including membrane bound v-src and v-fes as well as the CSF-1 receptor-related v-fms. Src1 was cloned from a transformed focus after infection with a murine retrovirus

containing v-src from Rous sarcoma virus into NIH-3T3 cells (10). Src2, on the other hand, was isolated as a G418 resistant colony obtained by infection with a retroviral vector containing v-src and the neomycin resistance gene (11). The v-fes and v-fms transformed cells were isolated from foci after transfection with plasmids containing cloned Gardner-Arnstein and McDonough strains of feline sarcoma virus, respectively (12,13). Cell lines not obtained through selection in G418 have been shown to contain and express their oncogene sequences as well as produce transforming virus upon rescue through transfection of helper virus (10,12,13). All four cell lines transformed by tyrosine kinase encoding oncogenes were very potent in the experimental metastasis assay (Table 8) as were the serine/threonine class of oncogenes. Although we have not formally excluded the possibility that the cell lines transformed by these kinase encoding oncogenes contain activated ras, it seems highly unlikely that all of the transfectants would have acquired this mutation and consequently express metastatic ability.

Histological examination of lungs revealed that the experimental metastases from cells transformed by either serine/threonine or tyrosine kinase encoding oncogenes were typical fibrosarcomas, similar to those formed after the injection of N(Z61.8) cells, which are transformed by a mutant c-H-ras gene (14)(Figure 11).

Lung colonizing ability after intravenous injection requires most of the characteristics essential for metastasis from a primary tumor except invasion and entry into the vasculature. The ability to form experimental metastases by H-ras transformed cells (2) and other tumors (15) have been found to correlate very well with their ability to induce spontaneous metastasis formation from a subcutaneous site. NIH-3T3 cell lines transformed by mos, raf, src, fes, fms and ras-control oncogenes were all capable of forming metastases from a subcutaneous tumor and are, therefore, fully metastatic in T-cell deficient nude mice (16).

All of the kinase-oncogene-transformed cell lines reported above (except Src2) were obtained through transfection of cloned viral DNA or viral infection of NIH-3T3 clone 7. This 3T3 subclone, which was selected for its flat morphology and contact inhibited growth properties (17), was found to be completely non-metastatic (Table 8). These cells were nontumorigenic following subcutaneous injection of 10^7 cells (16). We have also tested psi2 cells which were derived by transfection of defective cloned Moloney murine leukemia virus (MoMuLV) into NIH-3T3 cells (11). This retroviral packaging cell line as well as clone 7 infected with MoMuLV were controls, representing 3T3 cells transfected or infected with viral sequences lacking transforming oncogenes. Both psi2 and MoMuLV infected clone

7 were completely negative in the experimental metastasis assay (Table 8). However, uncloned NIH-3T3 cells when injected intravenously, produced a very low but detectable level of lung colonies, suggesting the existance of a small subpopulation of transformed and metastatic cells in this parental 3T3 line, as previously reported (1).

In contrast to the ability of kinase-encoding oncogenes to induce the metastatic phenotype, the most tumorigenic clones transformed by the nuclear oncogenes c-myc and p53 (18) were not significantly more metastatic than NIH-3T3 (Table 8). No gross spontaneous metastases were detected following subcutaneous injection of these two lines. Furthermore, lung tissue was free of micrometastases when cultured in G418, a sensitive method for detecting tumor bearing the neo^R gene (16). Both of these cell lines have been previously shown to contain and express their transfected oncogene (18). In addition, three cell lines were isolated in G418 through infection of clone 7 with a retroviral vector containing v-myc and the neomycin resistance gene (19). These three cell lines were completely non-metastatic in the standard experimental metastasis assay of 21 days (Table 8). VM5 was also completely negative in a 6-week assay, therefore, decreased latency is not likely responsible for the negative result. Although expression of myc or p53 did not significantly induce metastatic behavior, these genes may still be important in tumor progression through complementation with

other oncogenes. For example, N-myc amplification has been linked to tumor progression of neuroblastoma (5), and Bernards et al recently demonstrated that N-myc overexpression in the B014 rat neuroblastoma line profoundly affected MHC class 1 gene expression and metastatic behavior (20). The B014 tumor was obtained through ethylnitrosourea treatment of perinatal BDIX rats, and the carcinogenic insult was later shown to be an activation of the tyrosine kinase neu-oncogene (21). Therefore, N-myc may contribute to metastatic progression by complementation with the kinase neu-oncogene. A similar type of complementation has been suggested for myc and ras (22). It is also possible that overexpression or complementation of p53 will contribute to metastatic progression in the same way. With the observation that the kinase group of oncogenes described in this study, as well as the ras family (1,2) can produce metastatic conversion of NIH-3T3 cells, it is tempting to speculate that a common pathway may be involved in this process. In that regard, ras-mediated transformation can result in alterations of inositol phospholipid metabolism (23,24) including increased steady state levels of diacyl-glycerol (24). Recent work suggests that tyrosine kinase oncogenes also stimulate elevated phosphatidylinositol turnover possibly through modification of cellular phosphoinositide kinases (25,26). Furthermore, Smith et al have shown that transformation by the tyrosine kinase

encoding oncogenes src, fes and fms can be blocked by microinjection of antibodies to p21 (27) indicating that these genes act via a ras-dependent mechanism. While mos and raf mediated transformation are not dependent on ras p21 (9,27), these serine/threonine kinase oncogenes may act on the same metabolic pathway (28), although downstream of ras. This pathway may also utilize inositol phospholipid derived second messengers that regulate Ca^{2+} mobilization and protein kinase C activity (26). Both ras and mos, for example, suppress the transcription of $\alpha 2$ type I procollagen, which is also downregulated by the protein kinase C agonist PMA (29). Taken together these observations suggest that unregulated alterations in the membrane associated inositol second messenger system resulting from the action of the ras or kinase group of oncogenes are likely important, if not critical, events in the induction of metastatic behavior. In conclusion, we have shown that a single step conversion of NIH-3T3 cells by oncogenes encoding either serine/threonine or tyrosine kinases can induce the metastatic phenotype. It is now clear that mutation or abherant regulation of many structurally diverse oncogenes can effect metastatic dissemination.

Table 8.

Cell Line*	Transforming gene	Experimental Metastasis: Frequency	Experimental Metastasis: Lung Nodules (x ± SE)	Tumorigenicity: Frequency	Tumorigenicity: Latency (x ± days SE)
N(Z61.8)	c-H-ras (61-Leu)	6/6	32±21	5/5	7.6±0.2
Mos 1	v-mos	6/6	29±11	NT	NT
Mos 2	v-mos	5/5	62±27	5/5	7.4±0.6
Mos 3	v-mos	4/6	1.8±0.8	NT	NT
NIH/F4-3611	v-raf	4/6	4.7±2.3	5/5	5.0±0.0
NIH/9IV#5	A-raf	5/6	38±14	5/5	6.2±0.8
Src 1	v-src	6/6	59±25	6/6	7.3±0.5
Scr 2	v-src	5/5	6.8±1.3	NT	NT
Fes 1	v-fes	6/6	178±38	5/5	6.4±0.7
Fms 1	v-fms	6/6	110±45	5/5	6.2±0.7
NIH 3T3 clone 7 (MoMuLV)		0/5	0.0±0.0	NT	NT
NIH 3T3 clone 7		0/10	0.0±0.0	0/3	>50
Ψ2 (MoMuLV)		0/5	0.0±0.0	NT	NT
NIH 3T3		3/10	0.5±0.3	NT	NT
NIH/hmyc1	c-myc	2/5	1.0±0.6	6/6	13.7±0.3
NIH/p53.3	p53	1/5	0.2±0.2	5/5	8.0±0.6
VM1	v-myc	0/5	0.0±0.0	NT	NT
VM4	v-myc	0/5	0.0±0.0	NT	NT
VM5	v-myc	0/20	0.0±0.0	NT	NT
VM5+	v-myc	0/5	0.0±0.0	NT	NT

* Cell lines are as described (30). + Experimental metastasis assay terminated at 46 days.

Metastasis formation by NIH 3T3 cells transformed by oncogenes encoding tyrosine and serine/threonine kinases. Subconfluent and exponentially growing monolayer cultures were lightly trypsinized (0.05% trypsin in 0.5 mM EDTA), washed and resuspended in Hanks balanced salt solution. Aliquots of 3 x 10^5 cells in 0.2 ml were each injected into the lateral tail vein of 5- to 8-week-old BALB/c female nu/nu mice for the experimental metastasis assay. Twenty-one days later, mice were killed by ether anesthesia and Bouin's solution instilled directly in the trachea with a syringe. Lungs were removed and metastases counted under a dissecting microscope. Tumorigenicity of the lines was evaluated by monitoring tumor formation after subcutaneous injection of 3 x 10^5 cells into BALB/c nu/nu mice. All transformed cell lines produced large rapidly growing tumors. Latency was scored as the day at which a 2-mm tumor was detectable. NT, not tested.

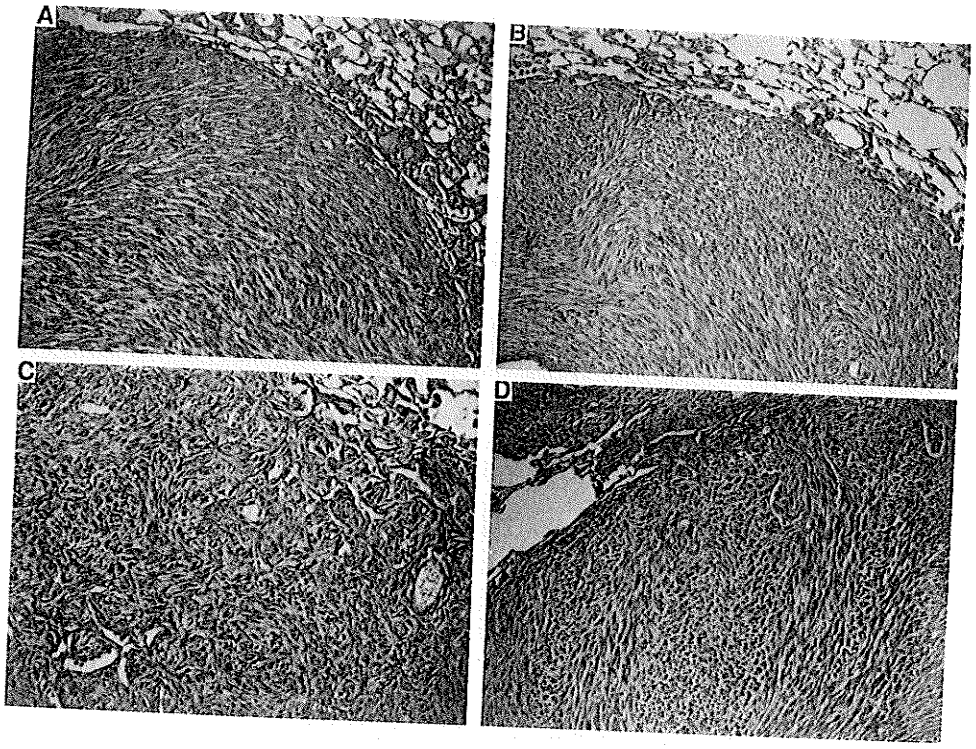

Figure 11- Histopathology of lung metastases of NIH-3T3 transformed by (A) v-mos, line Mos 2 (B) v-fms, line Fms 1, (C) v-src, line Src 1, and (D) c-H-ras (61-leu), line N (Z61-8). All lines produced typical fibrosarcomas.

REFERENCES

1. U.P. Thorgeirsson et. al., Mol. Cell. Biol. 5, 259 (1985); S.C. Bernstein and R.A. Weinberg, Proc. Natl. Acad. Sci. U.S.A. 82, 1726 (1985); R.G. Greig et. al., ibid., p. 3698.

2. S.E. Egan et. al., Mol. Cell. Biol., 7, 830 (1987).

3. K.H. Vousden, S.A. Eccles, H. Purvies, C.J. Marshall, Int. J. Cancer 37, 425 (1986).

4. A.P. Albino, R. LeStrange, A.I. Oliff, M.E. Furth, L.J. Old, Nature 308, 69 (1984).

5. G.M. Brodeur, R.C. Seeger, M. Schwab, H.E. Varmus, J.M. Bishop, Science 224, 1121 (1984); R.C. Seeger et. al., N. Eng. J. Med. 313, 1111 (1985); J. Yokota, Y. Tsunetsugu-Yokota, H. Battifora, C. LeFevre, M.J. Cline, Science 231, 261 (1986); M.M. Nau et. al., Proc. Natl. Acad. Sci. U.S.A. 83, 1092 (1986).

6. G. Kolata, Science 235, 160 (1987); D.J. Slamon et. al., ibid 235, 177 (1987).

7. Three lines of NIH/3T3 clone 7 cells were infected with Mo MuSV. Each of these non-producer cell lines is derived from a different v-mos infection and has been shown to contain v-mos sequences by Southern blot analysis. Northern blots revealed that Mos2 expressed more mos-related RNA than Mos1 which, in turn, expressed more than Mos3 (unpublished).

8. U.R. Rapp et. al., Proc. Natl. Acad. Sci. U.S.A. 80, 4218 (1983).

9. M. Huleihel, et. al., Mol. Cell. Biol. 6, 2655 (1986).

10. S.M. Anderson and E.M. Scolnick, J. Virol. 46, 594 (1983).

11. R. Mann, R.C. Mulligan, D. Baltimore, Cell 33, 153 (1983); The Src2 cell line was obtained through v-src/neo retroviral infection. The MX2122-B31/NEO plasmid was constructed by Dr. Mike Scott and Dr. Michael Verderame in Dr. Harold Varmus' lab. This retroviral vector contains v-src from the B77 strain of Rous Sarcoma Virus and the neomycin resistance gene. The plasmid was transfected into 2 cells and a producer cell line selected in G418. Supernatant from these src/neo virus-producing 2 cells was then used for infection of NIH-3T3. The Src2 cell line arose from a transformed colony selected in G418.

12. J. Even et. al., J. Virol. 45, 1004 (1983).

13. L. Donner, L.A. Fedele, C.F. Garon, S.J. Anderson, C.J. Sherr, J. Virol. 41, 489 (1982).

14. C.J. Der, B.-T. Pan, G.M. Cooper, Mol. Cell. Biol. 6, 3291 (1986).

15. A.L. Kripke, E. Gruys, I.J. Fidler, Cancer Res. 38, 2962 (1978); G.L. Nicolson and G. Poste, Int. Rev. Exp. Pathol. 25, 77 (1983).

16. Unpublished results.

17. NIH-3T3 clone 7 was isolated by limiting dilution cloning and generously supplied by Dr. D. Lowy, National Cancer Institute.

18. A. Kelekar and M.D. Cole, Mol. Cell. Biol. 6, 7 (1986).

19. G.P. Dotto, L.F. Parada, R.A. Weinberg, Nature 318, 472 (1985).

20. R. Bernards, S.K. Dessain, R.A. Weinberg, Cell 47, 667 (1986).

21. C. Shih, L.C. Padhy, M. Murray, R.A. Weinberg, Nature 290, 261 (1981); C.I. Bargman, M.-C. Hung, R.A. Weinberg, Cell 45, 649 (1986).

22. D.F. Stern, A.B. Roberts, N.S. Roche, M.B. Sporn, R.A. Weinberg, Mol. Cell. Biol. 6, 870 (1986).

23. L.F. Fleischman, S.B. Chahwala, L. Cantley, Science 231, 407 (1986); M.J.D. Wakelam et. al., Nature 323, 173 (1986).

24. J. Preiss et. al., J. Biol. Chem. 261, 8597 (1986); A. Wolfman and I.G. Macara, Nature 235, 359 (1987).

25. M.J. Berridge and R.F. Irvine, ibid., 312, 315 (1984); Y. Sugimoto and R.L. Erikson, Mol. Cell. Biol. 5, 3194 (1985); S. Jackowski, C.W. Rettenmier , C.J. Sherr, C.O. Rock, J. Biol. Chem. 261, 4978 (1986).

26. P.W. Majerus et. al., Science 234, 1519 (1986); R.M. Bell, Cell 45, 631 (1986); Y. Nishizuka, Science 233, 305 (1986).

27. M.R. Smith, S.J. DeGudicibus, D.W. Stacey, Nature 320,

540 (1986).

28. R.Jaggi, B. Salmons, D. Muellener, B. Groner, EMBO J. 5, 2609 (1986).

29. A. Schmidt, C. Setoyama, B. deCrombrugghe, Nature 314, 286 (1985); M.S. Rabin, P.J. Doherty, M.M. Gottesman, Proc. Natl. Acad. Sci. U.S.A. 83, 357 (1986).

30. The ras transformed cell line NZ61.8L was analyzed at passage 4. The serine/threonine kinase gene transformed cells Mos 1,2 and 3 were passage 6, while v-raf and A-raf lines were 19th and 9th passage, respectively. Tyrosine kinase gene transformed lines Src 1, Fes 1 and Fms 1 were low but undefined passage number. Src 2 was tested at passage 3. Nuclear oncogene transformed lines NIH/hmyc 1, NIH/p53.3 were passage 4 and 6, respectively, and VM 1,4 and 5 were 3rd passage. NIH/3T3 clone 7 was passaged a total of 16 times. Of the above cells, we isolated and tested lines transformed by serine/threonine kinases (Mos 1,2,3), tyrosine kinases (src 2) as well as nuclear oncogenes (VM 1,4,5). Data from these cell lines were identical with results from cells provided by other laboratories.

IV *myc*, *ras* COOPERATION IS INSUFFICIENT FOR METASTATIC TRANSFORMATION

This work is being prepared for submission as a manuscript in the format outlined by the journal Nature

myc/ras Cooperation is Insufficient for Metastatic Transformation.

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Abstract

Metastatic Transformation of 10T1/2 murine fibroblasts can be achieved in vitro through transfection with the human H-ras oncogene in the presence of fetal bovine serum (FBS). In contrast, these cells transformed by the combined action of myc and ras in calf serum (CS) are completely non-metastatic. Experiments in both CS and FBS indicate that the cooperative interaction of myc and ras is sufficient for benign transformation but an additional event, which can be stimulated by a factor present in FBS, is necessary for induction of the metastatic phenotype.

Introduction

Metastatic spread of cancer is the most life-threatening aspect of the disease. It is often associated with a poor clinical prognosis and lack of response to conventional therapeutical intervention. The metastatic process involves a complex cascade of events, normally initiated through tumor invasion into the lymphatics or vasculature. Metastatic cells must then survive immune surveillance, and hydrodynamic pressure drops in the circulation before implanting at a secondary site. This implantation requires binding to endothelium, and extravasation through the basement membrane which involves specific proteolysis and motility. Finally, the metastatic cell must be able to grow into an autonomous macroscopic tumor in its new environment. This complex phenotype is often acquired as a result of the multistep process known as tumor progression.

The multistep nature of tumorigenic transformation has been demonstrated in vitro through transfection of primary fibroblast cells by various cooperating oncogenes (1,2). These initial studies resulted in the dissection of in vitro transformation into two distinct steps. **Establishment** or **immortalization** was provided by a nuclear oncogene such as myc and **morphological transformation** could then result from expression of cytoplasmic oncogenes such as ras. Further study of this model has revealed that in vitro establishment

is not a sufficient prerequisite for transformation by the ras oncogene (3). In contrast, the single step transformation of primary cells has been observed under special culture conditions (4). Using this single step transformation model, it has also been demonstrated that primary cells transformed by ras alone are highly metastatic (5). This was later shown to be a result of co-selection for resistance to antibiotics such as G418 (6). Transformation of primary cells by ras alone in the absence of normal cells occurs at a high frequency but is not likely relevant to non-viral carcinogenesis in vivo (6). In order to understand the natural multistep process of tumor progression, the individual steps in metastatic transformation must be separated and studied.

We have previously shown that the ras oncogene can directly regulate the metastatic phenotype in two established fibroblast lines, 10T1/2 and NIH-3T3 (7). In order to study the cooperative interaction of myc and ras in the induction of metastatic behaviour we transformed 10T1/2 cells by both genes under conditions where either gene alone was ineffective. We report that 10T1/2 transformed by myc and ras in dialysed calf serum are tumorigenic but non-metastatic. In contrast these cells transformed by ras alone in fetal bovine serum (FBS) or a portion of the myc/ras transformants isolated in FBS were both tumorigenic and metastatic. Analysis of myc expression in the

metastatic ras transformants suggests that both oncogenes cooperate to regulate the metastatic phenotype in cells which are metastatic. 10T1/2 cells transformed by ras alone which are highly metastatic express higher levels of myc protein than poorly metastatic ras-transformants. The cooperative effects of myc and ras are sufficient for benign transformation but insufficient for metastatic transformation of the established fibroblast cell line 10T1/2. Additional events which can be induced by a factor present in FBS are essential to access the full metastatic phenotype. Both oncogenes likely cooperate in the regulation of the metastatic phenotype in cells which have undergone this additional event.

Myc/Ras Insufficient

10T1/2 murine fibroblasts were infected in 10%FBS/αMEM with a helper free v-myc/neo retrovirus (8) and cell lines 10T1/2myc-1#1, 10T1/2myc-1#2 and 10T1/2myc-1#4 selected as G418 resistant clones. Control G418 resistant cell lines SVX-1 and SVX-2 were generated through infection of 10T1/2 with the neo virus ZIPNEO/SVX (8,9). These lines were then used as recipients for infection by a ras containing virus (8). Infection by the v-H-ras-SVX virus was carried out in dialysed calf serum and, consistent with a previous report, the ras oncogene alone was unable to induce focus formation under these conditions (10). In contrast, focus formation

was reproducibly observed in the lines that contained v-myc (data not shown). In order to analyze the properties of ras-expressing derivatives of these myc/neo, or neo cells, we have co-transfected the human EJ-H-ras oncogene and the PY3 plasmid which codes for resistance to the antibiotic hygromycin B (11) into 10T1/2myc-1#1 or SVX-1. Hygromycin resistant cell lines derived from 10T1/2myc-1#1 were designated 10T1/2MRP clones A through I and SVX-1 derived lines named 10T1/2NRP 1 through 5. Analysis of these cells revealed that most myc/ras lines were both tumorigenic in syngeneic C3H mice and capable of anchorage independent growth (Table 9). In contrast, the neo/ras cell lines were non-tumorigenic and could not grow in soft agar (Table 9). The myc oncogene therefore is able, at least partially, to replace the requirement for transformation promoting signals, such as tumor promoting phorbol esters (12) or fetal bovine serum (10) which are required for ras-induced transformation of 10T1/2 cells.

We next analyzed the metastatic properties of these cell lines. All of the neo/ras lines were non-metastatic as assessed in the lung colonization or experimental metastasis assay. This is consistent with their lack of tumorigenic potential discussed above. Surprisingly, all of the myc/ras lines were completely non-metastatic (Table 9). Several lines were analyzed in long term assays to insure that micrometastasis were not overlooked in the three week assay.

Again no lung tumors were present. Almost all of these cell lines were both tumorigenic and capable of potent growth in soft agar yet, they were completely non-metastatic. This is in sharp contrast to our previous observation that foci obtained after transfection of ras alone in fetal bovine serum resulted in metastatic lines (7).

Transformation in FBS

We next repeated the experiment in fetal bovine serum. The myc/neo line 10T1/2myc-1#1 was again co-transfected with EJ-H-ras and PY3, and cell lines selected in hygromycin. Four drug resistant lines were obtained and designated 10T1/2M1RFCS-1 through 4. Biological properties of these lines were analyzed. As above, 10T1/2M1RFCS-1,2 and 3 were all able to grow in soft agar but were poorly tumorigenic and non-metastatic. In contrast to the lack of metastatic clones generated in dialysed calf serum, we found that the 10T1/2M1RFCS-4 was metastatic. This was not due to any alteration in myc-expression (data not shown) since all clones still expressed the viral myc gene.

In order to test whether the phenotype of 10T1/2M1RFCS-4 resulted from a transfection artifact or selection of drug resistant cells in the absence of normal cells, we have repeated this experiment using distinct myc/neo cell lines and through selection of transformed cells at confluence. Cell lines were established from foci after infection of

10T1/2myc-1#2 by the v-H-ras-SVX virus and these were named 10T1/2M2R-1,2 and 3. Similarly, 10T1/2M4R-1,2,3 and 4 were derived from foci after infection of 10T1/2myc-1#4 with the same ras virus. Once again most cell lines were found to be non-metastatic, poorly tumorigenic but able to grow in soft agar (Table 10). 10T1/2M2R3 and 10T1/2M4R3 were poorly and moderately metastatic respectively (Table 10). When transformed in fetal bovine serum the majority of cell lines were non-metastatic, as was the case with cells transformed by myc and ras in dialysed calf serum. In contrast, three of the eleven lines isolated after sequential introduction of myc and ras in fetal bovine serum were metastatic (Table 10).

We have previously reported that cell lines selected from transformed foci following transfection of ras into 10T1/2 in FBS were fully metastatic (7). Consistent with this we have found that cell lines obtained after infection of SVX-2 cells with the ras-virus (SVX2R1 and SVX2R2) were metastatic, indicating that the expression of neo prior to ras transformation was not inhibiting metastatic behaviour (Table 10). We have also isolated a cell line transformed by ras through co-transfection of ras and PY3 in FBS and subsequent selection for hygromycin resistance. This line (10T1/2RAS) was metastatic, indicating that expression of the hygromycin resistance gene did not inhibit the metastatic phenotype of ras-transformed cells (Table 10).

myc/ras Expression

We have previously described a series of ras-transformed 10T1/2 clones which were fully metastatic. Expression of ras-RNA in these clones correlated with their metastatic potential (7). Analysis of endogenous myc gene expression in these clones revealed that all lines contained equal levels of myc-RNA (data not shown). Analysis of myc protein, however, shows an increase in the steady state level in those cell lines selected from foci after ras-transfection (Figure 12). The metastatic lines CIRAS-1, CIRAS-2 and CIRAS-3 express more myc protein product than the control 10T1/2, non-metastatic NR3 or poorly metastatic NR4 lines. In summary, the highly metastatic cell lines (CIRAS-2 & CIRAS-3) expressed high levels of both myc and ras, the moderately metastatic line (CIRAS-1) expressed moderate levels of ras and high levels of myc, the poorly metastatic line (NR4) expressed moderate levels of ras and low levels of myc, whereas the non-metastatic controls (10T1/2 and NR3) expressed low levels of both oncogenes. This suggests that high levels of both myc and ras are important for expression of the highly metastatic phenotype, and that this phenotype can be regulated through the cooperative interaction of these two genes in cells that have become metastatic.

Discussion

Tumor progression in vivo is a multistep process which often occurs over extended periods of time. In 1983 cellular oncogene cooperation in transformation was described (1,2). It was later found that overexpression of a mutant ras oncogene alone was able to transform primary rat embryo fibroblasts (4). Pozzatti and colleagues have studied this system and found that the cells transformed by ras were highly metastatic (5). Malignant transformation of primary cells through the transfection of a single mutant oncogene does not mimic the multistep nature of cancer and tumor progression in vivo. It has also been found that in vitro establishment is not sufficient for susceptibility to transformation by ras (3). Why then can transfection of ras result in metastatic conversion of primary cells? This single step transformation can only occur under specific culture conditions, suggesting that other cooperating events which must occur, are being induced under such conditions. Single step transformation was found to be a result of co-selection for G418 resistance (6). Consequently, transformation occurred in the absence of normal cells which is not relevant to most in vivo situations (6). Transfection of primary fibroblasts with myc and ras was sufficient to transform these cells but they produced very poorly tumorigenic lines that were completely non-metastatic (1). Recently, Muschel and colleagues have studied

metastatic cell lines generated from the transfection of either ras and neo or myc/ras and neo together into primary rat embryo fibroblasts. This group reported that the transfected ras sequences had inserted into a region of chromosome 3 in all lines analyzed, suggesting that insertional mutagenesis of genetic material at this locus was essential for the metastatic transformation observed (13).

In an effort to separate the individual events necessary for metastatic transformation, we have transformed the established cell line 10T1/2 in dialysed calf serum through the cooperative interaction of myc and ras oncogenes. Under these conditions, neither gene alone is capable of transforming 10T1/2. All of the transformed cell lines generated were non-metastatic (Table 9). Transformation through the sequential addition of myc and ras in fetal bovine serum yielded mostly non-metastatic cells, but resulted in the metastatic transformation of approximately 25% of the lines. This is in contrast to cells transformed by the addition of ras alone in FBS which were all metastatic (Table 10 and reference 7). Fetal bovine serum has been shown to promote the transformation of 10T1/2 by ras (10). In addition, myc and ras cooperate in the transformation of 10T1/2 (14). Hsiao et.al. have reported that the effect of fetal bovine serum on transformation by ras is suboptimal and that the number of

transformed foci can be increased through the synergistic addition of the phorbol ester tumor promoter TPA (10). The event induced by fetal bovine serum is, therefore, stochastic. The introduction of myc into 10T1/2 cells allows for benign transformation by ras in the absence of the FBS promoted stochastic event (Table 9). Experiments performed in FBS suggest that since myc can increase the number of transformed foci following ras transfection in FBS (14), and most of the transformed lines are non-metastatic (Table 10), then expression of myc likely does not increase the frequency of an event necessary for metastatic transformation by ras. In addition, it is likely that the factor in FBS responsible for promotion of transformation by ras, which has been previously described (10), may be inducing the stochastic event necessary for promotion of metastatic transformation. In conclusion, the cooperative interaction of myc and ras is sufficient for benign transformation but insufficient for expression of the full metastatic phenotype. A stochastic event which can be promoted by fetal bovine serum must cooperate with ras for transformation of 10T1/2 in the absence of myc. This event is likely identical to the event necessary for metastatic-transformation by ras since all lines transformed by ras alone in FBS are metastatic. Highly metastatic ras-transformed lines express elevated levels of myc protein. This suggests that in cell lines in which the stochastic

event inducing metastasis has occurred, the metastatic potential is regulated through the cooperative interaction of myc and ras gene products. The characterization of this stochastic event constitutes an important challenge for future experimentation.

Table 9 legend; Tumorigenic and Metastatic Properties of 10T1/2 cells transfected in Dialysed Calf Serum.

10T1/2 cells were infected (15,16,17) with VM (v-myc/neo) (8) or SVX (neo) (8) retroviruses stocks using the PA317 line for viral packaging as described, in 10%FBS (Flow Laboratories: Mississauga,Ont.)/αMEM and selected for resistance to 400ug/ml of G418 sulphate (Gibco Laboratories; Grand Island,N.Y.). 10T1/2SVX1 and 10T1/2myc-1#1 were then switched to growth in 10% dialysed Colorado Calf Serum four days prior to transfection. pEJ and PY3 were transfected into these two cell lines at a weight ratio of 10:1 as per standard protocols (7), using the Pharmacia Cellfect transfection kit and following manufacturers instructions. Cell lines were cloned from colonies which had survived selection in 100ug/ml of Hygromycin B (Sigma Chemical Co.; St.Louis,MO.). These lines were maintained and analyzed in 10% Colorado Calf Serum/αMEM with the exception of soft agar growth assays which were performed as previously described in 10%FBS/αMEM (7). Tumorigenicity and experimental metastasis assays (5 mice per group) were performed on $3x10^5$ cell inoculum as previously described (7).

Table 9.

Cell Line	Anchorage Independent Growth (10^3 cells)	Colony size	Tumorigenicity Frequency	Tumorigenicity Latency (days±SE)	Metastatic Potential Experimental Metastasis no±SE
10T½ myc 1#1	217±18	S	0/3	N.A.	0.0±0.0
10T½ MRPA	165±6	M-L	2/3	40.5±9.5	0.0±0.0
10T½ MRPB	376±19	M-L	3/3	15.0±0.0	0.0±0.0
10T½ MRPC	302±29	M-L	3/3	8.0±0.0	0.0±0.0
10T½ MRPD	450±10	M-L	3/3	8.0±0.0	0.0±0.0
10T½ MRPE	540±48	M-L	3/3	8.0±8.0	0.0±0.0
10T½ MRPF	280±40	S-M	0/3	N.A.	0.0±0.0
10T½ MRPG	215±11	S	0/3	N.A.	0.0±0.0
10T½ MRPH	851±89	M-L	3/3	13.0±0.0	0.0±0.0
10T½ MRPI	115±17	S-M	3/3	71.0±0.0	0.0±0.0
10T½ SVX1	0.0±0.0	N.A.	0/3	N.A.	0.0±0.0
10T½ NRP1	0.0±0.0	N.A.	0/3	N.A.	0.0±0.0
10T½ NRP2	0.0±0.0	N.A.	0/3	N.A.	0.0±0.0
10T½ NRP3	0.0±0.0	N.A.	0/3	N.A.	0.0±0.0
10T½ NRP4	0.0±0.0	N.A.	0/3	N.A.	0.0±0.0
10T½ NRP5	0.0±0.0	N.A.	0/3	N.A.	0.0±0.0

N.A. = not applicable
S = small
M = medium
L = large

Table 10 legend; Tumorigenicity and Metastatic Properties of 10T1/2 Cell lines Transformed in Fetal Bovine Serum.
Cell lines were all derived and maintained in 10%FBS/αMEM through transfection or infection with retroviruses according to standard protocols as outlined in legend to Table 9 and discussed in text. Tumorigenicity and metastasis assays (5 mice per group) were performed on $3x10^5$ cell inoculum with noted exceptions.

Cell lines	neovirus	mycvirus	EJ-H-ras	PY3 rasvirus
10T1/2myc1#1,2,4	+			
10T1/2M1RFCS1-4	+	+	+	
10T1/2M2R1-3	+			+
10T1/2M4R1-4	+			+
10T1/2RAS			+	+
SVX2	+			
SVX2R1-2	+			+

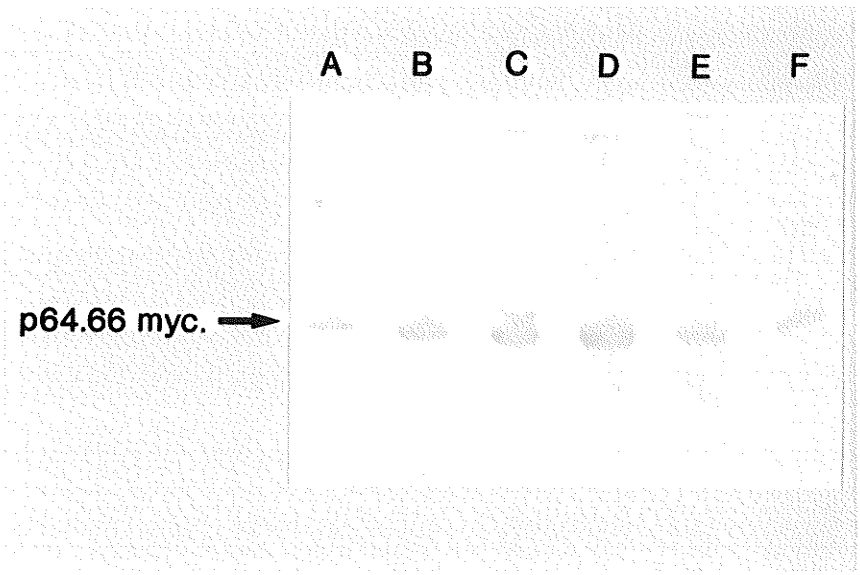
Table 10.

Cell Line	Anchorage Independent Growth (10^3 cells)	Colony Size	Tumorigenicity* Frequency	Latency (days±SE)	Metastatic Potential Experimental Metastasis C3H no.±SE	Balb/c nu/nu no.±SE
10T½ myc 1#1	217±35	S	0/3	N.A.	0.0±0.0	N.T.
10T½ MIRFCS-1	561±30	S-L	0/4	44±11	0.0±0.0	1.4±0.8
10T½ MIRFCS-2	359±11	S-M	2/4	18±5	0.0±0.0	0.2±0.2
10T½ MIRFCS-3	499±12	S-L	2/4	43±10	0.0±0.0	0.2±0.2
10T½ MIRFCS-4	287±14	S-L	4/4	14.3±.5	40.0±5.8	10.8±0.3
10T½ myc 1#2	0±0	N.A.	0/3	N.A.	0.0±0.0	N.T.
10T½ M2R1	284±25	S	0/4	N.A.	0.0±0.0	N.T.
10T½ M2R2	424±24	S-L	4/4	13±3	0.0±0.0	N.T.
10T½ M2R3	198±20	S-L	3/4	22±3	1.3±0.6	N.T.
10T½ myc 1#4	0±0	N.A.	0/3	N.A.	0.0±0.0	N.T.
10T½ M4R1	368±18	S	0/4	N.A.	0.0±0.0	N.T.
10T½ M4R2	434±15	S-L	2/4	10.0±0.0	0.0±0.0	N.T.
10T½ M4R3	236±18	S-M	3/4	10.0±1.0	10.8±5.0	N.T.
10T½ M4R4	168±10	S-L	4/4	10.5± .3	0.2±0.2	N.T.
10T½ RAS	268±32	S-M	4/4	7.3±0.5	48.6±6.3	N.T.
SVX2	0±0	N.A.	0/3	N.A.	0.0±0.0	N.T.
SVX2R1	426±18	S-L	3/3	18±2	42.0±4.3	N.T.
SVX2R2	257±14	S-M	3/3	9.7±0.9	201.0±28.0	N.T.

* Subcutaneous tumors from 10^6 cell inoculum
NA = not applicable S = small M = medium L = large
NT = not tested

Figure 12 legend; Western blot analysis of myc protein in 10T1/2 and ras-transformed derivatives. A)10T1/2 B)CIRAS-1 C)CIRAS-2 D)CIRAS-3 E)NR3 F)NR4.

Methods; Tissue culture cells were trypsinized, washed in phosphate buffered saline, counted and disrupted in lysis buffer (18) at a concentration of 10^7 cells/ml. Each lysate was passed through a 20 gauge needle approximately ten times and then clarified by centrifuging for five minutes at fourteen thousand rpm in an eppendorf microfuge. 10^5 cell equivalents were separated on a 10% PAGE gel and electrophoretically transferred to nitrocellulose. The c-myc protein was detected through incubation with the monoclonal antibody Myc1-8F9 (19) and subsequently visualized using the avidin/biotin-alkaline phosphatase antimouse-antibody detection kit (Vectastain ABC kit, Dimension Laboratories; Mississauga, Ont.) as per manufacturers instructions.


A
B
C
D
E
F
p64.66 myc.

References

1) Land,H., Parada,L.F., and Weinberg,R.A., 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. 304, 596-602.

2) Ruley,E.H., 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature. 304, 602-606.

3) Franza,B.R.Jr., Maruyama,K., Garrels,J.I., and Ruley,H.E., 1986. In vitro establishment is not a sufficient prerequisite for transformation by activated ras oncogenes. Cell. 44, 409-418.

4) Spandidos,D.A., and Wilkie,N.M., 1984. Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature. 310, 469-475.

5) Pozzatti,R., Muschel,R., Williams,J., Padmanabhan,R., Howard,B., Liotta,L., and Khoury,G., 1986. Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science. 232, 223-227.

6) Land,H., Chen,A.C., Morgenstern,J.P., Parada,L.F., and

Weinberg,R.A., 1986. Behavior of myc and ras oncogenes in transformation of rat embryo fibroblasts. Mol. Cell. Biol. 6, 1917-1925.

7) Egan,S.E., McClarty,G.A., Jarolim,L., Wright,J.A., Spiro,I., Hager,G., and Greenberg,A.H., 1987. Expression of H-ras correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in 10T1/2 and NIH-3T3 cells. Mol. Cell. Biol. 7, 830-837.

8) Dotto,G.P., Parada,L.F., and Weinberg,R.A., 1985. Specific growth response of ras-transformed embryo fibroblasts to tumour promoters. Nature. 318, 472-475.

9) Cepko,C.L., Roberts,B.E., and Mulligan,R.C., 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell. 37, 1053-1062.

10) Hsiao,W.-L.W., Lopez,C.A., Wu,T., and Weinstein,I.B., 1987. A factor present in fetal calf serum enhances oncogene-induced transformation of rodent fibroblasts. Mol. Cell. Biol. 7, 3380-3385.

11) Blochlinger,K., and Diggelmann,H., 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eukaryotic cells. Mol. Cell. Biol..

4, 2929-2931.

12) Hsiao,W.-L.W., Wu,T., and Weinstein,I.B., 1984. Oncogene-induced transformation of C3H 10T1/2 cells is enhanced by tumor promoters. Science. **226**, 552-555.

13) McKenna,W.G., Nakahara,K., and Muschel,R.J., 1988. Site-specific integration of H-ras in transformed rat embryo cells. Science. **241**, 1325-1328.

14) Taparowsky,E.J., Heaney,M.L., and Parsons,J.T., 1987. Oncogene-mediated multistep transformation of C3H10T1/2 cells. Cancer Research. **47**, 4125-4129.

15) Egan,S.E., Wright,J.A., Jarolim,L. Yanagihara,K., Bassin,R.H., and Greenberg,A.H., 1987. Transformation by oncogenes encoding protein tyrosine kinases induces the metastatic phenotype. Science. **238**, 202-205.

16) Miller,A.D., Trauber,D.R., and Buttimore,C., 1986. Factors involved in production of helper virus-free retrovirus vectors. Somatic Cell and Molecular Genetics. 12, 175-183.

17) Miller,A.D., and Buttimore,C., 1986. Redesign of retrovirus packaging cell lines to avoid recombination

leading to helper virus production. Mol. Cell. Biol.. 6, 2895-2902.

18) Egan,S.E., Broere,J.J., Jarolim,L., Wright,J.A., and Greenberg,A.H., 1989. Coregulation of metastatic and transforming activity of normal and mutant ras genes. International Journal of Cancer. In the press.

19) Evan,G.I., Lewis,G.K., Ramsay,G., and Bishop,J.M., 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol.. 5, 3610-3616.

V AUTOCRINE AND PARACRINE REGULATION OF THE METASTATIC PHENOTYPE BY GROWTH FACTORS

This work is being prepared for submission as a manuscript in the format outlined by the journal **Nature.**

Autocrine and Paracrine Regulation of the Metastatic Phenotype by Growth Factors.

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Abstract

Altered production and response to growth factors is often involved in neoplasia. Autocrine and/or paracrine regulation of metastatic behaviour by growth factors can be demonstrated by competence, progression and growth modulating factors. In addition, regulation of the metastatic phenotype by a specific growth factor can depend on the oncogenes responsible for cell transformation and the presence of other growth modulating factors.

Introduction

Metastatic dissemination of cancer represents the most serious challenge to therapeutic intervention. The metastatic cell exhibits a complex phenotype which enables it to specifically interact with basement membrane components. This interaction includes proteolytic degradation and tumor cell motility (1). In 1985, several groups reported that ras oncogenes could induce the metastatic phenotype in NIH-3T3 cells (2,3,4). We and others have also found that kinase encoding oncogenes including the growth factor receptor v-fms can induce metastatic behaviour (5,6). In addition, the nuclear oncogenes N-myc and p53 can increase the metastatic spread of neuroblastoma and bladder carcinoma cells respectively (7,8). In the case of ras and src, these two oncogenes can directly regulate the metastatic phenotype (9,10,6).

Polypeptide growth factors can regulate the proliferation and differentiation of cells (11). Similarly, oncogene products can alter the regulation of both growth and differentiation (12,13). In fact, some of the viral oncogenes or virally activated oncogenes encode polypeptide growth factors or their receptors (13). In addition, some growth factors such as basic fibroblast growth factor have strong oncogenic potential (14). Autocrine secretion of growth factors has been implicated in maintenance of the transformed phenotype in many tumor types (15). Aggressive

tumors also secrete many factors which are responsible for angiogenesis, modulation of the immune response and augmentation of stromal cell growth and differentiation (16,17). It is also clear that non-tumor tissue can secrete factors which are capable of modulating the behaviour and growth of tumors (18).

Autocrine Metastatic Transformation

In order to test for autocrine regulation of the metastatic phenotype we have analyzed the metastatic properties of NIH-3T3 cells transformed by a chimeric basic fibroblast growth factor (bFGF) gene (14). These cells produce bFGF with an immunoglobulin leader sequence which can target the molecule into the secretory pathway. Consequently these cells make bFGF which can meet its receptor and transform through chronic stimulation (14) (Table 11). Overexpression of bFGF without this leader sequence resulted in the accumulation of high levels of intracellular bFGF without transformation since the molecule was not secreted (14) (Table 11). Intravenous injection of bFGF-transformed cells into the tail vain of nude mice resulted in the formation of numerous experimental metastases or lung colonies in all five independent lines tested (Table 11). In contrast, intravenous injection of two cell lines which express high levels of the non-secreted form of bFGF or neomycin resistance gene did not result in

the formation of lung tumors. In order to insure that micrometastases were not being missed in the regular three week experimental metastasis assay, the control cell lines were tested in a six week assay. The bFGF transformed cells were also capable of completing the full metastatic cascade since clone #9460 disseminated to the lung from subcutaneous tumors in three out of three animals examined. Consequently, autocrine production of growth factors can induce the complete metastatic phenotype.

Paracrine Regulation of Metastasis

Since a growth factor can induce the full metastatic phenotype through autocrine mechanisms involving the normal endogenous receptor, we have analyzed the potential for paracrine regulation of tumor dissemination. Pretreatment of the ras-transformed 10T1/2 cell line Ciras-1 (9) with 10ng/ml of bFGF for twenty four hours prior to intravenous injection resulted in a thirteen-fold inhibition of experimental metastasis formation in immunocompetant C3H mice (Table 12). This effect was not limited to 10T1/2 cell lines since pretreatment with bFGF also blocked metastasis formation by the ras-transformed NIH-3T3 line Lys12Ala59 (10) (Table 12) in nude mice. Consequently, this factor can have both stimulatory (Table 11) and inhibitory (Table 12) effects on the metastatic properties of a single cell type depending on the conditions of treatment or accompanying

alterations present. In addition, pretreatment of the src-transformed NIH-3T3 line Src-1 (5) resulted in a seventeen-fold inhibition of lung tumor formation (Table 12), indicating that this inhibitory effect was not limited to cells transformed by ras. This assay, as above, was performed in nude mice which indicates that inhibition was not mediated through T-cell dependent immune rejection.

The inhibition of metastatic dissemination induced through bFGF treatment in these systems stands in marked contrast to the potent induction of the metastatic transformation induced through transfection of a chimeric bFGF gene in the previous section. There are several possible reasons for this result. It is quite likely that a transient pulse of bFGF will result in downregulation of the endogenous bFGF receptor and/or downregulation of the response pathway to this cytokine. In contrast, it is believed that the transforming bFGF gene produces a product which meets its receptor prior to membrane transport and therefore does not allow for receptor downregulation (14). If correct, this model may explain how downregulation to a 24 hour acute pulse of bFGF could induce an opposite effect on the metastatic behavior of cells to a chronic autocrine stimulation which does not allow for downregulation of the response. In support of this idea, transformation through expression of the mutant CSF-1 receptor v-fms which is not downregulated normally in response to ligand (19) results in

a CSF-1 induction of metastatic dissemination as a result of a 24 hour pretreatment (see below). In addition, the effects of bFGF may be opposite in the context of ras or src-mediated transformation as opposed to non-transformed NIH-3T3 cells which are recipients for the autocrine bFGF signal in the Ig-bFGF transformed cells.

Receptor Specificity

The v-fms oncogene is derived from cellular sequences encoding the M-CSF (macrophage-colony stimulating factor) or CSF-1 receptor (20). When expressed in NIH-3T3 cells it is capable of transforming these cells to the complete metastatic phenotype (5). In addition, the transformants become responsive to human CSF-1 (21). Treatment of v-fms transformed cells with recombinant human CSF-1 results in their enhanced growth in both monolayer culture and in soft agar (21). NIH-3T3 cells do not express endogenous murine CSF-1 receptors and are, therefore, not responsive to CSF-1 (22). We have pretreated the v-fms-transformed NIH-3T3 cell line FMS1 (5) with 10nM recombinant h-CSF-1 for twenty four hours prior to injection into the tail vain of nude mice. This treatment resulted in a forty-fold increase in the number of lung metastasis detected after three weeks (Table 12). No increase resulted from the treatment of ras-transformed cells with CSF-1 (Table 12), indicating that this stimulation was dependent on expression of the CSF-1

receptor or its derivative oncoprotein. Paracrine induction of metastatic behaviour is receptor specific and can occur through the inappropriate expression of growth factor receptors.

Growth Modulating and Progression Factors

Members of the TGF-ß group of growth regulators are produced by most tumor cells, and in some cases these molecules function as negative feedback inhibitors of cell growth (23). These molecules may also regulate immune response to tumor tissue, angiogenesis and extracellular matrix production by tumor stroma. Since TGF-ß can alter the interaction of tumors cells with matrix and is capable of either inducing matrix production or degradation, we have tested the potential for regulation of the metastatic phenotype by TGF-ß1. CIRAS-1 cells were grown in defined medium containing insulin and transferrin. Addition of 100pM TGF-ß1 for twenty four hours prior to injection resulted in an approximately four-fold inhibition of lung tumor formation (Table 12). This inhibition of metastatic behaviour by TGF-ß1 was blocked by the simultaneous addition of 800pM EGF (Table 12). Opposing effects of TGF-ß1 and EGF have been observed by others including regulation of 10T1/2 cell DNA synthesis in the defined medium used in the above experiment (24). It is unlikely that TGF-ß1 will have an inhibitory effect on metastasis in all cases since it is a

bifunctional regulator of cell proliferation (23). It is also clear that the effects of TGF-ß depend on cellular interactions with specific matrix components and with other hormones or cytokines (23).

Summary

Recent progress has been achieved in understanding the metastatic phenotype through transfection studies with dominant acting oncogenes. Many of the oncogene products have been characterized and found to interact with growth and differentiation factor response pathways. In addition, tumor cells often secrete numerous molecules capable of augmenting their own growth and differentiation as well as non-tumor tissue properties. Many tumors are under the influence of endocrine and paracrine factors produced by host tissues which can potently alter their behaviour. In this report it has been found that the metastatic phenotype can be induced through autocrine production of growth factors. The metastatic spread of tumor cells can be stimulated by growth factors through inappropriate expression of the receptor of a factor as in the case of the CSF-1 receptor derived oncogene v-fms. In addition, we have found that paracrine regulation of metastatic behaviour can be achieved through the action of competence, progression and growth modulating factors such as bFGF, EGF, and TGF-β1 respectively. The effect of a particular growth factor on

the spread of cancer, like its effect on proliferation and differentiation, will likely depend on many things including expression of oncogenes and presence of other growth factors. The signal transduction pathways which are altered in response to oncogenes are the same pathways on which growth factors can induce or inhibit metastatic dissemination through either autocrine or paracrine mechanisms.

Table 11 Legend; Autocrine Induction of the Metastatic Phenotype by bFGF. NIH-3T3 cells were maintained and transfected as described (14). In addition, the analysis of bFGF activity in cell extracts was performed as previously described (14). Experimental metastasis assays were performed as desribed in reference 9 except that cell lines transfected with contol neo or non-secreted bFGF vectors were analyzed in six week assays.

Table 11. Autocrine Induction of the Metastatic Phenotype by bFGF

	bFGF activity in cell extracts units/10^3 cells	n	Experimental Metastasis (3×10^5 iv) x ± SE
NIH-3T3 Igb FGF (transformed)			
NIH-N-IgbFGF-b-2M#8768	6.0	4	27 ± 11
NIH-N-IgbFGF-b-(F11,12)#8760	7.0	4	153 ± 44
NIH-N-IgbFGF-b-(G2,4)#8770	6.0	4	16 ± 5
NIH-N-IgbFGF-29-1M#9460	6.0	5	144 ± 45
NIH-N-IgbFGF-4-2M#8769	6.0	5	4.6 ± 2.4
NIH-N-IgbFGF-NM6.1	7.0	5	52 ± 28
NIH-3T3 - bFGF (non-transformed)			
NIH-BNM35	40	5	0.2 ± 0.2
NIH-BNM46	25	5	0.0 ± 0.0
NIH-3T3-neo			
NIH-N-A1	0.2	4	0.0 ± 0.0
NIH-CNM2	NT	5	0.0 ± 0.0

Table 12 Legend. Paracrine Regulation of the Metastatic Phenotype. All growth factors were incubated with cells for 24 hours before injection. bFGF (10ng/ml) (Collaborative Research Inc.,Bedford,MA) and r-Hu-CSF-1 (10nM) (Immunex Corp.,CA) were diluted in αMEM/10% FBS, while TGF-β1 (100pM in 1mg/mlBSA in 4nMHCL) (R&D Research,Minneapolis,MN) and EGF (800pM) (Collaborative Research,Bedford,MA) mixed in αMEM with 0.2mg/100mls insulin (Sigma Chemical Co.,St Louis,MO) and 0.4mg/100mls transferrin (Sigma Chemical Co.,St Louis,MO).

Table 12. Paracrine Regulation of the Metastatic Phenotype

Cell Line	Transforming Oncogene	Treatment	Mouse Strain	No.	Experimental Metastases ±SE
CIRAS-1	T24-H-ras	Nil	C3H/HeN	5	18.4±9.6
		bFGF	C3H/HeN	5	1.4±0.9
NIH-3T3-ras	H-ras (lys12 Ala59)	Nil	BALB/nu/nu	5	1.8±0.6
		bFGF	BALB/nu/nu	5	0.0±0.0
NIH-3T3-src1	v-src	Nil	BALB/nu/nu	5	147±39
		bFGF	BALB/nu/nu	5	8.8±5.2
NIH-3T3 Fms1	v-fms	Nil	BALB/nu/nu	5	0.2±0.2
		rhCSF-1	BALB/nu/nu	5	8.0±0.8
NIH-3T3 ras	H-ras (lys12 Ala59)	Nil	BALB/nu/nu	5	1.8±0.7
		rhCSf-1	BALB/nu/nu	5	2.2±1.5
CIRAS-1	T24-H-ras	Nil	C3H/HeN	5	113±21
		TGF-β1	C3H/HeN	5	29±6
CIRAS-1	T24-H-ras	EGF	C3H/HeN	5	25.6±6.1
		EGF+TGF-β1	C3H/HeN	5	25.0±4.6

References

1) Liotta,L.A., 1986. Tumor invasion and metastasis - role of the extracellular matrix: Rhoads memorial award lecture. Cancer Research. **46**, 1-7.

2) Thorgeirsson,U.P., Turpeenniemi-Hujanen,T., Williams,J.E., Westin,E.H., Heilman,C.A., Talmadge,J.E., and Liotta,L.A., 1985. NIH/3T3 cells transfected with human tumor DNA containing ras oncogenes express the metastatic phenotype in nude mice. Mol. Cell. Biol.. 5, 259-262.

3) Bernstein,S.C., and Weinberg,R.A., 1985/1988. Expression of the metastatic phenotype in cells transfected with human metastatic tumor DNA/Correction. Proc. Natl. Acad. Sci. USA. **82**, 1726-1730./85, 5581.

4) Greig,R.G., Koestler,T.P., Trainer,D.L., Corwin,S.P., Miles,L., Kline,T., Sweet,R., Yokoyama,S., and Poste,G., 1985. Tumorigenic and metastatic properties of "normal" and ras-transfected NIH/3T3 cells. Proc. Natl. Acad. Sci. USA. **82**, 3698-3701.

5) Egan,S.E., Wright,J.A., Jarolim,L., Yanagihara,K., Bassin,R.H., and Greenberg,A.H., 1987. Transformation by oncogenes encoding protein tyrosine kinases induces the

metastatic phenotype. Science. **238**, 202-205.

6) Chambers,A.F., and Wilson,S., 1985. Cells transformed with a ts viral src mutant are temperature sensitive for in vivo growth. Mol. Cell. Biol.. 5, 728-733.

7) Bernards,R., Dessain,S.K., and Weinberg,R.A., 1986. N-myc amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. Cell. **47**, 667-674.

8) Pohl,J., Goldfinger,N., Radler-Pohl,A., Rotter,V., and Schirrmacher,V., 1988. p53 increases experimental metastatic capacity of murine carcinoma cells. Mol. Cell. Biol., 8, 2078-2081.

9) Egan,S.E., McClarty,G.A., Jarolim,L., Wright,J.A., Spiro,I., Hager,G., and Greenberg,A.H., 1987. Expression of H-ras correlates with metastatic potential: evidence for direct regulation of the metastatic phenotype in 10T1/2 and NIH-3T3 cells. Mol. Cell. Biol.. 7, 830-837.

10) Egan,S.E., Broere,J.J., Jarolim,L., Wright,J.A., and Greenberg,A.H., 1989. Coregulation of metastatic and transforming activity of normal and mutant ras genes. International Journal of Cancer. In the press.

11) Sporn,M.B., and Roberts,A.B., 1988. Peptide growth factors are multifunctional. Nature. 332, 217-219.

12) Weinberg,R.A., 1985. The action of oncogenes in the cytoplasm and nucleus. Science. 230, 770-776.

13) Goustin,A.S., Leof,E.B., Shipley,G.D., and Moses,H.L., 1986. Growth Factors and Cancer. Cancer Research. 46, 1015-1029.

14) Rogelj,S., Weinberg,R.A., Fanning,P., and Klagsbrun,M., 1988. Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature. 331, 173-175.

15) Sporn,M.B., and Roberts,A.B., 1985. Autocrine growth factors and cancer. Nature. 313, 745-747.

16) Folkman,J., and Klagsbrun,M., 1987. Angiogenic factors. Science. 235, 442-447.

17) de Martin,R., Haendler,B., Hofer-Warbinek,R., Gaugitsch,H., Wrann,M., Schlusener,H., Seifert,J.M., Bodmer,S., Fontana,A., and Hofer,E., 1987. Complementary DNA for human glioblastoma-derived T-cell suppressor factor, a novel member of the transforming growth factor-β gene family. The EMBO Journal. 6, 3673-3677.

18) Dickson,R.B., McManaway,M.E., and Lippman,M.E., 1986. Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science. 232, 1540-1543.

19) Downing,J.R., Rettenmier,C.W., and Sherr,C.J., 1988. Ligand-induced tyrosine kinase activity of the colony-stimulating factor 1 receptor in a murine macrophage cell line. Mol. Cell. Biol. **8**, 1795-1799. (Addendum in Proof).

20) Sherr,C.J., Rettenmeir,C.W., Sacca,R., Roussel,M.F., Look,A.T. and Stanley,E.R., 1985. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell. **42**, 665-676.

21) Lyman,S.D., Park,L., and Rohrschneider,L.R., 1988. CSF-1 induced growth stimulation of v-fms transformed fibroblasts. Fourth Annual Meeting on Oncogenes; Proceedings, page 20.

22) Roussel,M.F., Dull,T.J., Rettenmeir,C.W., Ralph,P., Ullrich,A., and Sherr,C.J., 1987. Transforming potential of the c-fms proto-oncogene (CSF-1 receptor). Nature. 325, 549-552.

23) Sporn,M.B., Roberts,A.B., Wakefeild,L.M., and

deCrombrugghe,B., 1987. Some recent advances in the chemistry and biology of transforming growth factor-beta. The Journal of Cell Biology. **105**, 1039-1045.

24) Schwarz,L.C., Gingras,M,-C., Goldberg,G., Greenberg,A.H., and Wright,J.A., 1988. Loss of Growth factor dependence and conversion of transforming growth factor-β1 inhibition to stimulation in metastatic H-ras-transformed murine fibroblasts. Cancer Research. In the press.

CONCLUDING REMARKS

In the introduction and chapters of this thesis, the metastatic process has been defined and described in some detail. It is now important to readdress the problem of metastatic dissemination with respect to todays' knowledge and where this leads investigation in the future. The clinical perspective of metastatic spread remains unchanged despite the advances made in the field. To date we have no way to predict or alter metastatic spread of cells through the vasculature and lymphatics to secondary sites throughout the tumor host.

However, in the past four years our understanding of this complex process has been greatly enhanced. This has been achieved through advances in several areas. The most significant discovery was the identification of a role for dominantly acting oncogenes in the metastatic process. All such genes that have been examined can play a role in metastatic transformation of many cell types. The inescapable conclusion of this work is that all oncogenes can and do regulate metastatic dissemination. In addition, the effects of particular oncogene products are absolutely dependent on the specific interaction with a cell and its environment. Contrary to earlier expectations, there are no "metastasis" genes but simply genes which can effect the proliferation and differentiation of cells through regulation of signal transduction. The oncogene products which have been studied with respect to the metastatic

phenotype do not possess explicit biological functions that induce metastatic behaviour. Rather they affect biochemical activities such as phosphorylation of tyrosine residues and regulation of second messenger production or breakdown in response to environmental stimuli which may influence a variety of cellular functions depending on the cell and its microenvironment. For example, these biochemical activities when superimposed on B-cells can be responsible for differentiation to mature antibody-secreting plasma cells, while the same signals in non-metastatic tumor cells can result in expression of the metastatic phenotype. It is, therefore, essential that future experimentation consider more specifically the interaction of oncogene products with cells and their environment, to identify these regulatory pathways leading to the biological changes.

The work presented in this thesis establishes six main points which are discussed in the concluding sections of each chapter. These are: i) that many oncogenes can induce the metastatic phenotype, ii) the oncogene products can directly regulate metastatic dissemination, iii) environmental signals such as polypeptide growth factors can alter metastatic potential, iv) although dominant oncogenes can cooperate to transform cells, this cooperation is insufficient for metastatic transformation, v) factors such as the factor in fetal bovine serum present at the time of transformation can determine the metastatic potential of the

resulting cell, and vi) expression studies suggest that in cells that have become metastatic, oncogenes cooperate to regulate the metastatic phenotype.

These experiments on metastasis induction can now take us to more sophisticated questions than originally posed at the beginning of these studies.

I) The study of oncogene cooperation in transformation has led to the molecular characterization of complementing biochemical activities. As discussed in chapter IV, the metastatic phenotype is normally accessed through the multistep process known as tumor progression. Consequently, the molecular alteration which is responsible for the transition of a benign tumor into a malignant tumor does not act in isolation. This metastasis inducing molecular lesion is interacting with the previous alterations which have led to the benign tumor growth. In vitro examples of this phenomena are clear. The ras oncogene cannot induce focal transformation of normal primary fibroblasts. In cooperation with myc, the ras gene does induce transformation (Land et.al.,1983). The expression of transformation inducing activity of ras therefore, requires the simultaneous expression of myc induced alterations in the target cell. Demonstration of oncogene cooperation in the induction and regulation of the metastatic phenotype has yet to be achieved.

II) In this same vein, what role do recessive oncogenes or antioncogenes play in metastatic transformation? Can loss of genetic material result in the metastatic conversion of a benign tumor? A corollary of this has been demonstrated through the transfection of metastasis-suppressor genes. The dominant oncogenes E1a from Adenovirus type 2 and fos have been shown to suppress the metastatic phenotype when transfected into highly metastatic cells (Pozzatti et.al.,1988 & Feldman and Eisenbach,1988). What is the nature of this suppression? Can structural domains of these proteins which are responsible for this activity be identified and what are the molecules with which these domains interact?

III) Most of the oncogene products interact with or regulate signal transduction pathways. These oncogenes can regulate the metastatic phenotype presumably through the same pathways. What signal transduction pathways are important in the regulation of tumor dissemination? How are the essential metastasis-associated enzymes such as plasminogen activators and type iv collagenase deregulated by the oncogene products?

IV) The stochastic event induced by fetal bovine serum (FBS) as demonstrated in chapter 1v) requires further study. What is the molecule in FBS responsible for induction of the metastatic phenotype and what is the intracellular event

which is induced? Metastatic transformation of primary epithelial cells in vitro has not been demonstrated. There are likely several reasons for this. The growth of epithelial cells in FBS may suppress or fail to promote an event necessary for metastatic transformation. An event similar to the one demonstrated in FBS which is necessary for the metastatic transformation of 10T1/2 fibroblast cells may also be required for metastatic transformation of many cell types. This event may be induced by different factors in the case of epithelial or fibroblastic cells. Under what conditions does metastatic transformation of epithelial cells occur?

These are questions which can and likely will be answered in the very near future using existing concepts and technology. In the long term how will metastatic dissemination of cancer be treated? What properties of highly malignant cells can be exploited to selectively allow for their elimination? Therapy directed at non-specific properties of metastatic cells such as their proliferative activity are destined for failure due to effects on non-tumor cells or through the development of resistance to therapy. It is therefore essential that we understand the mechanisms responsible for induction and maintenance of the metastatic phenotype to design successful treatment without selection of resistant tumor.

References

Feldman,M., and Eisenbach,L., 1988. What Makes a Tumor Cell Metastatic? Scientific American. November 1988, 60-85.

Land,H., Parada,L.F., and Weinberg,R.A., 1983. Tumorigenic Conversion of Primary Embryo Fibroblasts Requires at least two Cooperating Oncogenes. Nature **304**, 771-778.

Pozzatti,R., McCormick,M., Thompson,M.A., and Khoury,G., 1988. The E1a Gene of Adenovirus Type 2 Reduces the Metastatic Potential of ras-Transformed Rat Embryo Cells. Mol. Cell. Biol. **8**, 2984-2988.