Université de Montréal

A tumoral and invasive phenotype independent of c-Met mutation

By

Giuseppe Giannini

Physiology Department

Faculty of Medicine

This thesis is presented to la Faculté des Études Supérieures

in the scope of obtaining a

Masters in Science (M.Sc.)

in Physiology

June, 2003

Université de Montréal

Faculté des Études Supéieures

This thesis entitled :

A tumoral and invasive phenotype independent of c-Met mutation

Presented by

Giuseppe Giannini

Has been evaluated by a jury consisting of the following individuals:

Jury president: Dr Angelino Calderone
Member of jury: Dr Lucie Parent
Study director: Dr Josette Noël

Thesis accepted on: _____

To my wife, Marianne, and my whole family, who provided support, love and encouragement throughout my academic journey…
And to the loving memory of my grandfather
who always emphasized that money and wealth can be lost at any time,
but your education stays with you until the end of your days.

ACKNOWLEDGEMENTS

For the passion she expresses in her work and for the support, trust and care she endows in her students, I express my deepest thanks to my study director, Dr. Josette Noël. Her generosity, constant encouragement, and valued advice throughout the period in attaining the Masters degree will always be greatly appreciated. Dr. Noël always made the impossible possible and for that I am forever grateful. Thank you Dr. Noël!

I would like to thank my laboratory co-workers for their constant support and help. Special thanks go out to Julie Vadnais and Yolaine Dodier for their direct contribution to Boyden chamber assays. In addition, I would like to thank David Germain for his valuable expertise and advice throughout my stay in the laboratory. Furthermore, I would like to thank Julie Verner from the tissue culture facility of the GRTM for her valuable help.

Additional thanks are intended for Dr. Ivan Robert Nabi in the Anatomy and Cellular Biology department of the Université de Montréal for the critical review of my manuscript and for his contribution (different MDCK cell lines) to my project.

Finally, I would like to take this opportunity to thank certain members of the department of physiology, namely Mrs. Hélène Klein, Mrs. Pierrette Fournel, Mrs. Joanne Payette, and Dr. Réjean Couture, for helping me make my studies both a pleasurable and successful experience.

SUMMARY

The hepatocyte growth factor receptor (HGF-R) otherwise known as the c-Met proto-oncogene is involved with various cellular responses such as morphogenesis, motogenesis, and mitogenesis. Phosphorylation and subsequent activation of the receptor's tyrosine kinase domain, through binding of hepatocyte growth factor (HGF), has been shown to trigger various cellular responses via the binding of different src homology domain 2 (SH2) containing proteins to phosphorylated tyrosine residues of the multi-functional docking site found in the receptor's C-terminal domain.

Various cancer cells express high levels of c-Met phosphorylation, which translates into high levels of c-Met activity. For this reason, much of the research performed today with regards to c-Met and its involvement in tumorigenesis revolves around the possible mechanisms behind this constitutive autophosphorylation. Some groups point to the presence of point mutations within the tyrosine kinase domain of c-Met which leads to its increased activity. Others suggest the existence of an autocrine activation loop. In fact, many of the cancers studied to date show evidence of these two possible phenomena.

In the present study, we wanted to analyze the tumorigenic character of an invasive variant of Moloney sarcoma virus-transformed MDCK cells (MSV-MDCK-INV) and to assess c-Met's role in this phenotype. Results demonstrated that in fact MSV-MDCK-INV cells have the capacity to form foci in soft agar similar in size and at a very similar efficiency to the tumorigenic NIH3T3/*ras* cell line. Although MDCK and MSV-MDCK cells formed foci in soft agar, the size and growth efficiency was small compared to MSV-MDCK-INV cells. Clones isolated from the soft agar were analyzed for their capacity to form colonies over monolayers of polarized MDCK cells. Invasive clones along with NIH3T3/*ras* clones were the only ones capable of forming these superficial colonies. Cells from soft agar clones illustrated different expression levels of certain proteins involved with focal adhesion complexes and cell-cell contacts, namely E-cadherin, FAK and paxillin. CAMI-4, one of the invasive

soft agar clones studied, was found to express a more phosphorylated c-Met and a 142% invasive capability when compared to the invasive capability expressed by MSV-MDCK-INV cells.

The results presented above support the fact that the MSV-MDCK-INV cell line expresses tumorigenic characteristics. Unfortunately though, no point mutations were observed in either the tyrosine kinase or C-terminal domains of c-Met that could explain the invasive and tumorigenic characteristics of these cells and the transformed phenotype observed for MSV-MDCK cells. Therefore, we assume the existence of an autocrine activation loop among MSV-MDCK-INV cells that can, at least in part, explain the auto-phosphorylation of c-Met and the subsequent phenotype observed.

Key words: HGF-R/c-Met, auto-phosphorylation, invasive, tumorigenic, soft agar, MDCK, mutation, tyrosine kinase.

SOMMAIRE

Le récepteur du facteur de croissance hépatique (HGF-R), reconnu aussi comme le proto-oncogène c-Met, est impliqué dans plusieurs réponses cellulaires telles que la morphogenèse, la motogenèse et la mitogenèse. La liaison du facteur de croissance hépatique (HGF) à son récepteur mène à la phosphorylation et à l'activation du domaine tyrosine kinase du récepteur. Cette activation amorce ensuite de nombreuses réponses cellulaires via l'interaction de différentes protéines contenant une séquence homologue aux domaines de liaison 2 de la protéine *src* (SH2) avec les résidus tyrosine phosphorylés du site de liaison multi-fonctionnel du domaine C-terminal.

Certains cancers et métastases expriment un c-Met hautement phosphorylé qui se traduit en un récepteur c-Met plus actif que dans les cellules normales. Pour cette raison, de nombreuses études effectuées sur c-Met et son implication dans la formation de tumeur et de métastases consistent à comprendre les mécanismes pouvant expliquer cette auto-phosphorylation constitutive. Certains groupes de recherche ont montré la présence de mutations dans le domaine tyrosine kinase de c-Met entraînant une augmentation de l'activité kinase et l'activation des cascades dépendantes de cette activation. D'autres groupes font référence à une boucle autocrine. Ces deux modes d'action peuvent aussi être impliqués simultanément dans certains cancers dépendant de l'activation de c-Met.

Dans la présente étude, nous avons étudié les caractéristiques tumorales d'une lignée de cellules invasives (INV) isolées de cellules MDCK transformées par le virus du sarcome de Moloney (MSV), les cellules MSV-MDCK-INV, ainsi que le rôle de c-Met dans l'établissement de ce phénotype. Nos résultats démontrent que les cellules MSV-MDCK-INV ont la capacité de former des foyers indépendants, lorsqu'ensemencées dans un milieu semi-solide tel que l'agar mou, de la même grandeur et avec la même efficacité que les cellules tumorales NIH3T3 transformées par l'oncogère *ras* (NIH3T3/*ras*). Malgré la capacité des cellules MDCK et MSV-MDCK à former des foyers dans l'agar mou, la grandeur de ceux-ci et l'efficacité de

croissance sont négligeables comparé à ce qui est observé pour les cellules MSV-MDCK-INV. Des clones isolés à partir de l'agar mou ont été analysés pour leur capacité à former des colonies de cellules lorsqu'ensemencées sur une monocouche de cellules épithéliales MDCK. Les cellules invasives et les cellules NIH3T3/*ras* étaient les seules capables de former des colonies superficielles sur la monocouche de cellules polarisées. Nous avons aussi montré que les cellules dérivées des colonies obtenues dans l'agar mou expriment à un degré différent, en relation avec leur phénotype, certaines protéines impliquées dans les plaques d'adhésion et les contacts cellules-cellules, telles la kinase présente dans les plaques d'adhésion (FAK), la paxilline une protéine de structure des plaques d'adhésion et la cadhérine-E une protéine nécessaire à l'établissement des contacts cellule-cellule. Les cellules CAMI-4, issues de l'une de ces colonies de cellules invasives tirées de l'agar mou, expriment un c-Met hautement phosphorylé et une capacité d'invasion de 42% supérieure à celle observée pour les cellules invasives MSV-MDCK-INV. Ces observations suggèrent un lien direct entre le degré de phosphorylation de c-Met et la capacité motile des cellules MSV-MDCK-INV, en accord avec certaines observations récentes du laboratoire.

Les résultats présentés dans ce mémoire montrent pour la première fois que les cellules MSV-MDCK-INV possèdent des caractéristiques de cellules tumorales. Malheureusement, aucune mutation n'a été observée dans le domaine tyrosine kinase ni le domaine C-terminal de c-Met qui pourrait expliquer le phénotype invasif des cellules MSV-MDCK-INV ainsi que le phénotype transformé des cellules MSV-MDCK. Ces observations nous portent à croire que la présence d'une boucle d'activation autocrine du récepteur, via la sécrétion de HGF par ces cellules, expliquerait le haut degré de phosphorylation de c-Met ainsi que le phénotype particulier de ces cellules MSV-MDCK-INV. Nous n'excluons cependant pas la possibilité que d'autres facteurs puissent aussi influencer l'état de phosphorylation de c-Met dans ces cellules.

Mots clés: HGF-R/c-Met, auto-phosphorylation, invasive, tumeur, agar mou, MDCK, mutation, domaine tyrosine kinase.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF TABLES

CHAPTER 1

LIST OF ABBREVIATIONS

AIG	Anchorage independent growth
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
bcr-abl	Fusion protein
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CaM	Calmodulin
cDNA	Complementary DNA
c-onc	Cellular/proto-oncogene
DNA	Deoxyribonucelic acid
ECM	Extracellular matrix
FAK	Focal adhesion kinase
Fyn	Kinase
Gab1	Grb-2 associated binder 1
Grb-2	Growth factor receptor-bound protein 2
GTP	Guanadine triphosphate
HGF	Hepatocyte growth factor
HGF-R	Hepatocyte growth factor receptor
HIR	Human insulin receptor
HOS	Human osteogenic sarcoma
HPRC	Human papillary renal carcinoma
Jak	Janus Kinases
kDa	KiloDalton
LTR	Long-terminal repeat
MBP	Myelin basic protein
MDCK	Madin Darby canine kidney
MNNG	*N*-methyl-*N*'-nitro-*N*-nitrosguanidine
mRNA	Messenger RNA
NGF	Neutrophins growth factor

NK cells	Natural killer cells
p85	Regulatory subunit of PI-3 kinase
Pi3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PRC	Papillary renal carcinoma
PRGF	Plasminogen related growth factor
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SF	Scatter factor
SH2	src homology domain 2
SH3	src homology domain 3
SHC	Gene which encodes an adaptor protein for cell signalling
SRC	Family of kinases
STAT-3	Signal transducers and activators of transcription
TGF-β	Transforming growth factor
TPR	Translocated promoter region
uPA	Urokinase-type plasminogen
uPA-R	Urokinase-type plasminogen receptor
VEGF	Vascular endothelial growth factor
v-*abl*	Protein tyrosine kinase encoded by Abelson murine leukemia virus
v-onc	Viral oncogene

CHAPTER 1

INTRODUCTION

1.1 Cancer

1.1.1 Definition of cancer and its development

A cancer can be defined as a group of cells among a total population of cells that have lost their normal control mechanisms and thus experience unregulated growth. Cancer can develop from any tissue or organ and as cancerous cells grow and multiply, they form a mass of cancerous tissue that invades adjacent tissues and spreads (metastasizes) throughout the body (Merck Manual, 1997). Cancer cells develop from normal cells in a process referred to as transformation. During transformation, a normal cell initially experiences a genetic change which primes it to become cancerous after exposure to agents referred to as carcinogens. Some examples of possible carcinogens include chemicals, virus, radiation, or sunlight. Not all cells are equally susceptible to carcinogens. A genetic flaw in the cell, its promoter, or even chronic physical irritation may make cells more susceptible to develop cancer. Changes in a cell's genetic composition are often hard to detect, but sometimes a change in the size or shape of one specific chromosome is a good indication of which type of cancer is involved. Moreover, these genetic changes may also lead to unscheduled cellular hyperproliferation, either through direct activation of cell cycle machinery or through signalling pathways that regulate the cell cycle (Merck Manual, 1997).

1.1.2 Oncogenes and their role in cancer development

Many genes involved in cellular transformation leading to the development of a cancerous phenotype have been discovered over the past few years. Cancer cells may possess oncogenes, otherwise known as tumour genes, which are involved in the induction of a cancer. At times, these oncogenes are abnormal versions of the genes responsible for growth and development before birth, which under normal circumstances are permanently deactivated after birth. Their reactivation later in life may result in the development of a cancer. Oncogenes were originally identified in cancer causing viruses. These viruses may be divided into two groups: the DNA tumor viruses that contain either linear or circular double-stranded DNA and the RNA-containing tumour viruses (also referred to as *retroviruses*). Stehelin and

collaborators, while studying the Rous Sarcoma virus (a retrovirus that causes sarcomas in chickens), were among the first to demonstrate that a viral transforming gene was derived from a normal cellular gene (Stehelin *et al.*, 1976). Since then, many other retroviruses have been discovered and shown to contain different oncogenes derived from and closely related to their cellular counterparts (Bishop, 1985).

The normal cellular genes from which viral oncogenes (v-*onc*) are derived are referred to as proto-oncogenes (or c-*onc*). The way in which proto-oncogenes incorporate into the viral genome and are converted to viral oncogenes with a high degree of transforming ability involves the recombination between the retroviral and cellular genomes. This usually follows the integration of a retrovirus adjacent to a cellular proto-oncogene in a process known as *transduction* that is associated with alterations in the structure and regulation of oncogene sequences (Varmus, 1988). **Figure 1.1** and **Figure 1.2** demonstrate how viral RNA, after penetrating the cellular plasma membrane, is reverse-transcribed to a double-stranded DNA form. Once in the double-stranded form, it can then proceed to migrating to the nucleus and integrating itself into the chromosomal DNA.

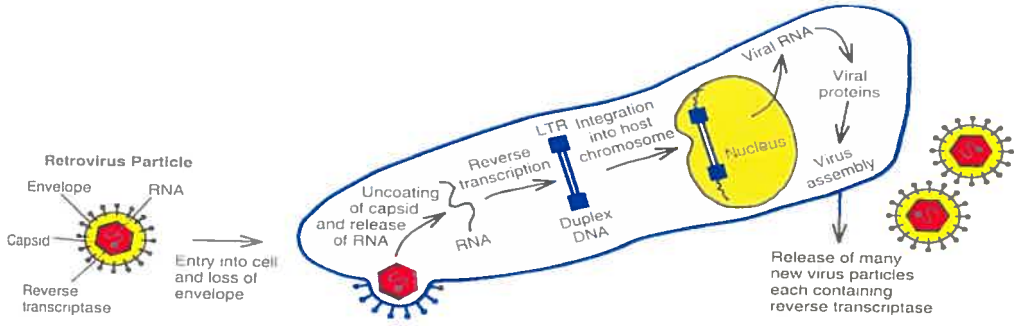


Figure 1.1: **Life cycle of a retrovirus.** Under normal circumstances a virus contains two identical RNA strands. For clarity, only one is shown. After penetrating the plasma membrane, the single-stranded viral RNA genome is reverse-transcribed to a double-stranded DNA form that contains a duplication at its ends referred to as the long terminal repeat (LTR). The viral DNA then migrates to the nucleus and integrates into the chromosomal DNA. The resultant single viral transcript can form the genome for progeny viruses or can be processed and translated to generate viral structural proteins.
Figure adapted from Tannock and Hill, 1998

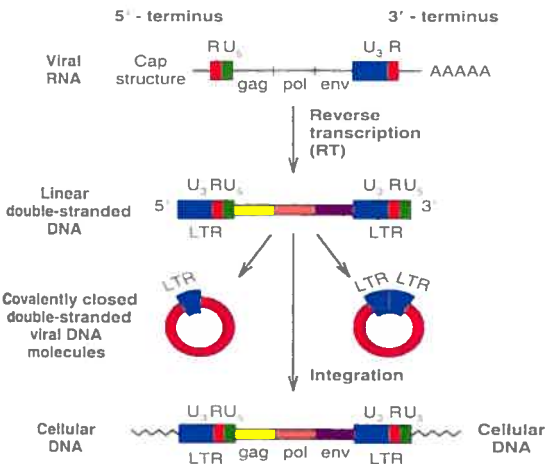


Figure 1.2: **Replication and integration of the retroviral genome.** RNA is reversed transcribed to form both linear and circular forms of viral DNA. It is the linear form of the DNA that integrates into cellular DNA, where it is referred to as a provirus. **Figure adapted from Tannock and Hill, 1998**

A large majority of the oncogenes found in transforming retroviruses have also been identified in spontaneously arising tumors of nonviral origin where they are activated via other possible mechanisms. Some of these mechanisms are point mutations, gene amplification, and chromosomal translocation. The activation of proto-oncogenes is associated with certain genetic alterations that result in either deregulation of (and an increase in) expression of the normal gene or alterations in the structure (and resulting function) of the encoded protein. In fact, all cells which make up the human body contain a set of genes that can participate in malignancy following appropriate activation or deregulation. Proto-oncogenes encode various proteins involved in the control of cell proliferation and differentiation, including many growth factors, growth factor receptors, components involved with signal transduction pathways and transcription factors that regulate the transcription of mRNA and therefore the expression of associated genes (Tannock and Hill, 1998).

1.1.3 Tumor suppressor genes and their role in cancer development

Tumor suppressor genes are also regarded as very important players in malignancy. They are genes that are likely to play a role in negatively regulating cell growth. The loss or inactivation of these tumor suppressor genes, as their name implies, is associated with malignancy (Weinberg, 1991; Knudson, 1993). Tumor suppressor genes portray different characteristics in comparison to oncogenes. One main difference is that oncogenes act in a dominant manner and the activating event is usually acquired. Tumor suppressor genes on the other hand act in a recessive manner. Mutations within a tumor suppressor gene would result in its inactivation and mutated forms of the gene may be inherited. Therefore, tumor suppressor genes appear very often in the development of familial cancers, but the presence of mutated forms of these genes in sporadic cancers is also very common (Tannock and Hill, 1998).

1.1.4 Neoplastic transformation of normal cells to cancer cells

Neoplastic transformation of human cells as well as cells from other species *in vitro* is seen as a complex multistep process by which normal cells acquire various phenotypic abilities. Four of these abilities are; the development of morphological transformation, growth in semisolid medium, immortality, and tumorogenicity. A large majority of human tumor cells of mesenchymal origin do portray morphological transformations that allow them to grow in conditions in which normal cells cannot.

In vitro assays used to demonstrate the transformed phenotype of a cell all involve three main aspects as depicted in **Figure 1.3**. Initially, transformed cells are examined for their ability to form foci. Normal cells will stop growing when coming into contact with nearby cells due to a phenomenon known as contact inhibition. Transformed cells are different because they are not contact-inhibited and continue to grow even when cells come into contact with one another. In fact, transformed or malignant cells grow by piling up over each other and forming bumps over the monolayer of cells. These bumps are referred to as foci (Tannock and Hill, 1998).

Anchorage-independent growth, the ability to grow in a semi-solid medium, constitutes another very important property of malignant cells (Pavelic *et al.*, 1980). Normal cells will not grow unless they adhere to a supporting matrix or substrate such as glass or plastic. By contrast, transformed and malignant cells do not require any adhesion or anchorage and are capable of growing when suspended in a semisolid medium such as soft agar (Tannock and Hill, 1998).

The last and seemingly most influential assay is the tumor formation assay following injection of tumor cells into nude animals. Normal cells will not form tumors when injected into syngeneic or immunologically deficient animals. Transformed cells on the other hand do possess this ability (Tannock and Hill, 1998). The phenomena and mechanisms involved in induction and progression of a tumor will be discussed later in this chapter.

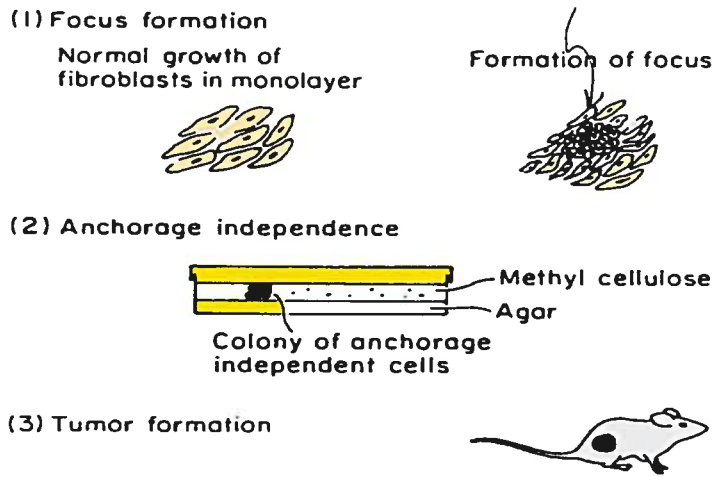


Figure 1.3: **Assays of the malignant phenotype.** Malignant transformation of a cell may be recognized in tissue culture **(1)** by cells stacking up on the bottom of the culture dish to form a focus; **(2)** by growth under anchorage-independent conditions in semisolid media such soft agar; or **(3)** by tumor formation in syngeneic or immune-deprived animals. **Adapted from Tannock and Hill, 1998**

1.1.5 Tumor progression and classification

Due to the unpredictable nature of cancer, tumors derived from different cancers display different characteristics. In general, tumors tend to show or become increasingly malignant with time. Some tumors depict an orderly progression from a benign state to a noninvasive state, followed by the development of pre-malignant lesions and ultimately malignancy. Highly malignant tumors on the other hand may arise de novo, without going through the different stages of progression. Thus, the degree of progression of any specific tumor is strongly dependent upon the type of tumor. In terms of classifying the severity of the tumors and deducing whether they are benign or malignant, it is common practice to assume that tumors that remain confined to a specific site with well differentiated cells are benign, whereas tumors composed of poorly differentiated cells that have spread beyond the local site to seed metastases are malignant (Tannock and Hill, 1998).

Tumor progression has for a long time been associated with the acquisition of permanent, irreversible qualitative changes in one or more characteristics of a neoplasm resulting in the tumor becoming more autonomous and malignant at the same time (see Heppner and Miller, 1998; Klein, 1998 for an integrated view). Recently, much more emphasis has been placed on genetic changes and how they can explain what occurs during tumor initiation and progression (Nowell, 1986). Genetic changes occur through various mechanisms, namely point mutation, deletion, gene amplification, and translocation. A cell's DNA can be damaged while exposed to various intracellular and extracellular agents, or through the errors made by polymerases whenever DNA is being replicated. In normal cells, this DNA damage is repaired through DNA repair mechanisms found within the cell or damaged cells. Those cells whose DNA cannot be repaired undergo apoptosis. Unfortunately, these mechanisms are not 100% fool proof, and therefore, this leads to a natural frequency of spontaneous mutations in cells (Simpson, 1997). In effect, many cancer cells appear to possess an increased frequency of mutations due to their deficiency in the repair of DNA lesions and their decreased activation of apoptosis. Therefore, these

mutated cells form a sub-population that can survive and continue proliferating (Loeb, 1998).

1.1.6 Metastasis

As tumors progress, they acquire the final and most malignant stage, metastasis. Tumors that have reached this point in their development are usually more difficult to treat than those that did not spread and invade other tissues. The cancerous cells could spread via the lymphatic vessels or via the blood vessels. It is for this reason that clinically, metastases can be divided into two categories: those found in the regional lymph nodes which have traveled through the lymphatic system, or those in more distant organs, which have traveled through the body's blood vascular system (Tannock and Hill, 1998).

The metastatic process involves four main steps. First, the metastatic cells must detach from the primary tumor mass and, depending upon the localization of the primary tumor mass, the cells will travel or spread via either the lymphatic or venous system. The inclusion of tumor cells into blood vessels may occur as a result of a prior invasion of the tumor mass into the blood vessels or due to the fact that the vasculature of some tumors may permit passage of the cells into the blood circulation (Tannock and Hill, 1998). **Figure 1.4** depicts an overview of the mechanism by which tumor cells from the primary tumor penetrate the blood vessels to enter the bloodstream and travel to distant organs to form metastases.

The initial detachment of the primary tumor cells from the primary tumor site is assumed to involve the decreased expression of adhesion molecules (e.g. cadherins) that are involved in the "homotypic" adhesion of cells to one another (Birchmeier and Behrens, 1994).

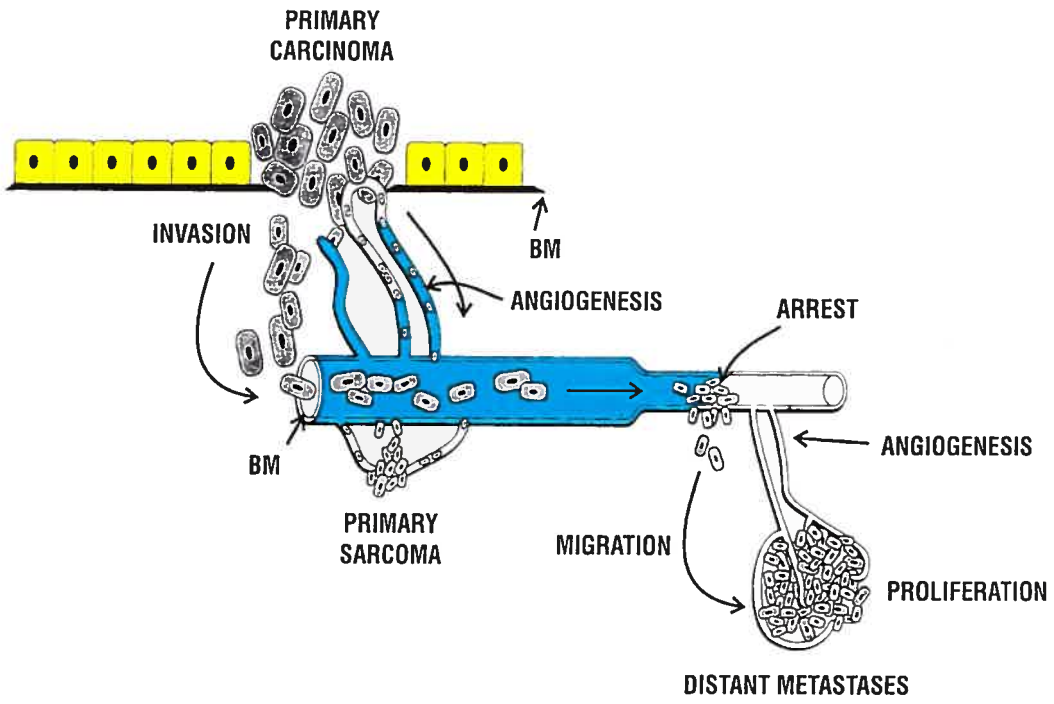


Figure 1.4: A model of the major steps of metastasis. Anchorage-independent growth of epithelial cells results in the formation of a primary carcinoma. The tumor induces the growth of blood vessels by angiogenesis. Some tumor cells separate from the primary tumor, invade through the basement membrane, enter the circulatory system, and eventually arrest in capillaries. At this point, they extravasate out of the blood vessels into the underlying connective tissue at the metastatic site; there, further cell growth and angiogenesis result in the formation of metastatic tumor growth. Angiogenesis is not seen as being a crucial step for the invasion and metastasis of sarcomas due to the fact that these tumors arise in the stroma in close vicinity to blood vessels.
Adapted from Tannock and Hill, 1998.

The second step in the metastasis process involves the host's immunologic mechanisms and how they are evaded during metastasis. There exists evidence in support of how some human tumors express weaker effects due to the presence of various cytokines and by nonspecific effector cells such as natural killer cells (NK cells) in the host. These two processes that represent the body's defense mechanisms and are in turn inhibited in the presence of a cancer, could reduce the incidence of metastasis formation (Tannock and Hill, 1998). In effect Hanna, by using transplanted rodent cells has shown an inverse correlation between the activity of NK cells in the host animal and the metastatic ability of injected tumor cells (Hanna,

1984). Therefore, a decrease in the number of effective NK cells may play an important role in the induction and development of metastases.

The third step in the development of a metastasis is rate-limiting and probably the most critical one. Tumor cells that have successfully detached from the primary tumor enter the blood stream where they travel for some time before reaching their destination. In the blood stream, hemodynamic forces eliminate the majority of these cells and only a small percentage of them survive. It is this small population of tumor cells that can extravasate successfully and lodge themselves into different organs that is responsible for the ongoing metastatic process. Before extravasation, tumor cells are trapped in the capillaries of the lung or liver because they are larger in comparison to the capillaries. The arrest of tumor cells within small blood vessels has been associated for quite some time with thrombus formation and involves the interaction of tumor cells with platelets and leukocytes. The role of thrombus formation remains unclear, but it has been hypothesized that the adhesive interactions between tumor cells and the endothelial cells lining the capillaries, which occur after the arrest of tumor cells, initiate a signal-transduction pathway that promotes the expression of various genes responsible for the next step of the metastatic process (Tannock and Hill, 1998).

The step preceeding the arrest of cells in the circulation, as touched upon previously, involves the escape of cells from the circulation into the host tissue. The first step of this procedure, outlined in **Figure 1.5**, involves the extension of tumor cell pseudopodia into the endothelial cell junctions or the induction of endothelial cell retraction that allows access to the basement membrane (Nicolson, 1982). These pseudopodia are sometimes referred to as *invadopodia* and contain varying concentrations of proteases and adhesive molecules that promote the extravasation and migration process. Direct attachment to an exposed portion of the basement membrane constitutes another route by which tumor cells may adhere. In fact, the subendothelial basement membrane is a much better adhesive for tumor cells than is the endothelial cell surface (Nicolson, 1982). The adhesion of cells to the basement

membrane involves the binding to certain membrane components such as laminin, fibronectin, vitronectin, type IV collagen, and proteoglycans (Liotta, 1986; Nicolson, 1988). The binding to these different components is mediated by certain cell-surface receptors, many of which are members of the integrin family. Following their adhesion to the basement membrane, tumor cells then digest the basement membrane through the use of various proteolytic enzymes which they usually produce or which they acquire from normal cells in the vicinity. Once tumor cells have managed to digest and penetrate the basement membrane, they can then migrate through it and escape once again into the interstitial space (Stetler-Stevenson *et al.*, 1993; Mignatti and Rifkin, 1993; Sloane *et al.*, 1994; MacDougall and Matrisian, 1995).

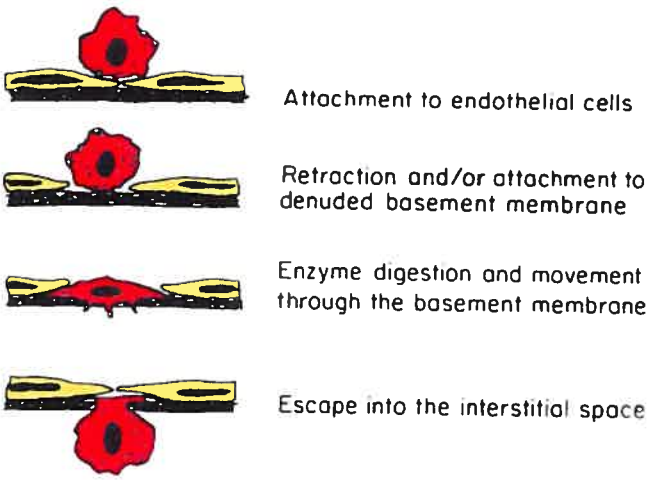


Figure 1.5: **Extravasation of a tumor cell.** Schematic representation of the processes involved in extravasation of a tumor cell through the wall of a capillary. The tumor cell first attaches to the surface of endothelial cells, which cover the basement membrane. It then attaches itself to the basement membrane through gaps left between endothelial cells, and using enzymatic digestion, moves through the basement membrane and into the interstitial space. **Adapted from Tannock and Hill, 1998.**

The last step in the progression of a metastasis is to stimulate proliferation among tumor cells which have successfully reached the target organs. One may wonder if there exists any specificity in terms of which organs are afflicted by the proliferating tumor cell. In fact, cells are known to require certain growth factors for successful

proliferation, and cellular interactions with the extracellular matrix also play a pivotal role in proliferation (Nicolson, 1988; Nicolson and Menter, 1995).

Aside from growth factors that encourage cellular proliferation among tumor cells, there also exist certain factors that inhibit metastasis such as TGF-β, and these factors also play a role in organ-specific metastasis. In fact, there is evidence which shows that as tumor cells become more malignant, they may switch from being growth inhibited by TGF-β to being stimulated by TGF-β (Wright *et al.*, 1993; Lu and Kerbel, 1994).

Another very important form of evidence that has been well documented over time is the fact that tumor cells do not require exogenous growth factors as compared to normal cells. This independence is in direct relation with their ability to form tumors in animals and to metastasize. In many instances, the independence of tumor cells for exogenous growth factors is related to autocrine production of such factors and the modification of the response to such factors (Tannock and Hill, 1998).

An extremely important property pertaining to the growth of a metastasis is the ability of tumor cells to induce angiogenesis. This is defined as the development of new blood vessels. Folkman was among the first to demonstrate that when tumor fractions were implanted on the cornea of the rabbit eye, new blood vessels formed (Folkman, 1976). There exist a number of growth factors such as the basic fibroblast growth factor (bFGF) (Folkman and Klagsbrun, 1987) and the vascular endothelial growth factor (VEGF) (Senger *et al.*, 1983) that are angiogenic and induce endothelial cell growth as well as morphologic differentiation. Without the development of new blood vessels to supply nutrients to the developing tumor cells, the tumor would not grow larger than a few millimeters in diameter.

1.2 Hepatocyte Growth Factor (HGF)

1.2.1 General aspects of HGF

The hepatocyte growth factor (HGF), also known as scatter factor, represents a typical example of how a regular growth factor critical during growth and development may trigger a change in cellular phenotype that in some instances could lead to the induction of a cancer. The hepatocyte growth factor is a cytokine that is ubiquitously expressed by cells of mesenchymal origin (Bottaro *et al.*, 1991). *In vitro* HGF stimulates cell motility, invasion of surrounding extracellular matrices, proliferation, survival and morphogenesis, and induces the expression of specific genes by binding to its receptor, a transmembrane tyrosine kinase encoded by the *MET* proto-oncogene in epithelial cells (Prat *et al.*, 1998).

1.2.1.1 Historical overview of HGF

Research groups working in the field of liver regeneration and epithelial/mesenchymal interactions were the first to identify the hepatocyte growth factor. Initially, HGF was identified as a potent mitogen of hepatocytes in culture. Studies based on whole animals demonstrated the presence of a certain growth factor found in serum, which mediated rapid regeneration of liver parenchyma *in vivo* (Moolten and Buchner, 1967; Fisher *et al.*, 1971). Following these findings, investigators partially purified this growth factor from the serum of hepatectomized animals and showed it to be the most potent mitogen found in hepatocytes in culture. This new growth factor displayed characteristics that set it apart from other known growth factors such as epidermal growth factor, fibroblast growth factor, platelet derived growth factor, somatomedin, thrombin, and transferin. The newly discovered polypeptide was named HGF (Michalopoulos *et al.*, 1984; Nakamura *et al.*, 1984).

At approximately the same time that work was being performed on liver regeneration, a different group of epithelial cell biologists investigating epithelial/mesenchymal interactions identified a new factor which was found to be produced by fibroblasts and stimulated movement of cultured epithelial cells *in vitro*. This factor derived from fibroblasts led to the disruption of cell-cell junctions and led to the scattering of

islands of non-confluent epithelial cells *in vitro* (Stoker and Perryman, 1985; Stoker *et al.*, 1987; Stoker, 1989). It was based upon these findings that the newly fibroblast-derived growth factor was referred to as scatter factor (SF). Initially, the biological activities of these two molecules set them as distinct from one another. Subsequent biochemical analysis and cDNA cloning demonstrated that HGF and SF represented the same molecule (Nakamura *et al.*, 1986; Gohda *et al.*, 1988; Zarnegar and Michalopoulos, 1989). Furthermore, these results helped classify this growth factor as a member of the Plasminogen Related Growth Factors family (PRGF's), due to its structural homology with enzymes of the blood-clotting cascade (Stella and Comoglio, 1999).

1.2.1.2 HGF's role in different cellular activities

As seen previously, cells of mesenchymal origin are responsible for the production and release of HGF. The resulting biological activities of HGF are targeted mainly toward epithelial cells that express its receptor, the HGF receptor otherwise known as c-Met. From this specific interaction existing between both cell types, the HGF was classified as a paracrine mediator in the interaction that exists between mesenchymal and epithelial cells *in vivo* (Stoker *et al.*, 1987; Birchmeier and Birchmeier, 1993). Epithelial cells, as targets of HGF, are thought to influence the production of HGF by mesenchymal cells in a paracrine fashion (Joseph *et al.*, 1995; Rosen *et al.*, 1994). The factors of epithelial nature, responsible for the stimulation of mesenchymal HGF production have not been well defined at this time. It is however known that HGF expression by fibroblasts is inhibited by several known peptide growth factors, such as transforming growth factor beta, epidermal growth factor, and transforming growth factor alpha (Seslar *et al.*, 1993).

HGF/SF, *in vivo,* plays a role in the neural system (Streit *et al.*, 1995; Ebens *et al.*, 1996), kidney (Santos *et al.*, 1994; Wolf *et al.*, 1995) and mammary gland (Niranjan *et al.*, 1995; Soriano *et al.*, 1995; Yang *et al.*, 1995) development, tissue regeneration (Matsumoto and Nakamura, 1993), angiogenesis (Bussolino *et al.*, 1992), tumor invasion and metastasis (Giordano *et al.*, 1993; Rong *et al.*, 1994). HGF is also very

important during embryogenesis where its signalling is essential for liver and placenta development (Schmidt *et al.*, 1995; Uehara *et al.*, 1995) as well as for migration of myoblast precursors from the limb buds (Bladt *et al.*, 1995; Maina *et al.*, 1996). In addition, this growth factor is involved not only in the stimulation of hepatocytes, but also of other primary cultured cells, including melanocytes and renal tubular cells (Igawa *et al.*, 1991; Kan *et al.*, 1991; Matsumoto *et al.*, 1991; Rubin *et al.*, 1991). Furthermore, serum HGF levels have been found to be elevated in patients suffering from chronic hepatitis and liver cirrhosis (Tomiya *et al.*, 1992) and in animals being treated with a hepato-promoter (Lindroos *et al.*, 1992).

1.2.1.3 Structural characteristics of HGF and its activation

In terms of its structural characteristics, the hepatocyte growth factor (HGF) is a heterodimeric protein containing a α chain composed of an N-terminal hairpin loop and four kringle domains, as well as a serine protease-like β-chain (Stoker *et al.*, 1987; Mizuno and Nakamura, 1993; Nakamura, 1991). It is synthesized and secreted as a biologically inactive single-chain 92 kDa precursor, which is converted into the bio-active dimer in the extracellular environment either by serine proteases activated during the coagulation process (Miyazawa *et al.*, 1994), or by the urokinase type plasminogen activator (uPA) bound to its receptor (uPA-R) at the cell surface (Naldini *et al.*, 1992). **Figure 1.6** illustrates how HGF goes from its inactive state where the α and β chains are attached, to its active mature form where it is cleaved and the resulting α and β-chains are attached via a disulphide bond. Moreover, stimulation by HGF induces the expression of both uPA and its receptor (Boccaccio *et al.*, 1994; Pepper *et al.*, 1992). UPA is also a very important component for its ability to catalyze extracellular matrix degradation (Blasi, 1993). Therefore, the activity generated by the coupling of the HGF/HGF-R and uPA/uPA-R pairs is directly involved in cell associated proteolytic processes required for tissue remodelling, cell migration and cell invasion during wound healing, angiogenesis and tumor metastasis (Blasi, 1993).

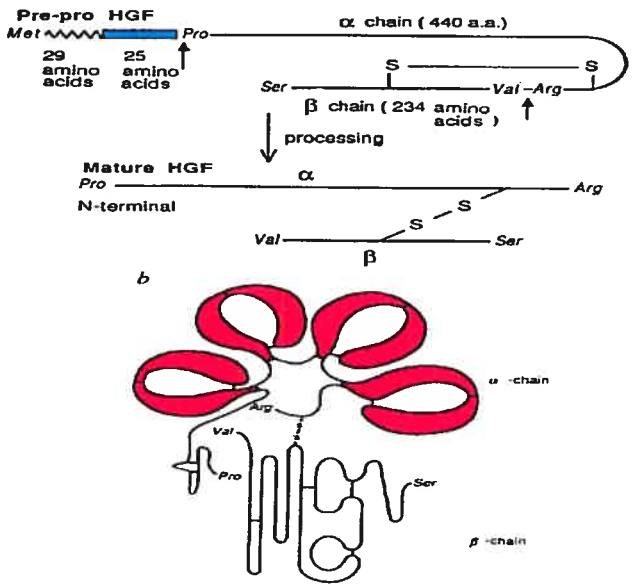


Figure 1.6: **Structure of HGF/SF.** HGF/scatter factor in its pre-form where both α and β-chains are attached and in its active form where it is cleaved and the α-chain is attached to the β-chain via disulphide bond (a). The α-subunit of HGF contains one hairpin loop and four kringle domains depicted in red that are triple-looped cysteine-rich motives involved in protein-protein interactions (b).
Figure adapted from Nakamura *et al.*, 1989.

1.2.1.4 HGF's role in cell scattering

HGF/SF's role in cell migration involves the initial recruiting of integrin receptors found on the cell surface and in contact with the extracellular matrix, cytoskeletal proteins, and $p125^{FAK}$ into focal adhesions that are strongly dependent on tyrosine kinase activity (Matsumoto *et al.*1994). Unlike the growth factor receptors, integrin receptors lack intrinsic tyrosine kinase activity. It is for this reason that one of the initial steps during integrin signalling is the tyrosine phosphorylation of the non-receptor tyrosine kinase, focal adhesion kinase (FAK), in response to cell adhesion (Schaller *et al.*, 1994; Richardson and Parsons, 1995; Parsons 1996; Chandra Kumar, 1998). All β_1 and α_v containing integrins share the ability to promote the assembly of focal adhesions and activate FAK. FAK is a non-receptor tyrosine kinase absent of any src homology (SH)2 and SH3 domains, and its phosphorylation is believed to result in the activation of a cascade of phosphorylation events and new protein interactions which are essential for adhesion-dependent signalling complexes (**Figure 1.7**).

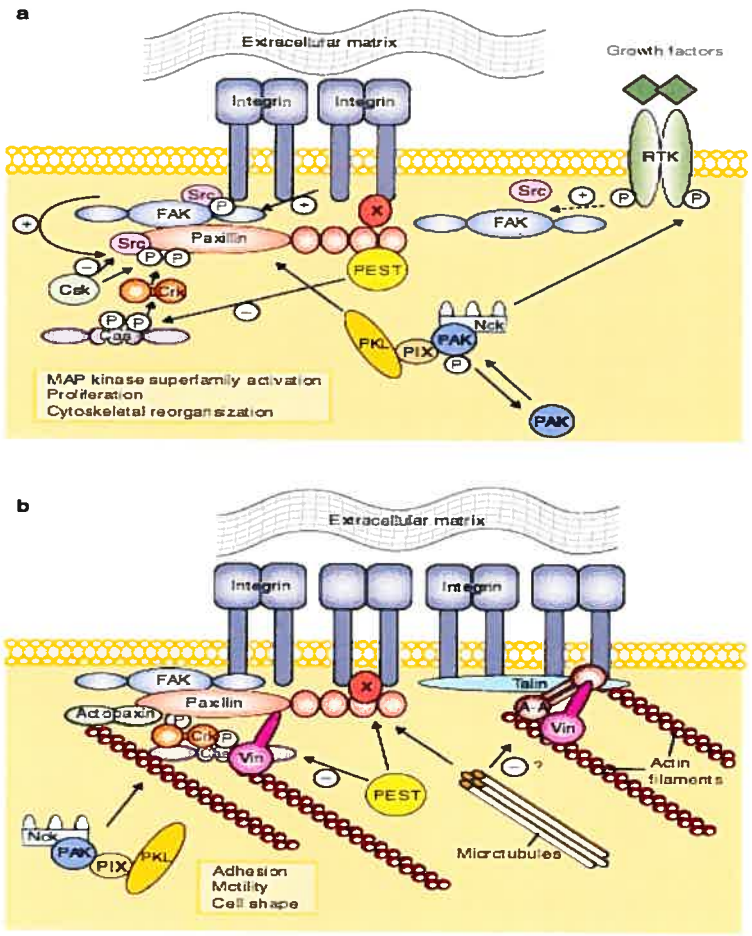


FIGURE 1.7: Integrin and growth-factor signalling through paxillin, and interactions with the actin cytoskeleton. A) Paxillin is involved with the recruitment of various signalling proteins to the plasma membrane, including the tyrosine kinases FAK and Src. The activation of these proteins is achieved through the interaction of integrins with the extracellular matrix, or after growth factor stimulation of receptor tyrosine kinases (RTK). The complex of proteins leads to the activation of the mitogen-activated protein kinase (MAPK) cascades, thereby stimulating gene expression. The signalling and cross-talk with integrins that is mediated by growth factor signalling, is enhanced by paxillin's ability to bind a PKL-PIX-PAK-Nck complex. **B)** Paxillin is seen bound to various proteins that help in the organization of the actin cytoskeleton at contact sites between integrin receptors and the extracellular matrix (focal adhesion points). Vinculin and actopaxin physically bind paxillin to actin filaments. The binding of the adaptor protein Crk to paxillin, which is phosphorylation-dependent, is necessary for the regulation of cell motility in certain cell types. Microtubule binding to paxillin also contributes somewhat to the ability of this filament system to promote cell motility via the disruption of focal adhesions. **Figure reproduced from Turner, 2000.**

Talin for one, is a protein that binds directly to the carboxy-terminal domain of FAK (Chen *et al.*, 1995) and also interacts with vinculin and subsequently paxillin (Brown

et al., 1996). Paxillin in turn binds to a distinct site in the carboxy terminus of FAK (Schaller *et al.*, 1995). Therefore, there is evidence to believe that the recruitment of FAK to activated integrins is an indirect process mediated by talin, and that once FAK has been recruited to the focal adhesion complexes, it undergoes a conformational change and interacts through its amino terminal domain with the β subunit tail (Richardson and Parsons, 1996). This conformational transition is therefore a prerequisite for FAK's catalytic activity because it leads to a trans-autophosphorylation on residue 397 which in turn results in the interaction with Src and Fyn through their SH2 domain (Schaller *et al.*, 1994). Src and Fyn are then responsible for the phosphorylation of a number of FAK-associated proteins including paxillin and tensin (Vuori *et al.*, 1996; Schlaepfer and Hunter, 1997; Schlaepfer *et al.*, 1997). Once in the phosphorylated state, FAK can then combine with and activate PI3Kinase (Chen *et al.*, 1996). Hence, FAK is associated with various intracellular signalling pathways, namely those involved with cell adhesion and cell motility. Embryonic fibroblasts derived from FAK knockout mice have shown to form numerous small focal contacts, but were unsuccessful in forming large peripheral focal adhesions and were found to migrate less efficiently than control cells (Ilic *et al.*, 1995). Another piece of evidence supporting FAK's role in cell migration is that overexpression of FAK stimulates cell migration. A dominant negative form of FAK completely inhibits cell migration (Cary *et al.*, 1996; Gilmore and Romer, 1996). These observations are contradicted by the report of Matsumoto and colleagues (Matsumoto *et al.*, 1994). He states, with regards to FAK, that a "Gradual loss of tyrosine phosphorylation coincided with disruption of focal adhesions and conversion to a motile phenotype". He also goes on to propose that "HGF/SF-induced phosphorylation of $p125^{FAK}$ may actually indicate the disassembly of the focal adhesions, a process apparently required to cell mobilization and locomotion" (Matsumoto *et al.*, 1994). Therefore, it would seem that according to Matsumoto, FAK would be over-expressed in motile cells, but phosphorylation on FAK would be greatly diminished.

The presence of HGF bound to its tyrosine kinase receptor (RTK) as seen in **Figure 1.7**, is essential in triggering the aforementioned cascade leading to cell scattering. Recruitment of integrin receptors on the cell surface as well as activation of different proteins involved with cellular motility and scattering is strongly dependent upon the tyrosine kinase activity produced when HGF activates HGF-R (c-Met). It is for this reason that the expression of certain proteins such as FAK and paxillin are examined extensively when working with invasive or motile cell lines.

1.2.2 Receptor tyrosine kinases and their role in cell growth

The process of cell growth and differentiation is triggered when extracellular substrates are recognized at the cell surface by a receptor, resulting in the activation of associated cytoplasmic and nuclear biochemical cascades. When deregulated, these normal growth-signalling pathways may be key to the malignant transformation of human cells. Growth factors were initially identified from culture medium for their ability to independently sustain cell growth. Virally transformed cells, unlike normal cells, depict lower requirements for exogenous growth factors. With the exception of TGF-β, which stimulates the proliferation of various mesenchymal cells, inhibits the proliferation of many epithelial cells and binds to a receptor with serine-threonine kinase activity (Derynck, 1994), most growth factors bind to specific transmembrane receptors belonging to the family of receptor tyrosine kinases (RTK's). These RTK's have been involved with human diseases such as developmental disorders and even cancer. Signal transduction by this family of RTK's is achieved through a series of steps including: a) ligand binding to the receptor and subsequent receptor dimerization, b) receptor trans autophosphorylation on tyrosine residues, c) recruitment to the receptor's cytoplasmic tail and activation of cytoplasmic signalling molecules that are mediators in the transmission of information to the nucleus (Longati *et al.*, 2001).

1.2.2.1 Historical overview of the hepatocyte growth factor (HGF-R)

HGF induced invasive growth is mediated by the HGF-R, or Met receptor. The HGF-R was identified as the *c-met* proto-oncogene (Bottaro *et al.*, 1991) and originally isolated as a transforming gene from a human osteogenic sarcoma cell line (HOS) that was treated *in vitro* with the chemical carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosguanidine (MNNG) (Blair *et al.*, 1982; Cooper *et al.*, 1984). Its activation in these HOS cells occurred via a DNA rearrangement that resulted in the fusion of sequences from the *TPR* (translocated promoter region) locus on chromosome 1 to sequences from the *met* locus on chromosome 7 (Park *et al.*, 1986; Dean *et al.*, 1987). Activation of the *met* oncogene via DNA rearrangement and fusion resembles to a great extent the activation process for several members of the tyrosine kinase family of oncogenes.

Nucleotide sequencing of a portion of the *met* oncogene showed that it was in fact a member of the tyrosine kinase family of oncogenes. It resembles to various degrees the *ron* and *sea* oncogenes, and its kinase domain is most homologous to the human insulin receptor (HIR) (Ulrich *et al.*, 1985) and the murine v-*abl* oncogene (Reddy *et al.*, 1983). Overexpression of the *met* proto-oncogene is evident in tumors of specific histotypes including thyroid (DiRenzo *et al.*, 1992), pancreatic carcinomas (DiRenzo *et al.*, 1995), and in hereditary papillary renal carcinomas (HPRC) (Schmidt *et al.*, 1997).

1.2.2.2 Structural characteristics of HGF-R

HGF-R is a heterodimer of 190 kDa comprising an α-chain of 50 kDa containing the HGF binding site. The α-chain is heavily glycosylated, found on the cell surface, and which is linked via a disulphide bond to a β-chain. The 145 kDa β-chain contains the kinase domain with the tyrosine autophosphorylation sites and the multifunctional docking site that comprises a specific stretch of amino acids located in the carboxy-terminal of the protein (Comoglio and Vigna, 1995). C-Met is synthesized as a glycosylated single chain precursor that undergoes proteolytic processing to form a mature heterodimer (Giordano *et al.*, 1989a).

The Met cytoplasmic domain can be divided into three distinct and functional domains as depicted in **Figure 1.8(A).**

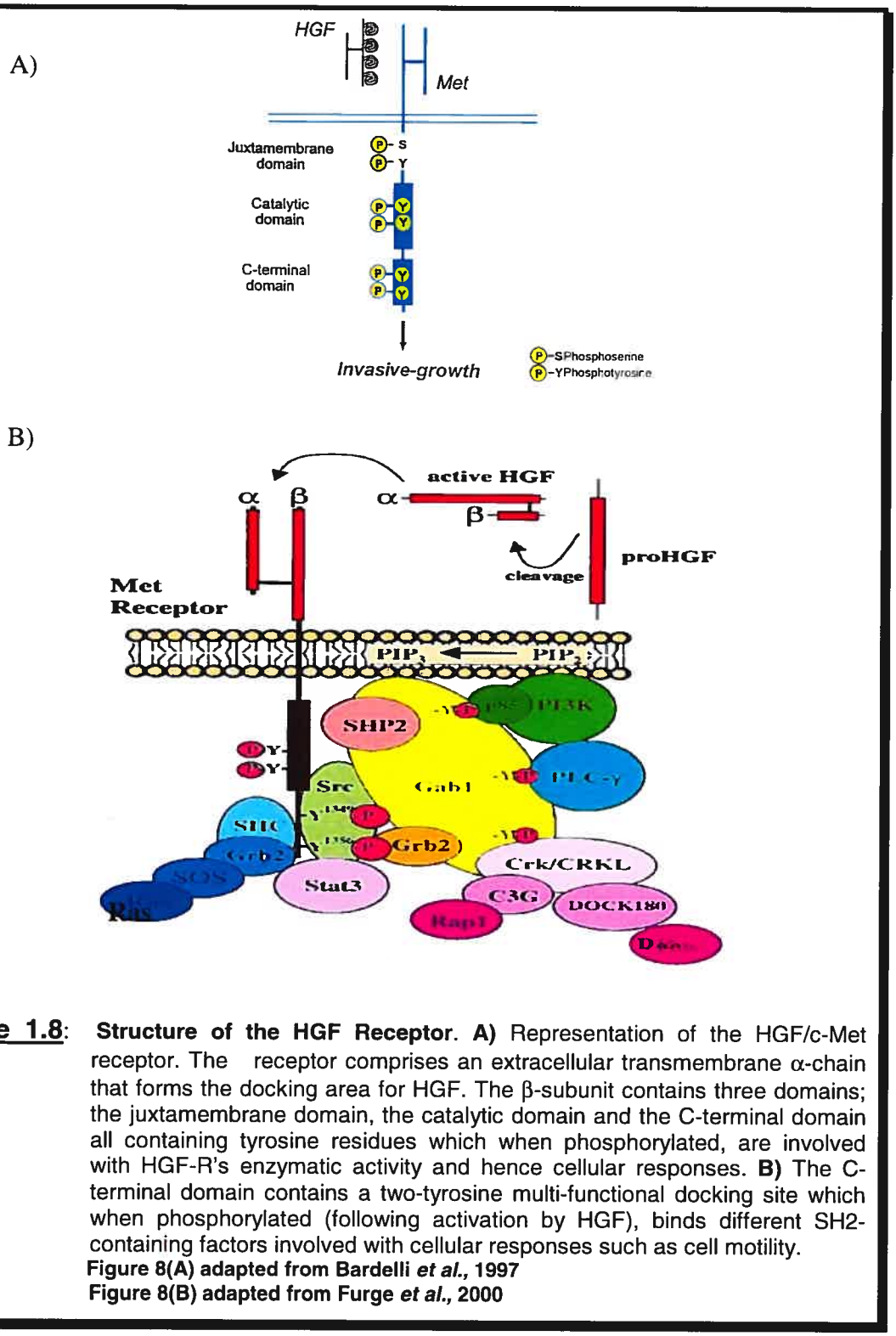


Figure 1.8: **Structure of the HGF Receptor. A)** Representation of the HGF/c-Met receptor. The receptor comprises an extracellular transmembrane α-chain that forms the docking area for HGF. The β-subunit contains three domains; the juxtamembrane domain, the catalytic domain and the C-terminal domain all containing tyrosine residues which when phosphorylated, are involved with HGF-R's enzymatic activity and hence cellular responses. **B)** The C-terminal domain contains a two-tyrosine multi-functional docking site which when phosphorylated (following activation by HGF), binds different SH2-containing factors involved with cellular responses such as cell motility.
Figure 8(A) adapted from Bardelli *et al.*, 1997
Figure 8(B) adapted from Furge *et al.*, 2000

The juxtamembrane domain, which immediately follows the transmembrane domain, negatively regulates Met activity. The phosphorylation of a serine in this domain, serine 975, by Protein Kinase C (PKC) or Ca^{2+}/ Calmodulin-dependent kinase, and/or the recruitment of a cytoplasmic tyrosine phosphatase by the phosphorylated tyrosine 1003, inhibit the Met kinase activity (Gandino *et al.*, 1990; Gandino *et al.*, 1994; Villa-Moruzzi *et al.*, 1993). Phosphorylation of two tyrosine residues in the kinase domain of the receptor (Y^{1234} and Y^{1235}) positively regulates the enzymatic activity of the Met kinase domain (Ferracini *et al.*, 1991; Longati *et al.*, 1994; Naldini *et al.*, 1991). This increased c-Met activity can promote the metastatic spread of cancer due to its stimulatory effects on a variety of processes such as angiogenesis, cellular motility, and protease secretion (Jeffers *et al.*, 1996). The C-terminal domain also contains a two-tyrosine region (Y^{1349} and Y^{1356}) that is referred to as the multi-functional docking site. Once both tyrosines become phosphorylated, this docking site, as depicted in **Figure 1.8(B)**, binds multiple SH2-containing transducers, including p85 the catalytic subunit of the phosphatidylinositol 3-kinase (PI3-kinase) (Graziani *et al.*, 1991), phospholipase C-γ, Src, the adaptors Grb2 (Growth factor receptor-bound protein 2) (Ponzetto *et al.*, 1994) and SHC (Pelicci *et al.*, 1995), the transcriptional factor STAT-3 (Signal transducers and activators of transcription-3) (Boccaccio *et al.*, 1998), and the docking protein Gab1 (Grb-2 associated binder 1) (Weidner *et al.*, 1996). This multi-functional docking site is crucial for all Met-mediated responses. Substitution of both tyrosines (Y^{1349} and Y^{1356}) with phenylalanine resulted in the abolishment of Met-mediated responses both *in vitro* and *in vivo* (Maina *et al.*, 1996; Ponzetto *et al.*, 1994).

A study by Gual and collaborators has recently demonstrated that the last 26 amino acids in the C-terminal domain positively regulate Met kinase activity and that their presence is critical for the transforming ability but not for Met-mediated invasiveness (Gual *et al.*, 2001). By constructing mutants that contained various deletions of the C-terminal domain and transfecting these mutants into NIH3T3 cells, they were able to establish that deletion of the last 47 amino acids in the C-terminal domain (the Δ47 mutant) displayed a lower level of phosphorylation. This was easily explained by the

fact that Met Δ47 lacks three auto-phosphorylation sites (Y^{1349}, Y^{1356} and Y^{1365}) (Gual *et al.*, 2001).

Following these findings, they proceeded to look at the enzymatic activity of the various mutants in comparison to the wild type Met. With the use of an exogenous substrate, the myelin basic protein (MBP), they measured the phoshorylating ability of the different receptors. The deletion of the last 47 amino acids resulted in a 46% increase in Met's ability to phosphorylate MBP *in vitro* as compared to the wild type. Thus, from these results it seems that this domain negatively regulates Met's enzymatic activity. In agreement to this, work from Bardelli and collaborators showed that a cell-permeable peptide derived from the Met tail (Ile-Gly-Glu-His-Tyr^{1349}-Val-His-Val-Asn-Ala-Thr-Tyr^{1356}-Val-Asn-Val-Lys-Cys-Val-Ala) and containing the docking site inhibits receptor activity. Once internalized with the help of the Antennapedia internalization domain at the amino terminus, the tail peptide blocked both ligand-dependent autophosphorylation and downstream Met signalling. One possible explanation for this inhibition as presented by Bardelli and his collaborators, is that the Met tail could impair receptor phosphorylation by interacting with another moiety of the catalytic domain. (Bardelli *et al.*,1999). The Δ26 mutant was also of extreme interest. The deletion of the last 26 amino acids resulted in a 35% reduction in Met's ability to phosphorylate MBP, compared to the wild type. These findings indicate that this portion of the receptor tail positively regulates the tyrosine kinase activity. Altogether, the findings by Gual and his collaborators propose that the C-terminal domain of Met contains two regulatory regions displaying opposite effects on the receptor kinase activity.

In a study from Giordano and collaborators, Met-mediated transformation was examined in NIH3T3 fibroblasts transfected with the full-length *met* cDNA (Giordano *et al.*, 1993). Exposing these transfectants to HGF resulted in a stimulation of tyrosine kinase activity and induced changes in cell shape, migration in Boyden chambers, and invasion of collagen matrices *in vitro.* In addition to these findings, it was observed that their motile and invasive phenotypes were solely

dependent upon the presence of the HGF/SF and that the factor promoted colony formation in soft agar in the presence of 5% calf serum.

Due to its seemingly influential role in cellular invasion and motility during early development, the possible role of c-Met in certain cellular transformations or even in certain cancers was examined. In fact, many research projects in the field of cancer research today aim to assess the peculiar ability of malignant tumor cells to escape from the primary tumor and settle at distant organ sites. The HGFR/*met* gene is physiologically expressed in many cell types, especially of epithelial nature (Tajima *et al.*, 1992), and is frequently over-expressed in many types of cancer.

1.3 Mutations in the *met* gene and their role in carcinoma development.

1.3.1 Met mutants in papillary renal carcinomas (PRC)

The identification of germ-line and somatic mutations in the Met gene in papillary renal carcinomas (Schmidt *et al.*, 1997; Schmidt *et al.*, 1999) and childhood hepatocellular carcinomas (Park WS *et al.*, 1999) has established the connection between Met and human cancers. All of the mutations found to date are located within the tyrosine kinase domain, either in the N-terminal portion, which contains the ATP binding site, or in the C-terminal portion, which includes both the catalytic and activation loops. Studies have demonstrated that all mutations reported so far seem to activate the catalytic activity of the receptor at different degrees (Jeffers *et al.*, 1997; Bardelli *et al.*, 1998; Giordano *et al.*, 2000). Moreover, Jeffers and his collaborators illustrate that different Met mutations identified in both hereditary and sporadic cases of human papillary renal carcinomas resulted in different levels of enzymatic activity in c-Met which translated into different levels of tumorigenicity (Jeffers *et al.*, 1997).

By stably transfecting NIH3T3 cells with some Met mutants, Jeffers and his team observed higher levels of Met phosphorylation among certain mutants and lower levels among others (Jeffers *et al.,* 1997) **(Table 1.1)**. In order to quantify the *in vitro*

transforming ability of the mutants, they performed a focus-formation assay among all mutants. Their results demonstrated that the most active mutants (i.e M1268T, Y1248H, D1246H, D1246N and Y1248C) produced a higher number of foci per μg of DNA when compared to their less active counterparts that failed to produce any foci. Tumor formation in athymic mice is an additional assay which demonstrates the functional consequences of c-Met mutations. The results obtained illustrate that cells expressing each of the mutant Met proteins form tumors, and that cells expressing the wild type protein failed to produce foci in soft agar as well as tumors in mice. As was observed with the focus–formation assay, a strong correlation exists between the Met mutants' enzymatic activity and their resulting tumorigenicity. Therefore, from the gathered results, it is evident that the M1268T mutation found in the tyrosine kinase domain of c-Met among this and other cancers, remains one of the most influential and important mutation.

Table 1. Activity of Met mutants

Met construct*	Met phos-phorylation†	Focus formation‡ #foci/μg DNA	Tumor formation§ # mice with tumors/# mice injected	Tumor formation§ Mean tumor size, mm^2
Wild type	–	0	0/14¶	0¶
M1268T(s)	+++	>300	8/8	216 ± 77
Y1248H(s)	++	156 ± 16	8/8	100 ± 40
D1246H(s)	++	119 ± 16	8/8	60 ± 52
D1246N(g)	++	147 ± 5	9/9	50 ± 25
Y1248C(g)	++	115 ± 11	7/8	77 ± 89
V1238I(g)	++	0	5/8	13 ± 15
V1206L(g)	+	0	6/6	50 ± 32
M1149T(g)	+	0	4/8	46 ± 56

Table 1.1: Activity of Met mutations when transfected in NIH3T3 cells.
Table reproduced from Jeffers *et al.*, 1997

Other research groups such as that of Giordano and collaborators revealed the presence of mutations in the c-Met of papillary renal carcinomas (PRC) different from those analyzed by Jeffers and collaborators (Jeffers *et al.*, 1997). Giordano and her team also demonstrated the presence of mutations within the tyrosine kinase

domain of c-Met (**Figure 1.9(A)**) which supports the fact that mutation M1250T displays the highest transforming ability in relation to the focus formation assay (**Figure 1.9(B)** bottom portion) as well as the highest mitogen activity as measured by a luciferase activity assay (**Figure 1.9(B)** top portion) (Giordano *et al.,* 2000). **Figure 1.10(A)** illustrates the precise localization of these mutations within the kinase domain of c-Met and the homology with residues mutated in the *RET* and *KIT* receptors. Both *RET* and *KIT* receptors are members of the receptor tyrosine kinase family (RTK). All members in this family of receptors promote invasive growth signaling through their own distinct version of a docking site (Bardelli *et al.*1997). Results obtained from their study illustrate that mutations that alter residues located in both the activation loop and in the N-terminus of the kinase domain are efficient enough at transforming mouse fibroblasts (Giordano *et al.,* 2000). An example of this can be found in **Figure 1.9(B)**, where it is clear that the MetL1195V mutation found in the N-terminus of c-Met's kinase domain expresses the same trancriptional activity and produces the same number of foci as for instance, the MetY1230C mutation found in the activation loop.

The Met receptor as seen previously, is expressed in epithelial cells and elicits various biological responses such as cell proliferation, protection against apoptosis, and invasion of the surrounding extracellular matrices. In order to examine the effect of some PRC mutations on these different biological properties, Giordano and her team transfected human Met DNA containing the various mutations into MLP 29 murine cells which display many biological properties in response to HGF treatment (Medico *et al.*, 1996). The cells having been successfully transfected with the PRC mutations displayed a "scattered" phenotype consisting of the gradual disassembling of tightly packed islands typical of epithelial cells. The introduction of exogenous HGF to these cells resulted in a higher degree of "scattering" in cells expressing the PRC mutants as opposed to cells expressing normal human Met (Giordano *et al.,* 2000).

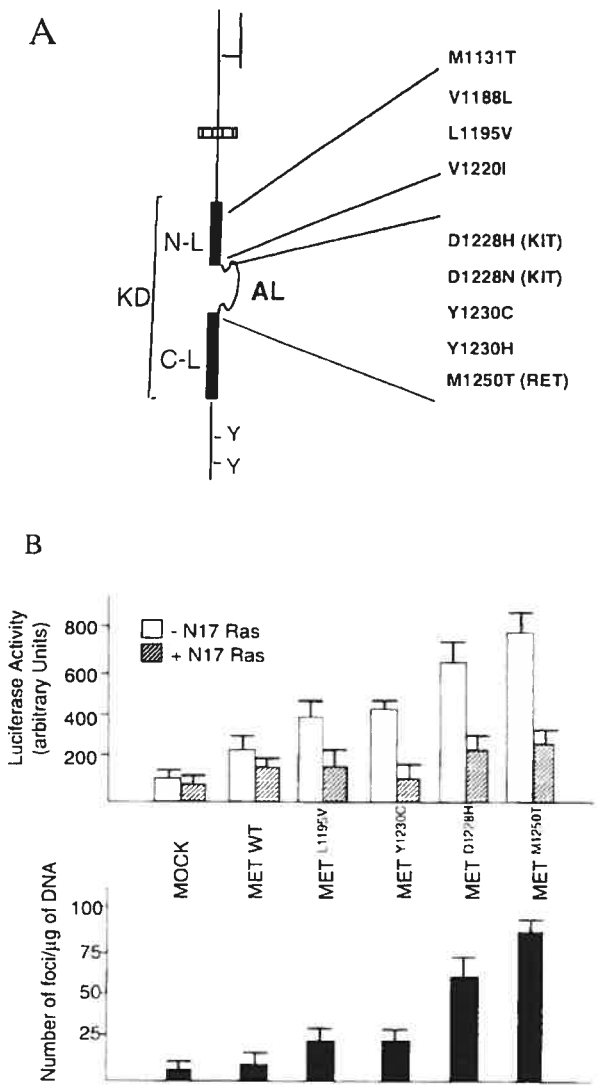


Figure 1.9: **Localization of Met mutations found in papillary renal carcinomas (PRC) and their respective activity. A)** Illustration of Met mutations found in papillary renal carcinomas (PRC). Blue boxes represent the tyrosine kinase domain (KD), which can be divided into N- and carboxyl-terminal lobes (N-L and C-L respectively) separated by a large loop referred to as the activation loop (AL). **B)** Upper portion: Transcriptional activity of Met mutants transfected in NIH3T3 cells in the presence (dashed bars) or the absence (open bars) of a dominant negative Ras (N17Ras). Lower portion: Transforming ability of MET^{PRC} mutants evaluated using the focus formation assay with MLP 29 murine epithelial liver oval cells harbouring different PRC mutations.
Adapted from Giordano *et al.*, 2000

1.3.2 PRC Met mutants and tubulogenesis

In addition to the characteristic scattering behaviour, cells transfected with PRC mutants, when stimulated with exogenous HGF, migrate in three-dimensional

collagen gels and form long and branched tubules (Khwaja *et al., 1998)*. When HGF is removed from the cells' environment, cells develop cystic structures with spikes projecting outwards. This was not observed among normal non-transfected cells. To further assess the capacity of these cells to display an invasive phenotype, Giordano and her team tested their ability to invade reconstituted basement membrane (Matrigel®) *in vitro*. According to previous studies, PI3Kinase plays a pivotal role in cellular invasion and tubulogenesis (Khwaja *et al., 1998*). For this reason, the interaction existing between the various PRC Met mutants and the p85 subunit of PI3Kinase was elucidated. Pull-down experiments of the Met^{PRC} mutants with the GST protein fused to the SH2 domain of the amino-terminal in p85 illustrated that mutants expressing the better invasive and tubulogenic ability (i.e. MetL1195V and MetY1230C) interact efficiently with PI3Kinase, whereas the mutants with decreased invasive and morphogenic ability (i.e. MetM1250T and MetD1228H) interact at a lower level with PI3Kinase. An imbalance between the activation of the Ras and PI3Kinase pathways could explain this variation among mutants due to the fact that activation of the Ras pathway results in disorganized growth that translates into reduced invasion and tubulogenesis (Khwaja *et al.*, 1998).

1.3.3 PRC Met mutants and apoptosis inhibition

The appropriate progression of a tumor is also very dependent upon the inhibition of apoptosis. Cells expressing the PRC Met mutations were exposed to staurosporin (apoptosis-inducing drug) to analyze the mutant response during apoptosis. Cells expressing mutants MetL1195V and MetY1230C were found to be more resistant to apoptosis and this resistance was further enhanced by applying exogenous HGF (**Figure 1.10**). Therefore, the results presented by Giordano and collaborators demonstrate that mutants with a low transformation potential *in vitro* and a strong invasive and tubulogenic potential, are more resistant to apoptosis.

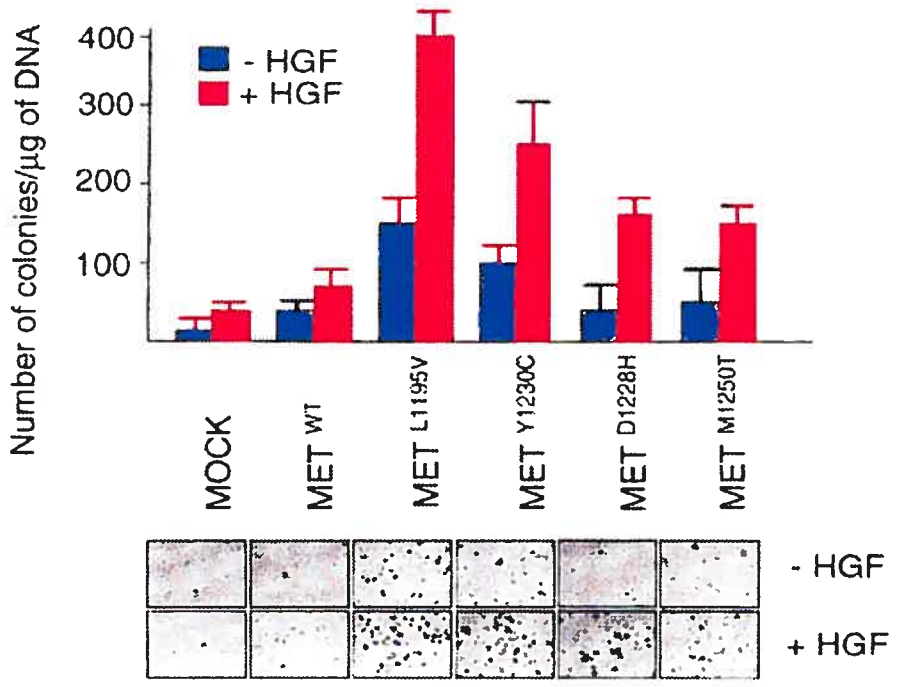


Figure 1.10: **Met^{PRC} mutants and their typical anchorage-independent growth and protection from apoptosis.** Stable transfectants with Met mutants, and their ability to form colonies in soft agar either in the presence (blue bars) or the absence (red bars) of Baculovirus-produced human HGF.
Figure adapted from Giordano *et al.*, 2000

In fact, HGF/Met plays an important role in apoptosis. Experiments performed on SK-LMS-1 leiomyosarcoma cells, demonstrate that the Akt kinase (kinase that protects cells from apoptosis) is activated by HGF in a time- and dose-dependent manner by PI3Kinase (Xiao *et al.*, 2001). Akt is also activated by tumorigenic forms of Met, truncated and constitutively dimerized forms of Met, and mutationally activated versions of Met corresponding to what is found in human hereditary papillary renal carcinomas. Furthermore, in NIH3T3 cells transfected with wild type Met, HGF was found to inhibit apoptosis induced by serum starvation and UV radiation (Xiao *et al.*, 2001). Thus, there is strong evidence that HGF could inhibit cell death through the Pi3-kinase/Akt signalling pathway.

1.3.4 PRC Met mutants and anchorage-independent growth

Another very important aspect of Met PRC mutant-transformed cells was their capacity to grow in an anchorage-independent environment. As seen earlier, this aspect of malignant growth remains one of its most important features. Thus, cells expressing the normal Met receptor formed very small colonies both in the presence and the absence of HGF. Cells expressing PRC Met mutants on the other hand formed numerous large colonies in the presence of HGF thus illustrating their capacity to grow in an anchorage-independent environment (Giordano *et al.*, 2000).

1.4 The Madin Darby Canine Kidney (MDCK) Cell Line

1.4.1 Historical Overview

The Madin Darby Canine Kidney Cell line (MDCK) was originally derived from normal dog kidney in 1958 and used in virology studies (Gausch *et al.*, 1966). The cells were then used as a model for studying the function of epithelial cells and more importantly, to investigate epithelial cell polarity (Mostov and Deitcher, 1986). In culture, these MDCK cells form a polarized sheet one cell thick with many of the properties that are normally found in canine kidney epithelium from which it is derived. When grown on porous filters, these cells have the ability to form a well-polarized monolayer and reconstitute a simple epithelial tissue with distinct apical and basolateral surface domains (Simons and Fuller, 1985). Like normal epithelial cells, MDCK cells form tight junctions that allow the epithelial sheet to function as a selective permeability barrier between the outside environment (above the cell monolayer) and the tissue compartments (under the cell monolayer). It is this permeability barrier that is responsible for the vectorial transfer from the external medium to the cell compartment.

1.4.2 The MDCK cell line and c-Met

Response from the MDCK cell line to HGF activation of c-Met is strongly dependent upon culture conditions and involves morphogenic and motogenic changes. These responses include cell scattering (Stoker *et al.*, 1987), tubulogenesis in collagen

(Montesano *et al.*, 1991a), and dedifferentiation of polarized cells grown on permeable filter supports (Balkovetz *et al.*, 1997). The HGF receptor is selectively located on the basolateral plasma membrane domain of polarized epithelial cells by direct delivery from the trans-Golgi network after synthesis (Niranjan *et al.*, 1995). The localization of c-Met at the basolateral membrane supports the role of HGF as an important mediator in epithelial/mesenchymal cell interactions.

The scattering of MDCK cells appears to occur in two distinct stages. In the first stage, the cells diverge away from each other while still maintaining contact points between each other. In the second stage, the cells separate completely and move away from one another. MDCK cell scattering in response to HGF was shown to result from cytoskeletal reorganization (membrane ruffling and lamellipodium extension, disappearance of peripheral actin bundles at the edge of colonies, and an overall decrease in stress fibers), the loss of intercellular junctions, and cell migration (Ridley *et al.*, 1995). Recent findings suggest that certain members of the Ras family of GTP-binding proteins may play a role in cellular movement. In fact, the Rac and Rho proteins have shown the ability to regulate actin reorganization in Swiss 3T3 fibroblasts in response to certain extracellular factors (Ridley and Hall, 1992; Ridley, 1994). Moreover, a microinjection of Ras, Rac, and Rho proteins and dominant negative mutants of the aforementioned poteins showed that HGF-induced movement requires activation of both Ras and Rac, whereas movement is inhibited when there is an increase in the Rho protein. Therefore, cell movements via changes in actin polymerization are a result of a downstream interaction or signalling process between Ras, Rac, Rho and c-Met **(Figure 1.8(B))**.

1.4.3 HGF and c-Met's role in MDCK cell tubulogenesis

Tubulogenesis is also a process essential for the proper development of various epithelial organs such as the kidney, intestine, pancreas, liver, and lung. Montesano and his group established the first *in vitro* model for epithelial cell movement in which tubular structures were formed (Montesano *et al.*, 1991a). In this study, they observed the development of tubular structures from cysts composed of MDCK cells

that were exposed to conditioned medium derived from MRC-5 human embryonic lung fibroblasts or otherwise known as NIH3T3 fibroblasts. In normal conditions where MDCK cells are not treated with conditioned medium derived from fibroblasts, they form hollow spherical cysts with no apparent tubular structures. Montesano, by first applying exogenous HGF and then completely eliminating the response by adding antibodies against HGF (Montesano *et al.,* 1991b), later demonstrated that it was in fact HGF which triggered this branching tubulogenesis from these cells in collagen. Due to the activation by c-Met's tyrosine kinase activity by HGF, tubulogenesis induced by HGF has been shown to be a phosphorylation-dependent process in MDCK cells (Santos *et al.,* 1993). Santos and others in the field demonstrated that HGF-induced tubulogenesis can also be modulated by other mechanisms involving phosphorylation and including protein kinase C (PKC), protein kinase A (PKA), and Ca^{+2}/ calmodulin (CaM)-dependent kinases. For example, more complex tubular structures have been observed when PKC was inhibited compared to what was observed with HGF activation alone. By contrast activation of PKA with a subsequent inhibition of calmodulin led to a decrease in tubule formation in the presence of HGF. Thus, based on these observations, it would be plausible to state that c-Met activation initiates epithelial tubulogenesis that can be subsequently modulated by PKC and/or PKA kinases as well as calmodulin (CaM) and CaM-dependent kinases. In addition to these modulators, extracellular matrix (ECM) proteins are also involved with, and regulate the formation of HGF-induced MDCK cell branching tubular structures (Santos and Nigam, 1993). Among these ECM proteins, laminin, entactin, and fibronectin induced the formation of tubules and increased their branching complexity. In contrast, ECM proteins such as type IV collagen, heparin sulfate proteoglycan, and vitronectin, substantially inhibited tubular formation. Interestingly, the opposing effects of fibronectin and vitronectin observed in the HGF-induced tubulogenesis parallels what is observed in HGF-induced MDCK cell scattering (Clark, 1994). This aspect assumes a very important role for these ECM receptor proteins, such as fibronectin, on processes involving epithelial cell movement.

Movement between epithelial cells also requires to some extent the regulation of cell-cell adhesion proteins. β-actin, for instance, was originally identified as a factor playing a role in cadherin mediated cell-cell adhesion junctions (Nagafuchi and Takeichi, 1989) and is involved with morphogenesis during embryonic development (McCrea *et al.,* 1993; Larabell *et al.,* 1997). β-catenin not only binds to E-cadherin but also to the adenomatous polyposis coli (APC) protein (Hulsken *et al.,* 1994) which is a cytoplasmic protein and the product of a tumor suppressor gene that was found to be mutated in colon cancer (Rubinfeld *et al.,* 1993). A study from Pollack and collaborators performed with the MDCK tubulogenesis model, illustrated that the interaction of β-catenin with the APC protein is essential for one of the very early steps in the initiation of tubule formation (Pollack *et al.,* 1997). Their observations demonstrated that not only is β-catenin involved with the formation of cell-cell adherens junctions, it also plays a role in cell migration during tubulogenesis through interactions with the APC protein. In fact, when the β-catenin protein is mutated to block interaction with the APC protein, cell migration, which is observed during tubulogenesis, is inhibited.

A more recent study has demonstrated that HGF-induced tubulogenesis in MDCK cells is also dependent on the transcriptional factor STAT-3. STAT proteins are phosphorylated by Jak kinases in the cytoplasm on tyrosine residues (Boccaccio *et al.,* 1998). This phosphorylation leads to STAT-3 protein dimerization and translocation into the nucleus where they express their function as transcription factors by binding to specific promoter sequences (Ihle, 1996). It is the binding of HGF to its c-Met receptor that leads to the recruitment of STAT-3 to the multifunctional docking site in the C-terminal domain, subsequent phosphorylation on STAT-3 tyrosine residues, nuclear translocation, and binding to its specific promoter element. The introduction of a tyrosine phosphorylated peptide, which interferes with the association between STAT and the receptor, as well as STAT dimerization, inhibited the HGF-induced tubule formation without affecting neither cell scattering or growth. Therefore, these finding are evidence that nuclear signalling is definitely required for HGF-induced branching tubulogenesis of MDCK cells.

1.5 Objectives

A model system consisting of invasive variants of Moloney sarcoma virus (MSV)-transformed MDCK cells, that exhibits decreased expression of E-cadherin, was selected for its capacity to pass through a Matrigel® coated filter unit (Behrens *et al.*, 1989; Simard *et al.*, 1996). The resultant invasive MSV-MDCK cell variants (MSV-MDCK-INV) exhibit increased expression of β-actin, the loss of actin stress fibers and the expression of multiple β-actin rich pseudopodia (Le *et al.*, 1998). The β-actin rich pseudopodia found in MSV-MDCK-INV cells express high levels of tyrosine phosphorylation. Furthermore, among those proteins found to be selectively phosphorylated on tyrosine in MSV-MDCK-INV cells, is a 160kDa protein identified as HGF-R or Met (Vadnais *et al.*, 2002).

In the present study, we examine the tumorigenic and transformed characteristics of MSV-MDCK-INV cells in comparison to their MDCK and MSV-MDCK counterparts. MSV-MDCK-INV cells express decreased levels of E-cadherin and possess multiple β-actin rich pseudopodia resulting in a motility that is not observed in either MSV-MDCK or MDCK cells. In addition, they express a highly phosphorylated c-Met which is involved in various cellular transformations leading to tumorigenic abilities and has been observed in different cancers, namely papillary renal carcinomas. In order to assess the tumorigenic properties, we cultured MSV-MDCK-INV, MSV-MDCK and MDCK cells in soft agar, and observed the respective focus-formation efficiencies and the size of resulting foci. Following the growth in soft agar, foci were isolated from the semi-solid environment and placed into culture in order to further examine other characteristics including their ability to grow over a monolayer of well-polarized MDCK cells. These characteristics, as seen previously, rank among the most important ones when classifying cells as having tumorigenic capacity.

Having gathered evidence pertaining to the tumorigenic character of these MSV-MDCK-INV cells, the next step was to investigate the presence of point mutations in

the tyrosine kinase as well as the C-terminal domains of c-Met. All studies pertaining to the analysis of Met's role in cancer, for example in hereditary papillary renal carcinoma, illustrate the presence of one or more point mutations in these domains leading to their constitutive activation and phosphorylation. This constitutive phosphorylation in turn leads to various cellular responses resulting in a transformed phenotype among different cellular models. In order to elucidate the presence of point mutations in both tyrosine and C-terminal domains of Met in MSV-MDCK-INV cells, we performed RT-PCR reactions using RNA isolated from all three cell lines (MSV-MDCK-INV, MSV-MDCK and MDCK) in conjunction with primers synthesized on the basis of the human c-Met DNA sequence. A subsequent comparison of the DNA and protein sequences among the three cell lines helped identify possible mutations that could account for the invasive and motile characteristics of MSV-MDCK-INV cells.

CHAPTER 2

Manuscript

A tumoral and invasive phenotype independent of c-Met mutation

Clinical Experimental Metastasis, in revision process

G. Giannini and J. Noël

Running head: Tumoral and invasive potential of c-Met

A tumoral and invasive phenotype independent of c-Met mutation

G. Giannini and J. Noël

Département de physiologie,
Groupe de recherche en transport membranaire,
Université de Montréal, Montréal, Québec, Canada H3C 3J7

To whom correspondence should be addressed:

Josette Noël
Chercheur agrégé
Département de physiologie
Université de Montréal
C.P. 6128, Succursale Centre-Ville
Montréal, Qué. H3C 3J7
Canada

Tel.: (514) 343-6111, ext. 4356
Fax: (514) 343-7146
[redacted]

2.1 Abstract

Various studies have outlined the importance of c-Met in cellular transformation, cell motility and invasion. In the present study we aimed to determine if the transformed, tumoral and invasive phenotypes observed in an invasive variant (INV) of the MDCK cell line transformed by the Moloney Sarcoma Virus (MSV-MDCK) presenting, under normal conditions, a highly phosphorylated c-Met were dictated by point mutations in the tyrosine kinase and C-terminal domains of c-Met. RT-PCR from total RNA extracts revealed that there is no point mutations in the tyrosine kinase or the C-terminal domains of MSV-MDCK-INV cells' c-Met when compared to sequences of parental MSV-MDCK and MDCK cells. Therefore, the invasive and tumoral character of MSV-MDCK-INV cells, as determined by their capacity to form foci in soft agar and colonies over a monolayer of well polarized epithelial cells, as well as the transformed phenotype of MSV-MDCK cells may not be attributed or explained by the presence of point mutations in either of these two very important domains.

Keywords

Anchorage independent growth

Autocrine HGF loop

Invasion

Transformation

Tumor

List of abreviations

Anchorage-independent growth (AIG)

Grb-2-associated binder 1 (Gab1)

Hepatocyte growth factor (HGF)

Hepatocyte growth factor receptor (HGF-R)

Invasive variant of Moloney sarcoma virus transformed epithelial MDCK cells (MSV-MDCK-INV)

Madin-Darby Canine Kidney (MDCK) cells

Moloney sarcoma virus (MSV)

Moloney sarcoma virus transformed epithelial MDCK cells (MSV-MDCK)

Phospholipase C gamma (PLC-γ)

Phosphotyrosine (P-Tyr)

Protein encoding the protooncogene *c-met* (c-Met or Met)

Regulatory subunit of PI3-kinase of molecular weight of 85 kDa (p85)

Reverse transcription-Polymerase chain reaction (RT-PCR)

Scatter factor (SF)

2.2 Introduction

Cancer is a progressive disease that follows a course of increasingly aggressive and treatment-resistant stages that involve the accumulation of molecular and genetic changes. These changes are depicted through tumor initiation that reflects unscheduled cellular hyper-proliferation, either through direct activation of cell cycle machinery or of signaling pathways that regulate the cell cycle [1].

Neoplastic transformation of human cells and cells from other species *in vitro* is seen as a complex multistep process by which normal cells acquire various phenotypic characteristics. Four of these major steps are involved and are namely, the development of morphological transformation, growth in semisolid medium, immortality, and tumorigenicity. A large majority of human tumor cells of mesenchymal origin do portray the ability to grow in semi-solid medium otherwise known as anchorage-independent growth or AIG [2].

The hepatocyte growth factor (HGF), also known as scatter factor (SF), is a pleiotropic cytokine which, by activating its receptor tyrosine kinase, stimulates cell motility, invasion, proliferation, survival and morphogenesis, and induces the expression of specific genes such as *ras,* PLC-γ, etc. [1]. Many groups have to date established the importance of the hepatocyte growth factor receptor (HGF-R) or c-Met as well as the consequences of its over-expression and its constitutive phosphorylation. Over-expression of the *met* proto-oncogene is evident in tumors of specific histotypes including thyroid [3], pancreatic carcinomas [4], and in hereditary papillary renal carcinomas (HPRC) [5]. Phosphorylation of two tyrosine residues in the kinase domain of the receptor (Y^{1234} and Y^{1235}) positively regulates the enzymatic activity of the c-Met kinase domain [6-8]. This increased activity can promote the metastatic spread of cancer due to its stimulatory effects on a variety of processes such as angiogenesis, cellular motility and protease secretion [9, 10].

Increased enzymatic activity in the tyrosine kinase domain of c-Met is translated into different cellular responses via the C-terminal domain. The C-terminal domain contains a two-tyrosine docking site (Y^{1349} and Y^{1356}) which, upon phosphorylation, binds multiple SH2-containing transducers including the p85 subunit of phosphatidylinositol 3-kinase [11], PLC-γ [12, 13], Src [14], the adaptors

Grb2 [15-17] and SHC [16, 18], the transcriptional factor Stat3 [12, 19], and the docking protein Gab1 (Grb-2-associated binder 1) [8, 9, 11, 20-22] (see [23] for an integrated view). A study from Gual et al. [24] has recently demonstrated that the last 26 amino acid portion of the C-terminal domain positively regulates c-Met kinase activity and that its presence is critical for the transforming ability but not for c-Met-mediated invasiveness. Furthermore, the introduction of the $M_{1250}T$ point mutation, which is known to up-regulate the kinase activity and interfere with the receptor's auto-inhibition mechanisms [10, 25], in the Met kinase domain can substitute for the role of this portion of the C-terminal domain [24].

The identification of germ-line and somatic mutations, like the $M_{1250}T$ mutation, in the *met* gene in papillary renal carcinomas [5, 26, 27] and childhood hepatocellular carcinomas [28] has established the connection between c-Met and human cancers. An even more direct relationship has been established between mutations and metastasis [10]. All of the mutations found to date are located within the tyrosine kinase domain, either in the N-terminal portion, which contains the ATP binding site, or in the C-terminal portion, which includes both the catalytic and activation loops. Studies demonstrate that all mutations reported so far activate the catalytic activity of the receptor to different extents [10, 25] with some inducing malignant transformation of NIH 3T3 cells [25].

Moloney sarcoma virus (MSV) transformants of the polarized epithelial MDCK (Madin Darby Canine Kidney) cell line were shown to exhibit a heterogenous phenotype [29]. Characterization of two sub-populations showed cells with a fibroblastoid phenotype possessing motile properties and a lack of E cadherin expression, or cells which were polarized, less motile and expressing E cadherin [29]. Selection of cells for their ability to pass through a Matrigel®-coated filter unit led to a pure population of cells of fibroblastoid phenotype called MSV-MDCK-INV for their invasive property [31]. This invasive MSV-MDCK cell variant exhibits multiple β-actin-rich pseudopodia, the loss of actin stress fibers, and a highly phosphorylated c-Met on tyrosines in position 1230, 1234, 1235 of the tyrosine kinase domain and in position 1349 and 1365 of the docking site [32]. Having demonstrated that an autocrine HGF loop could explain the motile phenotype of MSV-MDCK-INV cells

as well as the pseudopod formation [32], we examined the possibility of point mutations in both the tyrosine kinase and C-terminal domains of c-Met among MSV-MDCK-INV cells. We demonstrate that not a single point mutation in these domains could account for the high responsiveness of c-Met in MSV-MDCK-INV cells to secreted HGF. We also looked at the ability of the MSV-MDCK-INV cells as compared to their parental cell lines to grow in anchorage-independent environments, namely in soft agar and on a well-polarized monolayer of epithelial MDCK cells. As expected from their invasive property, we found that MSV-MDCK-INV cells are similar in terms of tumorigenicity to ras-transformed NIH 3T3 cells.

2.3 Material and methods

Cell Culture

MSV-MDCK and MSV-MDCK-INV cells, as well as all respective clones (MDCK (clone E16), MSV-MDCK (clone TE6) and MSV-MDCK-INV (clone 5.1) were obtained from Dr. Ivan Robert Nabi of the Pathology and Cellular Biology Department of the University of Montreal (Montreal, Quebec). MDCK Strain I cells were obtained from Dr. Guy Lemay of the Microbiology and Immunology Department of the University of Montreal (Montreal, Quebec). NIH 3T3 as well as NIH 3T3/ras cells were obtained from the laboratory of Dr. Victor Sandor at the Lady Davis Institute of the Jewish General Hospital (Montreal, Quebec). MDCK Strain I, MSV-MDCK and MSV-MDCK-INV cells, as well as the respective clones, were all cultured in High Glucose DMEM culture medium supplemented with 10% FBS, 1% non-essential amino acids and 1% vitamins (supplied by Gibco BRL Life Technologies, USA) under 5% CO_2 atmosphere. NIH 3T3 as well as NIH 3T3/ras cells were cultured in High Glucose DMEM culture medium supplemented with 10% FBS and 1% glutamine.

Soft Agar Assay

A basal agar layer was prepared one day prior to the assay in wells of a 6-well culture plate and consisted of 0.5% noble agar, 1X DMEM, 10% FBS, 1% non-essential amino acids and 1% vitamins. On the day of the assay, different cellular dilutions were prepared in duplicates in order to yield 5000 cells/well, 10,000 cells/well and 20,000 cells/well in culture medium containing DMEM, 10% FBS and 0.33% noble agar. Cells were then placed at 37°C under CO_2 atmosphere for a period of 10-14 days with the addition of fresh medium every two days before being observed. The growth efficiency was calculated from the number of resulting foci counted throughout the agar in relation to the number of cells plated for the 6 conditions, and expressed as percentage.

Phenotype analysis

MDCK cell monolayers were prepared by plating 500,000 cells/well in a 6-well culture plates containing coverslips. Once the monolayers were well polarized, 1000 cells were seeded over the monolayer and placed at 37°C under CO_2 atmosphere for a period of 7-10 days, or until superficial colonies became visible. This was performed in duplicates for MDCK, MSV-MDCK and MSV-MDCK-INV cells as well as for some MSV-MDCK-INV and NIH 3T3/*ras* clones isolated from soft agar. Following the observation of visible colonies on the monolayers, cells were fixed with a pre-heated solution (37°C) of 4% paraformaldehyde for 15 minutes then washed with PBS-CM (Phosphate-buffered saline containing 0.1mM $CaCl_2$ and 1mM $MgCl_2$). Cells were permeabilized with a PBS solution containing 0.075% saponin and 0.2% BSA for 10 minutes at room temperature, incubated in blocking solution (PBS containing 2% BSA and 0.2% gelatin) at room temperature for 20 minutes, then, with Phalloidine Texas-Red. The coverslips were mounted with a solution of 2% propyl-gallate in glycerol:Tris-HCl 200mM, pH 8.0 (1:1). Cells were visualized with a Zeiss AxioSkop fluorescence microscope equipped with a 63X Plan apochromat objective and selective filter for Texas red.

Isolation of RNA.

Total RNA from MDCK, MSV-MDCK and MSV-MDCK-INV cells cultured in supplemented DMEM medium , was prepared by using the GenElute Total Mammalian RNA Extraction Kit™ (Sigma Biotech, Ontario).

RT-PCR.

First strand cDNA was reverse transcribed from 2.5 µg of total RNA (Superscritpt II™ Invitrogen, Gaithersburgh, Md.) with an antisense primer (h-TKA; 5'-CACATTTTACATTCACATAAGTAGCGTTCA-3') specific to the human tyrosine kinase domain of c-met ([33], EMBL access code: #JO2958) chosen 20 nucleotides away from the 3'(antisense) end. The canine c-met cDNA was amplified using *Taq* DNA Polymerase®, a sense primer specific to the human tyrosine kinase domain of c-met chosen 20 nucleotides away from the 5' (sense) end (h-TKS; 5'-GGATAGGCTTGTAAGTGCCCGAAGTGTAAG-3') and the h-TKA primer.

To analyze the C-terminal domain of c-Met, an antisense primer, h-CTA, specific to the 3'-end of the human C-terminal domain and containing a poly-dT tail (5'-CTATGATGTCTCCCAGAAGGTTTTTTTTTT-3') was used for the initial reverse transcription reaction. The following PCR reaction involved the use of this h-CTA primer and a sense primer, c-TKIS, which was synthesized based on the internal canine tyrosine kinase domain sequence (5'- AAACATGGAGATCTTCGAAATTTCA-3'). The respective PCR reactions were performed using an MJ Research PTC-2000 Peltier Thermal Cycler, with 30 cycles at an annealing temperature of 65°C and an extension temperature of 72°C. Amplified DNA was analyzed through gel electrophoresis and purified using the GenElute PCR Clean-up kit (Sigma Biotechnology) before proceeding to the DNA sequencing.

DNA Sequencing.

Two sequencing reactions using purified DNA fragments and both sense and antisense primers were performed per DNA sample in order to ensure the complete sequencing of the entire tyrosine kinase domain as well as the entire C-terminal domain of the canine c-Met. All sequencing reactions were performed by The Sheldon Biotechnology Centre of McGill University, (Montréal, Québec, Canada).

2.4 Results and Discussion

Constitutive activation of the *met* proto-oncogene has for some time been elucidated as being involved with cellular transformation and responsible for invasive and metastatic properties in cancer cells [23, 34]. The constitutive auto-phosphorylation of c-Met, its over-expression and the presence of point mutations are all elements responsible for the conversion of physiologically active c-Met to oncogenic forms [3, 26, 27, 35-39].

The invasive variant of MSV transformed MDCK cells presents characteristics of tumoral cells.

Tumoral cells depict characteristics, which encompass a changed morphology and a capacity to grow under conditions where normal cells cannot. One such condition is anchorage-independent growth (AIG). Transformation of certain cell lines with viruses has been shown to induce AIG and the acquisition of this AIG in many immortal rodent cell lines has been shown to correlate well with tumorigenic growth [40]. To date, the invasive character of MSV-MDCK-INV cells was shown to be related to activation of an autocrine HGF loop [32] but the tumoral phenotype has of yet not been elucidated. When placed in an anchorage-independent environment, namely soft agar, MSV-MDCK-INV cells formed foci of comparable size to what was observed for NIH 3T3 cells transformed by the *ras* oncogene (a highly tumorigenic cell line) **(Figure 2.1 a).** In addition to size similarities, MSV-MDCK-INV cells grew at an efficiency quite comparable to what was observed for NIH 3T3/*ras* cells **(Figure 2.1 b-c)**. Colony formation in soft agar was also observed for MDCK as well as MSV-MDCK cells, but the size as well as the growth efficiency in relation to MSV-MDCK-INV cells was incomparable. This is quite interesting especially when considering that MDCK cells grow faster than MSV-MDCK and MSV-MDCK-INV cells under normal culture conditions and in the presence of 10 % FBS (data not shown). Moreover, when cells isolated from the soft agar foci were seeded over a monolayer of well polarized epithelial MDCK cells, only MSV-MDCK-INV cells (**Figure 2.2 A,B**) and NIH 3T3/*ras* cells (**Figure 2.2 E,F**) but not MDCK and MSV-MDCK cells, had the capacity to grow and form superficial

colonies. The original MSV-MDCK-INV (**Figure 2.2 C,D**) and NIH 3T3/*ras* cell populations (not shown) also possess the capacity to grow on a monolayer of epithelial cells, whereas MDCK and MSV-MDCK cells do not. This particular characteristic among MSV-MDCK-INV cells illustrates another property that serves as further evidence in the classification of these cells as tumoral and metastatic. Indeed, the first step involved in extravasation of a tumor cell through a capillary, when referring to the development of a metastasis, is the adhering of the cell to the basement membrane. It is only after the cells have adhered that they can proceed with the enzymatic digestion and movement through the basement membrane [34].

The invasive phenotype and the high level of c-Met tyrosine phosphorylation cannot be explained by point mutations in the tyrosine kinase and C-terminal domains.

Gual e*t al.* [24] associated the importance of the last 26 amino acids within the C-terminal domain of c-Met on the actual enzymatic activity of the tyrosine kinase domain which is critical for cell transformation but dispensable for invasive properties. Furthermore, they elucidated the importance of the last 47 amino acids in the triggering of various signalling pathways leading to cellular responses such as cell motility. This C-terminal domain contains the multi-functional docking site comprising two tyrosine residues, Y^{1349} and Y^{1356}, which when phosphorylated binds to and activates accessory proteins involved in the signal transduction of various cellular responses (see [23] for an integrated review). Based on this study, we assumed a possible role for this region within the C-terminal domain of c-Met and hypothesized the presence of mutations which could explain the invasive and tumoral properties of the transformed MSV-MDCK-INV cell line. To determine if mutations were in fact present, we performed a RT-PCR reaction on the C-terminal domain of c-Met using total RNA extracts from all three cell lines and then proceeded to automated DNA sequencing. Following the sequence analysis for the three respective cell lines, we were able to conclude the absence of point mutations in the C-terminal domain of c-Met for all three cell lines. A sequence analysis and comparison among species, as depicted in **Figure 2.3**, demonstrates that the C-terminal domain is somewhat homologous at the 5' end but loses its homology as one approaches the 3'

end. This is a phenomenon that seems to be fairly common among different species. Human c-Met antibodies that do recognize the canine c-Met were synthesized on the basis of the N-terminal portion of the protein as opposed to the C-terminal domain. However, existing human antibodies synthesized on the basis of the C-terminal domain do not function as well in terms of their canine sequence recognition.

In order to continue defining the mechanism behind the constitutive phosphorylation of c-Met among the MSV-MDCK-INV cell line [32], which is seemingly one of the main reasons why this transformed, invasive and tumoral phenotype is observed, we assessed the possibility of point mutations in the tyrosine kinase domain. To do so, we performed a RT-PCR reaction using the same conditions used for the C-terminal domain but with different primers, and then proceeded to automated DNA sequencing. The comparison of the three canine amino acids sequences (MDCK, MSV-MDCK and MSV-MDCK-INV) reveals no point mutations in the tyrosine kinase domain of c-Met. These results indicate that the transformed phenotype of MSV-MDCK cells as well as the tumoral and invasive phenotype of the MSV-MDCK-INV cell line cannot be explained by c-Met'mutations. Analysis of 25 cancer cell lines by others led to the same conclusion [10]. However, as Lorenzato et al. [10] found in a lung metastasis of a testicular germ cell tumor, we noticed the presence of silent bases changes between total populations of the three cell lines **(Figure 2.4)**. Given their nature of affecting only the nucleotide sequence of the gene and not the amino acid composition of the protein, we can only acknowledge their presence but not assume any particular role on their part with regards to the transformed phenotype observed among MSV-MDCK cells, nor the invasive phenotype observed among MSV-MDCK-INV cells.

In order to further analyze the obtained canine c-Met sequence and to assess the importance of these silent mutations, we performed the same set of experiments on clones derived from the three cell lines; MDCK (clone E16), MSV-MDCK (clone TE6) and MSV-MDCK-INV (clone 5.1). Interestingly enough, these cell lines' sequences, whether considered at the DNA level or the protein level, expressed no point mutations or silent base changes. Thus, this result enabled us to consider the possibility of a heterogeneous population among our previous total population cell

lines. It should be recalled that this total population is in fact composed of various invasive cells selected on the basis of their ability to pass trough the Matrigel® filter. Our results nonetheless establish a partial (kinase domain and C-terminal domain) canine c-Met DNA sequence that can prove to be important for many reasons. Two main reasons being; the cloning of the canine c-Met and the production of specific canine c-Met antibodies, which up to date remain unavailable.

Many cancers, as seen previously, do possess point mutations in the kinase domain of c-Met leading to its auto-phosphorylation [26-28, 35, 42]. On the other hand, certain human cancers, for example the human gastric carcinoma cell line GTL-16, possess an over-expressed c-Met without expressing any point mutations in the kinase domain [43]. Our findings that MSV-MDCK-INV cells expressed comparable amounts of c-Met proteins to the parental cell lines but highly phosphorylated [32] were thus suggestive of the presence of point mutations or the presence of an active autocrine HGF loop. The present study clearly demonstrated the absence of any change in the amino acid composition of the tyrosine kinase and C-terminal domains of c-Met in either MSV-MDCK or MSV-MDCK-INV cells. Point mutations in neither the C-terminal nor the tyrosine kinase domains may explain the transformed phenotype observed among MSV-MDCK cells and the invasive/tumoral phenotype observed among MSV-MDCK-INV cells. Recent studies by our group [32] as well as others have brought to light the presence of an autocrine loop, which allows the constitutive activation of c-Met through auto-phosphorylation of its kinase domain. MSV-MDCK-INV cells secrete their own HGF/SF in close proximity to the HGF-R, allowing an immediate and continuous activation of c-Met through an autophosphorylation. In turn, this activation leads to a cascade of signals leading to the triggering of various cell responses such as cell motility and invasiveness. The same was observed in murine mammary carcinoma (SP1) cells [44] where *in vitro* translation of mRNA and metabolic labelling confirmed expression and synthesis of HGF by SP1 cells. Moreover, in a study performed by Ferracini et al. [36] with human specimens of osteosarcoma, it was shown that the c-Met/HGF receptor was constitutively phosphorylated and treatment with suramin, a known blocker of autocrine loops which non-selectively interferes with the binding of growth factors to

their receptors and inhibits the secretion of growth factors [45], suppressed that phosphorylation. Therefore, it was suggested that c-Met/HGF receptor activation via a paracrine or autocrine route might play a very important role in the aggressive behaviour observed among these human osteosarcomas.

From this study, we demonstrated the tumoral phenotype of MSV-MDCK-INV cells through their capacity to grow in anchorage-independent environments. Based on the absence of point mutations in either the C-terminal or the tyrosine kinase domains of c-Met, we conclude that the major mechanism leading to the activation of the kinase domain of c-Met, to invasion and to the tumoral phenotype observed among MSV-MDCK-INV cells, would be the autocrine HGF activation loop [32]. The transformed phenotype of MSV-MDCK cells, being not explained by the autocrine HGF loop or the presence of mutations in c-Met, would involve another undefined mechanism.

Acknowledgements

We thank Claude Gauthier for his expert graphic support and Julie Vadnais for her technical assistance. This study was supported by grants from the Kidney Foundation of Canada and Canadian Research Institute for Health. J.N. is FRSQ Junior II research scholar of the Fonds de la Recherche en Santé du Québec. We acknowledge Dr I.R. Nabi for a critical reading of the manuscript.

2.5 References

1. McClatchey AI. Modeling metastasis in the mouse. Oncogene 1999;18:5334-9.

2. Pavelic ZP, Slocum HK, Rustum YM, Creaven PJ, Karakousis C, Takita H. Colony growth in soft agar of human melanoma, sarcoma, and lung carcinoma cells disaggregated by mechanical and enzymatic methods. Cancer Res 1980;40:2160-4.

3. Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pilotti S, et al. Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. Oncogene 1992;7:2549-53.

4. Di Renzo MF, Poulsom R, Olivero M, Comoglio PM, Lemoine NR. Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. Cancer Res 1995;55:1129-38.

5. Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet 1997;16:68-73.

6. Ferracini R, Longati P, Naldini L, Vigna E, Comoglio PM. Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. J Biol Chem 1991;266:19558-64.

7. Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, et al. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the *MET* receptor. EMBO.J. 1991;10:2867-2878.

8. Longati P, Bardelli A, Ponzetto C, Naldini L, Comoglio PM. Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene 1994;9:49-57.

9. Jeffers M, Rong S, Vande Woude GF. Hepatocyte growth factor/scatter factor-Met signalling in tumorigenicity and invasion/metastasis. J.Mol.Med. 1996;74:505-513.

10. Lorenzato A, Olivero M, Patanè S, Rosso E, Oliaro A, Comoglio PM, et al. Novel somatic mutations of the *MET* oncogene in human carcinoma metastases activating cell motility and invasion. Cancer Res. 2002;62:7025-7030.

11. Graziani A, Gramaglia D, Cantley LC, Comoglio PM. The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. J Biol Chem 1991;266:22087-22090.

12. Hung W, Elliott B. Co-operative effect of c-Src tyrosine kinase and Stat3 in activation of hepatocyte growth factor expression in mammary carcinoma cells. J Biol Chem 2001;276:12395-403.

13. Gual P, Giordano S, Williams TA, Rocchi S, Van Obberghen E, Comoglio PM. Sustained recruitment of phospholipase C-γ to Gab1 is required for HGF-induced branching tubulogenesis. Oncogene 2000;19:1509-1518.

14. Rahimi N, Hung W, Tremblay E, Saulnier R, Elliott B. c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. J Biol Chem 1998;273:33714-21.

15. Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, et al. A multifunctional docking site mediates signalling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell 1994;77:261-71.

16. Fixman ED, TM. F, DM. K, MA. N, M. P. Pathways downstream of Shc and Grb2 are required for cell transformation by the TPR-Met oncoprotein. J. Biol. Chem. 1996;271:13116-122.

17. Nguyen L, Holgado-Madruga M, Maroun C, Fixman ED, Kamikura D, Fournier T, et al. Association of the multisubstrate docking protein Gab1 with the hepatocyte growth factor receptor requires a functional Grb2 binding site involving tyrosine 1356. J. Biol. Chem. 1997;269:21936-21939.

18. Pelicci G, Giordano S, Zhen Z, Salcini AE, Lanfrancone L, Bardelli A, et al. The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. Oncogene 1995;10:1631-8.

19. Boccaccio C, Ando M, Tamagnone L, Bardelli A, Michieli P, Battistini C, et al. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature 1998;391:285-8.

20. Weidner KM, Di Cesare S, Sachs M, Brinkmann V, Behrens J, Birchmeier W. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature 1996;384:173-6.

21. Fan S, Ma YX, Gao M, Yuan RQ, Meng Q, Goldberg ID, et al. The multisubstrate adapter Gab1 regulates hepatocyte growth factor (scatter factor)-c-Met signalling for cell survival and DNA repair. Mol Cell Biol 2001;21:4968-84.

22. Rodrigues GA, Falasca M, Zhang Z, Ong SH, Schlessinger J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signalling. Mol Cell Biol 2000;20:1448-59.

23. Furge KA, Zhang Y-W, Vande Woude GF. Met receptor tyrosine kinase: enhanced signalling through adapter proteins. Oncogene 2000;19:5582-5589.

24. Gual P, Giordano S, Anguissola S, Comoglio PM. Differential requirement of the last C-terminal tail of Met receptor for cell transformation and invasiveness. Oncogene 2001;20:5493-502.

25. Jeffers M, Schmidt L, Nakaigawa N, Webb CP, Weirich G, Kishida T, et al. Activating mutations for the Met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci 1997;94:11445-11450.

26. Schmidt L, Junker K, Weirich G, Glenn G, Choyke P, Lubensky I, et al. Two North American families with hereditary papillary renal carcinoma and identical novel mutations in the MET proto-oncogene. Cancer Res. 1998;58:1719-1722.

27. Schmidt L, Junker K, Nakaigawa N, Kinjerski T, Weirich G, Miller M, et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene 1999;18:2343-2350.

28. Park CY, Hayman MJ. The tyrosines in the bidentate motif of the env-sea oncoprotein are essential for cell transformation and are binding sites for Grb2 and the tyrosine phosphatase SHP-2. J Biol Chem 1999;274:7583-7590.

29. Simard D, Nabi IR. Inverse relation of autocrine motility factor receptor and E-cadherin expression following MDCK cell transformation. Biochem Biophys Res Comm 1996;219:122-127.

30. Behrens J, Mareel MM, Van Roy RM, Birchmeier W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J Cell Biol 1989;108:2435-2447.

31. Le PU, Nguyen TN, Drolet-Savoie P, Leclerc N, Nabi IR. Increased β-actin expression in an invasive Moloney Sarcoma Virus-transformed MDCK cell variant concentrates to the tip of multiple pseudopodia. Cancer Res. 1998;58:1631-1635.

32. Vadnais J, Nault G, Daher Z, Amraei M, Dodier Y, Nabi IR, et al. Autocrine activation of the HGF-R/Met tyrosine kinase induces tumor cell motility by regulating pseudopodial protrusion. J. Biol. Chem. 2002;277:48342-48350.

33. Park M, Dean M, Kaul K, Braun MJ, Gonda MA. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc. Natl. Acad. Sci. USA. 1987;84:6379-6383.

34. Comoglio PM, Tamagnone L, Boccaccio C. Plasminogen-related growth factor and semaphorin receptors: a gene superfamily controlling invasive growth. Exp Cell Res 1999;253:88-99.

35. Schmidt L, Duh F-M, Chen F, Kishida T, Glenn L, Choyke P, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nature Genetics 1997;16:68-73.

36. Ferracini R, Di Renzo MF, Scotlandi K, Baldini N, Oliviero M, Lollini P-L, et al. The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. Oncogene 1995;10:739-749.

37. Rong S, Segal S, Anver M, Resau JH, Vande Woude GF. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci U S A 1994;91:4731-5.

38. Cooper CS, Park M, Blair DG, Tainsky MA, Huebner K, Croce CM, et al. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature 1984;311:29-33.

39. Park M, Dean M, Cooper CS, Schmidt M, O'Brien SJ, Blair DG, et al. Mechanism of met oncogene activation. Cell 1986;45:895-904.

40. Shin SI, Freedman VH, Risser R, Pollack R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proc Natl Acad Sci U S A 1975;72:4435-9.

41. Prat M, Crepaldi T, Pennacchietti S, Bussolino F, Comoglio PM. Agonistic monoclonal antibodies against the Met receptor dissect the biological responses to HGF. J Cell Sci 1998;111:237-247.

42. Maritano D, Accornero P, Bonifaci N, Ponzetto C. Two mutations affecting conserved residues in the met receptor operate via different mechanisms. Oncogene 2000;19:1354-1361.

43. Ponzetto C, Giordano S, Peverali F, Della Valle G, Abate ML, Vaula G, et al. c-met is amplified but not mutated in a cell line with an activated met tyrosine kinase. Oncogene 1991;6:553-9.

44. Rahimi N, Tremblay E, McAdam L, Park M, Schwall R, Elliott B. Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth & Differentiation 1996;7:263-670.

45. Benini S, Baldini N, Manara MC, Chano T, Serra M, Rizzi S, Lollini PL, Picci P, Scotlandi R. Redundancy of autocrine loops in human osteosarcoma cells. Int. J. Cancer 1999; 80: 581-588.

Figure legends

Figure 2.1

Growth efficiency in soft agar. a) Panels A to D represent typical colonies formed from each of the three canine cells lines (A: MDCK, B: MSV-MDCK, C: MSV-MDCK-INV) and the control NIH 3T3/*ras* cell line (D) grown in soft agar. Figure 1b depicts the cellular density of colonies from each of the four cell lines (A-D as above). Each of the respective cell lines were plated at a density of 10,000 cells/well and pictures were taken approximately 14 days following the plating date. Figure 1c illustrates the growth efficiencies of all four cell lines from two independent trials. The mean growth efficiency was obtained by dividing the number of colonies counted by the original number of cells plated, expressed as percentage, and taking an average of each independent replicate. Bar = 100 μ (a) and 2 mm (b), applicable for figures A,B,C and D.

Figure 2.2

Ability of MSV-MDCK-INV like NIH 3T3/ras cells to grow on a monolayer of epithelial cells. Illustrations A-D and E-F represent MSV-MDCK-INV cells and NIH 3T3/*ras* cells respectively, grown on a polarized monolayer of epithelial MDCK cells, strain I. Illustrations A, C and E were taken while focusing on the MDCK monolayer whereas illustrations B, D and F were taken in the same visual plane but focussing on the colony. Cells were labelled for F-actin with phalloïdine-TR.

Figure 2.3

Sequence comparison between the canine c-Met c-terminal domain and that of other species. To analyze the C-terminal domain of c-Met, an antisense primer specific to the 3'-end of the human C-terminal domain and containing a poly-dT tail (5'-CTATGATGTCTCCCAGAAGGTTTTTTTTTT-3') was used for the initial reverse transcription reaction with total RNA of wild type MDCK. Two sequencing reactions using purified DNA fragments and both sense and antisense primers were performed per DNA sample obtained from two independent RT-PCR. Sequence comparison of the canine c-Met with that of others species was performed with data obtained from Gene Bank

Figure 2.4

Silent mutations observed in the tyrosine kinase of c-Met in MSV-MDCK-INV cells. DNA sequences obtained for the tyrosine kinase domain of the three cell lines, MDCK (M), MSV-MDCK (MSV) and MSV-MDCK-INV (INV), were aligned to look for the presence of DNA mutations. DNA sequence were then translated to amino acid sequence. Although DNA mutations were reproducibly found in both MSV-MDCK and MSV-MDCK-INV as compared to the parental MDCK cell line, these DNA mutations do no translate in changes in amino acids. Only silent mutations were found.

Figure 2.1

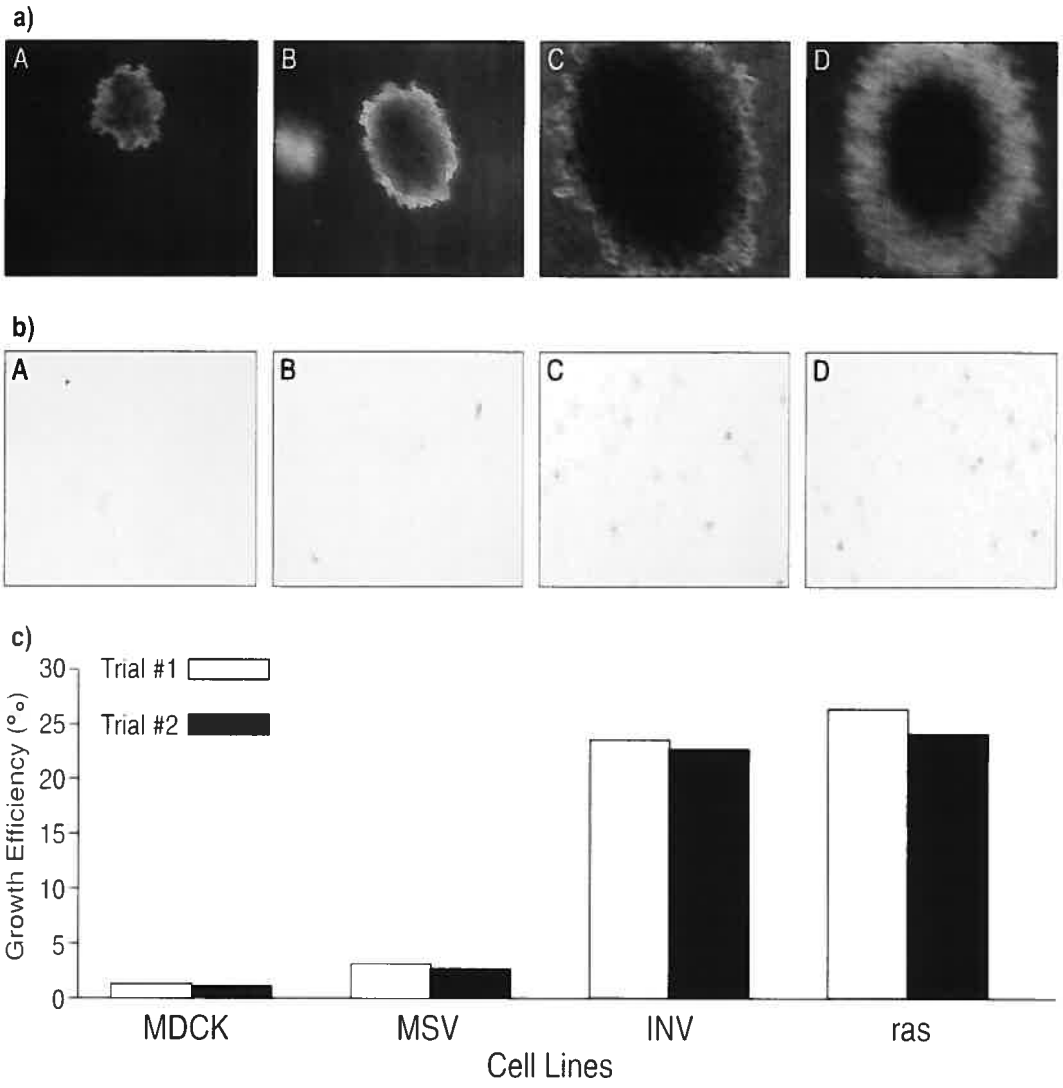

a)
A
B
C
D
b)
A
B
C
D
c)
Trial #1
Trial #2
Growth Efficiency (%)
30
25
20
15
10
5
0
MDCK
MSV
INV
ras
Cell Lines

Figure 2.2

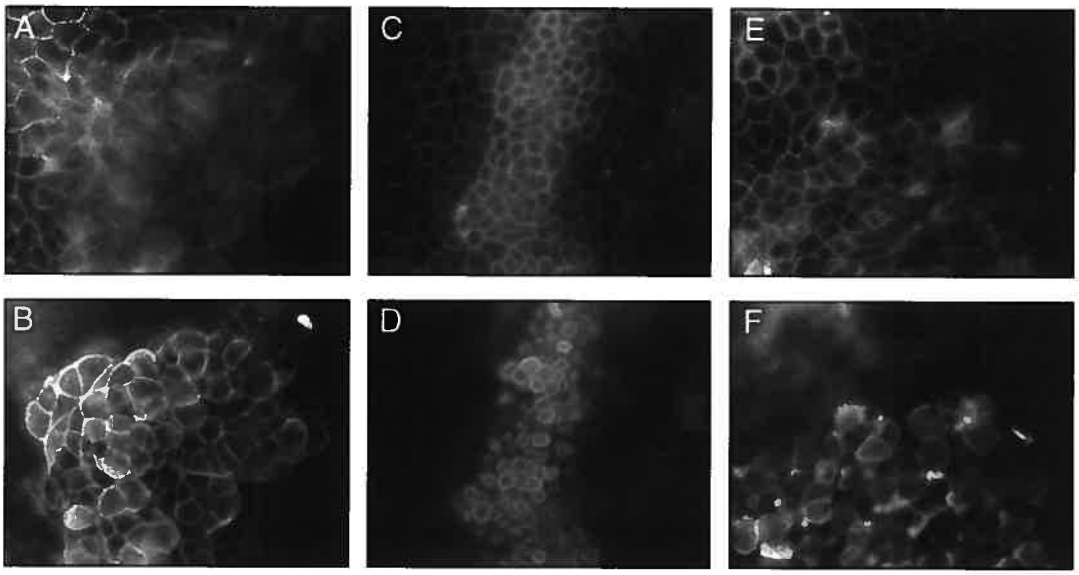
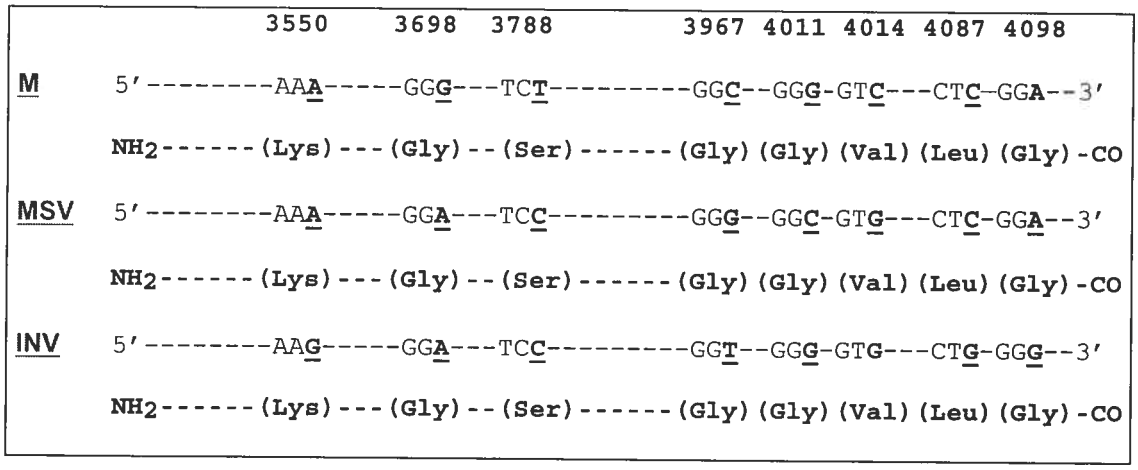


Figure 2.3

		Homology to canine sequence
Canine	5′–A------I---Y^{1349}-HV---Y^{1356}–V–V------SSQD-IDGEGDT-3′	
H.Sapiens	5 –A------I---Y^{1349}-HV---Y^{1356}–V–V------SSED-ADDEVDTRPASFWETS-3′	96%
M.Musculus	5′–S------I---Y^{1349}-HV---Y^{1356}–V–V------PSQD-IDGEGNT-3′	95%
R.Norveg.	5′–S------I---Y^{1349}-HV---Y^{1356}–V–V------PSQD-IDGEANT-3′	95%
G.Gallus	5′–T------I---Y^{1349}-HV---Y^{1356}–V–V------SSQD-TDMDVDT-3′	93%
X.Laevis	5′–S------L---Y^{1349}-LF---Y^{1356}–I–A------SPEG-IEFSIDT-3′	90%

Figure 2.4


3550 3698 3788 3967 4011 4014 4087 4098
M
5′--------AAA-----GGG---TCT---------GGC--GGG-GTC---CTC-GGA--3′
NH2------(Lys)---(Gly)--(Ser)------(Gly)(Gly)(Val)(Leu)(Gly)-CO
MSV
5′--------AAA-----GGA---TCC---------GGG--GGC-GTG---CTC-GGA--3′
NH2------(Lys)---(Gly)--(Ser)------(Gly)(Gly)(Val)(Leu)(Gly)-CO
INV
5′--------AAG-----GGA---TCC---------GGT--GGG-GTG---CTG-GGG--3′
NH2------(Lys)---(Gly)--(Ser)------(Gly)(Gly)(Val)(Leu)(Gly)-CO

CHAPTER 3
DISCUSSION

3.1 Study overview

Constitutive activation of the *met* proto-oncogene has for sometime been elucidated as being involved with cellular transformation and responsible for invasive and metastatic properties in cancer cells (Furge *et al.*, 2000; Comoglio *et al.*, 1999). The constitutive auto-phosphorylation of c-Met, its over-expression and the presence of point mutations are all elements reported to be responsible for the conversion of physiologically active c-Met to oncogenic forms (DiRenzo *et al.*, 1992; Schmidt *et al.*, 1997; Schmidt *et al.*, 1999; Schmidt *et al.*, 1998; Ferracini et al., 1995; Cooper *et al.*, 1984; Park *et al.*, 1986; Rong *et al.*, 1994). In this study, we examined the tumoral characteristics of cells expressing a highly phosphorylated c-Met (MSV-MDCK-INV) and the possible implications of c-Met, either through the presence of activating mutations or via other mechanisms. Like other research groups working with c-Met, we aimed to analyze the possible presence of point mutations in two very influential domains, namely the tyrosine kinase and C-terminal domains, which would help explain the highly phosphorylated and hence highly active c-Met in these MSV-MDCK-INV cells. In observing the cells' invasive phenotype and their ability to invade a Matrigel®-covered filter, we were immediately drawn to identifying specific mutations in c-Met that would help explain why these phenotypic characteristics exist only among these invasive cells and not their predecessors. Furthermore, explaining the transformed phenotype observed among the Moloney sarcoma virus-transformed MDCK cell line was also of interest to us. Indeed, while many mutations in c-Met were reported to be present in different cancers (Schmidt *et al.*, 1997; Schmidt *et al.*, 1999; Schmidt *et al.*, 1998; Park WS *et al.*, 1999; Jeffers *et al.*, 1997; Maritano *et al.*, 2000), there are only some mutations reported as being present in metastases of papillary renal carcinomas (Schmidt *et al.*, 1997; Schmidt *et al.*, 1999) and childhood hepatocellular carcinomas (Park WS *et al.*, 1999). These mutations were shown to induce metastasis when cells expressing the cDNA constructs were injected in mice (Jeffers *et al.*, 1997).

The main goal of our study is to contribute to the identification of mutations within the c-Met oncogene that, in long term perspective, would help in the possible

development of diagnostic kits which could be used in the identification of a mutated c-Met in patients before a possible cancer begins metastasizing. The most daunting prognosis for a cancer patient is to be told that the metastasis process has started. Treatment at this stage of the disease is quite difficult especially when considering that cancer cells travel and invade other tissues and/or organs.

Our working strategy for this study consisted of first identifying mutations in the canine c-Met of MSV-MDCK-INV cells and then transfecting MDCK cells and NIH3T3 cells with human c-Met containing the respective mutations. This would help assess the possibility of producing the invasive phenotype and functional changes observed among MSV-MDCK-INV cells in MDCK cells. In accordance to the results obtained from the first part of the project and depending on time constraints, the next step we set out to examine was the tumoral and metastatic ability of the transformed MDCK and NIH3T3 cells by injecting into nude mice.

Our efforts to sequence both the tyrosine kinase and C-terminal domains among the three cell lines as well as specific clones derived from the respective cell lines proved to be a success. By using primers synthesized on the basis of the human c-Met, we were able to amplify the same gene in canine cells. Unfortunately though, no mutations were found within the c-Met of MSV-MDCK-INV cells as compared to MSV-MDCK and MDCK cells. For this reason, we were unable to pursue this avenue of our research project. We did nonetheless report our findings in an article submitted to Clinical Experimental Metastasis (in revision) that acknowledged our hypothesis and efforts. In addition, we took the opportunity to characterize the tumoral properties of the MSV-MDCK-INV cell line in comparison to both the MSV-MDCK and MDCK cell lines. First, we placed all three cell lines in soft agar medium and analyzed their growth patterns. Next, we cultured cells from isolated soft agar colonies and examined their ability to form superficial colonies over a monolayer of epithelial cells.

3.2 Growth properties of MSV-MDCK-INV cells in soft agar and on a monolayer of epithelial cells.

Transformation of certain cell lines with viruses has been shown to induce AIG and the acquisition of AIG has been shown to correlate well with tumorigenic growth in many immortal rodent cell lines (Shin *et al.*, 1975). The invase character of the MSV-MDCK-INV cell model has been established previously (Le *et al.*, 1998; Vadnais *et al.*, 2002), but the tumoral phenotype has yet to be elucidated. Hence, one of the first objectives in this study was to assess the growth of these invasive cells in an anchorage-independent environment, soft agar, and compare it to the growth patterns observed for the parent cell lines MSV-MDCK and MDCK. We reported that foci formed from MSV-MDCK-INV and NIH3T3/*ras* cells (cells that were transformed by the *ras* oncogene and express highly tumorigenic characteristics) were similar in size **(Figure 2.1 a-b).** MSV-MDCK-INV cells also displayed growth efficiencies quite comparable to NIH3T3/*ras* cells **(Figure 2.1c)**. Although MSV-MDCK and MDCK cells did grow in soft agar, their growth efficiency and the size of their respective foci were much lower than observed for their invasive variant despite their overall comparable growth rates. In fact, these MSV-MDCK-INV cells do not grow faster than either MDCK or MSV-MDCK cells in culture. Therefore, their capacity to form large foci in soft agar at a fast rate may result from their ability to grow in an independent way, possibly through secretion of HGF.

The cells from each respective cell line grown on soft agar were subsequently isolated and kept in culture. In addition to growing in soft agar, tumoral cells should grow and strive over a monolayer of epithelial cells. The ability of MSV-MDCK-INV cells to replicate once seeded over a monolayer of MDCK epithelial cells was thus tested. Results illustrated in **Figure 2.2** showed that soft agar MSV-MDCK-INV cells, like the soft agar NIH3T3/*ras* cells, could replicate and form superficial colonies unlike the soft agar MSV-MDCK and MDCK cell lines. Furthermore, the original MSV-MDCK-INV and NIH3T3/*ras* cell populations (not shown) formed superficial colonies over the monolayer, whereas the MDCK and MSV-MDCK parent cell lines did not. In theory, the first step involved in extravasation of a tumor cell through a capillary, when referring to the development of a metastasis, is the

adhering of the cell to the basement membrane. It is only after the cells have adhered that they can proceed with the enzymatic digestion and movement through the basement membrane (Chambers and Hill, 1998). In this assay, epithelial MDCK cells form the tight monolayer onto which MSV-MDCK-INV cells adhere and grow. It is almost comparable to a tumor attaching to and penetrating through tissue. It would be of great interest to examine, would such an assay exist, whether or not these superficial colonies have managed to penetrate through the monolayer, or if cells forming the respective colonies just strive off of the cells forming the monolayer without penetrating. From the data shown in **Figure 2.2**, it is proposed that cells are able to penetrate through the monolayer and grow to form larger colonies.

The observation that MSV-MDCK-INV cells form foci in an anchorage-independent environment is a very good indication of their tumoral capacity. This observation though, does not specify any particular role of c-Met or HGF in the tumoral process. We already know that c-Met is involved with the invasive and motile phenotype of MSV-MDCK-INV cells (Vadnais *et al.,* 2000), but the role of activated c-Met in anchorage-independent growth has yet to be established. The best way to investigate this phenomenon would be to seed the cells in a normal culture medium containing the normal levels of growth factors, but to remove any trace of HGF through titration with a α-HGF antibody similar to what was performed by Vadnais and collaborators (Vadnais *et al.*, 2002). These results would definitely enable one to firmly establish c-Met and HGF's role in AIG among MSV-MDCK-INV cells. We however, did not proceed in performing these critical experiments. These experiments could become quite costly since anti-HGF would need to be added at high concentrations (5-20 μg/ml) and the culture medium (2ml) changed every two days for a period of 14 to 18 days. This procedure would have to be performed in triplicates in order to obtain one result.

Subsequently, cells grown on the epithelial cell monolayers should be studied in order to assess the role of HGF and/or c-Met in this phenotype. One feasible approach would be trypsinizing the cells and analyzing the level of c-Met

phosphorylation among the detached cells. If in fact c-Met is involved in their growth, then one may expect c-Met to display a higher level of tyrosine phosphorylation in MSV-MDCK-INV cells. Once again, as described in the preceeding paragraph, a α-HGF antibody should be used to remove any trace of HGF in the culture medium to establish whether HGF and/or c-Met activation were involved. This approach would also ensure that if the cells do produce HGF via an autocrine process (Vadnais *et al.*, 2002), and if this HGF secretion is essential for their growth on a monolayer, they would stop forming colonies and resemble their MDCK and MSV-MDCK parent cells.

As mentioned earlier, some of the assays described above can be quite costly. One other possibility would be to take advantage of the fact that these invasive cells secrete their own HGF via an autocrine activation loop (Vadnais *et al.*, 2002) and apply an inhibitor, such as suramin, that would interfere with both the secretion of HGF and binding of HGF to its receptor blocking the autocrine activity (Benini *et al.*, 1999). If the inhibitor succeeds at blocking the binding of HGF to its receptor and the subsequent activation of c-Met while simultaneously eliminating the capacity of these cells to form foci in soft agar and colonies on epithelial monolayers, then one may suspect involvement of c-Met activation.

A point worth remembering is that cells require certain growth factors and cellular interactions with the extracellular matrix for successful proliferation. For this reason, it would be of great interest to carry out the seeding of MSV-MDCK-INV cells over monolayers of cancer cells derived from different organs (e.g. colon cancer cells, liver cancer cells, etc…). If these invasive cells do in fact have the capacity to form colonies over the respective monolayers, analysis of the conditioned culture media would yield valuable information with regards to what growth factors or particular conditions are required for their growth. Therefore, it would help in understanding what allows these cells to proliferate under conditions where normal cells cannot.

One of the most important aspects that should be considered in future experimentation is the further classification of the MSV-MDCK-INV cell line as a tumorigenic cell line. Many of the preliminary steps like the growth in soft agar and the growth over a monolayer of epithelial cells have been analyzed already, but another important aspect worth examining would be the cells' capacity to form tumors *in vivo*. The initial stages of the *in vivo* study should involve the injection of MSV-MDCK-INV, MSV-MDCK, and MDCK cells into nude (athymic) mice to examine the incidence as well as frequency of tumor formation. A dissection of the resulting tumor and the culturing of tumoral cells for future analysis would also be of great value in understanding c-Met's role in tumor formation. If one would like to take this a step further, then another possibility would be looking at the culture medium in which these tumor cells grow, and determining its composition with regards to growth factors. The addition of autocrine activation loop inhibitors would also prove to be very useful especially in deducing whether or not the growth of cells that have metastasized can be blocked.

3.3 Absence of point mutation in the tyrosine kinase and C-terminal domains of c-Met

The tyrosine kinase domain of c-Met has for a long time been elucidated as being the enzymatic powerhouse of c-Met. In order to define the mechanism behind the constitutive phosphorylation of c-Met among the MSV-MDCK-INV cell line, which is seemingly one of the main reasons why this transformed, invasive and tumoral phenotype is observed, we assessed the possibility of point mutations in the tyrosine kinase domain, especially ones affecting the two tyrosine residues essential to the induction of enzymatic activity. We performed a RT-PCR reaction on the tyrosine kinase domain of c-Met using total RNA extracts from all three cell lines and then proceeded to automated DNA sequencing. A comparison between the primary nucleotide sequences obtained from the parental strain I MDCK cell line and those of other species reveals 96 % homology between the canine and the human sequence, 95% homology between the canine and the rat sequence, and 95 % between the canine and mouse sequences. It is clear from these numbers that evolution preserved

this amino acid sequence due to the unequivocal role of the tyrosine kinase domain in c-Met activity.

Sequence analysis of the tyrosine kinase domain of c-Met among MSV-MDCK and MSV-MDCK-INV cells as well as the sequence analysis of the tyrosine kinase domain of c-Met between MDCK and MSV-MDCK cells reveals no change in amino acid sequence for the 1100 nucleotides sequenced. These results indicate that the invasive phenotype observed from the MSV-MDCK-INV cell line cannot be attributed to a single point mutation within the tyrosine kinase domain of c-Met. Other regions of the c-Met protein, other proteins, or other mechanisms could however explain these phenotypes.

In the last few years evidence has been provided which demonstrates that the enzymatic activity of the tyrosine kinase domain of c-Met is controlled via other factors. Gual and collaborators pointed out the importance of the last 26 amino acids within the C-terminal domain of c-Met with the enzymatic activity of the tyrosine kinase domain which is critical for cell transformation but dispensable for invasive properties (Gual *et al.*, 2001). Furthermore, they examined the importance of the last 47 amino acids and the role this region plays in triggering different factors involved with various signalling pathways leading to cellular responses such as cell motility. As stated in Chapter 1, the C-terminal domain contains the multi-functional docking site comprising two tyrosine residues, Y^{1349} and Y^{1356}, which when phosphorylated bind to and activate accessory proteins involved in the signal transduction of various cellular responses. Based on these studies and considering that no mutations were found within the tyrosine kinase domain of c-Met between MSV-MDCK-INV and MSV-MDCK cells, and between MDCK and MSV-MDCK cells, we assumed a possible role for a specific region within the C-terminal domain of c-Met and hypothesized the presence of mutations which could explain the invasive and tumoral properties of the transformed MSV-MDCK-INV cell line. To do so, we performed RT-PCR using the same conditions like the ones used for the tyrosine kinase domain but with different primers. For this set of experiments as well, we used the human c-

Met sequence as a template for the design of the primers. Sequence analyses among the different species (data not shown) enabled us to observe that the 5' end of the C-terminal domain remains quite homologous. The 3' end on the other hand is much less homologous with regards to the various species. It is no wonder therefore, that the majority of human c-Met antibodies that recognize the canine c-Met protein were synthesized by using the N-terminal domain of the protein as a template instead of the C-terminal domain. In fact, all existing human antibodies produced on the basis of the C-terminal domain do not function appropriately with regard to recognizing the canine c-Met sequence (c-28 of Santa-Cruz and DQ-13 from Upstate Biotechnology). It is worth mentioning however that the DO-24 antibody (Upstate Biotechnology), which recognizes the N-terminal region of c-Met, yields reasonably good results when performing an immunoprecipitation assay, but fails to produce any signal on simple cellular lysates when performing a Western Blot (Vadnais *et al.*, 2002; Prat *et al.*, 1991). This emphasizes the importance, in some cases, of the native conformation of the epitope in addition to the primary amino acid sequence of the protein for antibody recognition. It was only by combining the biotinylation of surface proteins to a c-Met immunoprecipitation in the presence of the DO-24 antibody and revealing the biotinylated c-Met protein with streptavidin-HRP, that our laboratory was able to quantify the surface expression of c-Met (Vadnais *et al., 2002*).

Although the results obtained with regard to the sequencing of both the tyrosine kinase and C-terminal domains of c-Met among the three cell lines failed to demonstrate the presence of any point mutations which would help explain the invasive and transformed phenotypes observed among MSV-MDCK-INV and MSV-MDCK cells respectively, they did nonetheless help establish a partial (kinase domain and C-terminal domain) canine c-Met DNA sequence. There are quite a few advantages in knowing the canine c-Met sequence, especially to produce specific c-Met antibodies which up to date remain unavailable. This would help eliminate the dilemma presented previously where human antibodies recognize the N-terminal domain of the canine c-Met and not the C-terminal domain. When working with an antibody synthesized on the basis of the canine c-Met, the assay, whether it be an

immunoprecipitation or a Western Blot on simple cellular lysates, should yield similar results when working with canine cell lysates. Another important advantage is the cloning of the canine c-Met DNA sequence. To date, just over three thousand studies have been published with relation to MDCK cells and c-Met. Knowing the canine c-Met sequence would definitely be useful to research groups who want to study the effect of novel mutations in c-Met of canine cells, and to those interested in establishing a canine genomic sequence.

Having established the fact that no point mutations existed in c-Met between the MDCK, MSV-MDCK and MSV-MDCK-INV cell lines, our curiosity led us to further examine the DNA sequence of the respective cell lines. To our surprise, we discovered the presence of silent mutations between the total populations of the different cell lines. Silent mutations are referred to as "silent" because they do not induce a change in the amino acid sequence of a protein, thus not resulting in a mutation, but only result in changes of the nucleotide sequence. For this reason, we can only identify their positioning with respect to the canine c-Met tyrosine kinase domain. The importance of acknowledging the presence of silent mutations arises when one considers the synthesis of an antibody against the tyrosine kinase domain of the canine c-Met. In such a case, the areas on the sequence where most of these silent mutations are located may be considered sensitive or susceptible to mutation, especially after cells have been exposed to an environmental stress or transforming agents such as a virus.

Moreover, due to the fact that we were working with two strains of the original MDCK cell line (i.e. Strain I; from a tight epithelium and Strain II; from a loose epithelium), and because Strain II illustrated a more scattered phenotype, we wanted to assess the possibility of mutations between these two strains. Once again we performed a RT-PCR experiment with primers designed on the basis of the human c-Met sequence. Sequence analysis of the two strains did not show evidence of point mutations within either the tyrosine kinase domain or the C-terminal domain of these cells. Therefore, we believe that another mechanism exists which can explain the

more scattered phenotype observed among Strain II cells. Further experimentation would be required to examine the possible mechanism behind this difference in phenotype, but unfortunately, this would require a substantial amount of time.

In order to ascertain the results obtained with regards to the DNA sequences and the silent mutations identified, and in order to evaluate the importance of the silent mutations, we continued our analysis by performing the same assays on single clones derived from the MDCK cell line (clone E16), the MSV-MDCK cell line (clone TE6) and the MSV-MDCK-INV cell line (clone 5.1). The results we obtained with this set of experiments were very interesting and helped confirm certain aspects of our cell lines. A comparison of the sequences obtained from all three cell lines at the protein level and at the DNA level did not reveal any point mutations or silent mutations in the tyrosine kinase and C-terminal domains of c-Met. This is in comparison to the parental MDCK Strain I cell line. Therefore, this result was extremely valuable because it raised the possibility that the two transformed total population cell lines studied initially may be heterogeneous populations. In other words, if we take for example the total population MSV-MDCK-INV cell line, we can assume that it contains various clones all of which are invasive, but at different degrees.

RT-PCR amplification of the canine tyrosine kinase and C-terminal domains of c-Met from total RNA proved useful in determining the presence or absence of mutations among the three respective cell lines. As mentioned earlier in this report, the canine DNA sequence for c-Met has yet to be published. Therefore, we were obliged to use the human DNA sequence as a template for the design of primers. Another approach that one might consider taking is the actual cloning of the entire canine c-Met protein including the tyrosine kinase and C-terminal domains. Indeed, we cannot rule out the possibility that a point mutation or an amino acid deletion elsewhere within the protein could influence the autophosphorylation or subsequent activity of the receptor. Cloning the canine cDNA would of course take a substantial amount of time, but would be extremely useful for everyone working with canine cells. As seen previously, it could help in the synthesis of antibodies specific to the canine sequence

or even in assessing the effects of novel point mutations on cellular responses including motility and invasiveness. In addition, it would be a plausible start to the mapping of the canine genome.

The results demonstrating the absence of point mutations in both the tyrosine kinase and C-terminal domains of the canine c-Met are very important in helping us solve the molecular mechanism behind the invasive phenotype expressed by MSV-MDCK-INV cells. Indeed, two hypotheses were proposed initially and they involved the presence of point mutations in the tyrosine kinase and C-terminal domains of c-Met, an activating HGF loop, or both taken together. In the present study, we demonstrated that mutations in c-Met cannot explain the high level of c-Met autophosphorylation and hence the invasive phenotype. Therefore, this leaves us only with the autocrine HGF activation loop which has been examined extensively by Vadnais and collaborators as the possible mechanism behind c-Met's increased activity and the invasive character of MSV-MDCK-INV cells (Vadnais *et al.*, 2002).

The autocrine HGF activation loop, as its name implies, involves the secretion of HGF from the cell in close proximity to the cell surface receptor, HGF binding and c-Met activation. As a seemingly repetitive process, one may expect c-Met to be desensitized after some time due to the continuous presence of HGF. Indeed, some receptors possessing an intrinsic tyrosine kinase activity are downregulated following activation by ligand (Ullrich and Schlessinger, 1990). The downregulation or desensitization is present in cells to avoid overstimulation of the downstream signaling cascades which could lead to cellular transformation. Two main mechanisms are possible; the receptor internalization via coated pits and the ubiquitin-proteasome degradation. Jeffers and collaborators have shown that the proteasomal activity is crucial for c-Met degradation after an acute stimulation with hepatocyte growth factor (HGF) (Jeffers *et al.*, 1997). The ubiquitination followed by degradation which constitutes this proteasomal activity, ensures a short half-life for Met receptors and thus helps prevent any c-Met overstimulation. When lactacystin, an inhibitor of proteasome activity was used, degradation of c-Met was blocked.

Based on these observations and previous results illustrating that, in steady state conditions, there is an equal concentration of surface c-Met receptors for MSV-MDCK-INV cells (with a continuous activation of c-Met) as their parental MSV-MDCK and MDCK cells (Vadnais *et al.*, 2002), we can postulate that there is no proteasomal activity in the MSV-MDCK-INV cell line. This could help explain their tumoral phenotype. It would be interesting to demonstrate this hypothesis by studying the ubiquitination level and the c-Met half-life upon stimulation with HGF in the three cell lines, especially the MSV-MDCK cells. Ubiquitination and degradation of c-Met are expected in MSV-MDCK cells upon HGF treatment. Vadnais and collaborators have already observed a rapid tyrosine phosphorylation of c-Met followed by a decrease in phosphorylation 3 hours following treatment in these MSV-MDCK cells (Vadnais *et al.*, 2002). The loss of a receptor following proteasomal degradation could be involved in this phenomenon. Unfortunately, the level of c-Met receptors was not determined in the aforementioned series of experiments due to a lack of appropriate antibodies capable of recognizing c-Met through a Western Blot. For this reason, no conclusions can be drawn with absolute certainty. It would however be of great interest to investigate this aspect further.

The specific activation of downstream signalling events represents another possibility behind c-Met activation which results in tumorigenic properties. By using the specific inhibitor U0126 and LY294002, our research group has shown that both the ras-MEK-ERK and PI3kinase-Akt pathways are highly activated in MSV-MDCK-INV cells as compared to MDCK cells. Moreover, both pathways have been shown to be involved with cell motility and invasion (results not yet published). However, recent results obtained by treating MSV-MDCK-INV cells with high concentrations of anti-HGF to block c-Met activation have shown that, although fully active in the absence of serum, ERK1/2 activation (phosphorylation) is independent of c-Met activation. Furthermore, Akt activation in MSV-MDCK-INV cells is dependent on serum, suggesting that c-Met activation by the autocrine loop is not sufficient to activate the PI3-kinase-Akt pathway. These results thus suggest that the autocrine HGF loop is activating one or more undefined signalling pathway(s) such as rac and

MEK3,6/p38^{MAPK} that may be responsible, in large part, for the motile and invasive phenotype. A study performed by our research group in collaboration with I.R. Nabi has recently shown that p38^{MAPK} downstream of activated rho is involved with blebbing, pseudopod formation and cell motility (Jia *et al.*, 2003). Other similar pathways may exist and should be examined in future experiments.

Conclusion

In this study, we demonstrated the tumoral phenotype of MSV-MDCK-INV cells through their capacity to grow in anchorage-independent environments. In addition, our findings suggest that point mutations in neither the C-terminal or the tyrosine kinase domains may explain the transformed phenotype observed among MSV-MDCK cells and the invasive/tumoral phenotype observed among MSV-MDCK-INV cells. Our results lead to the conclusion that the major mechanism leading to the activation of the kinase domain of c-Met, to invasion and to the tumoral phenotype observed among MSV-MDCK-INV cells, although not exclusive, would be the autocrine HGF activation loop (Vadnais *et al.*, 2002). The mechanism involved in transformation by the Moloney sarcoma virus was however not elucidated.

Continued work on these cells with regards to their tumoral characteristics can prove to be very useful in the field of both fundamental research and clinical medicine. By establishing their capacity to form tumors in nude mice, we can introduce a new tumorigenic cellular model which can be used in research laboratories to study the mechanisms behind cancer and metastasis and as well, to assess the effect of anti-cancer drugs on reducing or eliminating the incidence of cancer. In addition, continued studies of c-Met can prove extremely valuable in understanding more deeply how this oncogene is involved with cancer. Due to the seemingly important role that mutations in c-Met play in certain cancers, it would be interesting to establish diagnostic kits which could help identify, from the beginning, mutations in c-Met, especially those involved with metastasis. This would lead to a more hopeful prognosis that could eventually present metastases spreading throughout the body.

REFERENCES

Balkovetz DF, Pollack AL, and Mostov KE (1997): Heptocyte growth factor alters the polarity of Madin Darby canine kidney cell monolayers. *J. Biol. Chem.* **272**, 3471-3477.

Bardelli A, Pugliese L, Comoglio PM (1997): Invasive-growth signalling by the *Met*/HGF receptor The hereditary renal carcinoma connection. Biochimica et Biophys. Acta. **1333**, M41-M51.

Bardelli A, Longati P, Gramaglia D, Basilico C, Tamagnone L, Giordano S, Ballinari D, Michieli P, Comoglio PM (1998): Uncoupling signal transducers from oncogenic MET mutants abrogates cell transformation and inhibits invasive growth. *Proc. Natl. Acad. Sci USA* **95**, 14379-14383.

Bardelli A, Longati P, Williams TA, Benvenuti S and Comoglio PM (1999): A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth. *J. Biol. Chem.* **274**, 29274-29281.

Behrens J, Mareel MM, Van Roy RM, Birchmeier W (1989): Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol.***108,** 2435-2447.

Benini S, Baldini N, Manara MC, Chano T, Serra M, Rizzi S, Lollini PL, Picci P, Scotlandi R (1999): Redundancy of autocrine loops in human osteosarcoma cells. *Int. J. Cancer* **80**, 581-588.

Berkow R (1997): The Merck Manual of Medical Information Home ed. Pocket Book Publishing. Section 15 page 865.

Birchmeier C and Birchmeier W (1993): Molecular aspects of mesenchymal-epithelial interactions. *Annu. Rev. Cell Biol.* **9**, 511-540.

Birchmeier W, Behrens J (1994): Cadherin expression in carcinomas: Role in the formation of cell junctions and the prevention of invasiveness. *Biochim. Biopys. Acta* **1198,** 11-26.

Bishop JM (1985): Viral oncogenes. *Cell* **42,** 23-38.

Bladt F, Riethmacher D, Isenmann S, Aguzzi A and Birchmeier C (1995): Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**, 768-771.

Blair DG, Cooper CS, Oskarsson MK, Eader LA & Vande Woude GF (1982): New method for detecting cellular transforming genes *Science* **218,** 1122-1125.

Blasi F. (1993): Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. *BioEssays* **15**, 105-111.

Boccaccio C, Ando M, Tamagnone L, Bardelli A, Michieli P, Battisitini C and Comoglio PM (1998): Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* **391**, 285-288.

Boccaccio C, Gaudino G, Gambarotta G, Galimi F and Comoglio PM (1994): Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. *J. Biol. Chem.* **269**, 12846-12851.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude G and Aaronson SA (1991): Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251**,802-804.

Brown MC, Perrotta JA, and Turner CE (1996): Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J. Cell Biol.* **135,** 1109-1123.

Bussolino F, Di Renzo MF, Ziche M, Bocchieto E, Olivero M, Naldini L, Gaudino G, Tamagone L, Coffer A and Comoglio PM (1992): Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J. Cell Biol.* **119**, 629-641.

Cary LA, Chang JF, and Guan JL (1996): Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J. Cell Sci.* **109,** 1787-1794.

Chambers AF and Hill RP (1998): Tumor Progression and Metastasis in: Tannock IF and Hill RP. **The Basic Science of Oncology**. 3rd ed. McGraw-Hill, pp. 219-239.

Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD, and Guan JL (1995): Interaction of focal adhesion kinase with cytoskeletal protein talin. *J. Biol. Chem.*, **270,** 16995-16999.

Chen HC, Appeddu PA, Isoda H, and Guan JL (1996): Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J. Biol. Chem.* **271,** 26329-26334.

Clark P (1994): Modulation of scatter factor/hepatocyte growth factor activity by cell-substratum adhesion. *J. Cell Sci.* **107**,1265-1275.

Comoglio PM, Tamagnone L, Boccaccio C (1999): Plasminogen-related growth factor and semaphoring receptors: a gene superfamily controlling invasive growth. *Exp. Cell Res.* **253,** 88-89. Review

Comoglio PM and Vigna E (1995): Structure and functions of the HGF receptor (c-Met). In *Liver Regeneration and Carcinogenesis* (ed. R. L. Jirtle), San Diego, Academic Press, pp. 51-70.

Cooper CS, Park M, Blair DG, Tainsky MA, Huebner K, Croce CM, & Vande Woude GF, (1984): Molecular cloning of a new transforming gene from a chemically transformed human cell line. *Nature (London)*, **311,** 29-33.

de Klein A, Van Kessel AG, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK & Heisterkamp N, Groffen J, Stephenson JR (1982): A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature (London)* **300,** 765-767.

Dean M, Park M & Vande Woude GF (1987): Characterization of the rearranged tpr-met oncogene breakpoint. *Mol. Cell. Biol.* **7,** 921-924.

Derynck R (1994): TGF-beta-receptor-mediated signaling (review). *Trends Biochem. Sci.* **19,** 548-553.

Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pilotti S, *et al.*(1992): Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. *Oncogene* **7,** 2549-53.

DiRenzo MF, Poulsom R, Olivero M, Comoglio PM & Lemoine NR (1995): Expression of the Met/Hepatocyte Growth Factor Receptor in Human Pancreatic Cancer. *Cancer Research* **55**, 1129-1138.

Ebens A, Brose K, Leonardo ED, Hanson MG, Bladt F, Birchmeier C, Barres BA and Tessier-Lavigne M (1996): Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. *Neuron* **17**, 1157-1172.

Ferracini R, Longati P, Naldini L, Vigna E, Comoglio PM (1991): Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. *J Biol Chem.* **266**,19558-19564.

Ferracini R, Di Renzo MF, Scotland K, Baldini N, Olivero M, Lollini PL, Cremona O, Campanacci M, and Comoglio PM (1995): The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. *Oncogene.* **10,** 739-749.

Fisher B, Szuch P, Levine M and Fisher ER (1971): A portal blood factor as the humoral agent in liver regeneration. *Science* **171,** 575-577.

Folkman J (1976): The vascularization of tumors. *Sci. Am.* **234,** 58-73.

Folkman J, Klagsbrun M (1987): Angiogenic factors. *Science* **235,** 442-447.

Furge KA, Zhang YW, and Vande Woude GF (2000): Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* **19,** 5582-5589.

Gandino L, Di Renzo MF, Giordano S, Bussolino F and Comoglio PM (1990): Protein kinase-c activation inhibits tyrosine phosphorylation of the c-met protein. *Oncogene* **5,** 721-725.

Gandino L, Longati P, Medico E, Prat M, and Comoglio PM (1994): Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. *J. Biol. Chem.* **269,** 1815-1820.

Gaush CR, Hard WL, and Smith TF (1966): Characterization of an established line of canine kidney cells (MDCK). *Proc. Soc. Exp. Biol. Med.* **122**, 931-935.

Gilmore AP, and Romer LH (1996): Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol. Cell Biol.***8**, 1209-1224.

Giordano S, Di Renzo MF, Narsimhan RP, Cooper CS, Rosa C, and Comoglio PM (1989a): Biosynthesis of the protein encoded by the c-met proto-oncogene. *Oncogene* **4**, 1383-1388.

Giordano S, Zhen Z, Medico E, Gaudino G, Galimi F and Comoglio PM (1993): Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. *Proc. Natl. Acad. Sci.USA* **90**, 649-653.

Giordano S, Maffe A, Williams TA, Artigiani S, Gual P, Bardelli A, Basilico C, Michieli P, and Comoglio PM (2000): Different point mutations in the met oncogene elicit distinct biological properties. *FASEB* **14**, 399-406.

Gohda E, Tsubouchi H, Nakayama H, Hirono S, Sakiyama O, Takahashi K, Miyazaki H, Hashimoto S, Daikuhara Y (1988)**:** Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure.**:** *J. Clin. Invest.* **81,** 414-419.

Graziani A, Gramaglia D, Cantley LC and Comoglio PM (1991): The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase.*J Biol. Chem.* **266,** 22087-22090.

Gual P, Giordano S, Anguissola S and Comoglio PM (2001): Differential requirement of the last C-terminal tail of Met receptor for cell transformation and invasiveness. *Oncogene* **20**, 5493-5502.

Hanna N (1984): Role of natural killer cells in host defense against cancer metastasis. In: Nicolson GL, Milas L eds. *Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects.* Raven Press, pp. 309-319.

Heppner GH, Miller FR (1998): The cellular basis of tumor progression. *Int. Rev. Cytology* **177,** 1-56.

Hulsken J, Birchmeier W, and Behrens J (1994): E-cadherin and APC compete for the interaction with beta –catenin and the cytoskeleton. *J. Cell Biol.* **127**, 2061-2069.

Igawa T, Kanada S, Kanetake H, Saitoh Y, Ichihara A, Tomita Y & Nakamura T (1991): Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. *Biochem. Biophys. Res. Commun.* **174,** 831-838.

Ihle JN (1996): STATs signal transducers and activators of transcription [Review]. *Cell* **84**, 331-334.

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T, and Aizawa S (1995): Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377,** 539-544.

Jeffers M, Rong S, and Vande Woude GF (1996): Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. *J Mol. Med.* **74,** 505-513.

Jeffers M, Schmidt L, Nakaigawa N, Webb CP, Weirich G, Kishida T, Zbar B, and Vande WG (1997): Activating mutations for the met tyrosine kinase receptor in human cancer. *Proc. Natl. Acad, Sci. USA* **94**, 11445-11450.

Jeffers M, Taylor GA, Weidner KM, Omura S, Vande Woude GF (1997): Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. *Mol. Cell Biol.* **17**, 799-808.

Jia Z, Barbier L, Vadnais J, Lu M, Pelech S, Noel J and Nabi IR (2003): Autocrine c-Met/HGF-R activation regulates cellular blebbing in tumor cell motility via a rho/ROCK/p38MAP kinase pathway. *J. Cell Biol.* (submitted in September 2003).

Joseph A, Weiss GH, Jin L, Fuchs A, Chowdhury S, O'Shaugnessy P, Goldberg ID and Rosen EM (1995): Expression of scatter factor in human bladder carcinoma. *J. Natl. Cancer Inst.* **87**, 372-377.

Kan M, Zhang G, Zarneger R, Michalopoulos G, Myoken Y, Mckeehan WL & Stevens JI (1991): *Biochem. Biophys. Res. Commun.* **174**, 331-337.

Khwaja A, Lehmann K, Marte BM, and Downward J (1998): Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. *J. Biol. Chem.* **273**, 18793-18801.

Klein G (1998) Foulds' dangerous idea revisited: The multistep development of tumors 40 years later. *Adv. Cancer Res.***72,** 1-23.

Knudson AG (1993): Antioncogenes and human cancer. *Proc. Natl. Acad. Sci. USA* **90,** 10914-10921.

Larabell CA, Torres M, Rowning BA, Yost C, Miller JR, Wu M, Kimelman D, and Moon RT (1997): Establishment of the dorso ventral axis in Xenopus embryos is presaged by early asymmetries in beta catenin that are modulated by the Wnt signalling pathway. *J. Cell Biol.* **136**, 1123-1136.

Le PU, Nguyen TN, Drolet-Savoie P, Leclerc N, Nabi IR (1998) : Increased β-actin expression in an invasive Moloney Sarcoma Virus-transformed MDCK cell variant concentrates to the tip of multiple pseudopodia. *Cancer Res.* **58,**1631-1635.

Lindroos P, Tsai WH, Zarnegar R & Michalopoulos GK (1992)**:** Plasma levels of HGF in rats treated with tumor promoters *Carcinogenesis* **13,** 139-141.

Liotta LA (1986): Tumor invasion and metastases-Role of the extracellular matrix. *Cancer Res.* **46,** 1-7.

Loeb LA (1998): Cancer cells exhibit a mutator phenotype. *Adv. Cancer Res.* **72,** 25-56.

Longati P, Bardelli A, Ponzetto C, Naldini L and Comoglio PM (1994): Tyrosines 1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). *Oncogene* **9**, 49-57.

Longati P; Comoglio PM, Bardelli A (2001): Receptor tyrosine kinases as therapeutic targets: the model of the MET oncogene. *Current Drug Targets* **1**, 41-55.

Lu C, Kerbel RS (1994): Cytokines, growth factors and the loss of negative growth controls in the progression of human cutaneous malignant melanoma. *Curr. Opin. Oncol.* **6,** 212-220.

MacDougall JR, Matrisian LM (1995): Contributions of tumor and stromal matrix mettaloproteinases to tumor progression, invasion and metastasis. *Cancer Metastasis Rev* **14,** 351-362.

Maina F, Casagranda F, Audero E, Simeone A, Comoglio P, Klein RA and Ponzetto C (1996): Uncoupling of grb2 from the met receptor in vivo reveals complex roles in muscle development. *Cell* **87**, 531-542.

Maritano D, Accornero P, Bonifaci N, and Ponzetto C (2000): Two mutations affecting conserved residues in the met receptor operate via different mechanisms. *Oncogene* **19,** 1354-1361.

Martin-Zanca D, Mitra G, Long LK, Barbacid M (1986): Molecular characterization of the human trk oncogene. *Cold Spring Harb Symp Quant Biol.***51**, 983-92.

Matsumoto K and Nakamura T (1993): Roles of HGF as a pleiotropic factor in organ regeneration. *In Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c*-met *Receptor*(ed. I.D. Goldberg and E.M. Rosen), pp. 225-249.

Matsumoto Kentaro, Matsumoto Kunio, Nakamura T, and Kramer RH (1994): Hepatocyte Growth Factor/Scatter factor Induces Tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$) and promotes migration and invasion by oral squamous cell carcinoma cells. *J. Biol. Chem.* **269**, 31807-31814.

Matsumoto K, Hashimoto K, Yoshikawa K & Nakamura T (1991)**:** Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. *Exp. Cell Res.* **196,** 114-120**.**

McCrea PD, Brieher WM, and Gumbiner BM (1993): Induction of a secondary body axis in Xenopus by antibodies to beta-catenin. *J. Cell Biol.* **123**, 477-484.

Medico E, Mongiovi AM, Huff J, Jelinek MA, Follenzi A, Gaudino G, Parsons JT, and Comoglio PM (1996): The tyrosine kinase receptors Ron and Sea control "scattering" and morphogenesis of liver progenitor cells *in vitro*. *Mol. Biol. Cell* **7**, 495-504.

Michalopoulos G, Houck KA, Dolan ML and Leutteke NC (1984): Control of hepatocyte replication by two serum factors. *Cancer Res.* **44,** 4414-4419.

Mignatti P, Rifkin DB (1993): Biology and biochemistry of proteinases in tumor invasion. *Physiol. Rev.***73,** 161-195.

Miyazawa K, Shimomura T, Naka D and Kitamura N (1994): Proteolytic activation of hepatocyte growth factor in response to tissue injury. *J. Biol. Chem.* **269**, 8966-8970.

Mizuno K and Nakamura T (1993): Molecular characteristics of HGF and the gene, and its biochemical aspects. *In Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c*-met *Receptor* (ed. I.D. Goldberg and E.M. Rosen), pp. 1-29. Basel: Birkhauser Verlag.

Montesano R, Schaller G, and Orci L (1991a): Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell* **66**, 697-711.

Montesano R, Matsumoto K, Nakamura T, and Orci L (1991b): Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67**, 901-908.

Moolten FL, Buchner NL (1967): Regeneration of rat liver: transfer of humoral agent by cross circulation. *Science* **158,** 272-274.

Mostov KE, and Deitcher DL (1986): Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA. *Cell* **46**, 613-621.

Nagafuchi A, and Takeichi M (1989): Transmembrane control of cadherin-mediated cell adhesion:a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul.* **1**, 37-44.

Nakamura T, Nawa K and Ichihara A (1984): Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* **122,** 1450-1459.

Nakamura T, Teramoto H & Ichihara A (1986): Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc Natl Acad Sci U S A.* **17**, 6489-6493.

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, et al. (1989): Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342**, 440-443.

Nakamura T(1991): Structure and function of hepatocyte growth factor. *Prog. Growth Factor Res.* **3**, 67-85.

Naldini L, Tamagnone L, Vigna E, Sachs M, Hartmann G, Birchmeier W, Daikuhara Y, Tsubouchi H, Blasi F and Comoglio PM (1992): Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. *EMBO J.* **11**, 4825-4833.

Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, Narsimhan RP, Hartmann G, Zarnegar R, Michalopoulos GK *et al.* (1991): Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J.* **10**, 2867-2878

Nicolson GL (1982): Cancer metastasis: Organ colonization and the cell-surface properties of malignant cells. *Biochim. Biophys. Acta* **695,** 113-176.

Nicolson GL (1988): Organ specificity of tumor metastasis: Role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev.* **7,** 143-188.

Nicolson GL, Menter DG (1995): Trophic factors and central nervous system metastasis. *Cancer metastasis Rev.* **14,** 303-321.

Niranjan B, Buluwela L, Yant J, Perusinghe N, Atherton A, Phippard D, Dale T, Gusterson B and Kamalati T (1995): HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. *Development* **121**, 2897-2908.

Nowell, P (1986): Mechanisms of tumor progression. *Cancer Res.* **46,** 2203-2207.

Park M, Dean M, Cooper CS, Schmidt M, O'Brien SJ, Blair DG,Vande Woude GF(1986): Mechanism of met oncogene activation. *Cell* **45**,895-904.

Park CY, Hayman MJ(1999): The tyrosines in the bidentate motif of the env-sea oncoprotein are essential for cell transformation and are binding sites for Grb2 and the tyrosine phosphatase SHP-2. *J Biol Chem.* **274,**7583-7590.

Park WS, Dong SM, Kim SY, Na EY, Shin MS, Pi JH, Kim BJ, Bae JH, Hong YK, Lee KS, Lee SH, Yoo NJ, Jang JJ, Pack S, Zhuang Z, Schmidt L, Zbar B, and Lee JY (1999): Somatic mutations in the kinase domain of the met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res.* **59**, 307-310.

Pavelic ZP, Slocum HK, Rustum YM, Creaven PJ, Darakousis C, and Takita H (1980): Colony growth in soft agar of muna melanoma, sarcoma, and lung carcinoma cells disaggregated by mechanical and enzymatic methods. *Cancer Res.* **40**, p. 2160-2164.

Pelicci G, Giordano S, Zhen Z, Salcini AE, Lanfrancone L, Bardelli A, Panayotou G, Waterfield MD, Ponzetto C, Pelicci PG and Comoglio PM (1995): The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. *Oncogene* **8,** 1631-1638.

Pepper MS, Matsumoto K, Nakamura T, Orci L and Montesano R (1992): Hapatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. *J. Biol. Chem.* **267**, 20493-20496.

Pollack AL, Barth AIM, Altschuler Y, Nelson WJ, and Mostov KE (1997): Dynamics of B-catenin interactions with APC protein regulate epithelial tubulogenesis. *J. Cell Biol.* **137**, 1651-1662.

Ponzetto C, Giordano S, Peverali F, Della Valle G, Abate ML, Vaula G, Comoglio PM (1991): c-met is amplified but not mutated in a cell line with an activated met tyrosine kinase. *Oncogene* **6**, 553-559.

Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, Graziani A, Panayotou G and Comoglio PM (1994): A multifunctional docking site mediates

signaling and transformation by the hepatocyte growth factor/scatter factor receptor family.*Cell* **77**, 261-71.

Prat M, Crepaldi T, Gandino L, Giordano S, Longati P, Comoglio PM (1991): C-terminal truncated forms of Met, the hepatocyte growth factor receptor. *Mol. Cell Biol.* **12,** 5954-5962.

Prat M, Crepaldi T, Pennacchetti S, Bussolino F and Comoglio PM. (1998): Agonistic monoclonal antibodies against the Met receptor dissect the biological responses to HGF. *J. Cell Sci.*, **111,** 237-247.

Rahimi N, Tremblay E, McAdam L, Park M, Schwall R, and Elliot B (1996): Identification of a Hepatocyte Growth Factor Autocrine Loop in a Murine Mammary Carcinoma. *Cell Growth & Differentiation* **7,** 263-270.

Reddy EP, Smith MJ & Srinivasan A (1983): Nucleotide sequence of Abelson murine leukemia virus genome: structural similarity of its transforming gene product to other onc gene products with tyrosine-specific kinase activity. *Proc. Natl. Acad. Sci. USA* **80,** 3623-3627.

Richardson A, and Parsons JT (1996): A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. *Nature* **380,** 538-540.

Ridley AJ and Hall A (1992): The small GTP binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.

Ridley AJ (1994): Membrane ruffling and signal trannsduction [Review] *Bioessays* **16**, 321-327.

Ridley AJ, Comoglio PM, and Hall A (1995): Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. *Mol. Cell Biol.* **15**, 1110-1122.

Rogriguez-Boulan E, and Nelson WJ (1989): Morphogenesis of the polarized epithelial cell phenotype. *Science* **245**, 718-725.

Rong S, Segal S, Anver M, Resau JH and Vande Woude GF (1994): Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. *Proc.Nat.Acad Sci. USA* **91**, 4731-4735.

Rosen EM, Joseph A, Jin L, Rockwell S, Elias JA, Knesel J, Wines J, McClellan J, Kluger MJ, Goldberg ID *et al.* (1994): Regulation of scatter factor production via a soluble inducing factor. *J. Cell Biol.* **127**, 225-234.

Rubin JS, Chan AML, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW & Aaronson SA (1991): A broad-spectrum human

lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc. Natl. Acad. Sci. USA* **88**, 415-419.

Rubinfeld B, Souza B, Albert I, Muller O, Chamberlain SH, Masiarz FR, Munemitsu S, and Polakis P (1993): Association of the APC gene product with beta catenin. *Science* **262**, 1731-1734.

Santos OF, and Nigam SK (1993): HGF induced tubulogenesis and branching of epithelial cells is modulated by extracellular matrix and TGF beta. *Dev. Biol.* **160**, 293-302.

Santos OF, Barros EJ, Yang XM, Matsumoto K, Nakamura T, Park M and Nigam SK (1994): Involvement of hepatocyte growth factor in kidney development. *Dev. Biol.* **163**, 525-529.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR and Parsons JT (1994): Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol. Cell Biol.* **14,** 1680-1688.

Schaller MD, Otey CA, Hildebrand JD, Parsons JT (1995): Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J. Cell Biol.*, **130,**1181-1187.

Schlaepfer DD, and Hunter T (1997): Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J. Biol. Chem.* **272,** 13189-13195.

Schlaepfer DD, Broome MA, and Hunter T (1997): Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol. Cell Biol.* **17,** 1702-1713.

Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, and Birchmeier C (1995): Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**, 699-702.

Schmidt L, Duh FM, Chen F, Kishida TL, Glenn P, Choyke SW, Scherer Z, Zhuang I, Lubensky M, Dean R, Allikmets A, Chidambaram UR, Bergerheim JT, Feltis C, Casadevall A, Zamarron M, Bernues S, Richard CJM, Lips MM, Walther LC, Tsui L, Geil ML, Orcutt T, Stackhouse J, Lipan L, Slife H, Brauch J, Decker G, Niehans MD, Hughson H, Moch S, Storkel MI, Lerman WM, Linehan and Zbar B (1997): Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nature Genetics* **16,** 68-73.

Schmidt L, Junker K, Weirich G, Glenn G, Choyke P, Lubensky I, Zhuang Z, Jeffers M, Vande Woude G, Neumann H, Walther M, Linehan WM, and Zbar B (1998): Two north american families with hereditary papillary renal carcinoma and identical novel mutations in the MET proto-oncogene. *Cancer Res.* **58,** 1719-1722.

Schmidt L, Junker K, Nakaigawa N, Kinjerski T, Weirich G, Miller M, Lubensky I, Neumann HPH, Brauch H, Decker J, Vocke C, Brown JA, Jenkins R, Richard S, Bergerheim U, Gerrard B, Dean M, Linehan WM, and Zbar B (1999): Novel mutations of the MET proto-oncogene in papillary renal carcinomas. *Oncogene* **18**, 2343-2350.

Senger DR, Galli S, Dvorak AM, *et al* (1983): Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219,** 983-985.

Seslar SP, Nakamura T and Byers SW (1993): Regulation of fibroblast hepatocyte growth factor/scatter factor expression by human breast carcinoma cell lines and peptide growth factors. *Cancer Res.* **53**, 1233-1238.

Shin SI, Freedman VH, Risner R, and Pollack R (1975): Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage-independent growth *in vitro. Proc. Natl. Acad. Sci. U.S.A.* **72,** 4435.

Simard D, Nabi IR(1996): Inverse relation of autocrine motility factor receptor and E-cadherin expression following MDCK cell transformation. *Biochem Biophys Res Comm* **219,**122-127.

Simons K, and Fuller SD (1985): Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* **1**, 43-88.

Simpson AJ (1997): The natural somatic mutation frequency and human carcinogenesis. *Adv. Cancer Res.* **71,** 209-240.

Sloane BF, Moin K, Lah TT (1994): Regulation of lysosomal endopeptidases in malignant neoplasia. In: Pretlow TG, Pretlow TP, eds. Biochemical and Molecular Aspects of Selected Cancers, New York, Academic Press, pp. 411-466.

Soriano JV, Pepper MS, Nakamura T, Orci L, and Montesano R (1995): Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J. Cell Sci.***108**, 413-430.

Stehelin D, Varmus HE, Bishop JM, Vogt PK (1976): DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260**, 170-173.

Stella Maria Cristina, Comoglio Paolo M, (1999)**:** HGF: a multifunctional growth factor controlling cell scattering. *Int J Biochem Cell Biol.* **12,**:1357-62. Review **.**

Stetler-Stevenson WG, Liotta LA, Kleiner DR Jr (1993): Extracellular matrix 6: Role of matrix metalloproteinases in tumor invasion and metastasis. *FASEB J.* **7,** 1434-1441.

Stoker M and Perryman M (1985): An epithelial scatter factor released by embryo fibroblasts. *J. Cell Sci.* **77,** 209-223.

Stoker M, Gherardi E, Perryman M, and Gray J (1987): Scatter factor is a fibroblast derived modulator of epithelial cell mobility. *Nature* **327,** 239-242

Stoker M (1989): Effect of scatter factor on motility of epithelial cells and fibroblasts. *J. Cell Physiol.* **139,** 565-569.

Streit A, Stern CD, Thery C, Ireland GW, Aparicio S, Sharpe MJ and Gherardi E (1995): A role for HGF/SF in neral induction and its expression in Hensen's node during gastrulation. *Development* **121**, 813-824.

Tajima H, Matsumoto K & Nakamura T (1992): Regulation of cell growth and motility by hepatocyte growth factor and receptor expression in various cell species. *Exp. Cell Res.* **202,** 423-432.

Tannock IF Hill RP (1998), The Basic Science of Oncology, 3rd ed. McGraw Hill Press, pp. 79-232.

Tomiya T, Nagoshi S & Fujiwara K (1992): Significance of serum human hepatocyte growth factor levels in patients with hepatic failure. *Hepatology* **15,** 1-4.

Turner CE (2000): Paxillin and focal adhesion signaling. Nature Cell Biology **2**, E231-E236.

Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T and Kitamura N (1995): Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373**, 702-705.

Ulrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM & Ramachandran J (1985): Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* **313,** 756-761.

Ullrich A and Schlessinger J (1990): Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

Varmus H (1988): Retroviruses. *Science* **240,** 1427-1435.

Vadnais J, Nault G, Daher Z, Amraei M, Dodier Y, Nabi IR, Noël, J. (2002). Autocrine activation of the HGF-R/Met tyrosine kinase induces tumor cell motility by regulating pseudopodial protrusion. *J. Biol. Chem.* **277,** 48342-48350.

Villa-Moruzzi E, Lapi S, Prat M, Gaudino G and Comoglio PM (1993): A protein tyrosine phosphatase activity associated with the hepatocyte growth factor/scatter factor receptor. *J. Biol. Chem.* **268,** 18176-18180.

Weidner KM, DiCesare S, Sachs M, Brinkmann V, Behrens J and Birchmeier W (1996): Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* **384,** 173-176.

Vuori K, Hirai H, Aizawa S and Ruoslahti E (1996): Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol. Cell. Biol.* **16,** 2606-2613.

Weidner KM, Behrens J, Vandekerckhove J and Birchmeier W (1990): Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J. Cell Biol.* **111**, 2097-2108.

Weinberg R (1991): Tumor suppressor genes. *Science* **254,**1138-1146.

Wolf DE, McKinnon CA, Daou MC, Stephens RM, Kaplan DR and Ross AH (1995): Interaction with TrkA immobilizes gp75 in the high affinity nerve growth factor receptor complex. *J. Biol. Chem.* **270**, 2133-2138.

Wright JA, Turley EA, Greenberg AH (1993): Transforming growth factor beta and fibroblast growth factor as promoters of tumor progression to malignancy. *Crit. Rev. Oncogene* **4,** 473-492.

Xiao GH, Jeffers M, Bellacosa A, Mitsuuchi Y, Vande Woude GF (2001): Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 247-252.

Yang Y, Spitzer E, Meyer D, Sachs M, Niemann C, Hartmann G, Weidner KM, Birchmeier W (1995): Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131**, 216-226.

Zarnegar R & Michalopoulos G (1989) : Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. *Cancer Res.* **49**, 3314-3320.