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Pseudopodial MSV-MDCK-INV glycolysis modulates the c-Met phosphorylation-dependent cell motility

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This thesis entitled:

Pseudopodial MSV-MDCK-INV glycolysis modulates the c-Met phosphorylation-dependent cell motility

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

Jury president: Dr Alfred Berteloot
Member of jury: Dr André Gougoux
Study director: Dr Josette Noël

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"Time flies," everyone says. It's frightening sometimes to look in the mirror and see the evidence of that. So often, we don't take a moment to say what's in our hearts, and then, when it's too late, we wish we had. I'm taking a moment now, Mom and Dad, to tell you this...
In the high seas of life, you have shown me the light. Thank you for guiding my way.
Thanks for always being my support.

To Mom, dad, Rody and Carla
Who always encouraged me to follow my dreams, I dedicate this thesis,
All its contents, the first word to the last success.

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I am greatly indebted to the entire secretarial and technical staff of the department, both past and the present. Gratitude is extended to Dr. Ivan Robert Nabi, in the Pathology and Cellular Biology department of the Université de Montréal for his unselfish collaboration, especially with pseudopod purification and confocal microscope work.

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SUMMARY

A metabolic imbalance exists in progressively malignant tumor cells that have a high aerobic glycolysis. The MSV-MDCK-INV cells are a Moloney sarcoma virus (MSV) transformed epithelial MDCK cells, which exhibit multiple β-actin rich domains where are localized the hepatocyte growth factor receptor c-Met, the glyceraldehyde-3-phosphate dehydrogenase and the Na^+/H^+-exchanger. NHE1. A previous study from the laboratory of Dr Noël had demonstrated that the autocrine activation of the hepatocyte growth factor receptor/c-Met tyrosine kinase induces tumor cell motility by regulating pseudopodial protrusion. We hypothesized that pseudopodial ATP produced from glycolysis and proton secreted by NHE1 drives pseudopodial formation, and modulates the motile character of MSV-MDCK-INV cells.

We present here strong indication of a pseudopodial ATP production by showing the expression and enrichment of the glycolytic enzymes hexokinase, phosphoglucose isomerase and aldolase, and the absence of mitochondrial proteins from β actin-rich pseudopodial domains. Glycolytic enzymes colocalize with β-actin and phosphorylated c-Met. Incubating cells in glucose-free medium with or without 2-deoxyglucose, or inhibiting glycolysis with iodoacetate in high glucose medium decreases the phosphorylation level of c-Met and leads to the loss of pseudopodia. In contrast, mitochondrial inhibitors such as oligomycin and antimycin-A do not affect this phosphorylation level in high glucose medium but decrease it in glucose-free medium. Pseudopodial glycolysis was found, in the present study, to be the main source of ATP required to phosphorylate the kinase domain of c-Met and lead to the actin remodelling, which were shown to modulate cell motility.

Inhibition of the Na^+/H^+-exchanger with ethylisopropylamiloride in the absence of bicarbonate and CO_2 atmosphere leads to a rapid reduction of p-c-Met signal. Secretion of protons produced by glycolysis may be a

necessary step to drive continuous ATP production required for autocrine c-Met phosphorylation. Our results suggest a functional link between pseudopodia-associated glycolysis, NHE1 proton secretion, and phosphorylation of c-Met that regulates motility and invasion of MSV-MDCK-INV cells.

Key words: c-Met phosphorylation, actin remodeling, proton secretion, invasion.

SOMMAIRE

Un déséquilibre métabolique existe dans les cellules tumorales ayant une forte glycolyse aérobie. Les cellules MSV-MDCK-INV sont des cellules MDCK transformées par le virus du sarcome de Moloney (MSV) sélectionnées pour leur propriété invasive. Elles présentent des domaines riches en actine β où sont exprimés le récepteur du facteur de croissance hépatocytaire c-Met, la glyceraldehyde-3-phosphate déshydrogénase et l'échangeur Na^+/H^+ NHE1. Des travaux antérieurs à la présente étude ont démontré que l'activation autocrine du récepteur c-Met module l'activité motile des cellules tumorales MSV-MDCK-INV en régulant la formation des pseudopodes. Nous avons fait l'hypothèse que l'ATP produit de l'activité glycolytique des pseudopodes ainsi que la sécrétion, par NHE1, des protons produit de l'activité glycolytique pseudopodiale sont impliqués dans la formation des pseudopodes et l'acquisition du caractère motile des cellules MSV-MDCK-INV.

Nous présentons ici des indices d'une production pseudopodiale d'ATP en montrant l'expression ou l'enrichissement des enzymes de la glycolyse (hexokinase, phosphoglucose isomérase et aldolase) et l'absence des protéines mitochondriales des domaines riches en actine β dans les fractions pseudopodiales purifiées. Les enzymes de la glycolyse colocalisent avec c-Met phosphorylé et l'actine β des pseudopodes. L'incubation des cellules dans un milieu sans glucose avec ou sans 2-deoxyglucose et l'inhibition de la glycolyse par l'iodoacétate dans un milieu riche en glucose diminuent le degré de phosphorylation de c-Met et entraînent la perte des pseudopodes. En revanche, les inhibiteurs mitochondriaux tels que l'oligomycine et l'antimycine A n'affectent pas ce degré de phosphorylation dans le milieu riche en glucose mais le diminuent dans le milieu sans glucose. Ces résultats suggèrent que la glycolyse pseudopodiale est la source principale d'ATP requise pour phosphoryler localement le domaine kinase de c-Met en réponse au facteur de croissance hépatocytaire et pour fournir l'énergie

nécessaire au remodelage d'actine impliqué dans le phénomène de motilité des cellules MSV-MDCK-INV.

L'inhibition de l'échangeur Na^+/H^+ par l'éthylisopropylamiloride en l'absence d'ions bicarbonate et de CO_2 entraîne une réduction rapide du degré de phosphorylation de c-Met. La sécrétion des protons issus de la glycolyse peut donc être une étape nécessaire à une production continue d'ATP, exigée pour l'activation autocrine de c-Met. Nos résultats suggèrent donc l'existence d'un lien fonctionnel entre la glycolyse associée aux pseudopodes, la sécrétion localisée des protons par NHE1 et la phosphorylation de c-Met pour la modulation des activités motile et invasive des cellules tumorales MSV-MDCK-INV.

Mots clés: phosphorylation de c-Met, remodelage de l'actine; sécrétion de protons, invasion.

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LIST OF ABBREVIATIONS

ALDO	Aldolase
ATP	Adenosine triphosphate
CB	Cell body
c-Met	Tyrosine kinase receptor for hepatocyte growth factor
2-DG	2-Deoxyglucose
EIPA	Ethylisopropylamiloride
ERK	Extra-cellular signal regulated kinase
FGF	Fibroblast growth factor
G3PDH	Glyceraldehyde-3-Phosphate dehydrogenase
HGF	Hepatocyte growth factor
HGF-R	Hepatocyte growth factor receptor
HK	Hexokinase
IAA	Iodoacetate
IGF	Insulin-like growth factor
INV	Invasive
kDa	Kilodalton
LDH	Lactate dehydrogenase

MDCK	Madin Darby canine kidney
MSV	Moloney sarcoma virus
NHE1	Isoform 1 of the Na^+, H^+ exchanger
Oligo	Oligomycin
PDK1	Mitochondrial pyruvate dehydrogenase kinase
PGI	Phosphoglucose isomerase
pHe	Extracellular pH
pHi	Intracellular pH
Pi	Inorganic phosphate
PK	Pyruvate kinase
PPF	Pseudopodia fraction
RTK	Receptor tyrosine kinase
SF	Scatter factor
TOM-20	20 kDa preprotein translocase of the outer membrane

Annex

Josette Noël My research supervisor. She directed all the experiments. With her advice and suggestions, she participated in the design and the correction of the manuscript.

I. Robert Nabi He allows us the use of the confocal microscopy. He made suggestions and correction on the manuscript.

Zong Jiang Jia He gave me advice concerning the pseudopod purification technique. He instructed me on how to use the confocal microscopy.

Yolaine Dodier and Zeinab Daher They made the experimentation concerning the inhibition of the Na^+/H^+ exchanger with EIPA (Figure 2.9)

Carlos El Hader I performed all the experiments including: cell culture, drug treatments, immunofluorescence, confocal microscopy, pseudopod and cell body purification, western blotting, ATP dosage by luminometry, c-Met quantification, and wound healing assays. I performed the writing of the manuscript designed in collaboration with my supervisor.

CHAPTER 1

INTRODUCTION

1.1 Cell proliferation and differentiation

1.1.1 Introduction

The biology of cell division and differentiation is exceedingly similar in normal and cancer cells. The cancer cell differs from its normal counterpart in that it is aberrantly regulated. Cancer cells generally contain the full complement of biomolecules necessary for survival, proliferation, differentiation, and expression of many cell-type-specific functions. However, failure to regulate these functions properly results in an altered phenotype, which often leads to cancer (1).

Three cellular functions tend to be inappropriately regulated in a neoplasm: First, the normal constraints on cellular proliferation are relaxed (2). This is a necessary but often insufficient requirement for tumor formation. Second, differentiation can be distorted. The tumor cells may be blocked at a particular stage of differentiation, or they may differentiate into an inappropriate or abnormal cell type (3). Third, chromosomal and genetic organization may be destabilized such that variant cells arise with high frequency. Some variants may have an increased growth advantage, others may be resistant to killing by chemotherapeutic drugs or radiation and others may have increased motility or production of enzymes that permit invasion and metastases (4).

1.1.2 Proliferation

1.1.2.1 Tumor growth and cell proliferation in vivo

In terms of population kinetics, the growth of any tissue depends on three parameters: 1) rate of individual cell division, 2) growth fraction of the cell population and 3) cell loss from the growing population through differentiation or cell death.

In an organism, the rate of cell division is a tightly regulated process that is intimately associated with growth, differentiation and tissue turnover. Generally, cells do not undergo division unless they receive signals that instruct them to enter the active segments of the cell cycle. Resting cells are said to be in the G_0 phase (quiescence) of the cell cycle **(Figure 1.1)**.

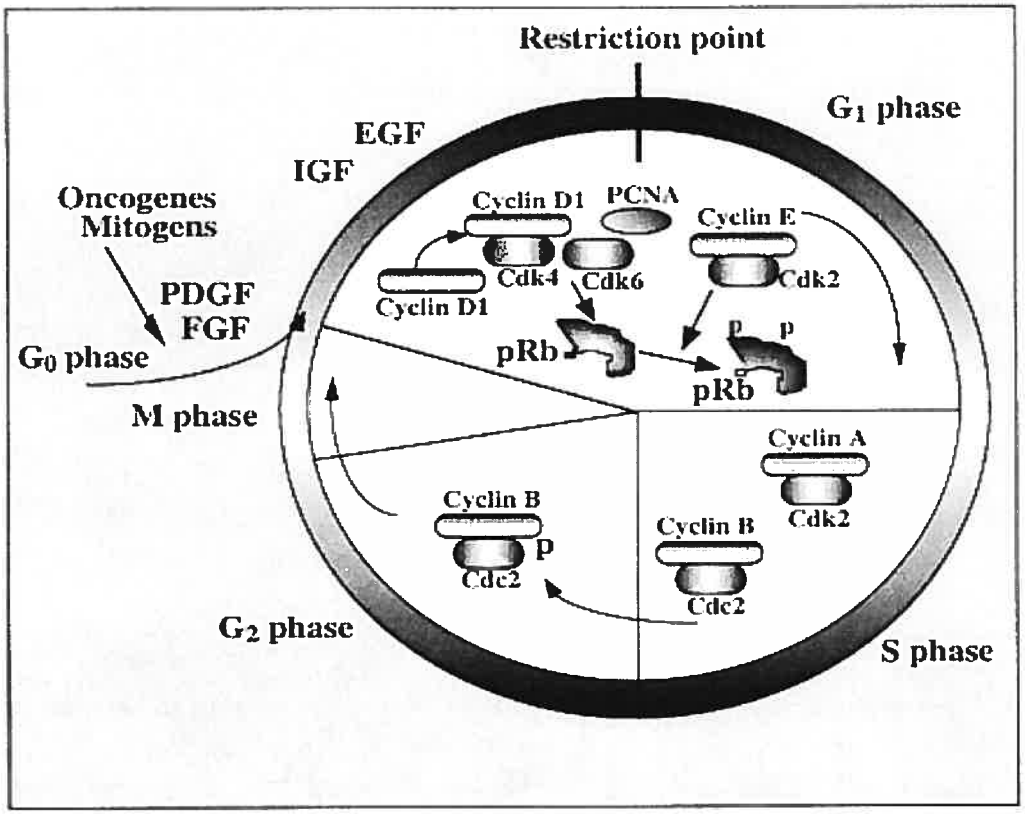

Figure 1. Schematic representation of the mammalian cell cycle. Competence factors such as PDGF and FGF promote entry into the early G_1 phase. Sequential treatment with progression factors, IGF or EGF, promote progression through the G_1 phase restriction point. Competence of the cyclin D1/Cdk4 complex is induced by mitogens. The cyclin D1/Cdk4 complex phosphorylates the pRB protein leading to sequential phosphorylation by cyclin E/Cdk2 and release of free E2F. The phosphorylation of pRB and relief of transcriptional repression by pRB induces genes involved in the induction

Figure adapted from (5)

The signals that induce cells to divide are diverse and trigger a large number of signal transduction cascades. Generally, signals that direct cells to enter the cell cycle are called growth factors, cytokines, or mitogens.

Normal cells reach a steady state of growth that provides a balanced economy for the body as a whole. Each organ maintains tight control over the growth rate, growth fraction, and cell loss. Some normal tissues grow faster than cancers under physiologic conditions, so it is not simply rapid growth at a single time and place that distinguishes neoplasia.

In the early phases of tumor cell growth, it is generally believed that neoplastic cells multiply exponentially, then as the tumor mass increases, the rate of growth declines (6). Several mechanisms have been invoked to explain this change in growth rate with larger tumors: 1) decrease in the growth fraction, 2) increase in cell loss or death, 3) nutritional depletion of tumor cells due to outgrowth of available blood supply, or 4) lengthening of cell cycle time (7). Experimental tumor models suggest that cell cycle time changes only slightly when tumor growth decreases (1). Under adverse conditions, tumor cells often leave the growth fraction and enter a non-growing state (G_0 or prolonged G_1) although these same cells can reenter the division cycle when conditions improve or when stimulated by growth factors.

1.1.2.2 Cells in culture

The importance of the individual cell in cancer is clear since a single cancer cell injected into an appropriate animal is sufficient to give rise to a tumor. Many studies have therefore been performed with isolated normal and tumor cells in culture. Both normal and tumor-derived mammalian cells can be grown and compared in culture, although many are not readily established in culture initially (8-11). Most studies have been done on fibroblasts since this cell type is easily cultured and is most likely to grow out of a tissue explants.

Cells are grown in a medium containing salts, amino acids, glucose, vitamins, and serum or growth factors. Normal cells, plated on a plastic surface to which they may attach, can grow until they have formed a confluent monolayer, whereupon growth ceases. Growth also ceases when cells have exhausted an essential nutrient or factor provided by serum, or when such substances are removed by changing to a deficient medium. Thus, growth can be manipulated in culture.

1.1.2.3 Molecular events in cell proliferation

When a quiescent cell in culture is stimulated, for example by the addition of serum, the activated chain of events leads eventually to formation of two cells. This requires duplication of a multitude of molecules in the original cell (12). Multiple growth factors provided in serum have been found to act sequentially following resumption of proliferation of fibroblasts: platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF-1), and hepatocyte growth factor (HGF) (13). These small polypeptides activate cells by binding to specific receptors on the cell surface (13, 14).

Growth factor receptors are complex large proteins that span the plasma membrane. On the outside of the cell they have a specific domain that recognizes the growth factor, and their cytoplasmic portion may have an enzymatic function, such as protein tyrosine kinase. Binding of a growth factor or ligand to its receptor can induce transmission of a signal to the cytoplasm through activation of the kinase (15) **(Figure 1.2)**. The next step is the transduction of the cytoplasmic signal to cell nucleus (16). This is accomplished by a heterogeneous group of molecules known as second messengers. They include various proteins that are phosphorylated by kinases such as transcription factors, small molecules such as inositol phosphates and cyclic AMP, and also ions, including Ca^{2+}, H^{+}, and Zn^{2+}. Within the nucleus, genes are then activated in response to these second messengers.

1.1.2.4 Properties of tumor cells in culture

Tumor and normal cells can be distinguished in culture by several tests. Tumorigenic cells are less sensitive to the presence of other cells in their immediate vicinity than are normal cells. Normal cells typically cease proliferation as the culture density increases, but tumor cells can reach several-fold higher densities in culture. If they are plated on a dense layer of non proliferating, homogenous normal cells, tumor cells can continue to grow and form foci of clustered cell colonies. Such colonies show altered interactions by the tumor cells, which grow randomly, criss-crossing one another and forming clusters of viable and necrotic cells. When these cultures are fixed and stained, number of tumor colonies is easily quantitied against the background population of normal cells. This is the basis of the commonly used "focus forming assay" also called "anchorage independent growth" for detecting transformation, e.g., the capacity of mutagens or oncogens to produce neoplastic transformation within a population of non tumor cells (3).

Cells of normal solid tissue lie on a secreted extracellular matrix (ECM), composed of various proteins that stimulate cell growth (17). Transformed cells are often partly or completely independent of ECM for optimal growth, and they may secrete little matrix material (18). In addition the cytoskeleton within tumor cells tends to be less well organized, and its actin filaments are less highly polymerized. Tumor cells often can be grown in the absence of a substratum, as within a semisolid medium containing agar. This formation of colonies in suspension is also used as a test of neoplastic transformation (3).

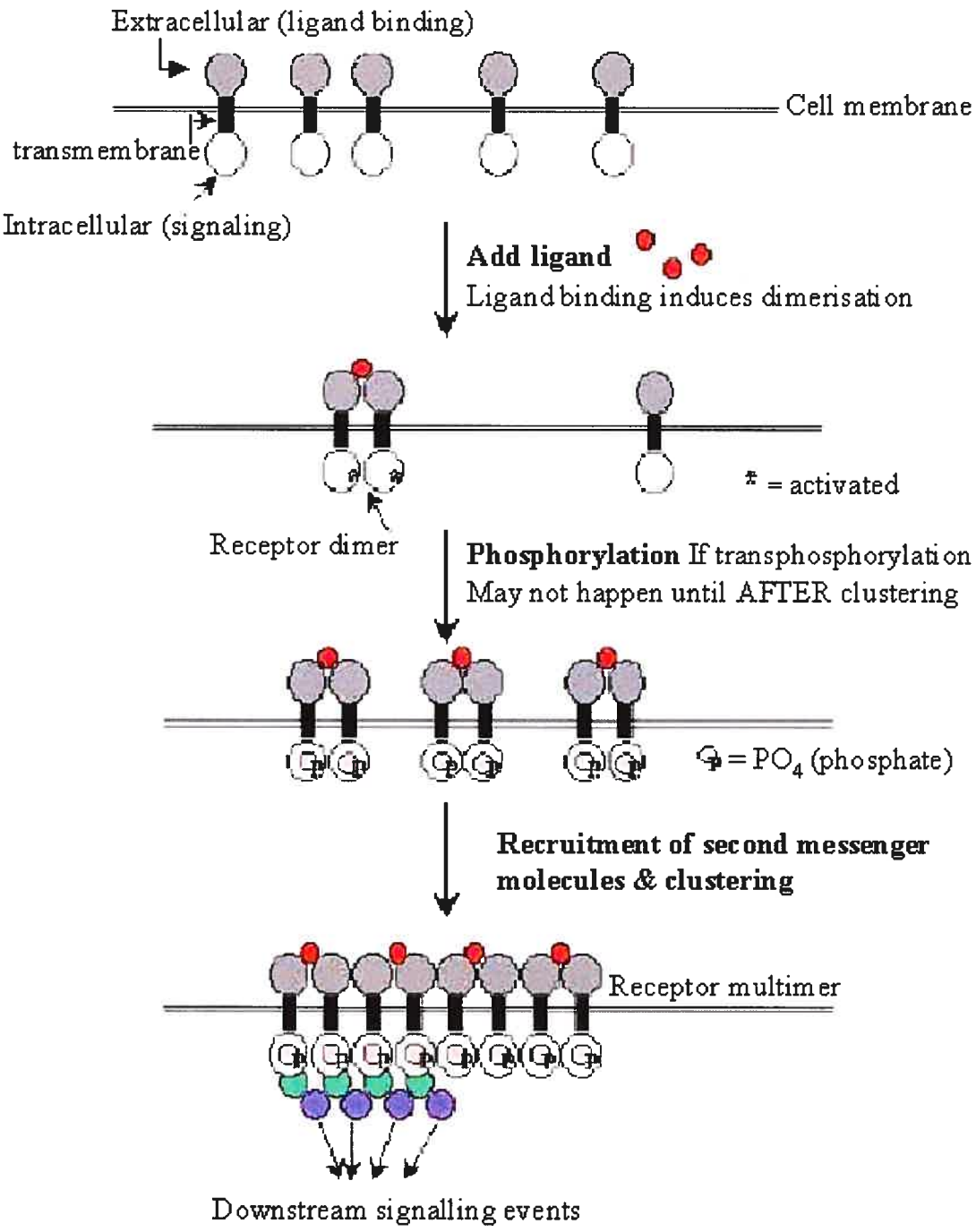

Figure 1.2: **Model of cell showing the sequence of events following ligand binding to growth factor receptors.**

Figure adapted from (19)

1.1.3 Differentiation

All tumor cells show abnormalities in the regulation of cell proliferation (neoplasia). In addition, most, if not all, tumor cells show abnormalities in differentiation (anaplasia). The anaplasia of tumors can provide insights into their etiology, degree of malignancy, prognosis, and sensitivity to therapeutic intervention by differentiation or maturation-inducing agents (3).

It is obvious that although somatic cells are genetically equal they are not phenotypically equal. Thus, skin fibroblasts are different from T lymphocytes, muscle cells differ from gastric mucosal cells, and so forth. However, within an organism, all cells have an identical complement of DNA. Differences in phenotype arise from differences in gene expression, not in gene content (3).

1.2 Growth factors

1.2.1 Introduction

The evolution of multicellular organisms has involved the development of intercellular communication required for such processes as embryonic development, tissue differentiation, as well as systemic responses to wounds and infections. These complex signaling networks are in large part mediated by growth factors, cytokines and hormones. Such factors can influence cell proliferation in positive or negative ways as well as inducing a series of differentiated responses in appropriate target cells. The interaction of a growth factor with its receptor by specific binding in turn activates a cascade of intracellular biochemical events that is ultimately responsible for the biological responses observed. Cytoplasmic molecules that mediate these responses have been termed second messengers. The eventual transmission of biochemical signals to the nucleus leads to effects on the expression of cassettes of genes involved in mitogen and differentiation responses (20).

Over the past few years it has become increasingly evident that the pathogenic expression of critical genes in growth factor signaling pathways can contribute to altered cell growth associated with malignancy. The v-sis oncogene of simian sarcoma virus, which encodes a growth factor homologous to the B chain of human platelet derived growth factor (PDGF-B), is the paradigm for such genes (21, 22). The normal homologs of other oncogenes have been shown to encode membrane spanning growth factor receptors (23, 24). Other genes that act early in intracellular pathways of growth factor signal transduction, for example ras, have been implicated as oncogenes as well. Present knowledge indicates that the constitutive activation of growth factor signaling pathways through genetic alterations affecting these genes contributes to the development and progression of most if not all human cancers.

1.2.2 Classification of growth factors

Growth factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Many growth factors are quite versatile, stimulating cellular division in numerous different cell types, while others are specific to a particular cell type (20).

1.2.2.1 Platelet-derived growth factor family

Platelet-derived growth factor is a cationic protein that consists of two related but non-identical polypeptide chains designated A and B (also called PDGF-1 and PDGF-2), which is the major growth factor in human serum (21, 22). PDGF molecules exist as AA and BB homodimers as well as an AB heterodimers (25, 26). PDGF-AB is the major PDGF form found in platelets and is released into serum upon blood clotting. Efforts to identify factors that control angiogenesis recently led to the identification of a new growth factor, the vascular endothelial growth factor (VEGF), that is a potent mitogen for vascular endothelial cells of small and large vessels, but has no effect on

fibroblasts, lens epithelial cells, corneal endothelial cells, keratinocytes, or adrenal cortex cells (21). VEGF was shown to be a critical factor which stimulates neovascularisation of tumor cells, in that way, favoring the development of metastasis .

1.2.2.2 Epidermal growth factor family

EGF purified from mouse sub-maxillary glands was found to promote precocious eyelid separation by enhancing epidermal growth and keratinization while it induced early incisor eruption by enhancing the differentiation of the lips of treated animals (27). Other members of the EGF family including tumor growth factor-α (TGF-α), amphiregulin (AR) and poxvirus growth factors share sequence similarities, high binding affinity to the EGF receptor and mitogenic effects on EGF-responsive cells. EGF is normally expressed in kidney and sub-maxillary glands and is produced in response to GI tract injury (27, 28) as well TGF-α appears to be normally expressed by a variety of epithelial cells (29).

1.2.2.3 Fibroblast growth factor family

There are seven known members of the fibroblast growth factor (FGF) family, whose targets include cells derived from mesoderm and neuroectoderm. Because heparin can bind to and modulate the biological activity of these proteins, they have also been termed heparin-binding growth factors (HBGFs) (30, 31).

1.2.2.4 The insulin family

The diversity of metabolic effects of insulin has been studied intensively for decades (32). Its primary in vivo functions involve the regulation of rapid anabolic responses such as glucose uptake, lipogenesis and amino acid and ion transport. Besides its effects on metabolism, insulin stimulates DNA synthesis and cell growth. The activities of insulin-like growth factors (IGF-I

and IGF-II) were first recognized as serum factors, antigenically distinct from insulin, that interacted with growth hormone in stimulating growth of skeletal tissues and were, as a result, termed somatomedins (33). Subsequently it was determined that somatomedin C is identical to IGF-I, while a polypeptide known as multiplication stimulating factor (MSA) is homologous to IGF-II.

1.2.2.5 Hepatocyte growth factor

A growth factor apparently specific for hepatocytes (HGF) was isolated from plasma (34) or platelets (35). Unexpectedly, the predicted amino acid sequence of HGF was found to be related to plasminogen (36). In addition to the 38% sequence identity to plasminogen, including its serine protease domain within the alpha chain, HGF was shown to possess disulfide bond-linked intrachain structures known as "kringles" which are typical of prothrombin. Neither plasminogen nor plasmin have HGF-like activity, and HGF is not likely to be a protease since the histidine and serine residues in the region corresponding to the catalytic site are replaced by other amino acids.

1.2.3 General aspects of HGF

Hepatocyte growth factor (HGF) is a heparin binding, secreted basic protein (37) initially identified as a potent hepatotrophic factor responsible for vigorous regeneration of the liver. It has become a well-characterized multipotent growth factor (more properly called a cytokine) with biological functions that reach far beyond the original identifications, operating in virtually every tissue of the body (38). Cellular targets include hepatocytes and other epithelial cells (e.g. lens epithelial cells) melanocytes, endothelial and haematopoietic cells.

1.2.3.1 Description of function

HGF, the natural ligand for the c-Met proto-oncogene product, is a mesenchymal- or stromal-derived multipotent polypeptide that mediates

epithelial-mesenchymal interactions (39). During embryogenesis, HGF stimulates cell proliferation (e.g. placental cytotrophoblasts), differentiation, motility and invasiveness via its membrane-spanning tyrosine kinase receptor, c-Met. HGF induces angiogenesis, is involved in haematopoiesis, chondrogenesis, and supports organogenesis and morphogenesis (40) of various tissues and organs, including the liver, kidney, lung, mammary gland, tooth, skeletal system, etc. In adult tissues, HGF elicits a potent organotrophic function, which supports regeneration of organs including the liver, kidney, and lung.

HGF has been shown to play a pivotal role in integrin-mediated adhesion and transmigration of neutrophils to sites of acute inflammation through cytoskeletal rearrangement (41). In neoplastic tissue, HGF is involved in tumor invasion and metastasis, through tumor-stromal interactions. Studies have shown anti-tumor activity for certain species of carcinoma cells, and in particular, growth of most hepatoma cells is inhibited by HGF.

1.2.3.2 Location and control

HGF is the ligand for the transmembrane tyrosine kinase (c-Met), and is initially secreted as a single-chain, biologically inert glycoprotein precursor (pro-HGF) (38). Under appropriate conditions, pro-HGF is converted into its bioactive form by proteolytic digestion; four proteases are reported to activate HGF within the so-called dibasic site (uPA) , a serine protease. This processing takes place in the extracellular environment; cleaving of the zymogens induces a conformational change in the ligand, and interaction/activation of the receptor follows. The HGF precursor can also be processed by a serum-derived serine protease. This soluble glycoprotein, known as HGF activator, may bring about quantitative activation of pro-HGF in response to the triggering of the blood coagulation cascade, as in the case of tissue injury, whereas uPA may effect a more restricted activation of the

precursor in the tissues and on the membrane of target cells under conditions other than trauma or injury (42).

Expression of HGF at the transcription level is regulated by various factors. Interleukin-1, platelet-derived growth factor, acidic and basic fibroblast growth factor, epidermal growth factor, and prostaglandin's are potent inducers of HGF expression (39). Polysaccharides, such as heparin and heparan-sulphate, stimulate HGF synthesis, but only affect post-transcriptional processes (39, 40). In fact heparin is crucial for inducing HGF dimerization (3-6 fold higher levels). Moreover, the fact that cellular responses to HGF depend on glycosaminoglycan composition of the cell membrane further supports the hypothesis that receptor activation requires a co-operative participation of multiple surface and soluble components (38). In contrast, transforming growth factor-β1 and glucocorticoids suppress the gene expression of HGF, acting transcriptionally (39).

1.2.3.3 Structural classification

Mature HGF is a heterodimer with a 69-kDa α-chain and a 34-kDa β-chain, linked by a single disulphide bridge (43). The α-chain contains the N-terminal hairpin structure and 4 homologous "kringle domains", the first of which carries receptor-binding determinants (38). Although the kringle domain (80 amino acids) is thought to play a role in protein-protein interaction, its physiological function is unknown (44). However, a hydrophobic core and a distinct folding pattern determined by 3 intramolecular disulphide bonds, characterize the dominant structural feature. The β-chain has serine protease-like motif (37) and thus has structural homology (38%) with plasminogen but 2 amino acid residues are different at the "protease active site" conferring it no biological activities.

1.2.4 Growth factor receptors with tyrosine kinase activity

Membrane spanning tyrosine kinase receptors contain several discrete domains including their extra cellular ligand binding, transmembrane, juxtamembrane, protein tyrosine kinase and carboxy-terminal tail domains (45, 46). Interaction of a growth factor with its receptor at the cell surface leads to a tight association, so that growth factors are capable of mediating their activities at low nanomolar concentrations. Following ligand binding, the growth factor-receptor complex is internalized leading to increased turnover of the receptor. It has been proposed that the growth factor activation signal might be mediated by its internalization (46). However, there is substantial evidence that activation of the receptor tyrosine kinase is the trigger for the biochemical cascade of events that follows. It is possible that conformational changes induced by ligand binding to the receptor's external domain are somehow transmitted through the transmembrane domain to induce the conformational alterations of the receptor kinase resulting in its activation (45, 46). In an alternative model more generally accepted, ligand binding induces receptor dimerization or oligomerization (46). By this latter mechanism, molecular interactions between adjacent cytoplasmic domains lead to activation of kinase function.

The tyrosine kinase domain is the most conserved among tyrosine kinase receptors and an intact protein tyrosine kinase domain is absolutely required for receptor signaling. For example, mutation of a single lysine in the ATP binding site (45), which blocks the ability of the receptor to phosphorylate tyrosine residues, completely inactivates receptor biological function. Yet, such kinase mutants retain the ability to bind ligand with high affinity and exhibit normal internalization and down regulation as well (45).

The carboxy terminal domain of the receptor is thought to play an important role in regulation of kinase activity. This region typically contains several tyrosine residues, which are phosphorylated by the activated kinase. In fact,

the receptor, itself, is often the major tyrosine phosphorylated species observed following ligand stimulation. Tyrosine phosphorylation of the carboxy terminal domain has been postulated to modulate kinase catalytic activity, and/or the ability of the kinase to interact with substrates. Thus, mutations, which alter individual tyrosine sites or deletions of the carboxy terminal domain, have the effect of attenuating kinase function in those receptors so far analysed (45, 47).

1.2.4.1 Hepatocyte growth factor receptor (HGF-R)

The receptor for HGF, the c-met protooncogene (48), is a transmembrane protein that is derived from a 170-kDa precursor. After processing by furin (49), the mature c-met is composed of a 50-kDa α subunit that is linked by 2 disulfide bonds to a 145-kDa ß subunit (50). The α subunit is extracellular and heavily glycosylated, whereas the ß subunit consists of an extracellular portion to which HGF binds, a membrane spanning domain, a distinctive juxtamembrane domain, a cytoplasmic tyrosine kinase domain and carboxy-terminal sequences acting as a docking site for adaptors **(Figure 1.3).**

Autophosphorylation of the tyrosine residues in positions 1230, 1234, 1235 has a positive regulatory effect on the enzyme activity whereas autophosphorylation of a serine residue in position 1003 in the juxtamembrane domain negatively regulates the kinase (38). The carboxy-terminal portion includes the tyrosine residues 1359 and 1365 that, when phosphorylated, together form a specific docking site for multiple signal transducers and adapters. Growth factor receptor-bound protein 2 (GRB2) binds preferentially to the second tyrosine residue and triggers the ras signal transduction pathway (38).

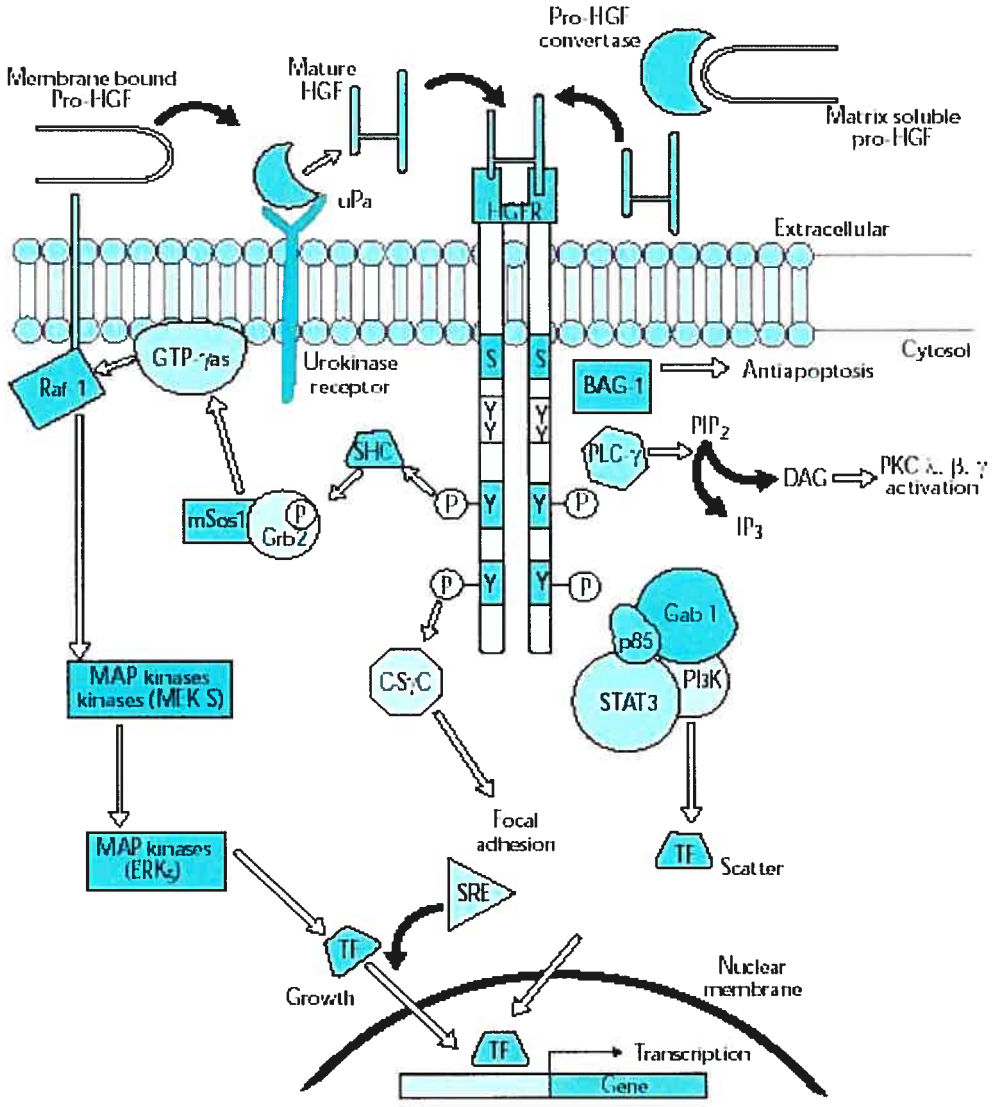

Figure 1.3: HGF binding to the HGF-R/Met tyrosine kinase receptor leads to activation of different signaling pathways. HGF, upon binding to its receptor c-met, induces its dimerization as well as autophosphorylation of tyrosine residues. Phosphorylation of tyrosine residues within multifunctional site triggers a pleiotropic response involving multiple signal transducers. The synchronous activation of several signaling pathways is essential to conferring the distinct invasive growth ability of the HGF receptor. In epithelial cells, HGF functions as a scattering (dissociation/motility) factor.

Figure adapted from (51)

The polypeptide growth factor hepatocyte growth factor/scatter factor (HGF/SF) (44, 52-54) and its receptor MET, the product of the c-*MET* proto-oncogene (55), play essential roles in the development of epithelial organs such as the placenta and liver (56, 57) and in the migration of myogenic precursor cells (58) and motor neurons (59, 60).

HGF/SF and MET are also involved in the spreading of a variety of epithelial tumors as a result of MET chromosomal rearrangements (61), somatic and/or germ line mutations in the MET kinase (62) or, more often, over expression in tumor cells of an unrearranged and unmutated *MET* gene (63).

Met signaling clearly has a role in normal cellular processes. When this signaling pathway is deregulated, it is implicated in tumor development and progression. Met signaling can increase tumorigenicity, induce cell motility, and enhance invasiveness *in vitro* and metastasis *in vivo* (56, 64, 65). In addition, Met signaling can increase the production of protease and urokinase, which are associated with extracellular matrix/basal membrane degradation and are important for metastasis (66).

Following c-Met activation by HGF in tumor cells, phosphorylation of beta-catenin occurs, together with loss of intercellular adhesion and a gain in the motile and invasive nature of the cell. It was shown that c-met is co-localized with beta-catenin and E-cadherin at regions of cell-cell contact in MCF7 and MDA MB 231 BCC lines (transformed mammary cell lines). Immunoprecipitation studies demonstrated an association between c-met and members of the cadherin adhesion complex in these epithelial tumor cells, along with the membrane tyrosine protein phosphatase, PTPmu (67).

Activating Met mutations leading to increased levels of tyrosine phosphorylation and enhanced kinase activity toward an exogenous substrate when compared with wild type Met could contribute to papillary renal carcinoma and other human malignancies (56, 68). Mutant Met induced

motility of Madin-Darby canine kidney cells and metastasis of NIH 3T3 cells while transgenic mice expressing the oncogenic form of Met developed metastatic mammary carcinoma.

1.2.4.2 Hepatocyte growth factor/receptor signaling pathways

Following c-Met dimerization and autophosphorylation of tyrosine residues, various adaptors and signal transducers bind to the docking site's phosphorylated residues. Interaction of growth factor receptor bound protein-2 (Grb2), gab1 (Grb-2 associated binder 1), p85-PI3kinase, phospholipase C γ (PLC γ), the isoform 3 of signal transducer and activator of transcription factor (STAT-3) and the Src homology/collagen (SHC) protein via Src homology domain 2 (SH2) triggers various signal transduction pathways (**Figure 1.3**). The synchronous activation of several signaling pathways is essential to conferring the distinct invasive growth ability of the HGF receptor.

HGF functions as a scattering (dissociation/motility) factor for epithelial cells, and this ability seems to be mediated through the activation of STAT-3 (Miller and Padanilam 2001). Phosphorylation of adhesion complex regulatory proteins such as ZO-1, β catenin, and focal adhesion kinase (FAK) may occur via activation of c-src. Another Bcl2 interacting protein termed BAG-1 mediates the antiapoptotic signal of HGF receptor by a mechanism of receptor association independent from tyrosine residues. Akt phosphorylation through the PI3kinase pathway also participates in the anti-apoptotic action of c-Met (51)

1.3 Energy metabolism in normal and tumor tissues

1.3.1 Aerobic glycolysis of neoplastic cells: The Warburg effect

A metabolic imbalance exists in progressively malignant tumor cells. The observation that rapidly growing tumor cells have a high aerobic glycolysis was first reported by Warburg (69), who considered that elevated glycolysis was a consequence of impairment in the respiratory mechanism of cells caused by carcinogens. Warburg noted that in all of the tumors he examined respiration was low and glycolysis was high. In Warburg's proposal, the carcinogenic agent is presumed to interfere with cell respiration and the cell either dies or adopts a fermentative mechanism to derive energy necessary for survival. The carcinogenic agent, whether it is a virus, anaerobiosis, X-ray, or chemical carcinogen, is presumed to damage respiration either by interfering with the extent of respiration or by impairing the effect of respiration, rendering it incapable of suppressing glycolysis. Warburg's theory implied that damage to respiration should be irreversible, but as yet there has not been an unequivocal demonstration that the respiration of tumors is distributed. Following Warburg's initial observation of a low rate of respiration in tumors, it was demonstrated that in some tumors the rate of respiration is normal and that several none-cancer tissues have appreciable rates of aerobic glycolysis, such as the retina, leucocytes, kidney, medulla and intestinal mucosa (70).

Mitochondria from neoplastic tissues have a full complement of the enzymes of the citric acid cycle and also functional respiratory pigments, which serve as the components of the electron transport chain (71). Further, as emphasized by Weinhouse (72), neoplasms exhibit a normal Pasteur effect when measured in terms of the Meyerhof oxidation quotient, that is, the oxygen, which is consumed by malignant cells, is just as effective in quantitatively inhibiting the formation of glycolysis end products as that consumed by normal tissues. One of the most potent arguments against the Warburg's hypothesis is the demonstration that the malignant cells respire at rates, which do not appear to

differ from that of their normal counterpart. Thus aerobic glycolysis is not necessarily an essential feature of malignancy.

Despite the failure to observe a unique biochemical pattern of energy metabolism in all tumor cells, a correlation of growth rate with increasing glycolysis is observed in the hepatoma series (73). However, the question as to whether this is related to the primary event in oncogenesis is unanswered.

Racker (74) has proposed a new hypothesis, which focuses on "the findings of Warburg as a significant feature of cancer pathogenesis". He proposes, "Tumors can be caused by a number of different primary lesions" all of which "have in common the ability to cause a persistent alteration in the intracellular pH thereby upsetting the normal regulatory mechanism that prevents uncontrolled growth". Thus the so-called Warburg effect remains, and, in this authors opinion, is deserving of further study in view of the reported correlation between growth rate and glycolysis (73). The increased aerobic glycolysis may reflect cellular alterations, which favor growing cells.

1.3.1.1 Relationship between respiration and glycolysis

Before discussing possible explanations for an elevated aerobic glycolysis, it is perhaps useful to discuss the regulation of carbohydrate metabolism, particularly the interrelationships of respiration and glycolysis. The fundamental controls, which operate on these processes at the cellular level, are part of the basic system on which the action of external controls, such as hormones and growth factors, is superimposed.

Inhibition of glycolysis by oxidative phosphorylation is known as the Pasteur effect. The uncouplers of oxidative phosphorylation reverse the inhibition of glycolysis, indicating that phosphorylation of ADP by inorganic phosphate (75) and not respiration is important to the control mechanism. As pointed out by Racker, the Pasteur effect is explained as follows: "the enzymes of glycolysis can metabolize glucose only when (a) inorganic phosphate and

ADP are available for the oxidation of glyceraldehyde-3-phosphate, and (b) the ATP: ADP ratio and the concentration of Pi and other allosteric effects of glycolytic enzymes are suitable for catalytic action" (74).

Some key enzymes of glycolysis are regulated allosterically by the ATP: ADP ratios as well as by Pi levels. As first demonstrated by Bucher and Russman (76), the key enzymes subject to regulation are those whose substrates and products are displaced from equilibrium in the cell. As an example, the phosphofructokinase reaction is inhibited by high concentrations of ATP but stimulated by Pi. Further, inorganic phosphate reverses product inhibition of hexokinase-1 by glucose-6-phosphate; also, inorganic phosphate as well as the NAD: NADH ratio regulates the oxidation of glyceraldehyde-3-phosphate. The NAD: NADH ratio is dependent on the availability of an electron acceptor such as pyruvate, and pyruvate formation from phosphoenolpyruvate is dependent on ADP availability. It is thus apparent that the levels of ATP, ADP, and Pi determine the rate of glycolysis by their interaction at several key steps in the fermentation scheme (70).

The rate of respiration is also markedly dependent on the availability of ADP and Pi and this dependence is known as respiratory control. Thus the cofactors of the phosphorylation system are common to both glycolysis and respiration. Further, there is competition between these two systems for ADP and Pi, which are in limited supply. As a chemical work (e.g., biosynthesis) or an osmotic work such as ion transport (e.g. Na^+ and K^+ by the Na^+ pump) is performed, energy is utilized and ADP and Pi become available for further generation of energy. The inhibition of glucose utilization by oxygen then is attributed in part to the lesser availability of cofactors of the phosphorylation system under aerobic conditions (70).

1.3.2 General aspects of glycolytic enzymes in cancer

One of the most notable developments in biochemistry during the past 50 years has been the recognition and description of the individual enzymes that mediate the various steps of intermediary metabolism in tissues.

1.3.2.1 The individual reactions of glycolysis

The pathway of glycolysis adapted from King et al., (**Figure 1.4)** can be seen as consisting of 2 separate phases :

The first is the chemical priming phase requiring energy in the form of ATP, and the second is considered the energy-yielding phase. In the first phase, 2 equivalents of ATP are used to convert glucose to fructose 1,6-bisphosphate (F1, 6BP). In the second phase F1, 6BP is degraded to pyruvate, with the production of 4 equivalents of ATP and 2 equivalents of NADH.

Some of the enzymes catalyze the reaction in one direction as, for example, glucokinase (or hexokinase IV) and hexokinase I, II, and III mediating the interaction of glucose with ATP to form glucose 6-phosphate, whereas the reverse reaction is mediated by glucose 6-phosphatase. Many other enzymes catalyze reversible reactions, and equilibrium is attained no matter from which direction the reaction is started.

The hexokinase reaction: The ATP-dependent phosphorylation of glucose to form glucose 6-phosphate (G6P) is the first reaction of glycolysis, and is catalyzed by tissue-specific isoenzymes known as hexokinase. The phosphorylation accomplishes two goals: First, the hexokinase reaction converts nonionic glucose into an anion that is trapped in the cytoplasm, since cells lack transport systems for phosphorylated sugars. Second, the otherwise biologically inert glucose becomes activated into a labile form capable of being further metabolized.

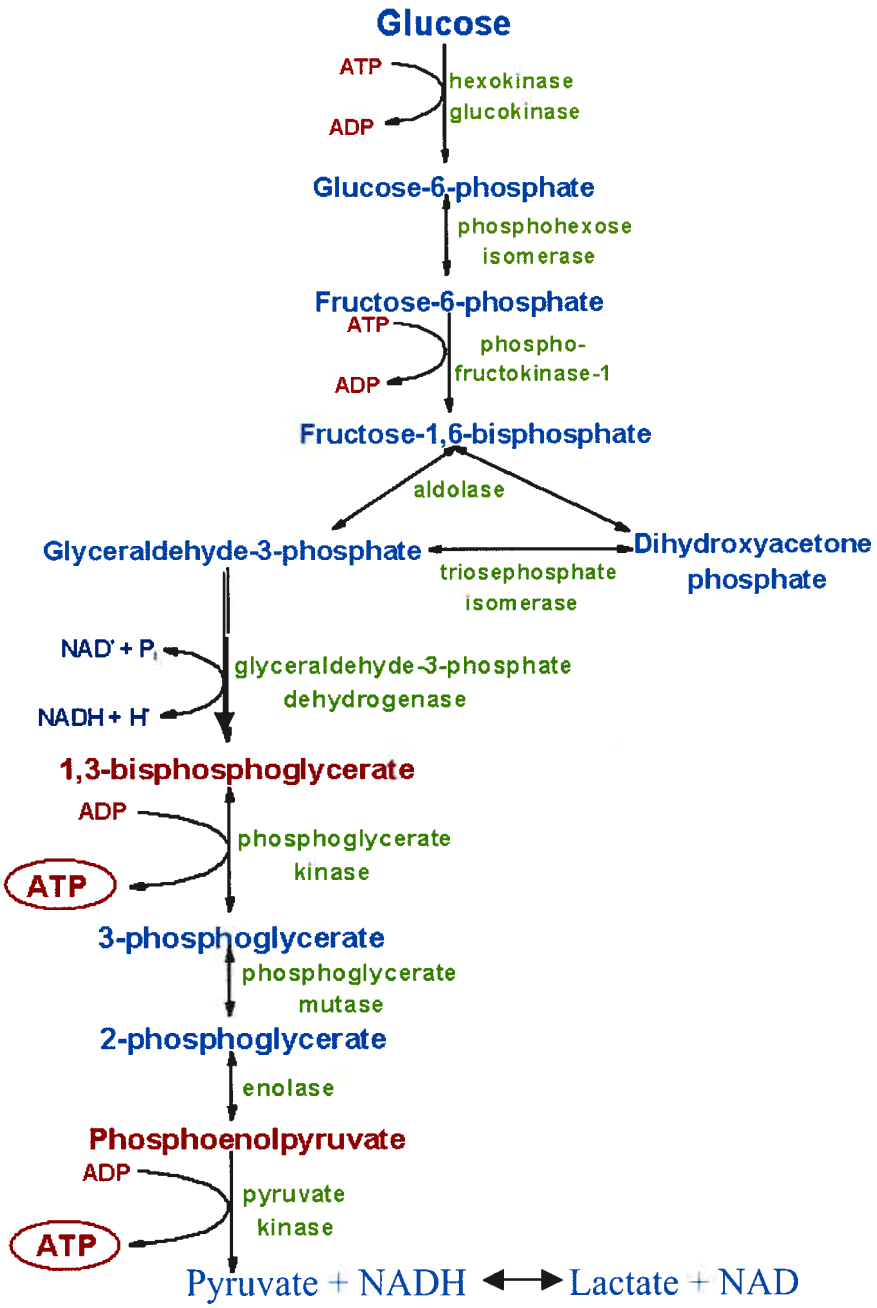

Figure1.4: Pathway of glycolysis from glucose to pyruvate. Substrates and products are in blue; enzymes are in green. The two high-energy intermediates whose oxidations are coupled to ATP synthesis are shown in red. Under aerobic conditions, pyruvate in most cells is further metabolized via the TCA cycle. Under anaerobic conditions pyruvate is converted to lactate by the enzyme ***lactate dehydrogenase*** **(LDH)**, and the lactate is transported out of the cell into the circulation. ***Figure adapted from (77)***

Phosphohexose isomerase: The second reaction of glycolysis is an isomerization, in which G6P is converted to fructose 6-phosphate (F6P). The enzyme catalyzing this reaction is phosphohexose isomerase (also known as phosphoglucose isomerase). The reaction is freely reversible at normal cellular concentrations of the two-hexose phosphates and thus catalyzes this interconversion during glycolytic carbon flow and during gluconeogenesis.

6-Phosphofructo-1-kinase (phosphofructokinase-1, PFK-1): The next reaction of glycolysis involves the utilization of a second ATP to convert F6P to fructose 1,6-bisphosphate (F1, 6BP). This reaction is catalyzed by 6-phosphofructo-1-kinase, better known as phosphofructokinase-1 or PFK-1. This reaction is not readily reversible because of its large positive free energy ($\Delta G_0^{'}$ = +5.4 kcal/mol) in the reverse direction. Nevertheless, fructose units readily flow in the reverse (gluconeogenic) direction because of the ubiquitous presence of the hydrolytic enzyme, fructose-1, 6-bisphosphatase (F-1, 6-BPase).

Aldolase: Aldolase catalyses the hydrolysis of F1, 6BP into two 3-carbon products: dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P). The aldolase reaction proceeds readily in the reverse direction, being utilized for both glycolysis and gluconeogenesis

Triose phosphate isomerase: The two products of the aldolase reaction equilibrate readily in a reaction catalyzed by triose phosphate isomerase. Succeeding reactions of glycolysis utilize G3P as a substrate; thus, the aldolase reaction is pulled in the glycolytic direction by mass action.

Glyceraldehyde-3-phosphate dehydrogenase: The second phase of glucose catabolism features the energy-yielding glycolytic reactions that produce ATP and NADH. In the first of these reactions, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) catalyzes the NAD^+-dependent oxidation of G3P to 1,3-bisphosphoglycerate (1,3BPG) and NADH. The G3PDH reaction is

reversible, and the same enzyme catalyzes the reverse reaction during gluconeogenesis.

Phosphoglycerate kinase: The high-energy phosphate of 1,3-BPG is used to form ATP and 3-phosphoglycerate (3PG) by the enzyme phosphoglycerate kinase. Note that this is the only reaction of glycolysis or gluconeogenesis that involves ATP and yet is reversible under normal cell conditions. Associated with the phosphoglycerate kinase pathway is an important reaction of erythrocytes, the formation of 2,3BPG by the enzyme bisphosphoglycerate mutase. 2,3BPG is an important regulator of hemoglobin's affinity for oxygen. Note that 2,3-bisphosphoglycerate phosphatase degrades 2,3BPG to 3-phosphoglycerate, a normal intermediate of glycolysis. The 2,3BPG shunt thus operates with the expenditure of 1 equivalent of ATP per triose passed through the shunt. The process is not reversible under physiological conditions.

Phosphoglycerate mutase and enolase: The remaining reactions of glycolysis are aimed at converting the relatively low energy phosphoacyl-ester of 3-PG to a high-energy form and harvesting the phosphate as ATP. The 3-PG is first converted to 2-PG by phosphoglycerate mutase and the 2-PG conversion to phosphoenolpyruvate (PEP) is catalyzed by enolase

Pyruvate kinase: The final reaction of aerobic glycolysis is catalyzed by the highly regulated enzyme pyruvate kinase (PK). In this strongly exergonic reaction, the high-energy phosphate of PEP is conserved as ATP. The loss of phosphate by PEP leads to the production of pyruvate in an unstable enol form, which spontaneously tautomerizes to the more stable, keto form of pyruvate. This reaction contributes a large proportion of the free energy of hydrolysis of PEP.

1.3.2.2 Glycolytic enzyme pattern in human cancer tissue

Because of the high glycolytic activity of tumors and the assumption that the activities of glycolytic enzymes might be elevated in tumors, Warburg and Christian (78) conceived the possibility that the blood leaving the tumor and entering the general circulation might show elevation of serum glycolytic enzymes. Indeed, they found that serum aldolase activity was elevated in rats bearing the Jensen sarcoma. These observations were confirmed by Sibley and Lehninger (79) who, in addition, extended the studies to human cancer.

Since these early studies, other enzymes in the glycolytic sequence and, indeed, in other metabolic sequences have been studied with regard to their appearance in human serum and their elevation in patients with cancer. Several factors may be involved in the elevation of a serum enzyme activity in cancer: 1- the damage of membranes of tumor cells or normal cells, so that one or more enzymes pass into the extracellular fluid and then into the circulation, 2- the size of the tumor or organ which is damaged and the concentration of enzymes in the tumor or normal organ and 3- the rate of disappearance of the enzyme from the circulation, whether by metabolism or by excretion. Elevations in serum enzyme activities may be characterized by varying degrees of specifity.

1.3.3 Anaerobic glycolysis of neoplastic cells

Under aerobic conditions, acetyl-CoA in most cells is further metabolized via the TCA cycle (**Figure 1.5**). Under anaerobic conditions and in erythrocytes under aerobic conditions, pyruvate is converted to lactate by the enzyme lactate dehydrogenase (LDH), and the lactate is transported out of the cell into the circulation. The conversion of pyruvate to lactate, under anaerobic conditions, provides the cell with a mechanism for the oxidation of NADH (produced during the G3PDH reaction) to NAD^+, which occurs during the LDH catalyzed reaction. This reduction is required since NAD^+ is a necessary

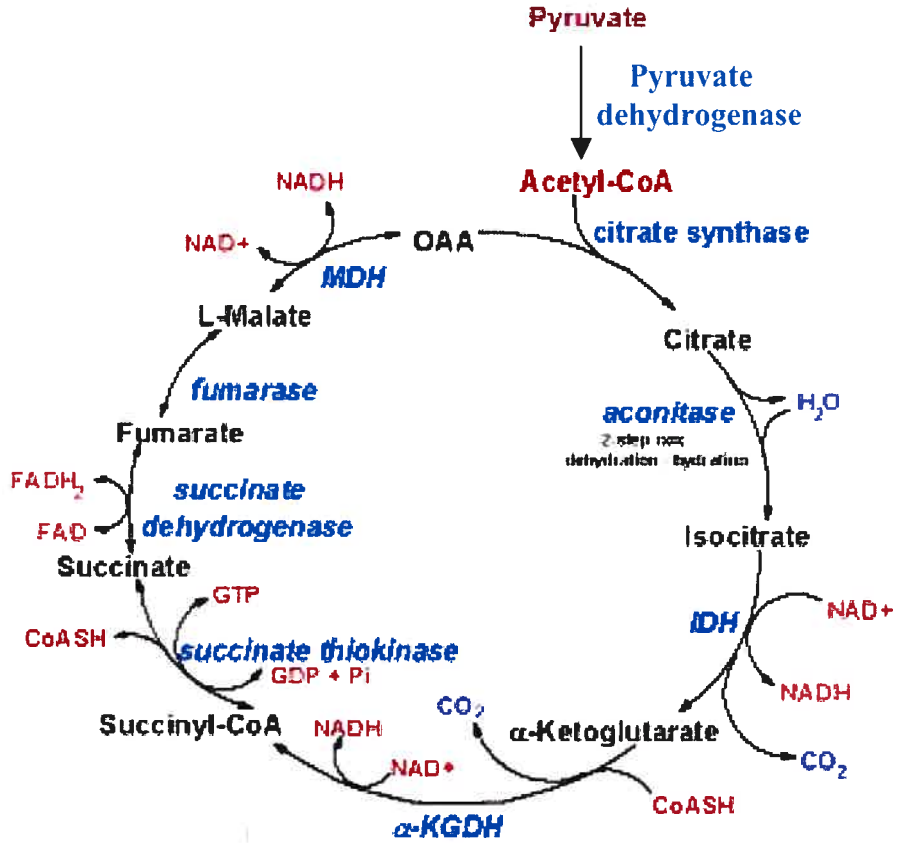

Figure 1.5: Schematic representation of the TCA cycle showing enzymes, substrates and products. The abbreviated enzymes are: IDH = *isocitrate dehydrogenase* and α-KGDH = *α-ketoglutarate dehydrogenase*. The GTP generated during the *succinate thiokinase* (succinyl-CoA synthetase) reaction is equivalent to a mole of ATP by virtue of the presence of *nucleoside diphosphokinase*. The 3 moles of NADH and 1 mole of $FADH_2$ generated during each round of the cycle feed into the oxidative phosphorylation pathway. Each mole of NADH leads to 3 moles of ATP and each mole of $FADH_2$ leads to 2 moles of ATP. Therefore, for each mole of acetyl-CoA, which enters the TCA cycle, 12 moles of ATP can be generated.

Figure adapted from (77)

substrate for G3PDH, without which glycolysis will cease. Normally, during aerobic glycolysis the electrons of cytoplasmic NADH are transferred to mitochondrial carriers of the oxidative phosphorylation pathway generating a continuous pool of cytoplasmic NAD^+.

Aerobic glycolysis generates substantially more ATP per mole of glucose oxidized than does anaerobic glycolysis. The utility of anaerobic glycolysis to a cell when it needs large amounts of energy stems from the fact that the rate of ATP production from glycolysis is approximately 100X faster than from oxidative phosphorylation. Cells do not need to energize anabolic reaction pathways. The requirement is to generate the maximum amount of ATP in the shortest time frame.

1.3.3.1 Control of glycolysis by proton secretion

There are several factors, which can contribute to the availability of the phosphorylating system and to the control of energy states in the cell. Aerobic glycolysis of glucose to pyruvate requires two equivalents of ATP to activate the process, with the subsequent production of four equivalents of ATP and two equivalents of NADH. Thus, conversion, of one mole of glucose to two moles of pyruvate is accompanied by the net production of two moles each of ATP and NADH and 2 protons (80).

1.4 Intracellular pH regulation in normal and tumor tissues

1.4.1 Secretion of protons by normal cells (pH homeostasis)

The maintenance of an appropriate pH within membrane-enclosed compartments is a constant challenge for all living beings, from the simplest prokaryotes to complex multicellular organisms. Accordingly, cells have evolved a variety of specialized proton-translocating devices (80).

In mitochondria, cytochromes exploit the flux of electrons to extrude protons, thus producing a proton gradient that is used to generate useful chemical energy in the form of ATP. Other organelles consume ATP to pump protons into the lumen to generate the acidic pH required for the maturation and processing of secretory proteins and for the dissociation and recycling of endocytosed materials. Proton-exchanger, ionophores of interest, allows an efficient extraction and/or release of cations by a simple control of the pH (80).

1.4.1.1 Intracellular alkalinization: normal vs tumor cells

Measurement of pH in vivo has shown that the microenvironment of tumors is generally more acidic than in normal tissue, with median pH values of about 7.0 for tumors and 7.5 for normal tissue (69, 81, 82). The studies of Warburg in the early part of the century (69) showed that tumor cells preferentially convert glucose and other substrates to lactic acid, even under aerobic conditions (83). Since then, positron emission tomography (PET) and magnetic resonance spectroscopy (MRS) have consistently demonstrated the increased use of glycolysis in tumors (84-87). The increased use of the glycolytic pathway matched with the compromised vasculature of a tumor results in the poor removal of lactic acid and is believed to be the main cause of acidity within solid tumors (88).

Traditionally, most estimates of pH in tissue were obtained by insertion of pH electrodes (81). These were usually quite large in comparison to a tumor cell (83), and primarily measured interstitial or extracellular tissue pH. For most purposes, the parameter of interest is intracellular pH (pHi), and since lactic acid production can lead to intracellular acidosis, tumors were thought for many years to have a more acidic pHi (89). However, with the advent of MRS imaging for non-invasive measurements of pHi (83), both human and animal tumor cells have been shown to have intracellular pH similar to normal cells, near neutrality (pH 7.0) (88, 90), or even slightly alkaline (pH

7.1-7.2) (89, 91). This gives rise to a reversed pH gradient across the cell membrane between normal tissues and tumors.

1.4.1.2 Extracellular acidification on tumor cell

Despite the more acidic tumor microenvironment, most *in vitro* experiments are still performed at the relatively alkaline extracellular pH (pHe) of 7.4. This is significant because slight changes in pHe can have profound effects on cell phenotype (92). It has been shown that the metastatic potential of tumor cells depends directly on the degree of acidification (93). More specifically studies by Martinez-Zaguilan and co-workers (92) have shown that the incubation of human melanoma cells under conditions of low pHe causes them to become more invasive and migratory. Furthermore, tumor angiogenesis may be regulated by pH. The switch to the angiogenic phenotype depends on a net balance of positive and negative angiogenic factors released by the tumor. Recent evidence from several laboratories (94-96) support a role for pH in this process, and indicate that tumor cells at low pH, increase the expression of positive angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8), probably in order to increase the vasculature of the tumor.

1.4.1.3 Importance of Na^+/H^+ exchange and intracellular pH in tumor cells

Proper regulation of intracellular pH (pHi) is critical for the optimal function of almost every biologic process. In many cell types, pHi is regulated primarily by a family of Na^+/H^+ exchangers (NHEs), transmembrane proteins that mediate the electroneutral exchange of an intracellular proton for an extracellular sodium ion. NHE1 was the first Na^+/H^+ exchanger isoform to be cloned and sequenced in 1989 (97). Since then, much effort has been directed to identifying novel members of the Na^+/H^+ exchanger family and

understanding their roles in a range of physiologic and pathophysiologic cellular processes, from cellular proliferation to apoptosis (98, 99).

The Na^+/H^+ exchanger has been recently examined in several models of human tumors. They suggest that, in at least some cell types, the Na^+/H^+ exchanger and intracellular pH regulation associated with this protein play an important role in tumor cell growth (100). Early studies showed that the Na^+/H^+ exchanger deficient cells (101) lost or severely reduced their capacity to grow tumors in vivo in immune deficient mice. In addition, a variety of evidence suggested that activation of the Na^+/H^+ exchanger and the resultant increases in intracellular pH may be required for mitogenesis in some cell types (98). In HCO_3^- free media, Na^+/H^+ exchanger deficient cells cannot proliferate in media of low external pH (101). In addition, extracellular Na^+ is limiting in growth factor-dependent proliferation (102), and amiloride and its analogs can block changes caused by growth factor stimulation, especially in HCO_3^- free or low Na^+ medium (103).

Because of the important role of the Na^+/H^+ exchanger in tumor and cell growth, amiloride and its analogs have been tested for use in tumor selective therapy.

1.4.2 Molecular structure of vertebrate Na^+/H^+ exchangers

Na^+/H^+ exchangers (NHE), also called antiporters, are vital transmembrane transporters involved in multiple cellular functions including the regulation of intracellular pH, the control of cell volume and transepithelial ion transport (104, 105).

1.4.2.1 Introduction

Na^+/H^+ antiport (exchange) is one of the primary mechanisms involved in the extrusion of H^+ from vertebrate cells. Originally described by Murer et al., (106) in vesicles from brush-border membranes of kidney tubules, the

transporter has since been identified in the plasma membrane of virtually all eukaryotic cells. Biochemical studies and, more recently, molecular cloning have provided increasing evidence about the structure, functional features and regulation of the Na^+/H^+ exchangers, referred to as NHE (107). Na^+/H^+ exchangers are integral plasma membrane proteins that catalyze the electroneutral exchange of extracellular Na^+ for intracellular H^+ with a stoichiometry of one for one. An essential feature of these exchangers is their allosteric activation by intracellular protons, which are presumed to interact at a 'modifier' site that is separate from the sites involved in Na^+ and H^+ transport (107).

The activity and expression level of the exchangers can be modulated by a remarkably wide variety of stimuli, including growth factors, tumor promoters, hormones (107) and chronic extracellular acidification (108), as well as by physical factors, such as changes in cell volume (109) or cell spreading (110). The most widely studied NHE isoform, NHE-1, is ubiquitously expressed and is involved in a variety of cellular functions by virtue of its ability to govern intracellular pH. It is inhibited by the diuretic compound amiloride and by the 5-amino-substituted derivatives of amiloride. Besides NHE-1, many other isoforms, initially characterized by their lower sensitivity to amiloride, have been identified and cloned, principally in epithelia where they perform more specialized ion transport (NHE-2, NHE-3, NHE-4, NHE-8). NHE-6 and NHE-7 are specialized isoforms involved in organelle pHi regulation and Na^+ transport (99).

1.4.2.2 Molecular identification of the Na^+/H^+ exchangers

Using a genetic strategy, Sardet et al., (1989) were the first to identify a Na^+/H^+ exchanger isoform fully by cloning a human cDNA encoding the amiloride-sensitive growth-factor-activatable Na^+/H^+ antiporter. This cDNA, cloned by complementation of a Na^+/H^+ antiport-deficient cell line, was shown to restore fully the biochemical and physiological features of the

transporter, namely pHi regulation, when transfected into antiporter-deficient cells. This human exchanger cDNA, now referred to as NHE-1, is ubiquitously expressed in tissues and cells and, in polarized epithelial cells, generally resides in the basolateral membrane, although exceptions have been reported (111). Pharmacological, kinetic and regulatory properties of Na^+/H^+ exchangers in various cell types, tissues and species had predicted the existence of multiple isoforms of Na^+/H^+ exchangers (112).

1.4.2.3 Structural features

On the basis of their hydropathy profiles, all Na^+/H^+ exchangers exhibit similar topologies. The molecule has two separate functional domains: an N-terminal hydrophobic domain (made up of 10–12 membrane-spanning domains) with approximately 500 amino acids, and a C-terminal hydrophilic domain with around 300 amino acids **(Figure 1.6).** The N-terminal transmembrane part of NHE-1 is necessary and sufficient to catalyze the ion exchange, whereas the cytoplasmic C-terminal domain determines the pH set point value of the exchanger and is crucial for mediating the activation of the exchanger by growth factors, hormones and hyperosmotic stress (113).

1.4.2.4 The NHE-1 isoform

Amiloride and its 5-amino-substituted derivatives are reported to inhibit the transporter by competing with Na^+ for its external binding site (114). The amiloride-binding site appears to be located in the N-terminal domain of the exchanger since NHE-1, after removal of the entire cytoplasmic domain, remains sensitive to amiloride (113). NHE-1 is the isoform that is most sensitive to amiloride and its analogues (103, 115).

Most mitogens activate the NHE-1 isoform, leading to an intracellular alkalization (107), most easily detectable in the absence of bicarbonate (116, 117). At least three groups have reported that mitogenic agents activate the antiporter by shifting the pH-dependence of the modifier site, adjusting the set

point upwards by 0.15–0.30 pH units (118-121). As a result, the exchanger is activated, but only temporarily, returning to near quiescence when intracellular pH (pHi) attains the new set point value.

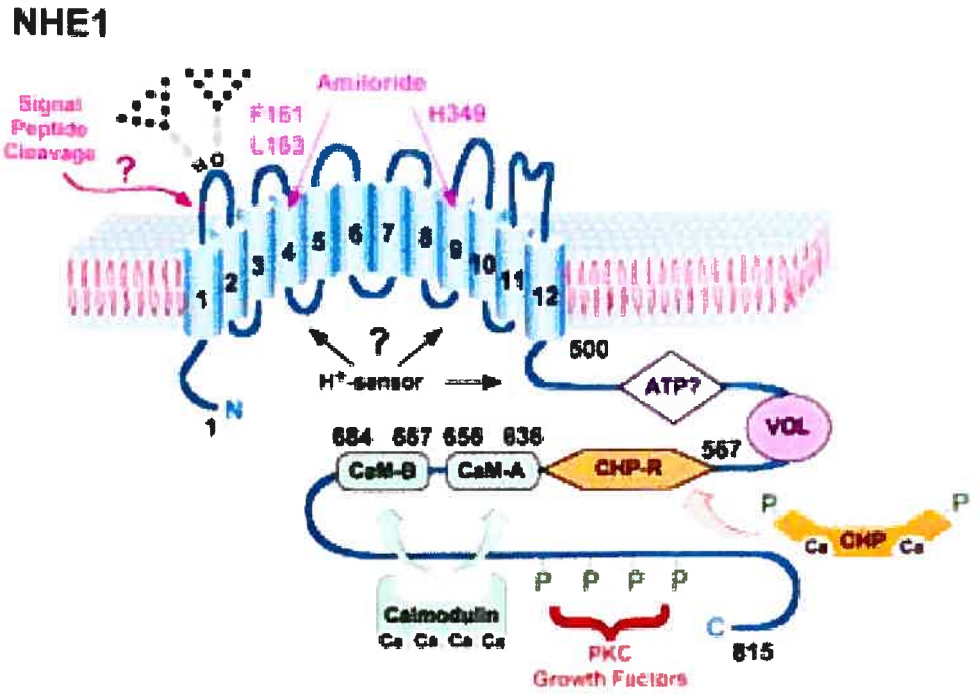

Figure 1.6: The general topology and regulatory sites of the Na+/H+ exchanger. The Na^+/H^+ exchanger is comprised of 12 transmembrane domains. The long cytosolic tail has 3 subdomains: one for regulation by internal ATP levels, which may involve changes in PIP2 levels in the adjacent membrane, a second that includes a "calmodulin autoinhibitory domain" and the last that contains phosphorylation consensus sequences which are either directly phosphorylated by cytoplasmic kinases or through accessory proteins. PIP2 = phosphatidylinositol 4,5-bisphosphate.

Figure adapted from (105)

1.4.3 Therapeutic potential of inhibitors of Na^+/H^+ exchanger

Since Na^+/H^+ exchange plays an important role in regulation of intracellular pH (pHi) and is essential for homeostasis of cells, inhibitors of the exchanger might influence the viability and/or proliferation of both normal and tumor cells.

Amiloride, a potassium-sparing diuretic, is known to inhibit the activity of the Na^+/H^+ exchanger by binding to the antiport at a site on the extracellular side of the membrane. Kleyman and Cragoe showed that analogs of amiloride such as EIPA or HMA were more potent and more specific inhibitors of Na^+/H^+ exchange activity than amiloride. EIPA was found to inhibit completely the Na^+/H^+ exchange activity (122).

1.5 Invasion and metastasis

1.5.1 Introduction

Metastatic spread of cancer cells greatly compounds the severity of the disease since it can result in secondary tumors developing in vital organs and could be one of the main contributing factors to cancer mortality (123)

1.5.2 Tumor-host interactions during the metastatic cascade

Clinically it has been observed that while large numbers of cancer cells can be shed into the circulation, only very few of these cells will result in clinically apparent metastases. Once cancer cells separate from the primary tumor and enter the circulation, the vast majority of them would quickly be destroyed in the circulation during transport to the secondary site and / or in the microcirculation after their arrest. Factors involved in this destruction could include homodynamic forces and the immune system in immunocompetent animals (124).

Various steps in the metastatic process have been believed to contribute significantly to this inefficiency of each tumor cells released to develop a secondary tumor. First, properties of the cells in the primary tumor can determine the likelihood of metastases developing, including the tendency and ability of cells to escape from the primary tumor and intravasate into the circulation. Second, the circulation has been believed to be a particularly hostile environment for cancer cells, the vast majority of which had thought to be destroyed by hemodynamic forces. Third, the ability to extravasate has been considered a major rate-limiting step in metastasis, which few cells that arrive in a new capillary bed thought to be able to escape from the circulation into the surrounding tissue. Fourth, cells that successfully extravasate need to be able to adhere to a different tissue and grow in the new site including a requirement for induction of new blood vessels in order for the tumor to grow beyond small size (124).

End point analysis, histological examination and electron micrographs have been used to formulate theories of metastasis. It was considered that cancer cells were arrested in venules that have diameters much larger than their own. Two different theories described how cancer cells moved into the surrounding tissues after becoming arrested in the microcirculation (125). One view is that cancer cells began dividing intravascularly used proteases to destroy the surrounding vascular basement membrane, and they literally divided their way into the surrounding tissues where they continued to replicate (125). A second view is that cancer cells behaved more like leukocytes and could extravasate singly without the destruction of micro vascular walls, using pseudopodial projections to move either between or through the endothelial cells (125).

1.5.3 The metastatic cascade

The inefficiency of tumor cells in completing the metastatic cascade(adapted from the Cancer principles and practice of oncology; 6^{th} edition) **(Figure 1.7)** is the result of the fact that successful formation of metastatic foci consists of

several highly complex and interdependents steps. Each step is rate limiting in that failure to complete any of these events completely disrupts metastasis formation. Metastasis is thought to be similar in all tumors and is characterized as follow:

1- Oncogenesis (tumorigenesis). After the initial neoplastic transformation, often by a virus, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential. The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate limiting for further growth.

2- Angiogenic switches. As the tumor grows and central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply. This process is called the angiogenic switch and involves the secretion of various angiogenesis inhibitors. Vascularization of the tumor is also associated with a dramatic increase in the metastatic potential of these tumors.

3-Clonal dominance and invasive phenotype. A continued genetic alteration in the tumor cell population results in selection of tumor cell clones with distinct growth advantage and acquisition of an invasive phenotype. Invasive tumor cells down–regulate cell-cell adhesion, alter their attachment to the extracellular matrix (ECM) by changing integrin expression profiles and proteolytically alter the matrix. Collectively, these changes result in enhanced cell motility and the ability of these invasive cells to separate from the primary tumor mass. These cells can detach from the primary tumor and create defects in the ECM that define tissue boundaries, such as basement membranes, thus accomplishing stromal invasion. Furthermore, the poorly formed tumor vasculature that forms in response to the angiogenic switch in phenotype of the primary tumor, as well as thin walled lymphatic channels in the surrounding stroma, are readily penetrated by these invasive tumor cells and offer ready conduits to the systemic circulation. Endothelial cells

responding to the angiogenic stimulus produced by the primary tumor also express an invasive phenotype.

4- Surviving in the circulation. Once the tumor cells and tumor clumps (emboli) have reached the vascular or lymphatic compartments, they must survive a variety of hemodynamic and immunologic challenges. Little is known about how these factors may impact on the inefficiency of the metastatic process in human cancers.

5- Tumor cell arrest. After survival in the circulation, tumor cells must arrest in distant organs or lymph nodes. This arrest may occur by size trapping on the inflow side of the microcirculation, or by adherence of tumor cells through specific interactions with capillary or lymphatic endothelial cells, or binding to exposed basement membrane.

6- Extravasation and growth at the secondary site. In most cases, arrested tumor cells extravasate before proliferating. Studies suggest that extravasation of tumor cells are not dependent on protease activity and are independent of metastatic potential. After exiting the vascular or lymphatic compartments, metastatic tumor cells may proliferate in response to paracrine growth factors or become dormant. After extravasation, tumor cell migrate to a local environment more favorable for their continued growth. Findings using in vivo videomicroscopy demonstrate that the poor growth of tumor cells after extravasation from the circulation is a major factor contributing to the inefficiency of the metastatic process.

7- Angiogenesis in metastatic foci. Finally continued growth of the metastatic foci is also dependent on angiogenesis. The development of this neovascular network at the metastatic site enhances the metastatic potential of these cells just as it does for the primary tumor.

8- Evasion of immune response. Metastatic foci of tumor cells must evade eradication by immune responses that may be either nonspecific or targeted directly against the tumor cells.

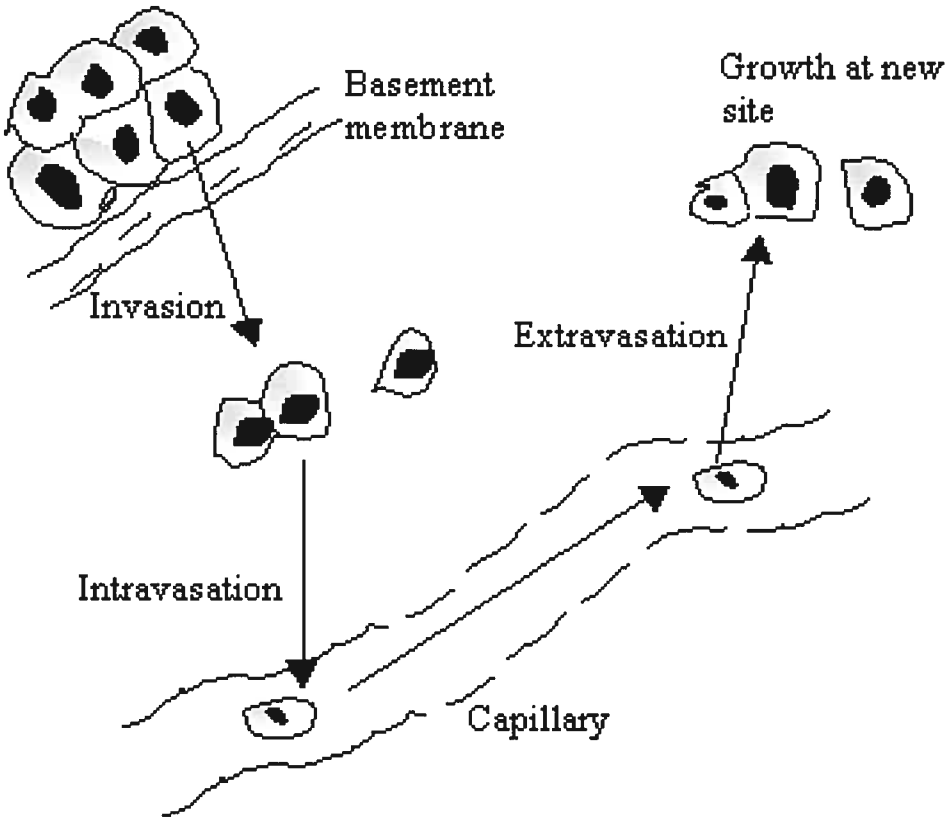

Figure 1.7 : Tumbling down the metastatic cascade. After breaking off from the primary tumor (far left), cancer cells travel through the blood vessels. Those that reach a secondary site such as the lung (right) may colonize it and form a metastasis.

Figure adapted from (126)

1.6 Objectives of the present study.

The MSV-MDCK-INV (INV) were selected from Moloney sarcoma virus (MSV) transformants of the polarized epithelial MDCK cell line for their capacity to pass through a Matrigel® –coated filter unit **(Figure 1.8)**. INV cells exhibit multiple pseudopodia, no visible actin stress fibers and β-actin rich domains at the tips of pseudopodia where is concentrated the hepatocyte growth factor receptor (HGF-R) otherwise known as the c-Met proto-oncogene (127). Moreover, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) **(Figure 1.9)** and the Na^+/H^+-exchanger NHE1 **(Figure 1.10)** from INV cells were found to colocalize with actin in pseudopodia, the site of dynamic actin polymerization.We hypothesized that a pseudopodial ATP would be involved in modulating c-Met activation, motility and invasion of INV cells.

Given the demonstrated role of glycolysis as a primary energy source for tumor cell motility, and the role of c-Met phosphorylation in modulating cancer cell invasion (128) and pseudopodial protrusion (128), we were interested in investigating the mechanism underlying the critical role of glycolysis in tumor cell motility, which is to drive the formation and protrusion of tumor cell pseudopodia. We took advantages of the improved pseudopod purification technique which gives hundred of μg of pseudopodial proteins to determine the content in glycolysis enzymes (hexokinase, aldolase, phosphoglucose isomerase) and mitochondrial proteins (PDK-1 and TOM 20) relative to β-actin and p-c-Met in cell bodies and total cell lysates. It was of interest to estimate the phosphorylation state of c-Met in conditions where cell ATP concentration was modulated by the use of different media and inhibitors. We performed several experiments intending to specifically decrease the concentration of ATP produced from glycolysis by using iodoacetate, or ATP produced from mitochondria by using antimycin A and

oligomycin. To assess whether ATP from mitochondrial respiration is also required for the formation of β actin rich pseudopodia, INV cells were incubated with oligomycin or antimycin in high glucose and glucose-free media . It was also of great interest to investigate the phosphorylation level of c-Met in the presence of an inhibited NHE-1. And finally to ascertain that the glycolytic ATP production within pseudopodia of INV cells is necessary to drive cell motility, wound-healing assays were realized to estimate the motile capability of INV cells in conditions shown to modulate c-Met phosphorylation level.

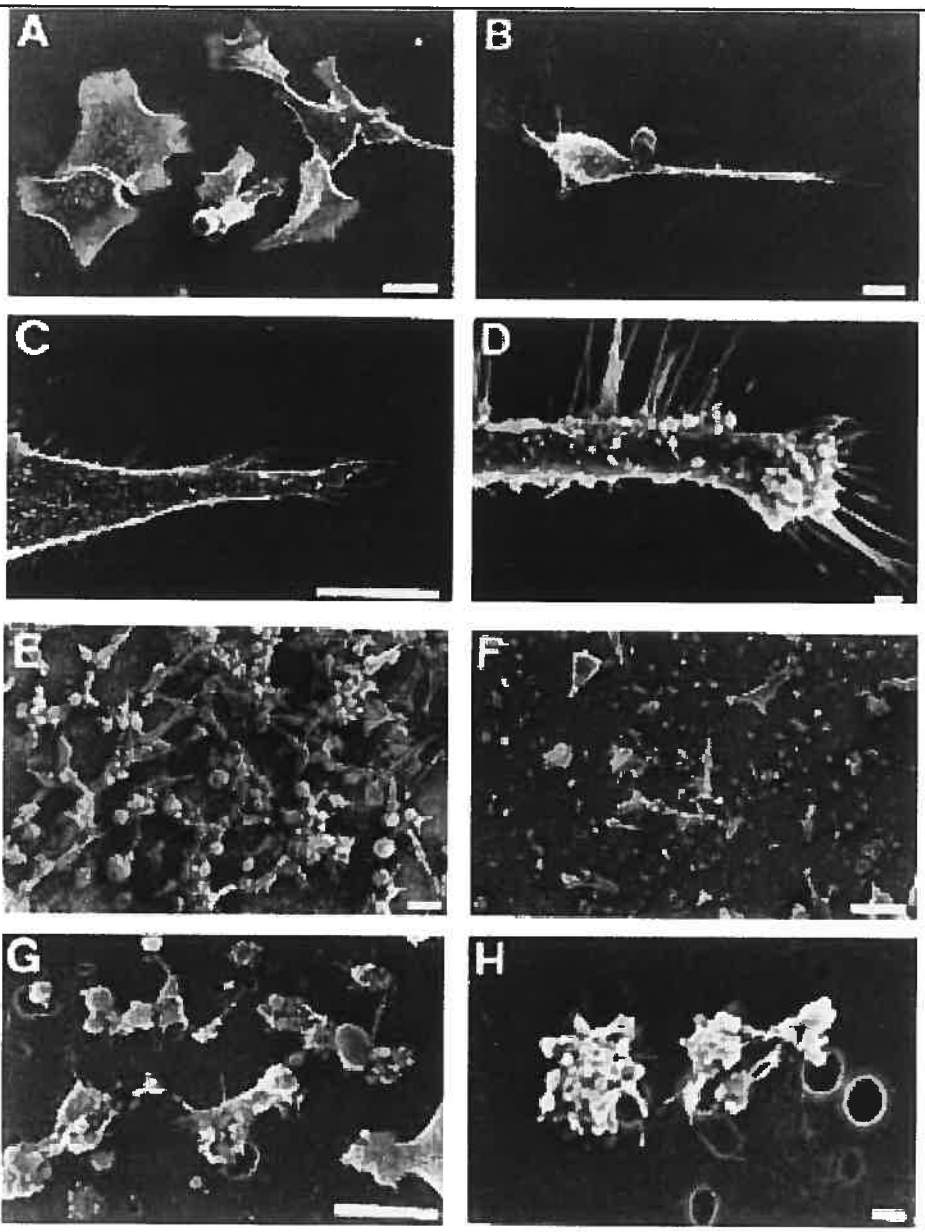

Figure 1.8: β-Actin-rich pseudopodia of MSV-MDCK-INV cells are highly blebbed as visualized by scanning electron microscopy. MSV-MDCK (A, C) and MSV-MDCK-INV (B, D–H) cells plated on a glass substrate (A–D) or on 1-μm-pore filters (E–H) were prepared for scanning electron microscopy. Distinctive blebbed pseudopodia are present in MSV-MDCK-INV cells (B, D) which are not present in MSV-MDCK (A, C) cells plated on a substrate. The upper side of the filter (E) presents numerous cell bodies while on the lower side of the filter (F–H) numerous protrusions have passed through the filter pores, the majority of which present the same blebbed morphology as the pseudopodia of MSV-MDCK-INV cells plated on a substrate. (A, E, F), bar, 25 μm; (B, C, G) bar, 10 μm; (D, H) bar, 1 μm.

Figure from (129).

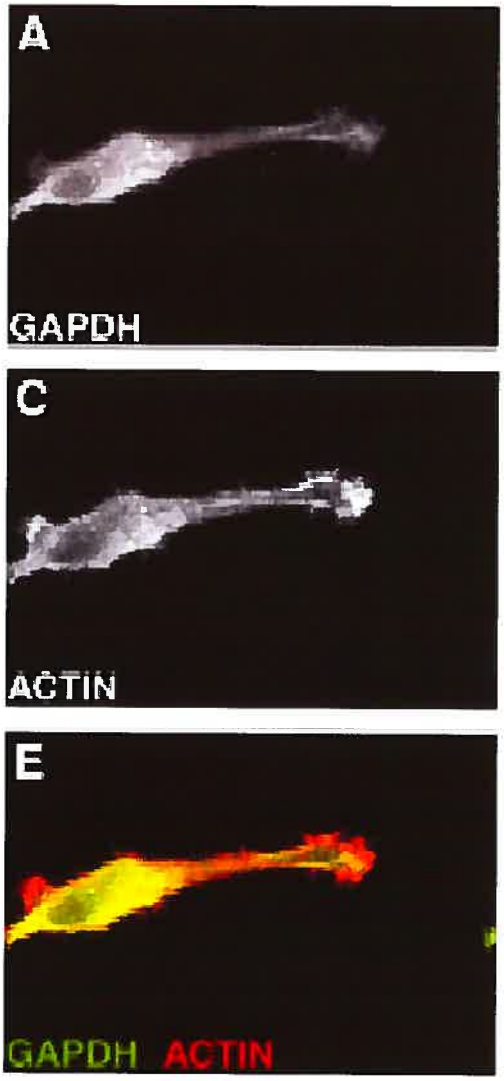

Figure 1.9: Immunofluorescent distribution of GAPDH in MSV-MDCK-INV cells. MSV-MDCK-INV cells were double immuno-fluorescently labeled for anti-GAPDH (A) followed by FITC-conjugated anti-mouse secondary antibodies together with actin using Texas red phalloidin (130). Merged images are presented with actin in red and GAPDH (E) in green as indicated. GAPDH labeling can be observed in the actin-labeled tips of MSV-MDCK-INV pseudopodia, although the majority of GAPDH labeling is present in the cell body.
Figure from (129)

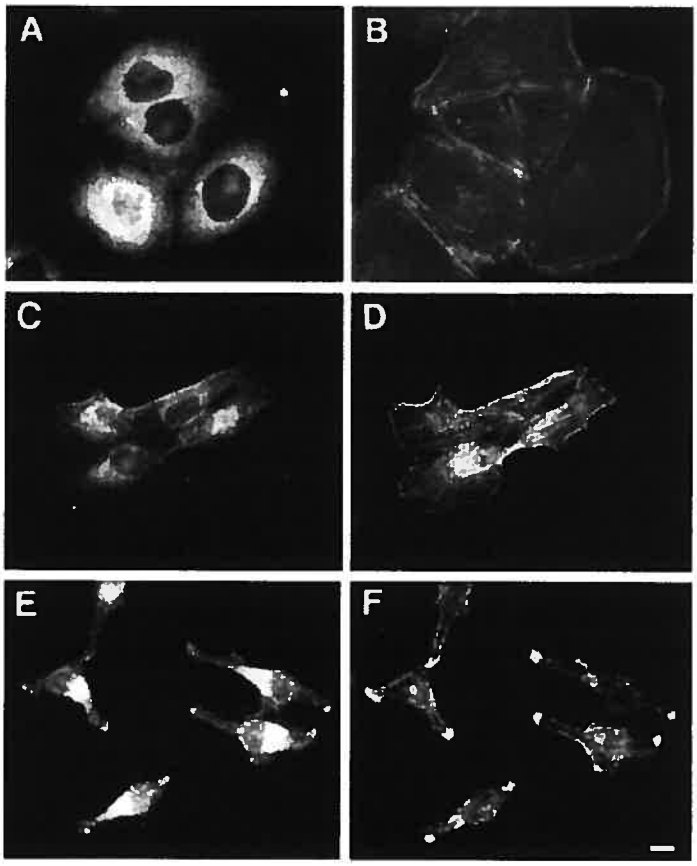

Figure 1.10: NHE1 is localized to β-actin-rich pseudopodia in MSV-MDCK-INV cells. (A,B) MDCK, (C,D) MSV-MDCK and (E,F) MSV-MDCK-INV cell lines were immunofluorescently labeled with polyclonal anti-NHE1 (A,C,E) and monoclonal anti-β-actin (B,D,F) antibodies. NHE1 is uniformly distributed in MDCK and MSV-MDCK cells, whereas in MSV-MDCK-INV cells it is also localized at the tips of pseudopodia, where it colocalizes with β-actin. Bar, 10 μm.
Figure from (131)

CHAPTER 2

Manuscript

Pseudopodial MSV-MDCK-INV glycolysis modulates the c-Met phosphorylation-dependent cell motility

Will be submitted to Cancer Research

Carlos El Hader, Yolaine Dodier, Zeinab Daher, Zongjian Jia, I.Robert Nabi and Josette Noël.

Running head : Pseudopodial ATP modulates INV motile character

Pseudopodial MSV-MDCK-INV glycolysis modulates the c-Met phosphorylation-dependent cell motility

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2.1 Summary

MSV-MDCK-INV (INV) cells were selected for their invasive character from a Moloney sarcoma virus-transformed cell line (MSV-MDCK). They present multiple β-actin rich pseudopodia where are localized the glycolytic enzyme GAPDH, the Na^+,H^+-exchanger NHE1, and the phosphorylated c- Met, activated via an autocrine HGF loop. We identified a pseudopodial ATP and we examined whether this ATP produced by glycolysis and the protons secreted by NHE1 drive pseudopodial formation and the motile character of INV cells. Hexokinase, phosphoglucose isomerase and aldolase were detected in pseudopodia and colocalized with β-actin and phosphorylated c-Met (p-c-Met). Mitochondrial proteins were absent from actin-rich pseudopodial domains. Incubating cells in glucose-free medium with or without 2-deoxyglucose, or inhibiting glycolysis with iodoacetate in high glucose medium decreases the phosphorylation level of c-Met and lead to the loss of pseudopodia. In contrast, mitochondrial inhibitors such as oligomycin and antimycin-A do not affect c-Met phosphorylation in high glucose medium but decrease it in glucose-free medium and more than that induce bleb formation. Together, these results indicate a preference for ATP production from glycolysis to maintain pseudopodial phosphorylation of c-Met and actin remodelling, which were shown to modulate cell motility (Vadnais *et al.*, J. Biol. Chem. 277: 48342-50, 2002). Inhibition of the Na^+,H^+ exchanger with ethyl-isopropy-amiloride in the absence of bicarbonate, reduce the pHi by 0.7 unit (Lagana *et al.*, J Cell Sci, 113: 3649-3662. 2000),and leads also to a rapid reduction of c-Met phosphoryaltion. Secretion of protons produced by glycolysis may be a necessary step to drive continuous ATP production required for the localized autocrine c-Met phosphorylation in pseudopodia. Our results suggest a functional link between pseudopodia-associated glycolysis, NHE1 proton secretion, and phosphorylation of c-Met to regulate cell motility and invasion of MSV-MDCK-INV cells.

2.2 Introduction

Up regulation of multiple glycolytic enzymes (132) and increase in glucose transport (133) is associated with tumor malignancy. Glycolysis was found to be the primary energy source in tumor cells (134), exceeding the capacity of mitochondrial oxidative energy metabolism (135). It was also shown to regulate the protrusion of the multiple pseudopodia, and thereby the motility, of tumor cells (129). Glycolytic enzymes have long been described to associate with the actin cytoskeleton enabling the compartmentalization of these energy-generating enzymes to selected cytosolic locations (136). Glycolytic enzymes distribution correlates with cell-cycle associated changes in the F-actin cytoskeleton suggesting that glycolytic activity may regulate actin cytoskeleton organization (137).

The Met receptor tyrosine kinase is the prototypic member of a small subfamily of growth factor receptors (138). The growth factor that binds and activates Met is commonly named HGF/SF as it was identified independently as both a growth factor for hepatocytes (HGF) and a fibroblast-derived cell motility factor, scatter factor (SF) (55, 139, 140). Following ligand binding and autophosphorylation, c-Met transmits intracellular signals using a unique multisubstrate docking site present within the C-terminal end of the receptor (138, 141-145). Autophosphorylation of the tyrosine residues at positions 1234-1235 has a positive regulatory effect on the enzyme activity; where as autophosphorylation of a serine residue in the juxtamembrane domain negatively regulates the kinase (38). Activated Met induces mitogenic, motogenic, and morphogenic cellular responses (138). In vivo, Met and HGF/SF likely play a key role in regulating many aspects of embryonic development including kidney and mammary gland formation (146-149), migration/development of muscle and neuronal precursors (58, 150) and liver and placenta organogenesis (56, 57). HGF/SF-Met signaling also promotes angiogenesis (151, 152) and has been described to facilitate wound healing (153) and tissue regeneration (154). Met signaling clearly has a role in normal

cellular processes; this signaling pathway has also been implicated in tumor development and progression. Met signaling can increase tumorigenicity, induce cell motility, and enhance invasiveness in vitro and metastasis in vivo (64, 65). In addition, Met signaling can increase the production of protease and urokinase, which are associated with extracellular matrix/basal membrane degradation and are important for metastasis (155). Activating MET mutations leading to increased levels of tyrosine phosphorylation and enhanced kinase activity toward an exogenous substrate when compared with wildtype MET could contribute to papillary renal carcinoma and other human malignancies (62, 75, 156-160). Particularly, Met activation via an autocrine HGF loop is directly responsible for pseudopodial protrusion and cell motility of tumor cells (128). Mutant MET induced motility of Madin-Darby canine kidney cells and metastasis of NIH 3T3 cells while transgenic mice expressing the oncogenic form of MET developed metastatic mammary carcinoma (68).

Under physiological conditions, the Na^+/H^+ exchanger NHE1 extrudes one H^+ ion in exchange for one extracellular Na^+ ion. NHE1 is a ubiquitously expressed membrane phosphoglycoprotein comprising 10-12 transmembrane segments (N-terminal) and a large cytoplasmic tail (C-terminal). NHE1 is involved in intracellular pH (pHi) homeostasis and cell volume regulation. An alkaline pHi together with an acidic extracellular environment are associated with a transformed or tumorigenic phenotype, suggesting that active proton extrusion capabilities provide a twofold advantage to tumor cells: the alkaline pHi favors metabolic processes associated with cellular proliferation, whereas the acidic extracellular environment, a consequence of H^+ extrusion, enhances the invasive capacity of transformed cells. The changes in pHi occuring during the migration of peridontal ligaments cells and human carcinoma and melanoma cells implicate pHi modulation in cell motility.

The MSV-MDCK-INV (INV) were selected from Moloney sarcoma virus (MSV) transformants of the polarized epithelial MDCK cell line for their capacity to pass through a Matrigel® –coated filter unit (161). INV cells exhibit

multiple pseudopodia, no visible actin stress fibers and β-actin rich domains at the tips of pseudopodia (161) where is concentrated the hepatocyte growth factor receptor (HGF-R) otherwise known as the c-Met proto-oncogene (127).

Moreover, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (129) and the Na^+, H^+-exchanger NHE1 (131) from INV cells were found to colocalize with actin in pseudopodia, the site of dynamic actin polymerization. We hypothesized that ATP produced from glycolysis in pseudopodia would be involved in modulating c-Met activation, motility and invasion of INV cells. We present here evidence of a pseudopodial ATP production by showing the expression and enrichment of glycolytic enzymes (hexokinase, phosphoglucose isomerase and aldolase) and the absence of mitochondrial proteins from β actin-rich pseudopodial domains. By using inhibitors of both the glycolytic and the mitochondrial ATP production, we made the demonstration that the pseudopodial ATP from glycolysis is essential for an adequate phosphorylation and local activation of c-Met, which modulates for the most part the motile character of INV cells. Furthermore, we show that NHE1 inhibition leads to a reduction of c-Met activation involving local pHi homeostasis in cell motility and invasion.

2.3 Experimental Procedures

Antibodies and reagents

Antibodies to phosphotyrosine (PT), the glycolytic enzymes hexokinase-1 (HK), aldolase (ALDO), phosphoglucose isomerase (PGI) and the mitochondrial pyruvate dehydrogenase kinase (PDK), the 20 kDa preprotein translocase of the outer membrane (TOM-20) were purchased from Santa Cruz Biotechnologies, Inc (Santa Cruz, CA). Phospho-specific polyclonal c-Met (p-c-Met) antibodies were from BioSource International (Medicorp, Montreal, Canada). Monoclonal antibodies to β-actin, D-luciferin, luciferase firefly and 2-deoxyglucose were purchased from Sigma Chemical (St.Louis, Montreal, Canada). Antimycin-A (Anti-A; mixture of the components A_1, A_2, A_3 and A_4), oligomycin (Oligo; mixture of the components A, B and C) and iodoacetate (IODO) were from Fluka Biochemika (Sigma division). Fetal bovine serum (FBS), glutamine, essential amino acids, vitamins, media, penicillin and streptomycin were obtained from Invitrogen (Burlington, ON). Filters for pseudopod purification were purchased from Becton Dickinson labware (B.F.K, USA) and the ring from Boulons Plus (Anjou, QC). Texas Red-conjugated phalloidin and secondary antibodies conjugated to either Alexa488, Alexa568 or Alexa633 were obtained from Molecular Probes (Eugene,OR). ECL reagent was from Mandel (St.-Laurent, Quebec).

Cell Culture

MSV-MDCK-INV cells were cultured in Dulbecco's minimum essential medium containing 25 mM $NaHCO_3$ (DMEM), 10% FBS, glutamine, 1% essential amino acids, vitamins, penicillin, and streptomycin under 5% CO_2 atmosphere at 37 ° C. Cells were seeded and cultured for 48 h at 2.5 x 10^5 cells / 100 mm plate for immunobloting, at 4 x 10^5 cells / 100 mm plate for ATP measurements and at 2 x 10^4 cells / 35 mm plate on glass coverslips for immunolocalisation experiments.

Drug treatments

MSV-MDCK-INV cells were treated for 15 min (or longer, as indicated) with 3 x 10^{-7} M oligo, 6μg/ml anti-A, 2 mM IOA and 5.5 or 20 mM 2-DG in the presence of high glucose (25mMglucose) or glucose free medium. In the latter, medium was supplemented with 10 % dialysed FBS to avoid any glucose source.

Pseudopods and Cell bodies purification.

MSV-MDCK-INV pseudopods and cell bodies were purified using a scaled-up procedure (127) similar to that previously described (129). Briefly, 10^7 MSV-MDCK-INV cells were plated on 100 mm 1μm pore filters mounted between two 3.5" custom-made washers in a 100 mm Falcon Petri dish and the exterior sealed with agarose to prevent cell leakage to the bottom of the filter. After 24 hours culture, the filter was washed 4 times with cold PBS/CM and both sides of the filter were scraped with a glass cover slip. Pseudopods (underside) and cell bodies (upperside) were recovered from the cover slip in ice-cold lysis buffer (lysis buffer consisting of cold PBS containing 1 % SDS, 5 mM EDTA, 0.2 mM orthovanadate, 40 mM β-glycerophosphate and protease inhibitors (1μg/ml aprotinin, 1μg/ml pepstatin, 1 μg/ml leupeptin and 0.1 mM PMSF). The recovered pseudopodia fraction (PPF) and cell bodies (CB) were sonicated twice for 15 seconds on ice, centrifuged for 20 minutes at 12,000 rpm, and the supernatant collected.

Immunoblot analysis of cell lysates and PPF.

MSV-MDCK-INV cells were grown to 60-80% confluence on Petri dishes in DMEM. Lysates for immunoblotting were prepared by adding 3X hot Laemmli (95°C) or SDS lysis buffer directly to plates. For the SDS lysis buffer technique, cell monolayers were washed at first three times with ice cold PBS/CM (140 mM NaCl, 2.7 mM $MgCL_2$), harvested and centrifuged at low speed. Lysis buffer consisting of cold PBS containing 1 % SDS, 5 mM EDTA, 0.2 mM

orthovanadate, 40 mM β-glycerophosphate and protease inhibitors (1μg/ml aprotinin, 1μg/ml pepstatin, 1 μg/ml leupeptin and 0.1 mM PMSF) . Cells were lysed for 20 min on ice, and DNA was broken by sonication.

Protein content of cell lysates, purified pseudopodial fraction (PPF) and cell bodies (CB) of MSV-MDCK-INV cells was determined using the BCA protein assay (Pierce, Rockford, IL). Laemmli or SDS lysates, CB and PPF were separated on 7.5 % or 12 % SDS-PAGE and transferred to Hybond C extra nitrocellulose membranes. The labeled bands were revealed by chemiluminescence (ECL reagent) and exposed to Kodak biomax light film.

Immunofluorescence labeling.

Cells were cultured for 48 h on glass coverlips until confluent and subjected to ATP depletion with or without a period of recovery. Cells were fixed with 2 % paraformaldehyde heated at 37°C for 15 min at room temperature and then permeabilized with 0.075 % saponin for 10 min, or with methanol/acetone (80%-20%) precooled at -80°C for 15 min at -20°C for β–actin labelling.

After labeling, cover slips were mounted in gelvatol and were viewed with a Zeiss Axioskop fluorescent microscope equipped with a 63X planapochromat objective and selective filters for fluorescein isothiocynate and Texas Red or with the 60X Nikon planapochromat objective of a Bio-Rad Radiance 2000 confocal microscope.

Confocal imaging.

Images of all different fluorescence probes were obtained simultaneously using a multichannel scanning procedure in which each line of the final image was scanned four times, using excitation and imaging filters specific for each individual fluorophore.

Measurement of ATP level concentration.

ATP was extracted in 5 % TCA . Briefly, plates were scraped, keeped on ice for 10 min then spun down at 4°C. Extracts were neutralized and ATP levels were measured by bioluminescence in a 96 well plates using the Luciferine-Luciferase assay (70 mM Tris-Acetate, 4 mM EDTA, 0.125% bovine serum albumin, 20 mM $MgCL_2$, 0.7 mM luciferine and 20 µl luciferase for each unit of luciferin.

Motility assay.

To measure cell motility, 250 x 10^3 MSV-MDCK-INV cells were plated for 48 h on glass coverslips until confluent and subjected to wound healing. Cells were treated for 3, 6 and 18 hours with 3 $.10^{-7}$ M oligo, 6 µg/ml anti-A, 20 mM 2-DG in the presence of glucose free medium supplemented with 10 % dialysed FBS to avoid any glucose source. Cells were fixed for 15 min with 2 % paraformaldehyde rewarmed at 37°C at room temperature and coverslips were viewed with 10X planapochromat objective.

2.4 Results

Confocal imaging was used to determine if glycolytic enzymes colocalize with β-actin and c-Met in protruding pseudopodia. Aldolase, hexokinase and PGI colocalize with actin-rich pseudopodia and with p-c-Met (**Figure 2.1**). In contrast, no colocalization was detected between the mitochondrial proteins PDK1 or TOM-20 with β-actin (**Figure 2.2**). This implicates a functional link between pseudopodia-associated glycolysis, and phosphorylation of c-Met that regulates cell motility and invasion of MSV-MDCK-INV cells.

The protrusive β-actin-rich pseudopodia of INV cells can be purified by plating the cells on filters containing 1 μm diameter pores for 24 hours which permit the passage of cellular protrusions but not of the cell body and nucleus. The isolated pseudopodial fraction (PPF) and cell body (CB) fraction were compared to the total INV cell lysate, and submitted to gel separation and immunoblotting (**Figure 2.3**). Hexokinase and PGI are present in MSV-MDCK-INV PPF although it is not concentrated in the PPF relative to cell lysate and CB. However, aldolase is clearly accumulated in the pseudopod fraction. For the same amount of proteins, TOM-20 and PDK-1 could not be detected in the pseudopodial fraction even in blots exposed for longer time periods. (**Figure 2.3**) indicating their absence in that localized cell membrane domain.

Glycolysis has been associated with cancer cells and shown to be the primary energy source for tumor cell motility (134). Given the demonstrated role of c-Met phosphorylation in modulating cancer cell motility (128) and pseudopodial protrusion (128), it was of interest to estimate the phosphorylation state of c-Met under conditions where cell ATP concentration was modulated by the use of different media and inhibitors. To demonstrate that glycolysis is required for the actin remodelling and kinase phosphorylation, which both take place in the pseudopod (127, 128), we incubated INV cells in glucose free medium and looked at the phosphorylation level of c-Met and actin labeling upon addition of glucose, respectively as an indication of the cell phosphorylative capacity and of

its motile character (**Figure 2.4**). Glucose deprivation induced a lost of actin-rich protruding pseudopodia and apparition of stress fibers, effects which were both reversed upon addition of a medium containing glucose (**Figure 2.4A**). The phosphorylation level of c-Met (p-c-Met) was clearly increased upon addition of glucose for 30 and 60 min (**Figure 2.4B**). These results suggest that glycolytic activity regulates the phosphorylation level of the tyrosine kinase c-Met receptor necessary to generate β-actin concentrations at the tips of the elongated pseudopodial projections.

The overall ATP concentration was then decreased by using 2-deoxyglucose (2-DG) (**Figure 2.4**). 2-DG is a metabolic inhibitor that competes with glucose for transport into the cells, and once having entered cells is phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate, which undergoes no further metabolism. The consequence is a rapid decrease in cellular ATP concentration. In the presence of 20 mM 2-DG, the β-actin rich pseudopodial protrusions of INV cells disappeared and extended lamellipodia formed (**Figure 2.4A**). Peripheral actin bundles and stress fibers became apparent. It has already been shown that the disruptive effect of 2-DG on pseudopodial expression could be prevented by the presence of glucose in the cell medium, either by adding 20 mM 2-DG to regular high glucose DMEM or by supplementing 2-DG-containing glucose free medium with 20 mM glucose (129). These results further suggest that the glycolytic activity modulates the actin dynamics. The addition of 5.5 or 20 mM 2-DG in the glucose free medium further decreases the level of p-c-Met in the first minutes of addition then the p-c-Met signal increases at the level observed in the absence of glucose (**Figure 2.4B**). The time-dependent increaement in c-Met phosphorylation and the disappearance of stress fibers is interpreted as a stimulation of the mitochondrial synthesis of ATP.

In order to further determine the importance of the glycolytic ATP production on c-Met phosphorylation and on actin remodelling, we performed several experiments intending to specifically decrease the concentration of ATP

produced from glycolysis using the G3PDH inhibitor, iodoacetate (2mM), of ATP produced from mitochondria using the ubiquinol-cytochrome c oxidase inhibitor antimycin A (6 µg/ml) and the Fo ATP synthase inhibitor oligomycin (3.10^{-7} M) . Figure 2.5 confirms that the level of c-Met phosphorylation is higher in high glucose medium compared to glucose-free medium. Iodoacetate in high glucose medium decreases significantly the phosphorylation level of c-Met proportionally to the decrease in the ATP concentration. However iodoacetate has no effect on both p-cMet and the ATP concentration in the absence of glucose.

In the presence of oligomycin and antimycin-A a 30-40 % decrease in ATP concentration is observed in high glucose medium but is not accompanied with a proportional decrease in p-c-Met level (**Figure 2.5**). In contrast, the important decrease in the ATP level induced by these inhibitors in the absence of glucose led to a major decrease in c-Met phosphorylation. These observations are in accord with a specific role of ATP produced from glycolysis for c-Met tyrosine phosphorylation.

In order to further demonstrate whether the glycolytic activity regulates the association of glycolytic enzymes with actin-rich protrusive domains, cells treated with iodoacetate were labelled for actin and hexokinase (**Figure 2.6**). Treatment with iodoacetate in high glucose medium results in a clear shortening and broadening of pseudopodia similar to that observed in low glucose medium. Iodoacetate treated INV cells demonstrated that active glycolysis regulates the formation of actin-rich protrusions and the association of glycolytic enzymes with actin.

To assess whether ATP from mitochondrial respiration is also required for the formation of β–actin rich pseudopodia, INV cells were incubated with oligomycin or antimycin in high glucose and glucose-free media (**Figure 2.7**). Treatment with oligomycin or antimycin in glucose-free medium compared to treatments in the high glucose medium induced over time the lost of

pseudopodia exhibiting β–actin-rich densities, and cells present multiple membrane blebs (**Figure 2.7a**). Washout of these inhibitors and plating cells in fresh medium led to recovery within a 2-hour period of their protrusive pseudopods indicating that oligomycin and antimycin-treated cells are not apoptotic (**Figure 2.7b**). Their cell cytoskeletton is specifically destroyed.

Given the similar morphologic aspect of oligomycin and antimycin A-treated cells to INV cells treated with EIPA (131), a specific inhibitor of the NHE1 isoform of the Na^+,H^+-exchanger (131), it was of great interest to investigate the phosphorylation level of c-Met in the presence of an inhibited NHE1. Cells were treated for 30 min to 3 hours with DMSO and 50 μM EIPA, a dose shown to be necessary to decrease by 80 % the specific $^{22}Na^+$ uptake in those cells (131). As above, cell lysates of INV were immunoblotted for p-c-Met. Figure 8 shows that treatment with EIPA induces a rapid and significant decrease in the c-Met tyrosine phosphorylation level of INV cells compared to DMSO- treated cells. These results are indicative of a functional link between the EIPA-induced intracellular acidification (131) and the phosphorylation level of c-Met, a tyrosine kinase receptor. The important cell acidification upon NHE1 inhibition being around 0.7 pH unit may inhibit by feedback the hexokinase enzyme activity and lead to decreased ATP production (162), which regulates the phosphorylation level of the tyrosine kinase c-Met receptor (**Figure 2.8**). Such that localisation of NHE1 in tumor cell pseudopodia may be specifically involved in regulating pHi in these regions of high glycolytic activity. NHE1 accumulates with β-actin at the tips of cell pseudopodia, its inhibition with EIPA induces significant intracellular acidification, which prevents pseudopodial protrusions and inhibits MSV-MDCK-INV cell motility. These results implicate NHE1 in the acquisition of motile and invasive properties by INV cells.

To determine that the glycolytic ATP production within pseudopodia of INV cells is necessary to drive cell motility, wound-healing assays were realized to estimate the motile capability of INV cells in conditions shown to modulate c-

Met phosphorylation level. The motile character of INV cells through the wound can be observed after 6 hours in high glucose and in glucose-free medium (**Figure 2.9**). The treatment with 2-DG inhibits the motility but its effect decreased over the time. This is consistent with the above observation (**Figure 3b**) where a rapid decrease in p-c-Met level was observed upon addition of 2-DG followed by a time-dependent p-c-Met recovery. Treatment with oligomycin and antimycin in high glucose medium has no effect on motility whereas these mitochondrial inhibitors nearly abolished the motile character of INV cells in the absence of glucose. The delayed inhibition of motility induced by oligomycin and antimycin in glucose-free medium as compared to 2-DG is consistent with the more immediate disruption of β-actin-rich pseudopodia by 2-DG than by oligomycin and antimycin. Wound healing assay on INV cells thus unraveals their glycolytic ATP-dependent motile phenotype.

2.5 Discussion

A metabolic imbalance exists in progressively malignant tumor cells. The observation that rapidly growing tumor cells have a high aerobic glycolysis was first reported by Warburg (69), who considered that elevated glycolysis was a consequence of an impairement in the respiratory mechanism of cells caused by carcinogens. Warburg noted that in all of the tumors he examined respiration was low and glycolysis was high.

The MSV-MDCK-INV cell line was selected from Moloney sarcoma virus-transformed epithelial MDCK cells for their enhanced invasive ability (161). Sequencing of a 37 kDa protein in their pseudopodia had identified this protein as GAPDH, a critical enzyme in the process of glycolysis (129). In the present study we were interested in investigating the mechanism underlying the critical role of glycolysis in tumor cell motility, which is to drive the formation and protrusion of tumor cell pseudopodia.

MSV-MDCK-INV cells were isolated by their enhanced capacity to traverse Matrigel-coated filters and exhibit multiple β-actin-rich pseudopodia (127, 129). We took advantages of the improved pseudopod purification technique which gives hundred of μg of pseudopodial proteins to determine the content in glycolysis enzymes (hexokinase, aldolase, phosphoglucose isomerase) and mitochondrial proteins (PDK-1 and TOM 20) relative to β-actin and p-c-Met in cell bodies and total cell lysates. Actin-labeled cell bodies could be visualized on the upper surface of the filter. The 1 μm size-filter pores therefore prevent passage of the cell body but permit passage of actin-rich protrusions (127, 129).

This allowed us to show that glycolytic enzymes are present or enriched in MSV-MDCK-INV pseudopodia relative to INV total cell lysate and that mitochondrial proteins are absent from the pseudopodial fraction indicating that pseudopodia are devoided of mitochondria.

The concentration of β-actin in the isolated pseudopodia of INV cells as demonstrated by immunoblot and immunolocalisation (161) provided the first biochemical evidence that β-actin concentrates in motile cellular regions. Association of glycolytic enzymes with the actin cytoskeleton is well-documented and implicated in the regulation of actin cytoskeleton dynamics and glycolytic activity (131). Interaction between actin and glycolytic enzymes may therefore provide an immediate and localized energy supply for the active assembly and desassembly of actin filaments which drives pseudopodial protrusions.

The selective increased phosphorylation of HGF-R in the invasive variant, MSV-MDCK-INV cells, but not in the parental MSV-transformed MDCK cell population (128) is highly consistent with the enhanced phosphorylation of HGF-R in metastatic tumor cells (163, 164). It further illustrates that constitutive phosphorylation of HGFc-Met is associated not with transformation but with the acquisition of motile and invasive properties by a variant of this transformed epithelial MDCK cell line (128). There are several factors that can contribute to the availability of the cell phosphorylating system and to the control of cell energy states in the cell. Glycolysis was found, in the present study, to be the main source of ATP required to phosphorylate the kinase domain of c-Met-dependent receptor signalling and activate its biological function.

Inhibiting glycolysis with iodoacetate or clearing available ATP with 2-DG reduces the cellular ATP concentration and disrupts the hexokinase accumulation in the actin-rich domains with clear morphological changes transforming the elongated pseudopodia to actin bundles and appearing of stress fibers. Furthermore it will decrease the phosphorylation of the tyrosine kinase residues of c-Met and will slow down its signaling pathways. All of the above events will result in the clear interuption of the motile character of INV cells.

Having specified that mitochondria are absent from protruding pseudopodia and that glycolysis enzymes are present, it is thinkable that protons are locally produced from anaerobic glycolysis. Thus, anaerobic glycolysis conversion of one mole of glucose to two moles of pyruvate then to lactate is accompanied by the net production of two moles of ATP, 2 NADH and 2 protons. To avoid cell acidification, cells have evolved many systems to regulate intracellular pH. Perhaps the best characterized are the Na-H exchangers (NHEs) (105, 165, 166) and the anion exchangers chlore-bicarbonate (AEs).

Studies have recently examined the Na^+/H^+ exchanger in several models of human tumors. They suggest that, in at least some cell types, the Na^+/H^+ exchanger and intracellular pH regulation associated with this protein play an important role in tumor cell growth. Early studies showed that the Na^+/H^+ exchanger deficient cells (101) cannot proliferate in HCO_3^- free media of low external pH and lost or severely reduced their capacity to grow tumors *in vivo* in immune deficient mice. In addition, several evidence have suggested that activation of the Na^+/H^+ exchanger and the resultant increase in intracellular pH may be required for proliferation in some cell types (107)

Because of the important role of the Na^+/H^+ exchanger in tumor and cell growth, amiloride and its analogues have been extensively tested for use in tumor selective therapy (100, 122, 167-173). The large amount of protons generated by the pseudopodial glycolysis would be extruded from the cell through the Na^+/H^+ exchanger found locally within the pseudopodia. We have previously shown that the INV cells present a proton-dependant $^{22}Na^+$ uptake sensitive to EIPA, identified with specific antibodies as the isoform 1 (NHE1) (131). NHE1 is localized to the pseudopodia of INV cells, where it colocalizes with β-actin (131). Pseudopodial protrusion is an essential aspect of cell motility (128) and the inhibition of pseudopodial protrusion with the NHE1 inhibitor EIPA identifies a necessary role for NHE1 in the regulation of the actin cytoskeleton dynamics that regulate pseudopodial protrusion and cell motility (131). We have hypothesized that the large amount of protons

generated by the pseudopodial glycolysis would be extruded from the cell pseudopodia through the Na^+/H^+ exchanger found locally within the pseudopodia, so the inhibition of proton secretion with EIPA will increase the H^+ concentration inside the cell, which will affect through retro-inhibition the glycolytic ATP production. Once reduced this ATP will not be enough to respond for the high demand of energy required for the motility processes of INV cells.

The demonstrated association of glycolytic enzymes, and not mitochondria with INV pseudopodia, the colocalization with c-Met and NHE1, the ability of 2-DG, EIPA and iodoacetate to disrupt the formation of β–actin-rich pseudopodia and decrease the phosphorylation level of c-Met implicate a functional link between actin, c-Met, NHE1 and glycolytic enzymes. We interprete these results as the localized pseudopodial glycolytic supply of energy is necessary to cell motility and invasion of MSV-MDCK-INV cells through activation of c-Met by tyrosine phosphorylation.

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Figure legends

Figure 2.1

Colocalization of glycolytic enzymes with p-c-Met in β-actin rich pseudopodia of MSV-MDCK-INV cells. MSV-MDCK-INV cells were fixed with 4 % paraformaldehyde and immunofluorescently labeled for hexokinase, aldolase, and phosphoglucose isomerase. Alexa 488 conjugated to anti-goat antibodies (HK, ALDO), alexa 588 conjugated to anti-mouse antibody (β-actin) and alexa 647 conjugated to anti-rabbit antibodies (p-c-Met, and PGI) were used. Confocal images from all fluorescent channels were superimposed and colocalization (merged images) appears in yellow (green and red) or white (green, red and blue).

Figure 2.2

Pseudopodia of MSV-MDCK-INV cells are divoided from Mitochondrial proteins . MSV-MDCK-INV were fixed with methanol/acetone (80/20) and immunofluorescently labeled with antibody directed against TOM20, PDK1, and β–actin. The following secondary antibodies were used: Alexa 488 conjugated to anti-goat antibodies (PDK1), alexa 488 conjugated to anti rabbit antibody (TOM20), alexa 588 conjugated to anti-mouse antibody (β-actin). Determination of the cellular distribution of mitochondrial proteins by immunofluorescent labeling confirmed the absence of mitochondrial proteins from the pseudopodia of MSV-MDCK-INV cells.

Figure 2.3

Glycolytic enzymes are present or concentrated in pseudopodial fraction, and mitochondrial proteins are absent. Glycolytic enzymes (HK, ALDO, and PGI), mitochondrial proteins (TOM-20 and PDK1), β-actin and p-c-Met expression were studied by immunoblot in fractionated MSV-MDCK-INV cells. Total cell lysates of INV, PPF and CB were isolated as described in Methods and were

blotted with the corresponding antibody on fresh nitrocellulose membranes. Hexokinase, PGI and aldolase are present in MSV-MDCK-INV PPF, CB and cell lysates and colocalize with p-c-Met and β-actin. For the same amount of proteins, TOM 20 and PDK-1 could not be detected in the pseudopodial fraction even in blots exposed for longer time periods. These blots were all obtained on the same preparation.

Figure 2.4

Glycolytic activity regulates the actin dynamics and the phosphorylation level of c-Met. INV cells incubated in glucose free medium were treated with 5.5 or 20 mM of glucose (30' and 60') and with 5.5 or 20 mM 2-DG (30' and 60'), then A) either immediately fixed for rhodamin conjugated phalloidin labeling or B) ice-cold stopped for c-Met phosphorylation determination; Cell lysates were prepared and blotted as described with p-c-Met antibody. Cells incubated in glucose-free medium (A), supplemented with 20 mM glucose for 30 min or 60 min retained β-actin concentrations at the tips of elongated pseudopodial projections. In the presence of 20 mM 2-DG for 30 or 60 min , the β-actin rich pseudopodial protrusions of INV cells disappeared and extended lamellipodia formed. Peripheral actin bundles and stress fibers became apparent, and correlated with the decrease in c-Met tyrosine phosphorylation.

Figure 2.5

Phosphorylation degree of c-Met is higher in high glucose medium compared to a glucose-free one. MSV-MDCK-INV cells were treated for 15 min with 3 x 10^{-7} M oligomycin (oligo), 6-μg/ml antimycin-A (Anti), and 2 mM iodoacetate (IOA) in the presence of high glucose medium (25 mM glucose) or glucose free medium. The low glucose medium was supplemented with 10 % dialysed FBS to avoid any glucose source. Ice-cold cells were lysed in lysis buffer immediately after treatments and deproteinized in 5 % trichloroacetic acid for ATP determination by luminescence or lysates blotted for p-c-Met and phosphorylation level was quantified.

Figure 2.6

Treatment with iodoacetate in high glucose medium results in a clear shortening and broadening of pseudopodia similar to that observed in low glucose medium. Cells were fixed immediately after treatment with warm paraformaldehyde to determine the consequences on actin cytoskeleton and hexokinase cell distribution following iodoacetate treatment in high glucose medium compared to glucose-free one.

Figure 2.7

Treatments with oligomycin or antimycin in glucose-free compared to high glucose medium induced the loss of pseudopodia, and appearance of multiple membrane blebs. The disappearance of β-actin-rich pseudopodial protrusions of INV cells in glucose-free medium treated cells (A) indicates that glycolytic ATP is important for the maintenance and the dynamic remodelling of actin during invasion. Cells washed from oligomycin and antimycine (B) and plated in fresh medium recovered with time their protrusive pseudopod indicating that oligomycin and antimycin-treated cells are not apoptotic.

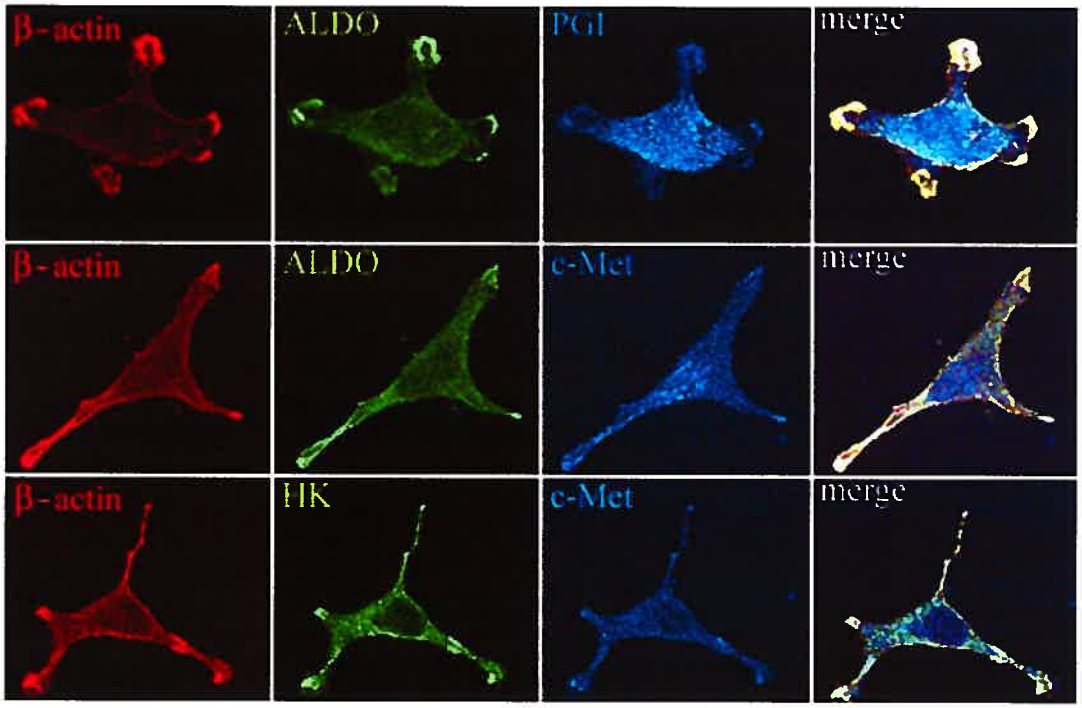
Figure 2.8

Inhibition of Na^+, H^+-exchange activity decreases c-Met phosphorylation. MSV-MDCK-INV cells were incubated in a high glucose medium containing no $NaHCO_3$ but 50 μM EIPA for 5', 30', 60' and 180' at 37 C or DMSO in HEPES medium for 15' and 180'. Control cells were let in their original medium for another 180'. Inhibition of NHE1 by EIPA induces a pronounced decrease in INV c-Met tyrosine phosphorylation level .

Figure 2.9

***The wound healing linked to the motile character of INV cells is significantly decreased in the absence of glucose*.** The migratory rate of MDCK-MSV-INV cells in a wounding assay was determined by measuring wound width as function of time for cells plated on gridded coverslips. Data are expressed as the mean (SEM) of three experiments.Wound assays of INV cells in high glucose medium alone (30' and 6h) or supplemented with 3 $.10^{-7}$ M oligo; 6 µg/ml anti-A compared with INV cells in glucose-free medium alone (30' and 6h) or supplemented with 3 $.10^{-7}$ M oligo; 6 µg/ml anti-A; 20 mM 2-DG.

Figure 2.1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ALDO
PGI
merge
β-actin
ALDO
c-Met
merge
β-actin
HK
c-Met
merge

Figure 2.2

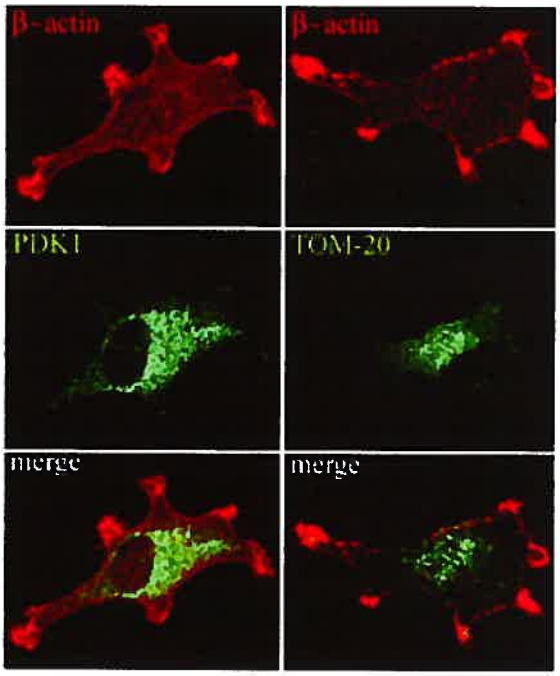
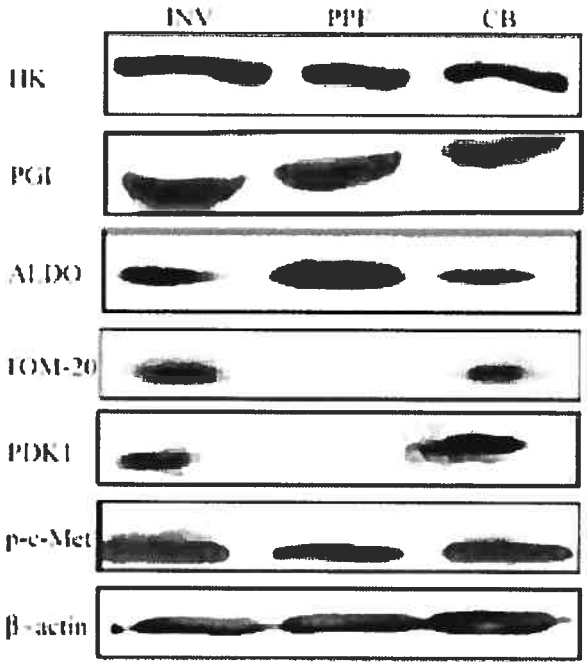


Figure 2.3



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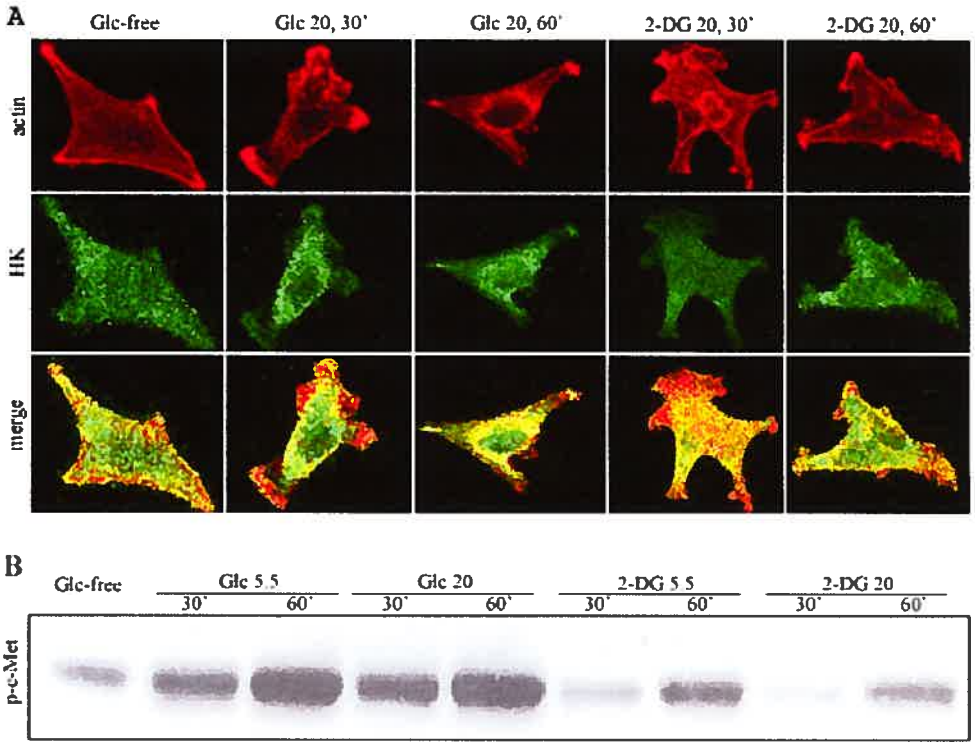

A
Glc-free
Glc 20, 30'
Glc 20, 60'
2-DG 20, 30'
2-DG 20, 60'
actin
HK
merge
B
Glc-free
Glc 5.5
Glc 20
2-DG 5.5
2-DG 20
30'
60'
30'
60'
30'
60'
30'
60'
p-c-Met

Figure 2.5

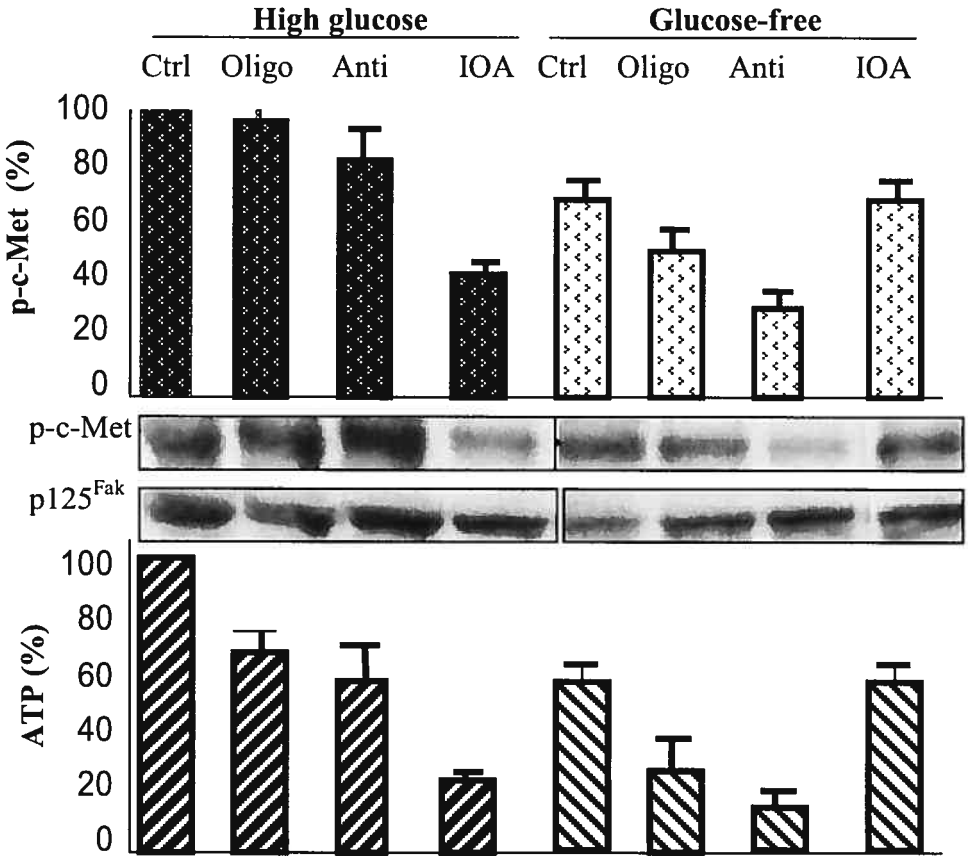

High glucose
Glucose-free
Ctrl
Oligo
Anti
IOA
Ctrl
Oligo
Anti
IOA
p-c-Met (%)
100
80
60
40
20
0
p-c-Met
p125Fak
ATP (%)
100
80
60
40
20
0

Figure 2.6

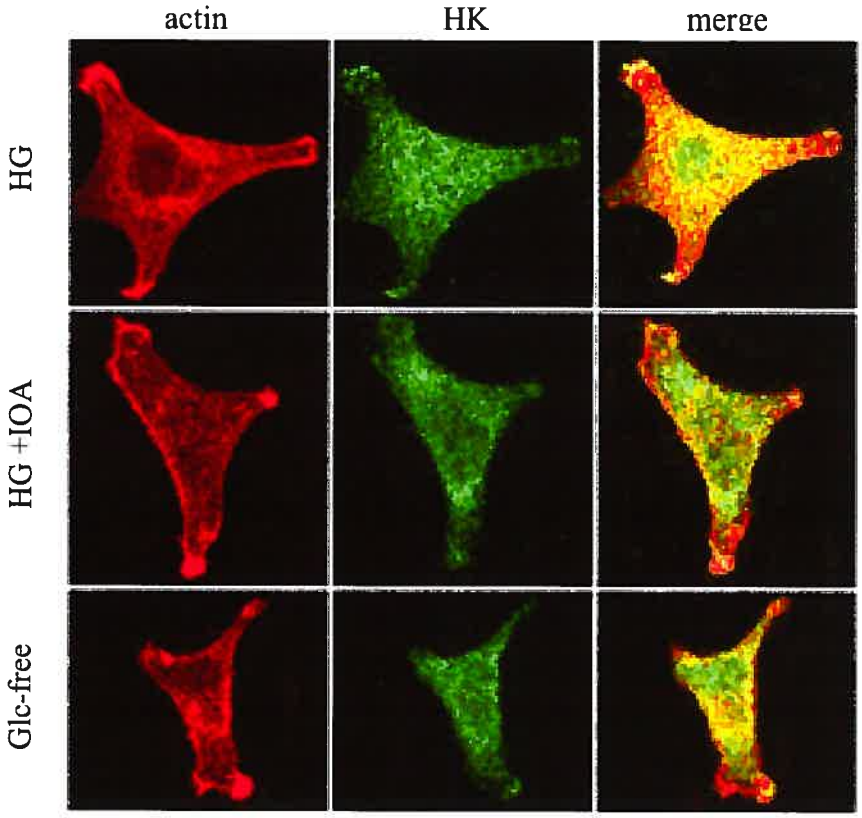
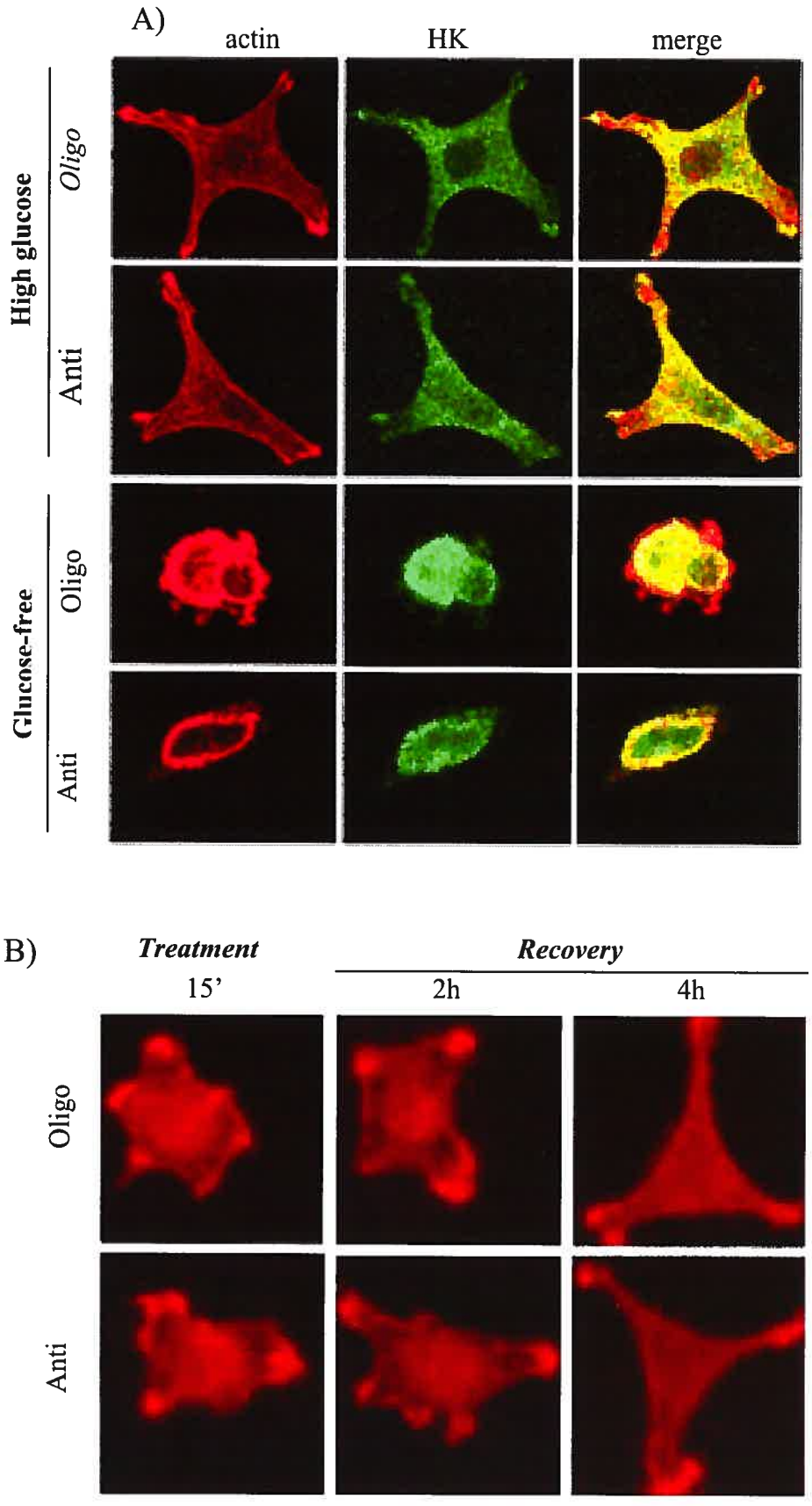


Figure 2.7


A)
actin
HK
merge
High glucose
Oligo
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Glucose-free
Oligo
Anti
B)
Treatment
Recovery
15'
2h
4h
Oligo
Anti

Figure 2.8

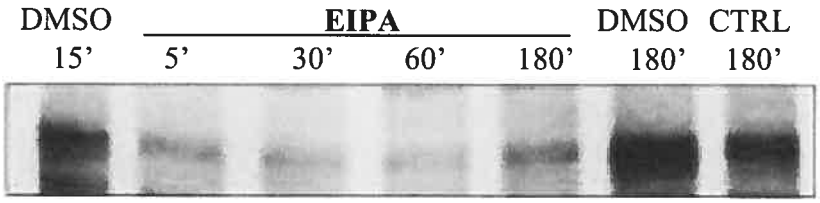
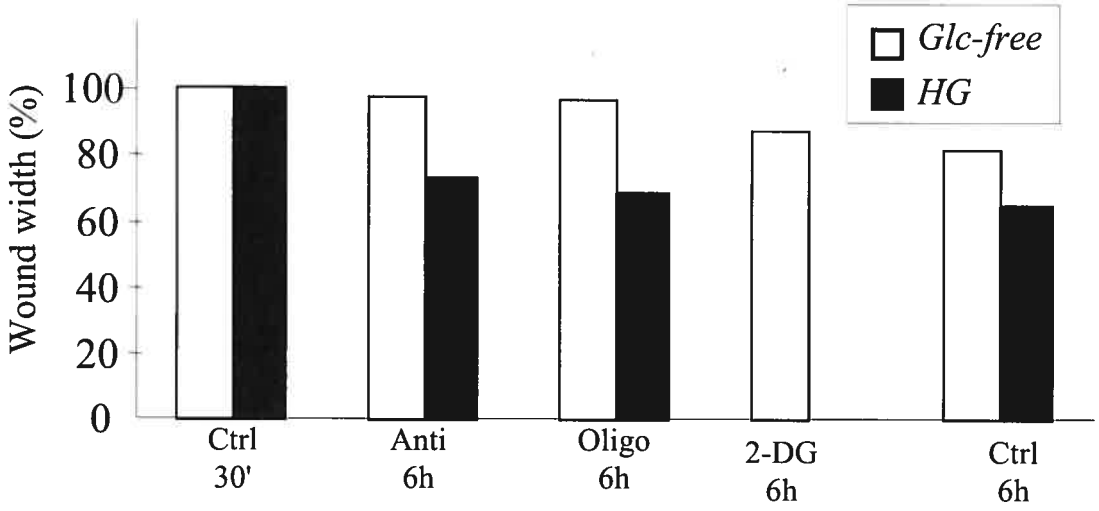


Figure 2.9


Glc-free
HG
Wound width (%)
100
80
60
40
20
0
Ctrl
30'
Anti
6h
Oligo
6h
2-DG
6h
Ctrl
6h

CHAPTER 3

DISCUSSION

3.1 Study overview

A high rate of proliferation is characteristic of malignant tumor cells. This by itself does not account however for the ability of the cells to leave the primary tumor mass and metastasize to secondary sites. Tumor cells from the primary mass must aggressively migrate through stromal matrix and vessel walls following the metastatic process (174, 175).

The MSV-MDCK-INV cell line was selected from Moloney sarcoma virus-transformed epithelial MDCK cells for their enhanced invasive ability and exhibits multiple β-actin-rich pseudopodia (161). The MSV-MDCK-INV cells migrate in response to self-generated autocrine motility factor (129) and hepatocyte growth factor in a receptor-mediated process (128). HGF stimulates chemotaxis and may play a role in the homing of invasive cells to secondary sites (174, 175).

In the present study we were interested in investigating the mechanism underlying the critical role of glycolysis in tumor cell motility, which is to drive the formation and protrusion of tumor cell pseudopodia.

3.2 Biochemical characterization of tumor cell pseudopodia

Malignant cells are known to exhibit a high rate of glycolysis even under aerobic conditions (69). Glucose utilization through glycolysis, which is the primary energy source in cancer cells, is known to be controlled by allosteric regulators, as well as by reversible binding of glycolytic enzymes to cytoskeleton enabling the compartmentalization of these energy-generating enzymes to specific cytosolic locations. Sequencing of a 37-kDa protein in the MSV-MDCK-INV cells pseudopodia had identified this protein as GAPDH, a critical enzyme in the process of glycolysis (129). GAPDH is present in pseudopodial protrusions, its expression in these regions is reduced compared to the rest of the cell (129). GAPDH is therefore not concentrated in MSV-MDCK-INV pseudopodia. However, the fact that this protein is detected by the

Coomassie blue staining within the pseudopodial fraction identifies it as one of the major proteins present within pseudopodia and is indicative of a functional role for this protein in pseudopodial protrusion (129).

We took advantages of the improved pseudopod purification technique developed by Zongjian Jia from the Nabi's laboratory which gives hundred of μg of pseudopodial proteins to determine the content in glycolytic enzymes (hexokinase, aldolase, phosphoglucose isomerase) and mitochondrial proteins (PDK-1 and TOM 20) relative to β-actin and p-c-Met in cell bodies and total cell lysates. The 1 μ size-filter pores prevent passage of the cell body but permit passage of actin-rich protrusions (129, 176). Parrallele immunoblots of the PPF, CB and cell lysates revealed that hexokinase and PGI are present in INV PPF although it is not concentrated in the PPF relative to cell lysate and CB (**Figure 2.3**), as was previously observed for GAPDH. However, aldolase, the fourth enzyme in the glycolysis cascade, is clearly accumulated in the pseudopod fraction compared to the INV cell lysate or cell body fraction. These results confirm Sibley and Lehninger's (1948) observations, who extended the studies to human cancer, and Warburg and Christian's (1943) observations who conceived the possibility that the blood leaving the tumor and entering the general circulation might show elevation of serum glycolytic enzymes. Indeed, they found that serum aldolase activity was elevated in rats bearing the Jensen sarcoma. By contrast, the fact that in our model the two mitochondrial proteins, TOM-20 and PDK-1, could not be detected in the pseudopodial fraction (**Figure 2.3**) indicates an extremely low amount of these proteins or their absence in that localized cell membrane domain. These results are also indicative of a very good purification process for the pseudopodia. Considering the large amount of mitochondrial PDK-1 and TOM-20 proteins into the cytoplasm, the clear distribution of glycolytic enzymes and mitochondrial proteins respectively in and out of pseudopodia reflects a quite pure pseudopodial fraction.

β-actin is not as concentrated as should be expected from the clear pseudopodial β-actin accumulation observed upon immunolocalisation (128, 161). The lack of a net accumulation of β-actin within the pseudopodial fraction led us to compare these three fractions. We first thought that for the same amount of proteins (50 μg) loaded onto the gel, a protein which is abundantly expressed within the pseudopodia, as is the case for β-actin, should be highly enriched in PPF as compared to CB fraction. However, only a 1.5 to 2.5 enrichment factor is repeatedly found. A possible explanation for this observation is that a comparable amount of proteins is present within the pseudopodial fraction (regrouping 2-6 pseudopodia per cell) and within the cell body fraction, given that the pseudopodia are a very well developed cell domain. An enrichment factor of 1,5-3 for β-actin in PPF compared to INV cell lysate would then be normal. BSA protein may present another explanation if BSA protein present in the culture medium contaminates proportionnaly more PPF than CB or INV lysates. This however does not seem to be the case since a Ponceau coloration of the three fractions does not reveal a major band corresponding to 68 kDa, the molecular weight of albumin. Our results are clear and indicate a very good pseudopodia purification, but there is a need for a better quantification process. Quantification of phospholipids seems to be problematic since the dosage is very sensitive to the presence of phosphate; the fact that INV cells need to be washed with a phosphate-containing buffer avoids us the use of this technique to quantitate the respective fractions. Quantification of cholesterol could be another method. However, for the moment, we estimate that this technique will not be optimal neither. Indeed, the fact that mitochondria lack cholesterol will avoid them to be quantified in the total cell lysates, increasing falsely the enrichment factors of accumulated pseudopodial proteins. This technique would nevertheless merit to be explored.

3.3 β-actin rich tumor cell pseudopodia: Role of glycolysis

Association of glycolytic enzymes with the actin cytoskeleton is well documented and implicated in the regulation of actin cytoskeleton dynamics and glycolytic activity (136). *In vivo* glyceraldehyde-3-phosphate dehydrogenase has been shown to be associated with F-actin in synaptosomes and at post-synaptic sites (137) Moreover, it has recently been localized to the actin-rich pseudopodia of MSV-MDCK-INV cells (129). In very confluent cultures of epithelial MDCK cells, glyceraldehyde-3-phosphate dehydrogenase was triton X-100 insoluble and exhibited ATP-dependent cytoskeleton association. Immunolocalization of hexokinase, aldolase and phospho-glucose isomerase to actin rich domains of MSV-MDCK-INV cells therefore identifies other glycolytic enzymes that are associated with actin-rich pseudopodia and suggests that multiple glycolytic enzymes are expected to be associated with motile actin-rich pseudopodial domains.

For the tumor cell, up regulation of multiple glycolytic enzymes is associated with tumor malignancy (133) and glycolysis is the principal supply of energy for cell motility (129) . Glycolysis was indeed shown to regulate the protrusion of the multiple pseudopodia, and thereby the motility of MSV-MDCK-INV cells (129) . Localized actin polymerization responsible for the protrusion of motility-associated cellular structures is a critical element of cell movement. Interaction between actin and glycolytic enzymes may therefore provide an immediate and localized energy supply for the active assembly and desassembly of actin filaments which drive pseudopodial protrusions (129).

Glycolytic activity correlates with cell cycle associated changes in the F-actin cytoskeleton suggesting that glycolytic activity may regulate actin cytoskeleton organization (129). The ability to disrupt the interaction of hexokinase with the actin cytoskeleton by using 2-deoxyglucose (**Figure 2.4**) demonstrates that the local concentration of ATP can regulate the association of glycolytic enzymes with the actin cytoskeleton. Formation of a localized glycolytic cytomatrix at

the site of pseudopodial protrusion is therefore involved in active actin-mediated pseudopodial protrusion. Increased expression of glycolytic enzymes in tumor cells may act to enhance tumor malignancy in two ways: 1) by enhancing glycolytic activity and stimulating pseudopodial activity. 2) via the phosphorylation and activation of the hepatocyte growth factor receptor, c-Met, which drives pseudopodial protrusion and motility (128) .

3.4 Phosphorylated c-Met induces tumor cell motility by regulating pseudopodial protrusion.

Induction of several biological activities critical for development and maintenance of normal cellular functions such as proliferation, motility, survival, and differentiation are mediated by protein growth factors and their associated receptor tyrosine kinases. The c-Met receptor tyrosine kinase is a member of a small subfamily of growth factor receptors that when activated induces mitogenic, and morphogenic cellular responses. The ligand for Met is hepatocyte growth factor/scatter factor (HGF) and while normal HGF/SF-Met signaling is required for embryonic development, abnormal Met signaling has been strongly implicated in tumorigenesis, particularly in the development of invasive and metastatic phenotypes. Following ligand binding and autophosphorylation, Met transmits intracellular signals using a unique multi-substrate docking site present within the C-terminal end of the receptor (138).

While the importance of the multidocking site in Met-mediated proliferation, cell movement/invasion, and branching morphogenesis has been appreciated for several years, only recently have more complete models been developed for how this site organizes a signaling complex following HGF/stimulation (138). Disruption of the signaling complex by either inhibiting the ATP production (this study and (129) or blocking the Na^+/H^+ exchanger (177) leads to loss of cell pseudopodia (**Figure 2.6; 2.7; 2.8**).

Clarification of the metabolic events involved in tumor cell motility could lead to improvements in therapy and diagnostic tests for aggressive malignancies with metastatic potential. There are several factors that can contribute to the availability of the cell phosphorylating system and to the control of cell energy. Glycolysis was found, in the present study, to be the main source of ATP required to phosphorylate the kinase domain of c-Met, due probably to the localized expression of c-Met within the pseudopodia (**Figure 2.4**). Once ATP binds to its site, the receptor will autophosphorylate the tyrosine residues in position 1230, 1234, 1235 of the human c-Met tyrosine kinase domain, then induces phosphorylation of tyrosine in position 1349 and 1365 of the multidocking site to activate its biological functions (145). Pseudopodial glycolysis appears to play a critical role in activating signal complexes, not only by phosphorylating the tyrosine residue of c-Met, but also by recruiting the ATP for the actin remodeling.

Signals that derive from c-Met activation to the extracellular matrix (ECM) regulate important physiological events including cell motility and growth, and most often involve changes in the organization of the actin cytoskeleton (138). Cells interact with the ECM via different signal transduction pathways, and signaling molecules to the sites of matrix provide links to the actin cytoskeleton. Our results suggest that glycolysis first affects c-Met activation, then the regulation of the actin cytoskeletal dynamics. The HGF-dependent phosphorylation of c-Met on its tyrosine kinase residues elicits the generation of pseudopodia (128) . Inhibition of glycolysis propagates the formation of actin stress fibers and loss of β-actin rich-elongated pseudopodia (129).

3.5 Glycolysis as primary energy source in tumor cell chemotaxis

Tyrosine phosphorylation is generally associated with cellular transformation and increased cellular motility of tumor cells More specifically, tyrosine phosphorylation of c-Met was shown to regulate the formation of the β-actin-

rich pseudopodia of INV cells (128). In order to determine the importance of the glycolytic ATP production on c-Met phosphorylation and on actin remodelling, we performed several experiments intending to specifically decrease the concentration of ATP produced from glycolysis by the G3PDH inhibitor iodoacetate ATP produced from mitochondria by the ubiquinol-cytochrome c oxidase inhibitor antimycin A and the Fo ATP synthase inhibitor oligomycin.

A critical role for glycolysis-dependent tyrosine phosphorylation in the acquisition of the motile phenotype of INV cells was determined using the G3PDH inhibitor, iodoacetate , and the tyrosine phosphorylation level in position 1230, 1234 and 1235 of the tyrosine kinase receptor c-Met. Tyrosine phosphorylation of the 160 kDa protein identified as c-Met decreased significantly on iodoacetate treatment (**Figure 2.5**). Treatment of MSV-MDCK-INV cells with iodoacetate resulted in a gradual disappearance of the β-actin rich pseudopodia. A glycolysis ATP production coupled to the activity of cellular tyrosine kinases is therefore required for pseudopodial protrusion found in MSV-MDCK-INV cells, supporting a role for HGF-R/c-Met tyrosine phosphorylation in this process.

Figure 2.5 confirms that the level of c-Met phosphorylation is higher in the high glucose medium compared to the glucose-free one. Iodoacetate in high glucose medium decreases significantly the phosphorylation level of c-Met proportionally to the decrease in the ATP concentration. However iodoacetate has no effect on both p-c-Met and the ATP concentration in the absence of glucose, thus ruling out a non-specific effect of iodoacetate on these parameters.

While the glycolytic activity is an important source of energy in cancer cells, the mitochondrial oxidative phosphorylation is also a major site of ATP production. The 30-40 % decrease in the ATP concentration observed with oligomycin and antimycin A in high glucose medium is not accompanied with a proportional decrease in p-c-Met level (**Figure 2.5**). In contrast, the important

decrease in the ATP level induced by these inhibitors in the absence of glucose led to a major decrease in c-Met phosphorylation. These observations are in accord with a specific and major role of ATP produced from glycolysis for c-Met tyrosine phosphorylation.

We characterized the energy metabolism in proliferating INV cells using inhibitors of both glycolysis and mitochondrial respiration, and we determined the relative contributions of these pathways to motility. Inhibiting glycolysis with iodoacetate reduces the cellular ATP concentration and results in a clear shortening and broadening of pseudopodia similar to that observed in low glucose medium. Iodoacetate treated INV cells demonstrated that active glycolysis regulates the formation of actin-rich protrusions and the association of glycolytic enzymes with actin **(Figure 2.6)**.

To assess whether ATP from mitochondrial respiration is also required for the formation of β–actin rich pseudopodia, INV cells were incubated with oligomycin or antimycin A in high glucose and glucose-free media (**Figure 2.7**). Treatments with oligomycin or antimycin in glucose-free medium compared to treatments in the high glucose medium induced over time the lost of pseudopodia exhibiting β–actin-rich densities, and cells present multiple membrane blebs (**Figure 2.7a**). Cells washed from these inhibitors and plated in fresh medium recovered within a 2-hour period their protrusive pseudopods indicating that oligomycin and antimycin-treated cells are not apoptotic (**Figure 2.7b**). Their cell cytoskeletton is specifically destroyed.

3.6 Cell migration requires both ion translocation and cytoskeletal anchoring by the Na-H exchanger.

Studies have examined the roles of Na^+/H^+ exchanger in several models of human tumors (101). They suggest that, in at least some cell types, the Na^+/H^+ exchanger and intracellular pH regulation associated with this protein play an

important role in tumor cell growth. Early studies showed that the Na^+/H^+ exchanger deficient cells cannot proliferate in HCO_3^- free media of low external pH and lost or severely reduced their capacity to grow tumors *in vivo* in immune deficient mice (101). A lot of observations have suggested that activation of the Na^+/H^+ exchanger and the resultant increases in intracellular pH may be required for proliferation in some cell types (107).

Because of the important role of the Na^+/H^+ exchanger in tumor cell growth, amiloride and its analogues have been extensively tested for use in tumor selective therapy (100, 122, 167-173). Pseudopodial protrusion is an essential aspect of cell motility (128) and the inhibition of pseudopodial protrusion with the NHE1 inhibitor EIPA identifies a necessary role for NHE1 in the regulation of the actin cytoskeleton dynamics that regulate pseudopodial protrusion and cell motility (131). NHE1 is localized to the pseudopodia of INV cells, where it colocalizes with β-actin (131) and where are also localized four enzymes of the glycolytic pathway (**Figure 2.3**).

Having specified that glycolysis enzymes are present within protruding pseudopodia, that mitochondria are absent, and having indirectly demonstrated that a significant glycolytic activity takes place within pseudopodia, it was expected that protons would be locally produced from glycolysis. We have hypothesized that the large amount of protons generated by the pseudopodial glycolysis would be extruded from the pseudopodia through the Na^+/H^+ exchanger, so the inhibition of proton secretion by EIPA would increase the H^+ concentration inside the cell, and significantly more within the specialized pseudopod membrane compartment, which would affect through retro-inhibition the glycolytic ATP production. Once reduced this ATP might not be in sufficient concentration to respond for the high demand of energy required for the motility processes of INV cells.

The decrease in the phosphorylation level of c-Met (**Figure 2.8**) upon EIPA treatment may be a consequence of the reduced glycolytic metabolism and

suggests an essential role for NHE1 to secrete protons generated from glycolysis, to regulate the phosphorylation level of the tyrosine kinase c-Met receptor and to retain the β-actin concentration at the tips of elongated pseudopodial projections. A more direct effect of cell acidification on tyrosine kinase activity of c-Met should also be envisaged.

3.7 Glycolysis and tumor cell motility.

The increased phosphorylation level of INV c-Met compared to MDCK c-Met is relative to the enhanced phosphorylation of c-Met in metastatic tumor cells (128). Further it makes the link between the phosphorylation of this tyrosine kinase receptor and the acquisition of the motile character of INV cells. Localization to the plasma membrane of constitutively activated Met oncogene with a c-Src myristoylation signal induces enhanced MDCK cell transformation as well as pseudopodial protrusions (178). Similarly, constitutive localized activation of the HGF-R/Met tyrosine kinase may be responsible for the rapid pseudopod formation of the MSV-MDCK-INV variant and acquisition of motile and invasive capabilities (128).

INV cells are highly invasive as shown by their ability to migrate through a collagene-coated filter (Y. Dodier, J. Noël and collaborators, in preparation). In the latter study, it was shown that both the PI3kinase and the MEK-ERK pathway are involved in directing the invasive character of INV cells (179) The cellular mechanisms underlying their motile behavior are further clarified in the present study by the demonstration of energy mobilization via glycolysis and mitochondria, with glycolysis favored as the major pathway for *in vitro* motility. The energy requirements via glycolytic pathways were directly demonstrated in migrating INV cells (**Figure 2.9**). The motile character of INV cells through the wound can be appreciated after 6 hours in high glucose and in glucose-free media. Treatment with oligomycin and antimycin in high glucose medium has no effect on motility whereas these mitochondrial inhibitors nearly abolished the motile character of INV cells in the absence of glucose. In

glucose-free conditions, HGF-dependent motility was dependent on mitochondria-derived energy and represented only a fraction of what could be achieved in the presence of glucose. Thus, our results clearly showed that motility of the invasive MDCK cell line, the MSV-MDCK-INV cells, depends on pseudopodial glycolysis in the presence of glucose as its principal source of energy.

3.8 Conclusions and perspectives

The major purpose of our research was to understand the link between the pseudopodial β-actin, glycolytic enzymes, c-Met and NHE1 in regulating cell motility. Localized actin polymerization drives the protrusion of motility-associated cellular structures, and pseudopodial protrusion is a critical element of cell movement (180). Glycolytic enzymes had been localized to the actin-rich pseudopodia of an invasive variant of Moloney sarcoma virus-transformed MDCK cells and glycolysis shown to regulate the protrusion of the multiple pseudopodia, and thereby the motility, of those cells (129) Our hypothesis suggests that to avoid any intracellular acidification by protons produced from the glucose metabolism, protons would be directly extruded via the local membrane Na^+/H^+ exchanger NHE1 and ATP produced from glycolysis would serve to polymerize the actin monomers and also, to phosphorylate the HGF receptor (**Figure 3.1**). As EIPA was shown to inhibit the Na^+/H^+ exchanger activity and induce a significant intracellular acidification (131), we propose that the high intracellular H^+ concentration would affect the glycolytic pathway and decrease the ATP production, necessary to phosphorylate c-Met and other components of the signalling machinery such as PI3kinase, Akt, MEK, ERK etc. As these pathways are implicated in activating the motogenic pathway, through modulation of the remodelling of actin, an intracellular acidification would also perturb the protrusion of pseudopodia, as previously observed (131). The Na^+/H^+ exchanger NHE1 localized close to the site of ATP production within the pseudopodia would be there to insure the continuous secretion of

protons produced locally by the glycolysis pathway, and to avoid feedback inhibition of hexokinase by accumulated protons.

Recent findings on this colocalization will lead to exciting new areas for future investigations. Findings that pseudopodial glycolytic enzymes and NHE1 activities contribute to migratory responses indicated the need for glycolytic flux measurements in the presence or the absence of EIPA as well as in the presence of different glycolysis and mitochondrial inhibitors to firmly establish the relationship between the glycolytic flux and the need for proton secretion. Preliminary results obtained by J. Noël since my departure from the laboratory are really encouraging and nicely support our hypothesis. The results showed that a treatment of INV cells with 50 μM EIPA for different periods of time (0 (DMSO), 30, 60, 90 and 120 min) decreases by more than 50 % both lactate production and glucose utilization (data not shown). This result supports the idea that NHE1 function is closely linked to the glycolytic flux. The laboratory will extend the study to include measurements of the metabolic flux (lactate production) within the purified pseudopod fraction as compared to the total cellular lactate production. Measurement of lactate production within purified pseudopodia will also prove that a functional glycolysis occurs in this cellular compartment independently from the cytoplasm glycolytic enzymes. This result would also add to the originality of the present work.

Identification of MSV-MDCK-INV glucose transporters specially found in the pseudopodia, alterations in glucose transport kinetics, and studies in the presence of specific inhibitors of signal transduction components, should get an important insight into the comprehension of the mechanisms by which glucose transport is regulated in these cells, and will indicate the extent to which deregulation of this transport contributes to the inappropriate migration of INV cells.

The present work brings some hope that glycolysis enzymes of tumor cells will be in the future a target for pharmaceutical agents to inhibit cell movement, and so, the metastatic progression of the cancer.

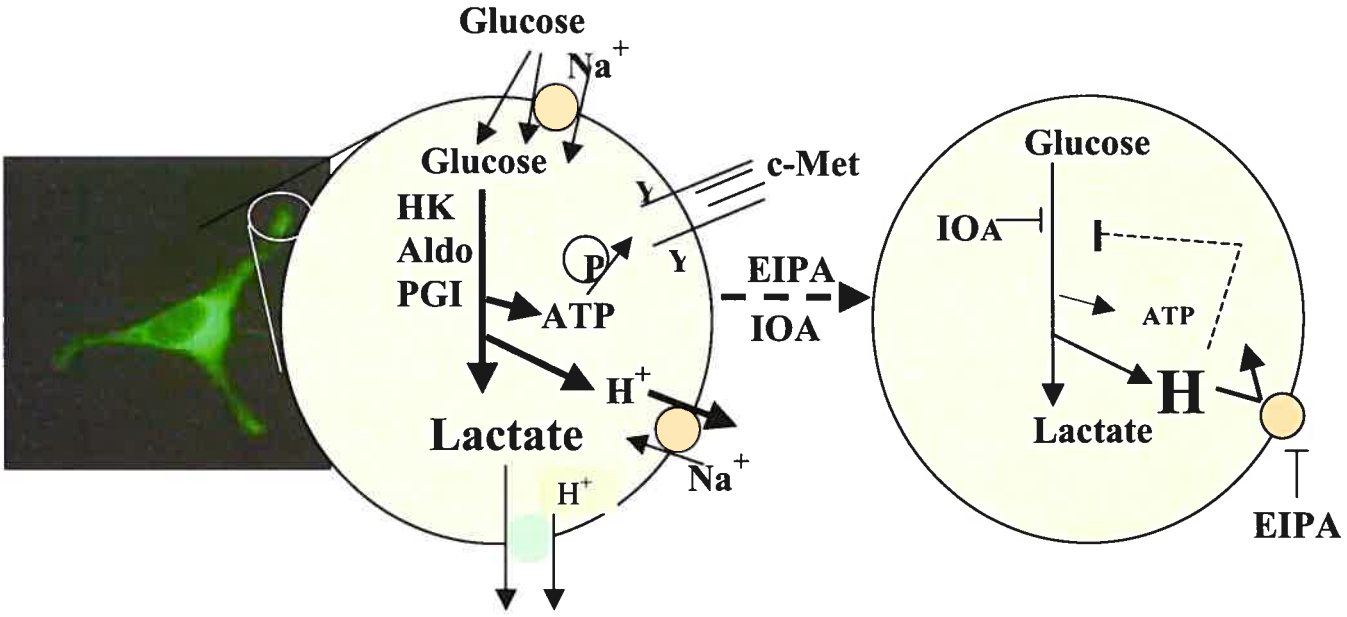


Figure 3.1: Our hypothesis suggests that under anaerobic conditions glucose will be metabolized in the pseudopodia via the glycolytic pathway but leading to lactate production and cell acidification. Based on this hypothesis our model suggests that lactate will be extruded via a lactate/ proton cotransporter, protons will be extruded via the Na^+/H^+ exchanger and ATP will serve to polymerize the actin and phosphorylate the c-Met receptor. EIPA inhibits the Na^+/H^+ exchanger activity and decreases the intracellular pH. In that condition, the high intracellular H^+ concentration will affect, through retroinhibition, the glycolytic pathway and decrease the ATP production, which will induce a reduction in c-Met activation and pseudopod protrusion.

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