Investigation of the Role of IQGAP1 in Metastasis

by

Sarah Elizabeth Ann Frew

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Carnegie Mellon University, 1998

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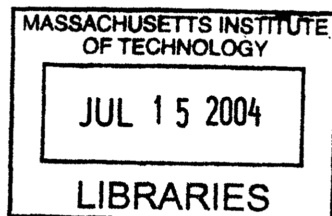
Signature of Author: ____________________
Department of Biology
May 21, 2004

Certified By: ____________________
Richard O. Hynes
Professor of Biology
Thesis Supervisor

Accepted By: ____________________
Alan D. Grossman
Professor of Biology
Graduate Committee Chairperson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Submitted to the Department of Biology on May 21, 2004 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Abstract

Metastasis, or the spread of a primary tumor to distal sites in the body, is the major cause of human cancer-related morbidity and mortality. Metastasis requires a complex series of cellular events that remain poorly understood at the molecular level. Recently, advances in microarray technology have allowed cancer biologists to globally survey metastatic progression and define patterns of gene expression that correlate with progression to a metastatic phenotype. By using such a genomic approach, our laboratory identified a subset of genes that regulate the actin cytoskeleton whose enhanced expression correlates with metastasis. This thesis describes the characterization of IQGAP1, a key regulator of the cytoskeleton, as a potentially critical player in metastatic progression. Here I show a strong positive correlation between IQGAP1 expression levels and metastatic progression in both *in vivo*-selected human metastatic melanoma cells and other human tumors. In addition, I have experimentally analyzed the role of IQGAP1 in metastasis using two different dominant-negative mutants. The results suggest that IQGAP1 may play a functional role in metastatic progression, particularly in the processes of cell migration and invasion. This work lays a scientific framework by which cancer biologists can look at global gene expression analyses and then probe deeper into individual genes to define the molecular mechanisms underlying their roles. In addition, this work contributes to a deeper understanding of the molecular pathogenesis of metastasis, and identifies in IQGAP1 a potential molecular target for future tumor metastasis therapies.

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Title: Professor of Biology

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Chapter 1

Introduction

Metastasis is a deadly disease

Metastasis, the spread and relentless growth of tumor cells from the primary neoplasm to distant organs, is the most fearsome aspect of cancer (Fidler 2003). Unlike primary tumors, which can often be treated successfully when caught early, most distant metastases are resistant to conventional therapies. Despite significant improvements in diagnosis, surgical techniques, and local and systemic therapies, the acquisition of metastatic ability of most cancers leads to clinically incurable disease. Little progress has been made in treating metastatic disease because our understanding of systemic cancer progression is remarkably poor. For instance, it is not known how many and which genes are expressed at the time of tissue invasion and dissemination. We do not know which metastatic cells survive at ectopic sites nor which interactions with the specific organ microenvironment are necessary to initiate the metastatic outgrowth of a disseminated cell (Klein 2000). Clearly, a deeper understanding of the systemic, cellular and molecular pathogenesis of metastasis is necessary for new diagnostic and therapeutic strategies.

Metastasis is a multi-step process

Metastasis of a primary tumor to a distant site consists of a series of selective steps, all of which must be successfully completed to give rise to a metastatic tumor (Fidler 1991, 2003). These essential events include: proliferation of the primary tumor, establishment of a pro-angiogenic environment within the primary tumor; detachment, motility and invasion through the extracellular matrix (ECM) and vascular or lymphatic basement membrane for entry into the circulation (intravasation); survival in the circulation; cell arrest in distant capillary beds and extravasation through the endothelium and basement membrane for nonrandom homing to distant organs; response to local growth factors; and vascularization of the metastatic tumor for maintenance of growth at the distant site (Chambers et al. 2002).

Angiogenesis is an essential process for both primary tumor growth and metastasis. The primary tumor requires angiogenesis for the transport of nutrients and the removal of waste products. Tumor cells can secrete several angiogenic factors, including fibroblast growth factor (FGF) family, transforming growth factor-alpha (TGF-α) and vascular endothelial growth factor (VEGF) (reviewed in (Denijn and Ruiter 1993)). The resulting increase in vascularization increases the chance for tumor cells to enter the circulatory system and metastasize, perhaps because the newly formed blood vessels are more permeable to tumor cells (Denijn and Ruiter 1993). The requisite interactions between tumor cells, endothelial cells and the extracellular matrix for angiogenesis to occur are thought to be mediated by both serine proteases and metalloproteinases (discussed below).

Cancer cells that will ultimately metastasize must initially acquire changes in their ability to adhere to their surrounding cells and extracellular matrix. The cadherin and integrin receptor families, respectively, modulate the cell's adhesive properties (Hynes 1992; Nagafuchi 2001). The homodimeric transmembrane classical cadherins mediate homotypic Ca^{+2}-dependent cell-cell adhesion via their extracellular domains. In addition, they are indirectly linked to the actin cytoskeleton via interactions between their cytoplasmic domains and catenins (Mareel et al. 1992). Underexpression of E-cadherin is a prognostic marker of poor clinical outcome in many tumor types, and germline mutations have been identified in certain familial cancers (Bracke et al. 1996; Guilford et al. 1998; Guilford et al. 1999). Loss of expression or adhesive function of E-cadherin has been shown to correlate with an aggressive phenotype in invasive cell lines and carcinomas (Behrens et al. 1989; Perl et al. 1998). In addition, direct evidence has shown that E-cadherin is involved in the suppression of invasion (Vleminckx et al. 1991) and osteolytic bone metastasis (Mbalaviele et al. 1996). These experiments illustrate the importance of E-cadherin-mediated cell-cell adhesion for the suppression of invasion and metastasis.

Integrins are a ubiquitously expressed family of cell adhesion receptors that support and modulate a variety of cellular functions that are required for tumor metastasis, including tumor cell attachment, migration and invasion, and arrest within the vasculature of target organs (Felding-Habermann 2003). The integrin family consists of 18 alpha and 8 beta subunits, which dimerize to yield at least twenty-four different heterodimeric transmembrane proteins, each of which have distinct ligand binding and signaling properties (Hynes 2002). These proteins bind to different extracellular matrix (ECM) molecules with their extracellular domains and connect directly or indirectly to the actin cytoskeleton with their intracellular domains, thereby linking the ECM to the cytoskeleton. During tumor development, changes in integrin expression and signaling modulate the ability of tumor cells to interact with their environment. Crosstalk between integrins, signaling proteins, cytoskeletal adaptor proteins, growth factor receptors and metalloproteinases converts sessile tumor cells to a migratory and invasive phenotype (Felding-Habermann 2003). Although integrins offer attractive drug targets to fight tumor growth and metastasis, much more needs to be learned about the variability of their functions, cross-talk with other signaling pathways and tumor-specific roles.

Metastatic cells must acquire the ability to invade, or translocate across extracellular matrix barriers. Invasion requires local remodeling of the extracellular matrix, pseudopodial extension, and cell migration (Liotta and Rao 1986). At the biochemical level, malignant invasion appears to use similar molecular mechanisms as normal physiological processes, including neovascularization, wound healing, and neurite outgrowth during embyogenesis. Unlike these, however, malignant invasion is not tightly controlled and malignant cells perpetually stimulate host stromal and vascular cells. In addition, activation of the local invasive environment seems to create a permissive field for the malignant cell (Liotta and Kohn 2001).

Basement membranes constitute barriers that are overcome for tumor cell invasion, so remodeling of the extracellular matrix is a necessary step for local invasion (Liotta et al. 1991a; Werb 1997). The ECM may also contain stored latent proteinases and cytokines

that can be activated by the invading cell pseudopodia. The principal enzymes that are believed to degrade the ECM and cell-associated proteins are: the matrix metalloproteinases (MMPs), a family of secreted and membrane-bound zinc-binding proteinases; the adamalysin-related membrane proteinases; the bone morphogenetic protein-1-type metalloproteinases; and tissue serine proteinases (Liotta and Kohn 2001). These enzymes are tightly regulated by a series of activation steps and specific inhibitors, many of which are themselves important regulators of metastasis. For example, overexpression of the MMP inhibitor TIMP-2 in breast carcinoma cells is correlated with a decrease in the number of osteolytic bone lesions (De Clerck et al. 1992).

The pseudopodium, or leading edge, of the invading cell contains the degradative enzymes and adhesion receptors that coordinate sensing, protrusion, burrowing and traction (Guirguis et al. 1987). Actin cytoskeleton structures provide the mechanical basis for pseudopodial extension of invading cells (Stossel 1993; Dong et al. 1994). Control of cell migration depends on cytoskeletal rearrangements and an ordered redistribution of integrin molecules at the cell surface, as cells establish and de-establish matrix contact while they move forward and constantly change their shape. As the invading cell moves forward through the ECM, the leading-edge complex of enzymes, inhibitors and receptor molecules cycle through adhesion, de-adhesion and proteolysis, and previous attachment sites at the rear of the cell detach (Liotta et al. 1991b).

The direction of tumor cell invasion and migration can be influenced by three groups of chemoattractants: local attractants, proteolysed matrix fragments and cytokines or growth factors. Local attractants include autocrine motility factors that are secreted by the tumor cells themselves, and include hepatocyte growth factor/ scatter factor (HGF/SF), which binds to the Met receptor (c-Met) (Gherardi 1991; Naldini et al. 1991). Matrix proteins that can induce motility are vitronectin, fibronectin, laminin, type I collagen, type IV collagen and thrombospondin (Tchao 1982; McCarthy and Furcht 1984; Basara et al. 1985; McCarthy et al. 1985; Taraboletti et al. 1987; Klominek et al. 1993). These proteins stimulate chemotaxis (motility toward a chemical gradient) and haptotaxis

(motility stimulated toward a bound substrate) mostly through their integrin receptors. This represents a positive feedback loop for tumor cell motility. As tumor cells secrete enzymes that degrade the ECM, those same fragments can stimulate the tumor cells to migrate. Finally, host-secreted growth factors can stimulate tumor cells to move toward the organs that produce them. These motility factors can contribute to motility by eliciting changes in cell shape, cell adhesion or cytoskeletal rearrangements (Woodhouse et al. 1997).

Metastatic cells often use the bloodstream to colonize distant target organs. To do this, tumor cells must detach from the primary tumor, gain access to blood vessels and survive and manage the unique conditions in the vasculature (Felding-Habermann 2003). In this environment, tumor cells are confronted with plasma proteins, erythrocytes, leukocytes and platelets, as well as shear forces that are generated by blood flow and physically oppose cell attachment. Tumor cells may express cell-surface adhesion molecules to aggregate to other cells in the circulation. For example, CD44 and VLA-4 play a role in the adhesion to bone marrow fibroblasts and endothelial cells, whereas ICAM-1 is involved in the binding of tumor cells to bone marrow macrophages (Okada et al. 1995). It is generally accepted that a fairly large number of cells from a primary tumor enter the circulation, however, relatively rare metastases will arise, either as a result of getting stuck in the vasculature or via recruitment to a distant organ (Humphries et al. 1986). To colonize their target organs successfully, tumor cells must attach to vascular endothelial cells. This is mediated by specific adhesive interactions between tumor cell receptors, including integrins, and endothelial counter receptors. For example, *in vivo* studies have shown that in certain tumor cell types, expression of integrin $\alpha v\beta 3$ is required for hematogenous metastasis and inhibition of $\alpha v\beta 3$ function on circulating tumor cells strongly inhibits colonization of target organs (Pozzi et al. 2000; Dumin et al. 2001). Metastatic cells that manage to arrest within microvessels of their target organs either extravasate or start to proliferate at the attachment site (Horton and Davies 1989). Cells that cannot proliferate within the vasculature undergo rapid apoptosis (Liapis et al. 1996)

while non-proliferating extravasated cells may remain dormant for extended periods of time (Matsuura et al. 1996).

Once tumor cells extravasate from the endothelium into a distant organ, they must recruit survival and growth factors to form a secondary tumor (Steeg 2003). Integrin supported adhesion of circulating tumor cells not only represents the first critical step toward target organ colonization, but also contributes to tumor cell survival and proliferation at the secondary site (Felding-Habermann 2003). Factors that stimulate metastatic cells to proliferate at secondary sites, including insulin growth factor-1 and epidermal growth factor (Blat et al. 1989; Kurtz et al. 1990; de Wit et al. 1992) have also been identified. However, how metastatic cells in the process of colonizing a secondary site respond to stress conditions and paracrine signals from the local microenvironment is not well understood. The failure of most tumor cells to proliferate within target organs is controlled in part by metastasis suppressor genes (Steeg 2003).

The existence of metastasis suppressor genes was first suggested by the observation that fusion of non-metastatic with metastatic cells suppressed the metastatic potential of the latter (Turpeenniemi-Hujanen et al. 1985). A few years later, the first putative "metastasis suppressor" gene was identified when differential colony hybridization was used to compare seven cell lines derived from a single murine K-1735 melanoma (Steeg et al. 1988). RNA levels of the nm23 gene were ten times higher in two low colonization lines than in five related high colonization lines, and correlated with metastasis inhibition. The data, which were highly contested at the time, suggested that consistent changes in gene expression occur in metastasis, and that the expression of a specific gene was reduced in tumors with high metastatic potential. The comparison of metastatic tumor cells with tumorigenic (but nonmetastatic) tumor cells in model systems has since been used to identify additional metastasis suppressors, or genes that significantly reduce tumor metastatic potential with no effect on primary tumor size.

Early techniques used to identify metastasis suppressor genes include differential display and subtractive hybridization (Steeg 2004). Microcell-mediated chromosome transfer was also used to identify a chromosomal region that has suppressive activity *in vivo.* In this approach, well-characterized donor cells carrying a single human tagged chromosome are used to transfer the chromosome into recipient metastatic cells, and the *in vivo* metastatic behavior is then determined. If found to suppress metastasis, the region of the chromosome is successively narrowed to candidate genes, which are then studied for expression patterns and function in transfection experiments (Steeg 2004). The transfer of chromosome 6 into the human melanoma cell lines C8161 and MelJuSo, for example, suppressed their ability to metastasize from intradermal injections in nude mice, or to colonize the lungs following tail vein injections, but had no effect on tumorigenicity (Welch et al. 1994). The metastasis suppressor gene Kiss-1 was eventually shown to map to this region of chromosome 6 (Lee et al. 1996; West et al. 1998).

To date, only twelve confirmed metastasis suppressors have been identified (Steeg 2003). The specific biochemical activities and signaling pathways responsible for the suppression of metastasis by these genes are currently being investigated. Kiss-1, for example, was reported to encode a precursor of a secreted neuropeptide for an orphan G-protein-coupled-receptor (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). However, the mechanism of its metastasis-suppressing activity has not yet been elucidated. The advent of DNA microarrays has accelerated the discovery of metastasis suppressors and enhancers, and this technology will be discussed in detail below.

Metastatic heterogeneity

A single malignant cell must acquire the ability to complete all the aforementioned steps to successfully metastasize to a distant organ. Although doctors and pathologists had known for decades that metastasis was a rare event, the advent of tumor cell radioactive labeling in the 1960s provided the first quantitative evidence to support this observation. Using ^{125}iodine-iodo-deoxyuridine labeled tumor cells, Josh Fidler showed that within

twenty-four hours after entry into the circulation, less than 0.1% of these cells were still viable, and that less than 0.01% of those cells, when reintroduced into the circulation, survived to produce metastases (Fidler 1970).

To understand better why only rare cells are capable of metastasis, Fidler analyzed the metastatic properties of a heterogenous population of B16 murine melanoma cells in an *in vivo* experimental metastasis assay (metastasis assays are explained in greater detail below). The poorly metastatic murine melanoma cells were injected intravenously into syngeneic mice, and secondary site metastases were assessed, harvested, and expanded in culture. The metastatic capacity of the *in vivo*-selected cells was compared to and shown to be greater than that of the original parental tumor (Fidler 1973). Additional studies with B16 melanomas showed that subclones isolated from individual cells of a primary tumor that did not undergo selection also varied significantly in their metastatic capacities (Fidler and Kripke 1977). Taken together, these results suggest that metastasis is a selective process that arises from rare, highly metastatic variants within the primary tumor. Several labs have since repeated these results to derive metastatic cell lines from commonly studied experimental tumors. In addition, it has been shown repeatedly for a wide range of tumors of different histological origin that subpopulations with differential metastatic potential exist within the same primary tumor (Poste and Greig 1982).

The clonal nature of metastatic lesions has been described for several tumor types, including breast cancer and melanoma. Experimental analyses have shown that when two metastatic cell lines are intravenously injected into mice as heterogenous clumps, karyotypic patterns of abnormal marker chromosomes suggest that the resulting lung metastases each originated from a single progenitor cell (Fidler and Talmadge 1986). This result directly supports the conclusion that metastasis is a rare event resulting from a highly selective process.

The Seed and Soil Hypothesis

The rare metastatic event requires the interaction between tumor cells and a specific organ microenvironment. The nonrandom propensity of different primary tumors to metastasize to distant organs was originally described in 1889 by Stephen Paget (Paget 1889), whose "seed and soil" hypothesis suggested that the patterns of tumor cell homing and metastasis depended on crosstalk between selected cancer cells (the 'seeds') and specific organ microenvironments (the 'soil'). This idea was later challenged in 1929 by James Ewing, who proposed that the anatomical structure of the vasculature between a primary tumor and a distant metastasis was sufficient to account for organ-specific metastasis (Ewing 1928). After much scientific inquiry, we know today that the two theories are not mutually exclusive, and that both factors influence metastatic spread. The pattern of regional venous drainage can partly but not completely predict the distribution pattern of metastases. A series of autopsy studies of sixteen primary tumor types and eight target organs revealed that 66% of metastases develop in the first capillary bed encountered after discharge from the primary tumor (Weiss 1992). Therefore, the remaining 34% of tumors metastasize to distant locations that are not predictable based on blood flow patterns, supporting the hypothesis that additional factors besides blood flow are necessary for tumor cell homing to secondary sites. Experimental data on the preferential invasion and growth of murine B16 melanoma metastases has also shown that metastatic sites are determined not only by the characteristics of the neoplastic cells, but also by the microenvironment of the host tissue (Hart and Fidler 1980).

Identifying the molecular factors that define the "soil" microenvironment is a key goal for cancer biologists. However, the specific mechanisms that actually promote organ-specific metastasis are only beginning to be elucidated. A recent study of breast cancer cells, for example, revealed that the specific expression of chemokine ligands and their receptors was essential for the precise and preferential destination of metastatic tumor cells to specific organs (Muller et al. 2001). Clearly, much clinical value stands to be gained from these and other similar analyses.

Experimental modeling of metastasis

Much remains to be learned about the biology of the metastatic process in part because metastasis is a "hidden" process, which occurs inside the body and so is inherently difficult to observe (Chambers et al. 2002). Therefore, to begin to understand the myriad cellular and molecular changes necessary for metastatic progression, the process has been modeled experimentally. Two classes of transplantable *in vivo* metastasis assays have been developed to measure metastatic ability (Welch 1997). In "spontaneous metastasis assays," tumor cells are implanted into experimental animals subcutaneously, intramuscularly or directly into tissues (orthotopically). Spontaneous metastases from this primary site have completed all the steps of the metastatic process are monitored. In "experimental metastasis assays," tumor cells are injected directly into the circulation (for example, into the tail vein for lung metastases, into the left heart ventricle for bone metastases and into the portal vein for liver metastases). These assays circumvent the first steps in the metastatic process and reflect the ability of tumor cells to survive in the circulation, arrest in a distant capillary bed, and grow in a distant organ (Fidler 1986).

In recent years, transgenic mouse models of endogenous (spontaneous) tumorigenesis have been generated. These mice are usually designed to overexpress activated forms of oncogenes or carry targeted mutations in tumor suppressor genes, and are powerful tools for analyzing the mechanisms behind specific tumor processes, such as proliferation, apoptosis, angiogenesis or metastasis (Macleod and Jacks 1999). These mice can then be interbred to assess the extent of cooperativity between different genetic lesions in disease progression, leading to a better understanding of the multi-stage nature of tumorigenesis. The polyoma middle T oncoprotein mouse, for example, is an excellent model of human breast cancer. These mice develop lesions that undergo morphological changes and express biomarkers analogous to those seen in progressive human breast cancer. In addition, the malignant transition in these mice is followed by a high frequency of distant metastasis (Lin et al. 2003).

Depending upon where along the progression to metastasis cells are analyzed, these assays tend to measure endpoint metastases, making it difficult to assess which individual steps in the total metastatic process are being manipulated. In addition to the *in vivo* metastasis assays, several *in vitro* assays have been developed to model specific cellular processes required for metastasis, including cell adhesion, migration, invasion and colonization. For example, tumor cell colonization in soft agar has been used as an *in vitro* measure of metastatic colonization. Similarly, tumor cell migration and invasion through Transwell pores and matrigel, respectively, are used as *in vitro* measures of malignant migration and invasion. These assays are interpreted with some caution, however, because they may not be representative of the true tumor environment.

Expression analysis of metastatic progression

A comparison of primary tumors and metastatic lesions provides useful information about tumor progression to metastasis. Several methods have been used for these kinds of comparative analyses, including differential display (Liang et al. 1992), serial analysis of gene expression (SAGE) (Velculescu et al. 1995), subtractive hybridization (Diatchenko et al. 1996), and representational difference analysis of cDNA (Hubank and Schatz 1994). Indeed many molecular factors that contribute to the progression of metastasis have been identified using these methods. However, it was the advent of DNA microarray expression analysis around the start of the twenty-first century that has transformed the field of metastasis research and unveiled a wealth of new information relevant to the question of metastatic progression. These genome-wide expression analyses allow a nearly complete genetic characterization of tumor samples, and contribute to a more complete understanding of the molecular changes that lead to metastasis.

Several groups have used DNA microarray analyses to compare the gene expression profiles of different classes of primary human tumors. Computational algorithms were then used to cluster the analyzed tumor samples on the basis of their gene expression

profiles, or signatures. The result of these bioinformatic analyses was the prediction of molecular signatures for "good" and "bad" prognosis. A comparison of the gene expression profiles of adenocarcinoma metastases of multiple tumor types to unmatched primary adenocarcinomas identified a gene expression signature that distinguished primary from metastatic adenocarcinomas (Ramaswamy et al. 2003). Notably, the authors identified the same "poor prognosis" signature in solid tumors originating from different tissues, perhaps implying that different tumor types show similar pathways to metastasis and suggesting the possible existence of therapeutic targets common to different tumors (Liotta and Kohn 2003). These analyses suggest that the clinical outcome of individuals with cancer can be predicted using the gene-expression profiles of primary tumors at diagnosis (Perou et al. 2000; Alizadeh et al. 2001; Sorlie et al. 2001; van 't Veer et al. 2002; van de Vijver et al. 2002).

An interesting result from the Ramaswamy analysis was that a number of primary tumors resembled metastatic tumors with regard to their gene expression signature. The authors and others suggested that this data challenged the long standing hypothesis that metastatic cells are rare and arise late during tumor progression (Bernards and Weinberg 2002; Ramaswamy et al. 2003). A recent report from Kang *et al.*, however, helped reconcile the two opposing views (Kang et al. 2003). This group used an *in vivo* metastasis assay to select highly metastatic breast cancer cells that specifically metastasized to bone. They showed that these metastatic variants preexist in the parental tumor cell population and have a distinctive "bone metastasis" gene expression signature. Formation of bone metastases resulted from the selection and enrichment of these preexisting variants that highly expressed the "bone metastasis" profile. When the Kang group used the "poor prognosis" gene signature previously identified by the van't Veer group to analyze their own samples, they found that all of their breast cancer isolates, whether of high or low metastatic potential to bone, fitted with the "poor prognosis" signature. Therefore highly metastatic rare variants that expressed the "bone metastasis" signature could be selected *in vivo* from a heterogeneous population of breast cancer that already expressed the "poor prognosis" signature.

If proven reliable for diagnosis, “poor prognosis” signatures can have a tremendous impact on treatment of disease. Most stage I solid tumors are treated as if they will metastasize, although only 10-20% of these tumors will ultimately disseminate. A “poor prognosis” gene expression signature that could reliably predict the subset of individuals with cancer for whom treatment would be beneficial would spare unnecessary treatment for 80% of these patients. Clinical trials to evaluate these prognosticators are clearly warranted (Liotta and Kohn 2003).

Identifying how the genes that are overexpressed in a “poor prognosis” signature function is a logical starting point for understanding the molecular mechanisms of metastasic progression. However, because the human tumor sample were not microdissected away from the surrounding stroma before the gene expression patterns were analyzed, the genes that make up several of these signatures could represent genes that are either expressed by the tumor cells themselves, or by the surrounding stroma cells, including vasculature, connective tissue or immune cells (Hynes 2003). Whether the expression profile is a cause or local consequence of the metastatic process is unclear, and may explain why metastasis signatures are common among several different tumor types. Experimental analyses of human tumors in transplantable mouse models allow the investigation of additional aspects of the metastatic process that cannot be addressed above. Together these two complementary approaches, experimental metastasis assays in mice and retrospective analyses of human tumor samples, will undoubtedly provide many more insights into the cellular and molecular aspects of metastatic progression (Hynes 2003).

Conclusions

Metastasis remains the leading cause of cancer morbidity and mortality today because the cellular and molecular mechanisms responsible for its progression remain poorly understood. Clearly, the systemic, cellular and molecular pathogenesis of metastasis must be more clearly elucidated before better therapies can be designed. The advent of

DNA microarray expression analysis has transformed the field of metastasis research and unveiled a wealth of new information relevant to the question of metastatic progression. These genome-wide expression analyses allow for a nearly complete molecular picture of the cellular events that lead to metastasis. The identification of additional molecules that are required for the growth of tumor cells in specific target organs is imperative for the development of new therapeutic strategies.

More than one hundred years later, the revised statement of Paget's "seed and soil" hypothesis can be summarized by three principles (Fidler 2002). First, the primary tumor contains a heterogenous population of cells with very different angiogenic, invasive and metastatic properties. Second, the multi-step process of metastasis is highly selective, favoring the survival and growth of only a subset of cells that can accomplish all the steps. Third, growth at a distant secondary site depends on multiple interactions between metastatic cells (seed) and homeostatic mechanisms (soil). Our knowledge of these three principles must be further developed to gain a deeper understanding of the molecular pathogenesis of metastasis. With this increased understanding, successful rational strategies for treatment of metastatic disease can be developed.

Chapter 2

Differential Expression of IQGAPs in Cancer Progression

Sarah E. Frew, Lei Xu and Edwin A. Clark

My contributions to this chapter include everything except the selection of metastatic cell lines and the microarray expression analysis. The B16 F1, F2, F3 and A375 M1, M2 cells lines were generated and analyzed via Affymetrix microarray by Edwin Clark, published in Nature, 406: 532-535 (2000). The A375 MA-1, MA-2, MC-1 and MC-2 cells were selected and analyzed via Affymetrix microarray by Lei Xu, unpublished results.

Introduction

Metastasis is the process whereby a primary tumor spreads to distant parts of the body (for review, see (Ruoslahti 1996)). Metastatic cells must detach from their surrounding cells, migrate away from the original tumor and invade into a blood or lymphatic vessel. Only about one in ten thousand cells will survive in the circulation long enough to attach to the vessel wall, extravasate from the vessel and proliferate in its new surroundings, forming a secondary tumor at a site distant from the original tumor (Fidler 1970). Although this event is very rare, it is the primary cause of cancer-related morbidity and mortality. Cancer cells must lose some characteristics of normal cell behavior and acquire several new capabilities to elicit the changes in cell adhesion, polarity, migration, invasion, and survival that are necessary to complete this process.

To explore the molecular pathways involved in the progression to a metastatic phenotype, our lab recently utilized oligonucleotide arrays (GeneChip, Affymetrix) to measure changes in gene expression on a genomic scale (Clark et al. 2000b). Poorly metastatic human and murine melanoma cell lines (A375P and B16F0, respectively) were used in an *in vivo* selection scheme (see below) to derive metastatic variants of the parental cell line (Fidler 1973). Human (HUM 6.8K or U133A) and murine (MUR 6K) gene chips were used to compare gene expression levels between tumors derived from the parental cell line and its highly metastatic derivatives. As a result, several genes that are differentially expressed between non-metastatic and metastatic tumors were identified. The genomic approach allows one to identify families of genes involved in a process, not just single genes, and can indicate which molecular and cellular events might be important in a complex biological process such as metastasis. In this regard, we found that the progression to a metastatic phenotype involves the enhanced expression of several genes involved in extracellular matrix assembly and of a second set of genes that regulate, either directly or indirectly, the actin-based cytoskeleton ((Clark et al. 2000b), Xu, L. unpublished results).

A cancerous cell escapes many controls, and often uses the extracellular matrix to its own

advantage. When a cancerous cell does this, it triggers programs of gene expression and protein activity that are not appropriate for that particular cell type or that particular stage of development. In this regard, the microarray expression analyses described above revealed that the progression to a metastatic phenotype is associated with changes in the expression of genes encoding not only cell-cell interactions, but also cell-matrix interactions.

In order to invade through the basement membrane, tumor cells use proteinases to remodel the extracellular matrix (Liotta and Rao 1986). The ECM, in turn, may contain stored latent proteinases and cytokines that can be activated by the invading cell pseudopodia. Proteolyzed fragments of several matrix molecules can also act as chemoattractants. These proteins stimulate chemotaxis and haptotaxis mostly through their integrin receptors (Woodhouse et al. 1997). This represents a positive feedback loop for tumor cell motility. As tumor cells secrete enzymes that degrade the ECM, those same fragments can stimulate the tumor cells to migrate.

We found that a number of extracellular matrix proteins, and molecules that regulate their assembly, were expressed at higher levels in highly metastatic melanoma cells. These molecules included: fibronectin, two collagen subunits alpha2(I) and alpha1(III), the matrix Gla protein, fibromodulin and biglycan. Previous studies have correlated fibronectin expression with tumorigenesis and metastasis (Zhang et al. 1997b; Maniotis et al. 1999). Similarly, collagen expression has been correlated with the invasive potential of ocular melanomas and the matrix Gla protein has been shown to be overexpressed in breast carcinoma cell lines relative to normal breast epithelial cells (Chen et al. 1990; Maniotis et al. 1999). Our finding that progression to a metastatic phenotype involves the enhanced expression of several genes involved in extracellular matrix assembly supports the hypotheses that enhanced expression of ECM proteins may promote tumor cell survival or angiogenesis (Ruoslahti 1999).

We also found that several genes whose products regulate the actin cytoskeleton showed

enhanced expression in highly metastatic metastatic cells. These molecules included: RhoC, thymosin β4, α-catenin, α-actinin 1, α-centractin and IQGAP1. RhoC is a member of the Rho family of small GTPase regulatory proteins that coordinate remodeling of the actin cytoskeleton in response to various stimuli (Ridley 2001). In the active, GTP-bound form, they interact with and activate target proteins that regulate actin polymerization, cell motility and gene expression. Enhanced RhoC expression has been reported to correlate with the progression of pancreatic adenocarcinomas to a metastatic phenotype (Suwa et al. 1998). Our group went on to show that RhoC enhances metastasis when overexpressed in A375P cells, whereas a dominant-negative Rho inhibits metastasis in the A375M highly metastatic melanoma cells (Clark et al. 2000b). Thymosin beta4 is an actin-sequestering protein that regulates actin polymerization and has since been shown to stimulate tumor metastasis by activating cell migration and angiogenesis (Cha et al. 2003).

Another actin-binding protein that was upregulated in several of the independently derived metastatic variants is IQGAP1. This scaffolding protein directly binds to a number of proteins, including F-actin, the plus-end microtubule-binding protein CLIP-170, active GTP-bound Rac1 and Cdc42, E-cadherin, β-catenin, and calmodulin (Mateer et al. 2003). Via its numerous binding partners, IQGAP1 is a component of the signaling networks that regulate cell-cell adhesion, cell polarity, and cytoskeleton organization. For example, IQGAP1 can disrupt cell-cell adhesion by directly binding to E-cadherin and β-catenin at sites of cell-cell contact (Kuroda et al. 1998). This interaction disrupts the interaction between α-catenin and β-catenin and unlinks the cadherin/catenin adhesion complex from the actin cytoskeleton.

Previous studies have shown that IQGAP1 is overexpressed in human colon carcinoma, compared with normal colonic epithelia, and is upregulated as a result of gene amplification of 15q26 in two invasive cell lines derived from human diffuse-type gastric carcinomas (Sugimoto et al. 2001; Nabeshima et al. 2002). Although IQGAP1 directly interacts with a number of proteins that have been implicated in cancer progression, its

role in metastasis has not yet been elucidated.

We therefore decided to analyze further the role of IQGAP1 in metastatic progression. We found that IQGAP1 expression is enhanced in several *in vivo*-selected highly metastatic melanoma variants at both the RNA and protein level, independent of the cells' microenvironment. In addition, a survey of the ONCOMINE cancer microarray database provided additional evidence of increased IQGAP1 expression in progressive human cancers, and further implicates IQGAP1 in the initiation or maintenance of a metastatic phenotype. Together these data implicate enhanced IQGAP1 expression as a positive correlate of metastasis.

Materials and Methods

Cell lines

The A375 human (ATCC# CRL-1619) and B16 murine (ATCC# CRL-6322) melanoma cell lines, and their derivatives, were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 units/ml penicillin (Gibco/Invitrogen, Carlsbad, CA), and 100ug/ml streptomycin (Gibco/Invitrogen). Cells were split 1:5 every three days.

Experimental metastasis assay

A375 cells (500,000 cells in 0.2ml) were injected intravenously into the lateral tail vein of 5- to 8-week-old athymic nude mice (Strain CBy.Cg-*Foxn1*^{nu}) (The Jackson Laboratory, Bar Harbor, ME). Likewise, B16 cells (50,000 cells in 0.2ml) were injected into syngeneic C57BL/6J mice. Mice were sacrificed three (for B16) or five to eight weeks (for A375) post injection. The lungs were removed and washed in Phosphate Buffered Saline (150mM NaCl plus 10mM sodium phosphate, pH 7.4) (PBS). Macroscopic pulmonary metastases were quantified by visualization under a dissecting microscope, removed aseptically from the surrounding stroma, and either snap-frozen in liquid nitrogen or immediately homogenized for RNA extraction. All mouse work was done with the approval of the Institutional Animal Care and Use Committee of Massachusetts Institute of Technology.

RNA extraction

Total RNA was isolated from melanoma cells grown in tissue culture and from freshly dissected or flash-frozen tumors using the RNeasy kit (Qiagen Inc, Valencia, CA). Briefly, samples were lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC) containing buffer. Ethanol was then added to provide appropriate conditions for RNA binding to a silica-gel-based membrane contained within

a spin column. After contaminants were washed away, RNA was eluted in RNAse-free water. Total RNA was then quantified and used for microarray analysis and RNAse Protection assay.

In vivo Selection of Metastatic Variants

The experimental metastasis assay was used to select tumor-derived cell lines with an enhanced ability to form experimental pulmonary metastases (Fidler 1973). A375P cells used in retroviral gene transfer studies were transfected with a plasmid containing the ecotropic receptor (a gift from H. Lodish, MIT, MA) (Pear et al. 1993) and selected for neomycin-resistance. The lines MA-1 and MC-1 were derived from individual metastases isolated from mice injected intravenously with the A375P($^{eco+}$) cells. The successively derived lines A375M1, M2 and B16F1, F2, F3 highly metastatic melanoma cells were selected in the same way and have been previously described (Clark et al. 2000b).

In vivo Proliferation

A375 melanoma cells (250,000 cells in 0.1ml) were injected subcutaneously into the rear flank of athymic nude mice. Five to six weeks later, tumors were dissected free, washed in PBS and weighed.

In vitro Proliferation

A375 melanoma cells (50,000 cells/well) were seeded into the wells of a six-well tissue culture dish. Every 24 hours for the next five successive days, cells were trypsinized and counted using a Coulter counter. Assays were performed in triplicate.

Microarray Analysis

Total RNA extracted from subcutaneous tumors and pulmonary metastases was treated with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) to synthesize cDNA. cRNA was subsequently synthesized and labeled using BioArray HighYield RNA Transcript Labeling kit (Enzo Life Sciences, Inc., Farmingdale, NY). After fragmentation, the cRNAs were hybridized to HU133A chips (Affymetrix, Santa Clara, CA) and images were scanned with GeneArray Scanner (Affymetrix). Differential expression data were analyzed by dCHIP software (Li and Wong 2001). Microarray expression analysis of the A375M1, M2 and B16F1, F2, F3 highly metastatic melanoma cells has been previously described (Clark et al. 2000b).

RNAse Protection Assay

A 1053bp fragment of human IQGAP1 3' untranslated region (UTR) was generated by Polymerase Chain Reaction (PCR) using the forward primer 5'-GTAATTGATCGTTTGCTGCCC-3' and reverse primer 5'-GCAACACTGCAGATAAATCC-3' and cloned into the pCR-BluntII TOPO vector using a Zero Blunt TOPO Cloning Kit (Invitrogen). The pCR-BluntII-IQGAP1 vector was linearized with ApaLI to generate a template for the synthesis of a 296-nt antisense IQGAP1 transcript. The alpha[-^{32}P] UTP-labeled antisense probe was synthesized *in vitro* with T7 RNA polymerase. The 127-nt β-actin control template was purchased from Ambion (Austin, TX). The RNAse Protection Assay was performed essentially as described (DeSimone et al. 1992). 5ug of total RNA was hybridized to 50,000 cts/minute of purified probe overnight at 45°C. Following hybridization, samples were digested with RNase A and RNase T1, electrophoresed on a 5% polyacrylamide gel, dried and exposed to film. Autoradiographic films were quantified using an Is-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

Western Blot Analysis

Cells were washed in PBS and lysed in either radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150mM NaCl, 10mM Tris-Hcl, pH 7.2) or Laemmli sample buffer (0.0625M Tris-HCl pH6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) plus Complete protease inhibitor (Antithrombin III, Aprotinin, 3,4-Dicholorisocoumarin, phenylmethylsulfonyl fluoride, Prefabloc SC, Leupeptin, EDTA-Na_2, E-64, Phosphoramidon, Bestatin, TIMP-2) (Roche, Basel, Switzerland). Snap-frozen tumors were lysed in RIPA buffer plus inhibitors and Dounce-homogenized. All lysates were clarified by centrifugation at 13,000g for 15 minutes at 4°C, and protein concentrations were determined using a Pierce Micro BCA protein assay kit (Pierce Chemical Co, Rockford, Il). Fifty/twenty-five micrograms of protein was loaded per well onto a 7.5% SDS-polyacrylamide gel, separated and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell BioScience, Inc., Keene, NH) at 0.75Amp for 1 hour. The membrane was blocked in 5% nonfat dried milk in PBS + 0.1% Tween-20 (PBST) and incubated with monoclonal primary antibody against IQGAP1 (Clone 24), Nucleoporin (Clone 53), GFP (Clone JL-8) or c-myc (Clone 9E10) primary antibody (BD Biosciences, San Diego, CA). The membranes were washed with PBST and incubated with peroxidase-conjugated AffinPure F(ab')$_2$ fragments donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). After washing a second time with PBST, the membranes were developed with Western Lightning Chemiluminescence Reagent (Perkin Elmer Life Sciences, Boston, MA) and exposed to film. Films were quantitatively analyzed using an Is-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

Immunofluorescence

Immunfluorescence analysis was performed essentially as described (Clark and Hynes 1996). Cells were plated on glass coverslips overnight at 37°C. Adherent cells were rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 10' at room temperature (RT), and then permeabilized with 0.5% NP-40 in PBS for 10' at RT. Cells were stained for

filamentous actin using rhodamine-labeled phalloidin. After blocking the cells with 10% normal goat serum in PBS (NGS/P) for 30' at 37°C, they were incubated with primary anti-serum to IQGAP1 (rabbit antiserum, kind gift of G. Bloom, University of Virginia, VA), diluted 1:250 in NGS/P for 1 hour at 37°C. The cells were washed 3 times in PBS and then incubated with AlexaFluor 488 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR) that was diluted 1:200 in NGS/P. The coverslips were washed three times in PBS, dried and mounted on glass slides using Gelvatol. The cells were analyzed with a Zeiss Axiophot.

Results

Selection of highly metastatic melanoma cells

To better understand the cellular and molecular mechanisms of metastasis, our lab adopted an *in vivo* selection scheme using the experimental metastasis assay to select highly metastatic cells with enhanced ability to form experimental pulmonary metastases from a population of poorly metastatic parental cells (Figure 2-1). Poorly metastatic human A375P and murine B16F0 melanoma cells were injected into the lateral tail vein of athymic nude mice and syngeneic C57BL/6J mice, respectively. These parental cells formed rare pulmonary metastases that were individually dissected free from the lungs, minced, and expanded in tissue culture. The *in vivo*-selected population of metastatic melanoma cells was reinjected into the tail vein of host mice for an additional round of *in vivo* selection and formed more pulmonary metastases than the parental cells (Figure 2-2A). Many of the *in vivo*-selected metastatic variants were significantly more metastatic than the parental cells (Figure 2-2B). In addition, the metastatic capacity of *in vivo*-selected cells tended to increase with each additional round of *in vivo* selection, although this was not true in all instances. (The experimental dataset presented here for MC-2 cells is quite variable, and is not fully representative of the highly metastatic character of these cells. I will discuss this point further in the Chapter 3 discussion.) These data agree with previous studies that have demonstrated that highly metastatic cells can be selected *in vivo* from a heterogeneous population of poorly metastatic tumor cells (Fidler 1973; Kozlowski et al. 1984).

To confirm that we were selecting melanoma cells with differences in metastatic, rather than growth, properties, we analyzed proliferation of the A375 MC series of metastatic melanoma cells both *in vitro* and *in vivo* (Figure 2-3). Although there was a significant difference in metastatic potential, there was no statistically significant difference in cellular proliferation between the metastatic and the parental cells either *in vitro* or when grown *in vivo* as subcutaneous tumors. In addition, it has been reported previously that the A375 M1, M2 and the B16F1-3 series of metastatic melanoma cells do not demonstrate an observable difference in growth rate compared to the parental cells (Clark

et al. 2000b). These data demonstrate that the *in vivo*-selected melanoma cells have enhanced metastatic, rather than tumorigenic, capabilities. Therefore, the highly metastatic variants and the poorly metastatic parental cells represent a valuable experimental system for analyzing cellular and molecular changes associated with metastasis.

IQGAP1 expression is enhanced in metastatic melanomas

We used Affymetrix Genechip expression arrays to analyze genome-wide differences in gene expression between the poorly and highly metastatic melanoma cells. The expression profiles of total RNA extracted from pulmonary metastases of the selected variants was compared to the expression profile of total RNA extracted from the parental cells grown as subcutaneous tumors. [The complete data sets from these analyses are discussed elsewhere ((Clark et al. 2000a), Xu, L. unpublished results), and will not be discussed in detail here.] At first we used relatively stringent conditions to analyze the expression data, and came across IQGAP1 as a gene whose expression was enhanced greater than 3-fold in all three B16 metastatic variants (Figure 2-4A). We then narrowed our focus to IQGAP1 alone, and analyzed its gene expression in all the A375 samples. We found that IQGAP1 expression was not only enhanced in B16 metastatic variants, but also in all A375 metastatic variants as compared with the parental cells. Although consistent, the differences in gene expression were small, less than 3-fold in all samples (Figure 2-4A, B). Therefore, small increases in IQGAP1 expression positively correlate with the metastatic phenotype of human melanoma cells.

The stroma surrounding growing tumors can actively influence gene expression in malignant cells (Liotta and Kohn 2001). We analyzed whether or not enhanced IQGAP1 expression in the pulmonary metastases was dependent on the surrounding microenvironment by also growing the metastatic variants as subcutaneous tumors. We then compared the expression profiles of total RNA extracted from the parental and metastatic cells grown as subcutaneous tumors. IQGAP1 expression was similarly

enhanced in subcutaneous tumors in three of the four metastatic variants analyzed (Figure 4C). Although relative IQGAP1 expression levels were increased in these three metastatic variants (MA-2, MC-1, MC-2) independent of the microenvironment, the absolute levels of expression did vary, suggesting that total IQGAP1 expression may be influenced by the extracellular environment. These results show that from within a heterogeneous population of poorly metastatic A375P cells, we have selected several independent metastatic variants that intrinsically express increased levels of IQGAP1.

IQGAP1 and IQGAP2 are Differentially Expressed in Human Cancers

Our microarray expression analysis identified a positive correlation of IQGAP1 with metastasis. To analyze further IQGAP1 expression in other human tumors, with a particular interest in progressive and metastatic disease, we utilized the web-based resource ONCOMINE (www.oncomine.org) (Rhodes et al. 2004). This cancer microarray database and data-mining platform integrates information from several bioinformatics resources, including Swiss-Prot, LocusLink, and Unigene. As of April 1, 2004 ONCOMINE contained catalogued information from 180 cancer microarray studies, of which 65 studies were available and compiled, totaling 47,489,773 gene measurements from 4702 microarray experiment. All of these gene measurements were independently log-transformed, normalized and assessed for differential expression using t-statistics, allowing users to assess and visualize the differential expression of a selected gene across a wide number of differential expression analyses.

In addition, the ONCOMINE database compiles all the available microarray study samples into five analyses of interest: cancer versus respective normal tissue; high-grade (undifferentiated) cancer versus low-grade (differentiated) cancer; poor outcome (metastases, recurrence, or cancer-specific death) versus good outcome (long-term or recurrence-free survival) cancer; primary cancer versus metastatic disease; and disease subtype 1 versus subtype 2.

We utilized ONCOMINE to analyze the expression profile of IQGAP1 in human tumors. A comparison of "cancerous versus normal tissue" gene expression profiles from several independent studies revealed that IQGAP1 was significantly overexpressed in a number of human tumors (Figure 2-5A), including lung cancer and prostate cancer. In one study, IQGAP1 expression was significantly downregulated in salivary gland adenocarcinoma (data not shown). Next we similarly compared "advanced versus less-advanced tumors" gene expression profiles and found that IQGAP1 expression was significantly upregulated in metastatic prostate cancer and grade three breast adenocarcinoma, compared with the less progressive stages of the disease (Figure 2-5B). IQGAP1 is also differentially expressed in various leukemia subtypes (data not shown).

IQGAP2 is a closely related member of the IQGAP family that is 62% identical and 77% similar to IQGAP1 over its entire length and contains all the protein-protein interaction domains identified in IQGAP1 (Brill et al. 1996). The cellular function of IQGAP2 is just beginning to be elucidated and as yet, no reports analyzing IQGAP2 expression in human cancer have been published. Although our microarray analysis revealed that IQGAP2 was not significantly expressed in the *in vivo*-selected metastatic melanoma cells, we used the ONCOMINE database to survey its expression in other cancer microarray analyses. IQGAP2 expression was significantly enhanced in two tumor types, however, its expression was significantly reduced in a number of other human carcinomas (Figure 2-6A). Next we compared "advanced versus less-advanced tumors" gene expression profiles and found that IQGAP2 expression was similarly significantly reduced in metastatic prostate cancer and advanced lung and ovarian cancers, compared with less progressive stages of the diseases (Figure 2-6B). This pattern of IQGAP2 expression is in marked contrast to that of IQGAP1.

IQGAP1 RNA Expression in Human Metastatic Melanoma Cells

Given the positive correlation of IQGAP1 expression with metastasis, we decided to analyze further IQGAP1 expression levels in the highly and poorly metastatic A375

melanoma cells. RNAse Protection Assay was used as an independent measure of IQGAP1 RNA expression. RNA was isolated from A375 cells grown in tissue culture, as subcutaneous tumors, and as pulmonary metastases. The RNAse Protection Assay results were nearly identical to the expression profiling results, confirming that IQGAP1 RNA is enhanced in the metastatic variants (MA-1, MA-2, MC-1, MC-2) compared to the parental (P) cells (Figure 2-7). In addition, the RNAse protection analysis of cells grown in tissue culture and tumors grown both subcutaneously and as pulmonary metastases confirmed that the *in vivo*-selected metastatic melanoma cells exhibit enhanced IQGAP1 RNA expression independent of their microenvironments.

IQGAP1 Protein Expression in Human Metastatic Melanoma Cells

We measured IQGAP1 protein expression in poorly and highly metastatic A375 melanoma cells by Western blot analysis. SDS lysates were prepared from cells grown in tissue culture. RIPA lysates were prepared from subcutaneous tumors and pulmonary metastases. Compared with the parental cells, all metastatic variants exhibited increased IQGAP1 protein expression. Consistent with the results from the microarray expression analysis and RNAse protection assay, IQGAP1 protein expression was enhanced in the metastatic variants independent of their microenvironment (Figure 2-8).

IQGAP1 Cellular Localization in Human Metastatic Melanoma Cells

We next explored where IQGAP1 is localized in poorly (P) and highly metastatic (MA-2, MC-2) A375 cells using immunocytochemistry (Figure 2-9). IQGAP1 was evenly distributed along cell-cell junctions in A375P cells, and also showed diffuse cytoplasmic staining. In polarized cells, IQGAP1 also localized to lamellipodia and filopodia at the leading edge. This pattern is similar to what has been previously reported in other cell types, including MDCK and Vero fibroblasts (Kuroda et al. 1998; Fukata et al. 2002b). IQGAP1 localization in the metastatic cells was similar but less organized. Although some cells expressed IQGAP1 evenly along cell-cell contacts, many cells expressed

IQGAP1 in punctate membranous spots, particularly among MC-2 melanoma cells. IQGAP1 also localized to the leading edge of polarized metastatic cells (data not shown). It has previously been suggested that cellular redistribution of IQGAP1 in metastatic cells may play a consequential role in the metastatic phenotype (Nabeshima et al. 2002).

Discussion

In this chapter, we have identified a positive correlation of IQGAP1 expression with both experimental and clinical metastasis. First, we used an in vivo experimental metastasis assay to select for cells with an enhanced ability to metastasize from a heterogeneous population of poorly metastatic melanoma cells. Microarray expression analysis revealed that all of the independently derived, highly metastatic variants expressed higher levels of IQGAP1 than the poorly metastatic parental cells. We independently confirmed the microarray expression results at the RNA and protein levels by RNAse protection and Western blot analysis, respectively. In addition, analysis of IQGAP1 expression levels in cells grown in tissue culture, as pulmonary metastases or as subcutaneous tumors revealed that several of the metastatic variants overexpress IQGAP1 independent of their microenvironment. This result suggests that increased IQGAP1 expression is intrinsic to many of the *in vivo*-selected cells.

To analyze further IQGAP1 expression in human tumors, we utilized the ONCOMINE cancer microarray database. A survey of this database identified several studies in which either IQGAP1 expression is enhanced or IQGAP2 expression is reduced in human tumors compared with normal tissue. The results of the ONCOMINE analysis show that differential expression of both IQGAP1 and IQGAP2 may play a role in the tumorigenesis of several different human tumors. We are investigating the relevance of IQGAP1 expression in metastasis, however, so the results of the "advanced versus less-advanced tumors" expression profile comparison are more relevant to our analysis. These results reveal that IQGAP1 expression is increased in other examples of progressive and metastatic disease and support our hypothesis that increased IQGAP1 expression may play a positive role in metastatic spread.

In recent years, several groups have described IQGAP1 expression in other human tumors. (Sugimoto et al. 2001) showed that IQGAP1 RNA and protein is upregulated as a result of gene amplification of 15q26 in two invasive cell lines derived from human diffuse-type gastric carcinomas. IQGAP1 is also overexpressed in human colon

carcinoma, compared with normal colonic epithelia (Nabeshima et al. 2002). Immunohistochemical analysis of the carcinoma samples revealed intense cytoplasmic and membranous IQGAP1 staining at the invasion front. When the carcinoma invaded deeper into the colonic wall, IQGAP1 expression was more frequent and intense in the deeper portions of the carcinoma, including the invasion front, than in the superficial portions (Figure 2-10). In addition, there was a strong positive correlation between pronounced IQGAP1 expression at deeper portions of the invading carcinoma and a higher incidence of nodal metastasis.

IQGAP1 expression in intestinal-type and diffuse-type human gastric cancer has also been analyzed (Takemoto et al. 2001). Intestinal-type gastric cancer is more differentiated than diffuse-type and maintains E-cadherin-mediated cell-cell adhesion, whereas membranous expression of E-cadherin, and concomitant cell-cell adhesion, tends to be lost in diffuse-type gastric cancer. Although there was no overall difference in IQGAP1 expression levels between the two tumor types, there was a clear difference in IQGAP1 cellular localization. IQGAP1 was particularly localized to the cytoplasm in intestinal-type gastric cancer cells, but relocalized to the cellular membrane in diffuse-type gastric cancer cells. The expression of IQGAP1 at the cell membrane showed a significant inverse correlation with E-cadherin expression. In addition, this group also reported that IQGAP1 was expressed at the invading edge of diffuse-type gastric cancers.

In addition, other groups have reported a shift in IQGAP1 cellular localization from the cytoplasm to the membrane in migratory and invasive cells. For example, the heterologous expression of metastasis-associated protein 1 (MTA1) in PANC-1 pancreatic carcinoma cells markedly enhances the cellular motility and the invasive penetration of epithelial barriers by the cells. Interestingly, IQGAP1 localization shifted to the membrane in these cells, whereas it was localized to the cytoplasm in control cells (Hofer et al. 2004). Hepatocyte growth factor (HGF) treatment of human colon carcinoma cells also induced a similar shift in intracellular IQGAP1 localization (Shimao et al. 2002). IQGAP1 can colocalize and directly interact with E-cadherin and β-catenin

at sites of cell-cell contact, resulting in the disruption of interactions between α-catenin and E-cadherin/β-catenin (Kuroda et al. 1998; Li et al. 1999). IQGAP1 localizes to cell-cell junctions, as well as lamellipodia and filopodia in both the poorly and highly metastatic melanoma cells, which is similar to previous reports of IQGAP1 cellular localization (Hart et al. 1996; Fukata et al. 2002b; Mataraza et al. 2003). However, the status of cadherin-mediated cell-cell adhesions in the highly metastatic melanoma cells remains unclear, and is currently being assessed.

The progression to invasiveness for epithelial tumors like the ones listed above requires that cells undergo initial morphological transformations known as epithelial mesenchymal transition (EMTs). EMTs are transient changes in cell structure that are often associated with the acquisition of motile properties. Normal physiological EMTs that occur during development are tightly regulated. However, EMTs in cancer represent the first indication that a cell does not recognize its neighbors and provide a vehicle for its propagation through the organism (Arias 2001). Acquisition of a spindle-shaped morphology and migratory properties during EMT require the concomitant down-regulation of E-cadherin (Takeichi 1995; Huber et al. 1996). A variety of experiments indicate that E-cadherin can act as a tumor suppressor and that there is a good correlation between the processes of invasion and metastasis and the loss of E-caherin in cancerous cells (Perl et al. 1998). In some instances, introduction of E-cadherin in tumor cells can stop their proliferative and invasive abilities. Therefore the regulation of E-cadherin activity and expression might provide some clues about how cells acquire some of their cancerous traits.

Snail, a member of the slug family of transcription factors, is associated with EMTs of the neural crest of mouse embryos, where a negative correlation can be observed between its expression and that of E-cadherin at the time of EMT (Nieto et al. 1994). This is mediated at least in part by the binding of Snail to the E-cadherin promoter, where it acts as a strong transcriptional repressor (Cano et al. 2000). The overexpression of Snail in different epithelial cells leads to a dramatic conversion towards a fibroblastic phenotype

at the same time that E-cadherin expression is lost and tumorigenic and invasive/migratory properties are acquired. Futhermore, invasive tumor cell lines and primary tumors induced in mice display expression of Snail at invasive fronts where E-cadherin has been lost. Similar observations were made in cancerous cells from patients with invasive breast carcinomas (Cano et al. 2000). The expression pattern for IQGAP1 in invasive primary tumors as described above is strikingly similar to that described for Snail.

As regulators of the actin cytoskeleton and cadherin junctions, the Rho family of small GTPases is commonly implicated in the processes leading to EMT and invasion (Bishop and Hall 2000). Microinjection of RhoA, Rac1 or Cdc42 into fibroblasts triggers the formation of stress fibers, lamellipodia or filopodia, respectively (Ridley and Hall 1992; Ridley et al. 1992). In the active, GTP-bound form, they interact with and activate target proteins that regulate actin polymerization, cell motility and gene expression. Rac1 and Cdc42 are involved in the establishment and maintenance of epithelial intercellular adhesions (Braga et al. 1997; Hordijk et al. 1997; Takaishi et al. 1997; Zhong et al. 1997); in contrast, RhoA activation is implicated in the reversion of the epithelioid phenotype toward a migratory, fibroblastoid morphology of NIH 3T3 cells (Sander and Collard 1999). While the molecular mechanisms by which small GTPases affect cell-cell adhesion are still poorly understood, they likely involve regulating actin cytoskeletal organization and adherens junction formation (Fukata and Kaibuchi 2001).

TFG-β plays an essential role in a wide array of cellular processes. The most well studied response in normal epithelial cells is growth inhibition, although in some cell types, TGF-β can induce an EMT and act as a promoter of tumor progression during later stages of tumorigenesis (McLeod et al. 1990; Han et al. 1993; Wrana et al. 1994; Pierce et al. 1995; Cui et al. 1996; Oft et al. 1996; Oft et al. 1998; Portella et al. 1998). Bhowmick *et al* have shown that TGF-β regulates at least two components of EMT progression through RhoA-dependent pathways, the regulation of the actin cytoskeleton and stability of adherens junctions (Bhowmick et al. 2001). Microarray analyses that

identified TGF-β-regulated genes in NMuMG cells, a nontransformed mouse mammary gland epithelial cell that exhibits both a growth inhibitory response and an EMT response to TGF-β, revealed that TGF-β induces upregulation of several cell adhesion-related genes, including IQGAP1 (Xie et al. 2003).

The observations that IQGAP1 expression at the membrane strongly correlates with loss of E-cadherin at the invasive edge in diffuse-type gastric carcinomas and that IQGAP1 is upregulated by TGF-beta during induction of EMT in murine mammary gland epithelial cells provide more correlative evidence that this protein is important in tumor progression, perhaps for both local invasion and distant metastasis. In addition, our results show that IQGAP1 expression is enhanced in a number of *in vivo*-selected highly metastatic melanoma variants. Collectively, these data present a positive correlation of enhanced IQGAP1 expression and cellular relocalization with EMT, invasion and metastasis. Through its many binding partners, IQGAP1 could influence changes in cellular behavior that are necessary for metastasis, including adhesion, polarity and migration. Based upon our preliminary results and the IQGAP1 body of literature, we decided that IQGAP1 was worthy of further study. The next chapter describes our functional analysis of the role of IQGAP1 in metastasis.

Figure 2-1

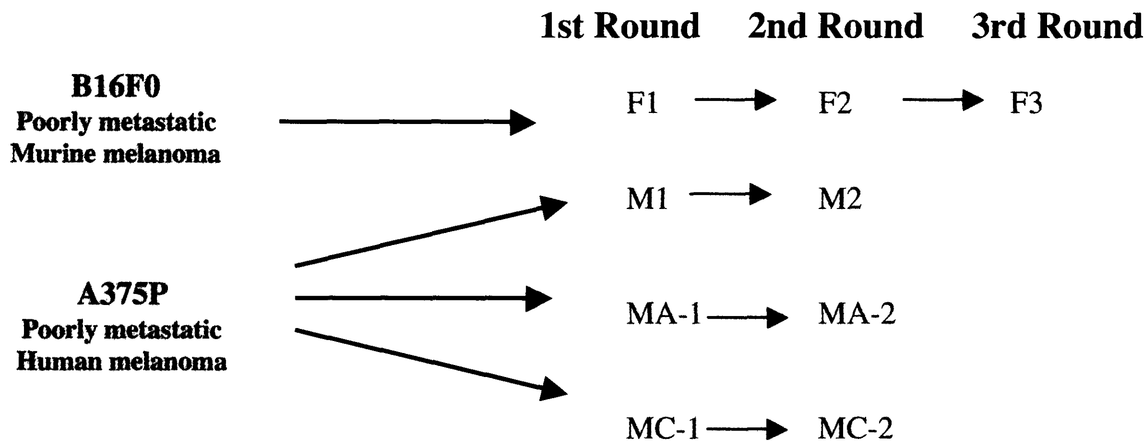


Figure 2-1: *In vivo* selection of highly metastatic melanoma cells from a population of poorly metastatic melanoma cells. Poorly metastatic human A375P or murine B16 F0 melanoma cells were injected intravenously into the lateral tail vein of nude mice or syngeneic C57BL/6J mice, respectively. These parental cells formed rare metastatic lesions in the lung. Individually isolated metastases were removed from the lungs, minced, grown in tissue culture and reinjected into the tail veins of host mice. This selection was serially repeated one time for A375 cells and twice for B16 cells. Individual pulmonary metastases isolated from A375P and B16F0 cells in the first round of *in vivo* selection yielded the variants A375M1, MA-1, MC-1 and B16F1, respectively. After being expanded in tissue culture, these cells were used in a second round of *in vivo* selection to generate A375M2, MA-2, MC-2 and B16F2 cells, respectively. B16F2 cells were used in a third round of selection to generate B16F3 cells.

Two different parental populations of A375 cells were used for these studies. The MA and MC series of A375 metastatic variants were derived from A375P cells that stably express the ecotropic envelope protein, and were used in subsequent retroviral gene transfer studies. The M1 and M2 variants were selected from A375P cells that did not express ecotropic receptor.

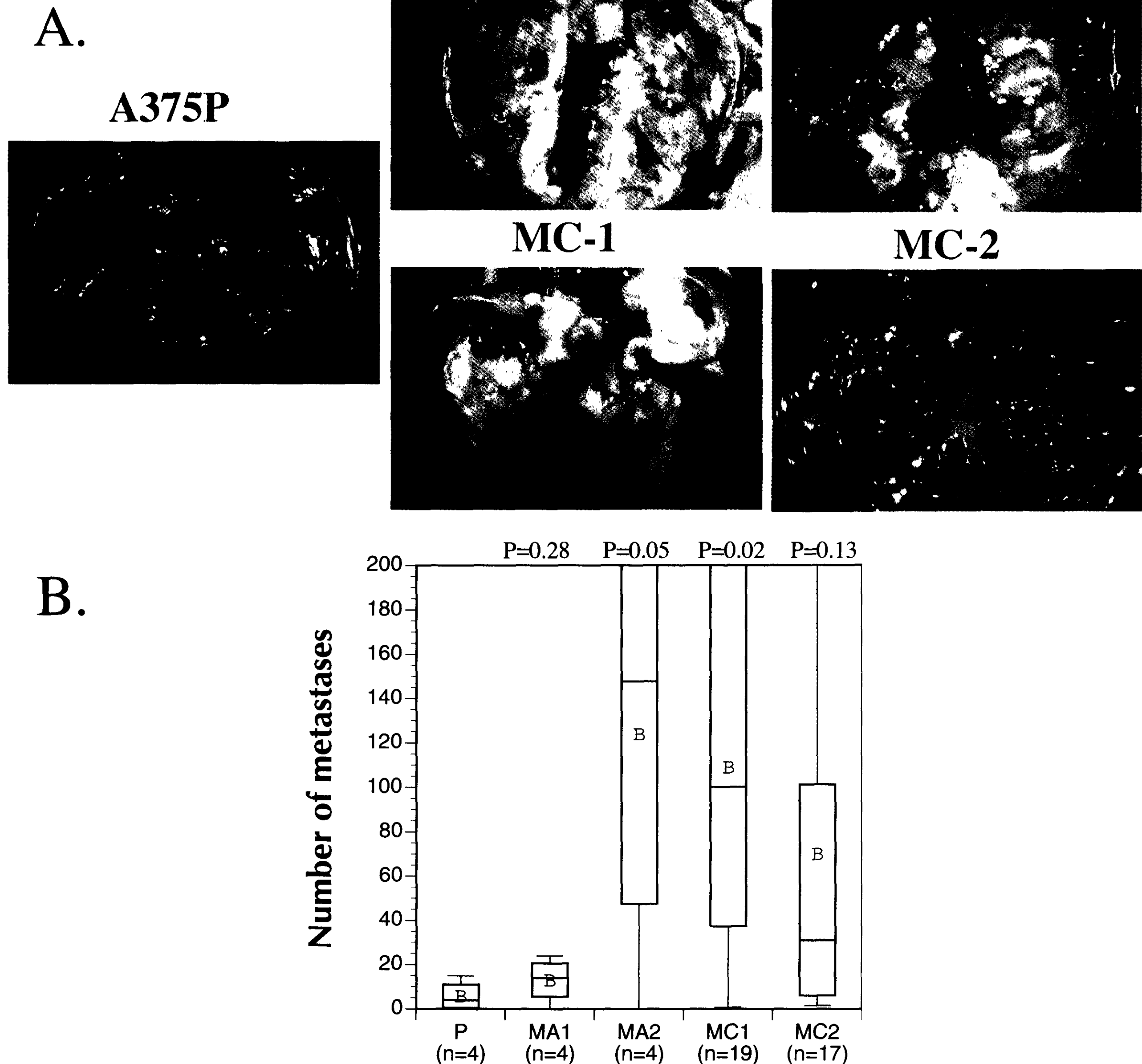


Figure 2-2: Pulmonary metastases in host mice injected with A375 human melanoma cells. A. MA-1 and MC-1 melanoma cells were selected from individual A375P lung metastases and expanded in cell culture. When reinjected into host mice, the *in vivo*-selected cells yielded more metastases than the parental line. The experimental metastasis assay was repeated, and a second round of selection generated additional metastatic variants: MA-1 -> MA-2 and MC-1 -> MC-2. B. Quantification of pulmonary metastases in host mice injected with A375 human melanoma cells. Host mice were injected with poorly or highly metastatic A375 melanoma cells and sacrificed five-eight weeks later. Macroscopic pulmonary metastases were quantified by visualization under a dissecting microscope. The *in vivo*-selected variants MA-2 and MC-1 are significantly more metastatic than the parental cells.

Figure 2-3

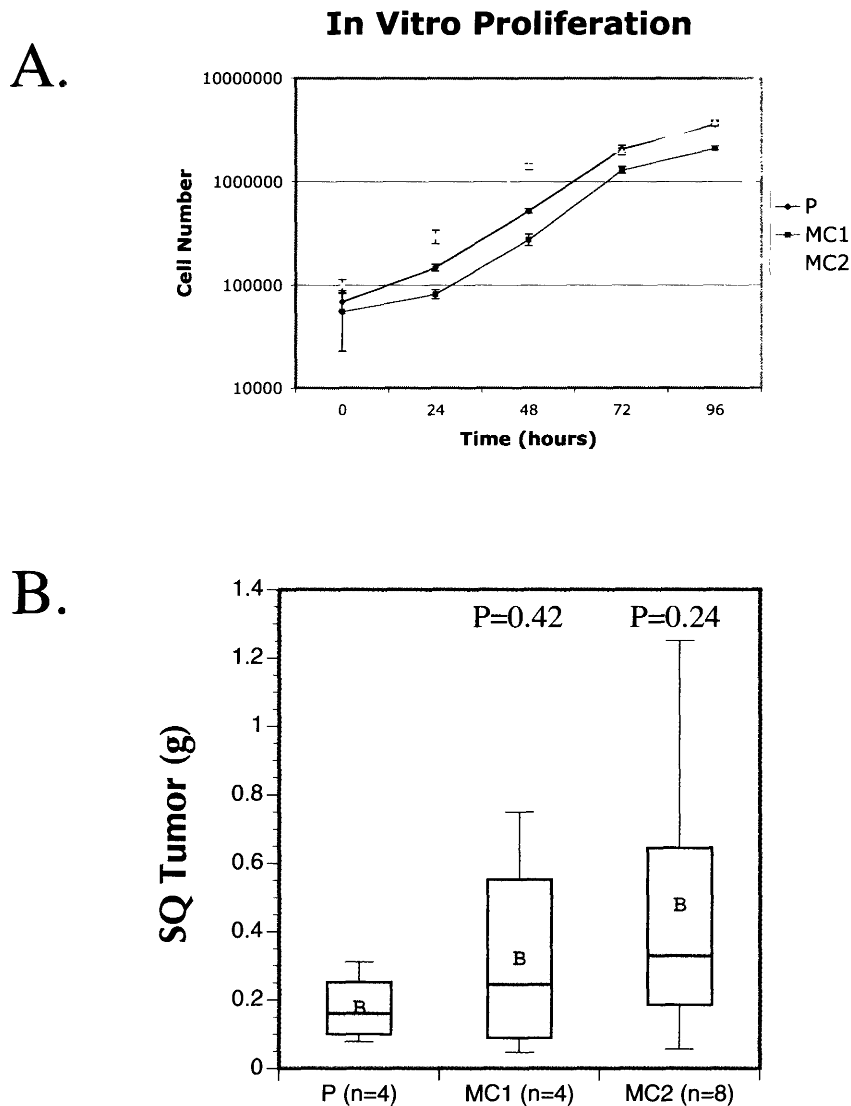
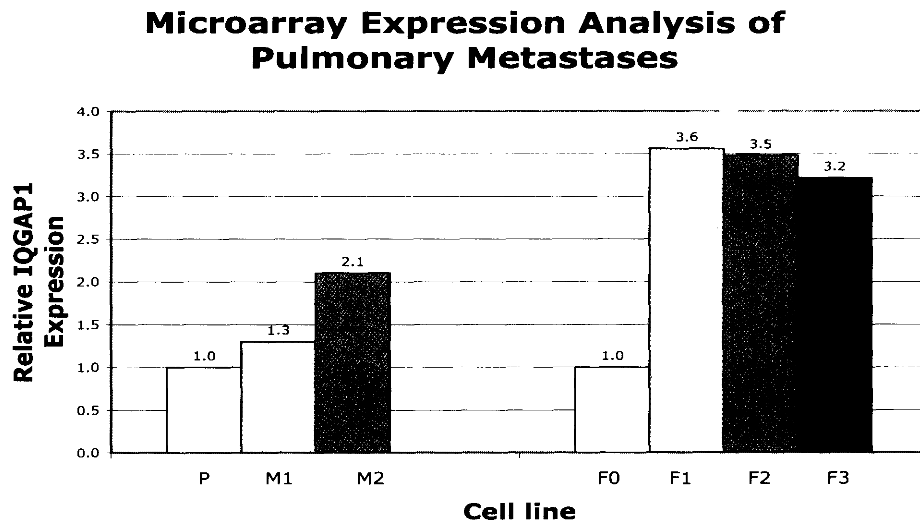


Figure 2-3: Cell proliferation of poorly and highly metastatic melanoma cells.
A. A375 cells were plated at $5x10^4$ cells on Day 0. Cells were trypsinized and counted on days 1-5. Results are expressed as absolute cell numbers +/- SD (n=3).
B. A375 cells ($2.5x10^5$) were injected into the rear flank of nude mice. Mice were sacrificed five to six weeks later, and tumors were removed and weighed. Results are represented as box and whisker plots. The top, middle and bottom of the box corresponds to the 75th percentile (top quartile), 50th percentile (median) and 25th percentile (bottom quartile), respectively. The whiskers extend from the 10th percentile (bottom decile) to the 90th percentile (top decile.) The mean is represented by the letter "B". Differences in tumor weight are not statistically significant, as determined by an unpaired Student's *t* test.

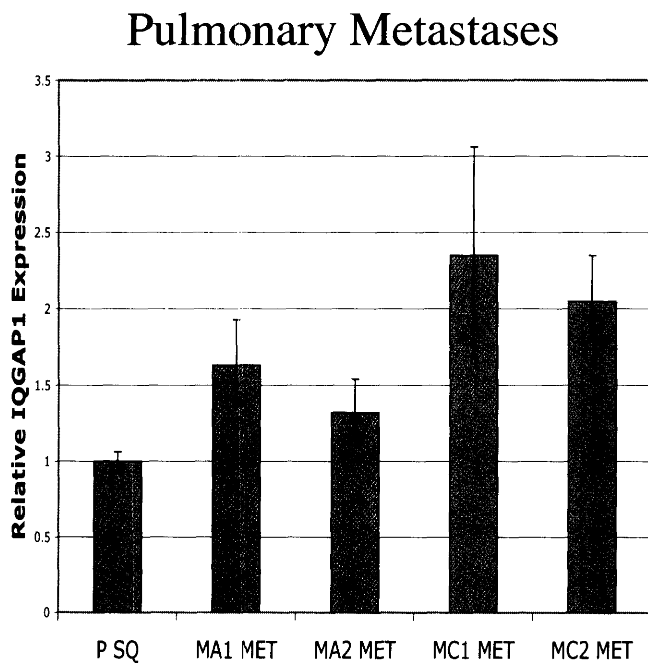
Figure 2-4

A.



B.

C.



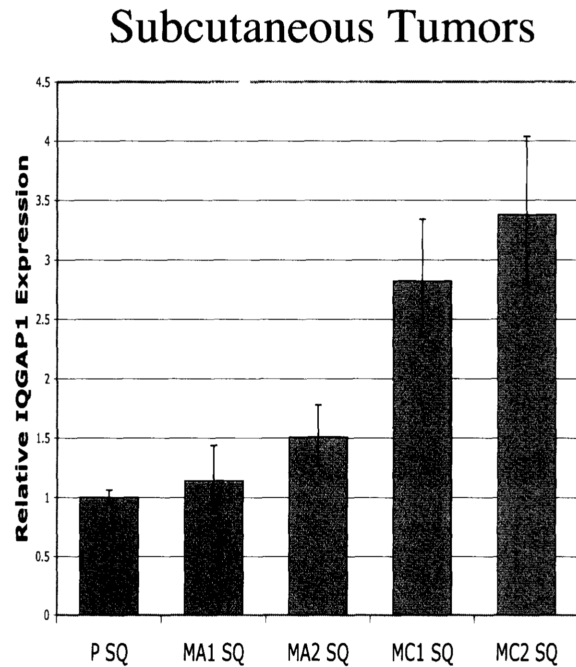


Figure 2-4: Affymetrix GeneChip microarray analysis of IQGAP1 expression in metastatic melanomas.

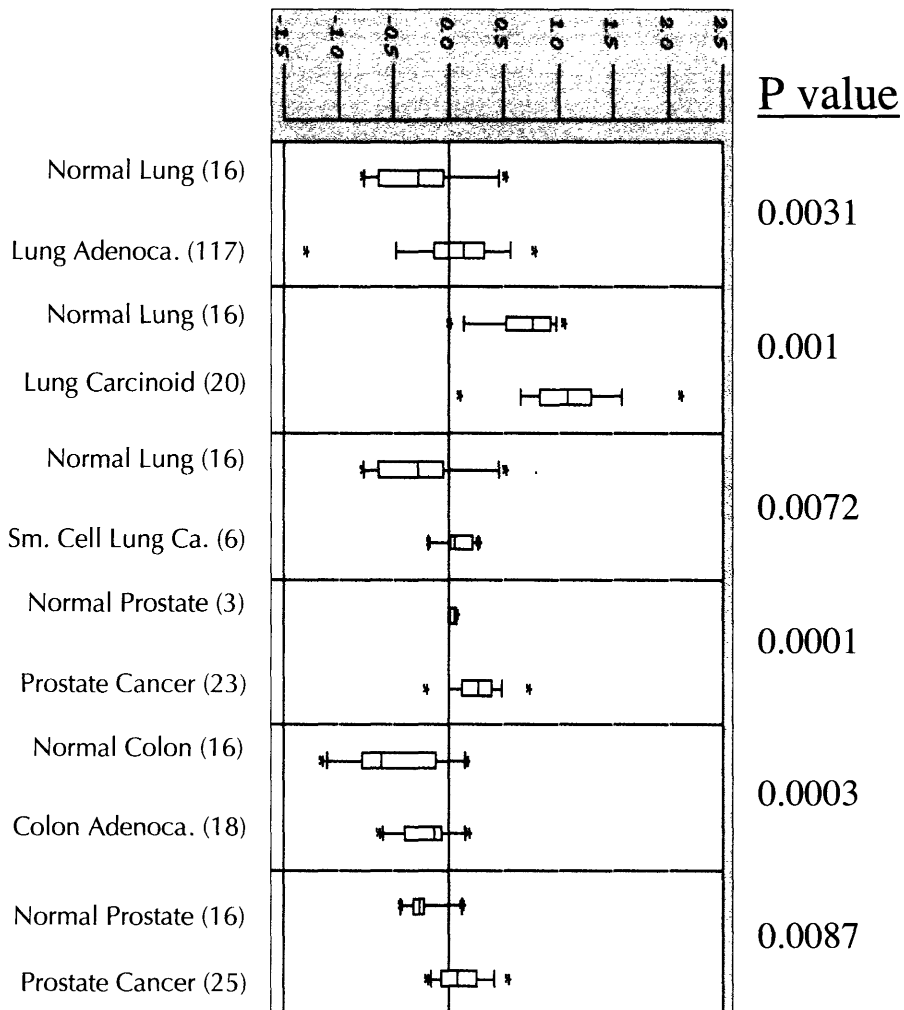
A. IQGAP1 expression is enhanced in A375 (M1, M2) and B16 (F1, F2, F3) pulmonary metastases compared with parental cells (A375P and B16F0) grown as subcutaneous tumors. A375 samples were hybridized to Affymetrix HUM6.8K arrays and B16 samples were hybridized to Affymetrix MUR 6K arrays (Clark *et al*, 2000). The values for P and F0 are the average of two experiments. Data are presented as fold expression compared with the parental tumors.

B. IQGAP1 expression is enhanced in A375 (MA-1, MA-2, MC-1, MC-2) pulmonary metastases compared with parental cells (A375P) grown as subcutaneous tumors. A375 samples were hybridized to Affymetrix HUM U133 arrays (Xu, L., unpublished results). The value for P is the average of six experiments and all metastatic samples were analyzed in triplicate. Data are presented as fold expression compared with the parental tumors.

C. IQGAP1 differential expression analysis in highly (MA-1, MA-2, MC-1, MC-2) and poorly (P) metastatic A375 cells grown as subcutaneous tumors. A375 samples were hybridized to Affymetrix HUM U133 arrays (Xu, L., unpublished results). The value for P is the average of six experiments and all metastatic samples were analyzed in triplicate. Data are presented as fold expression compared with the parental tumors.

Figure 2-5

A.



B.

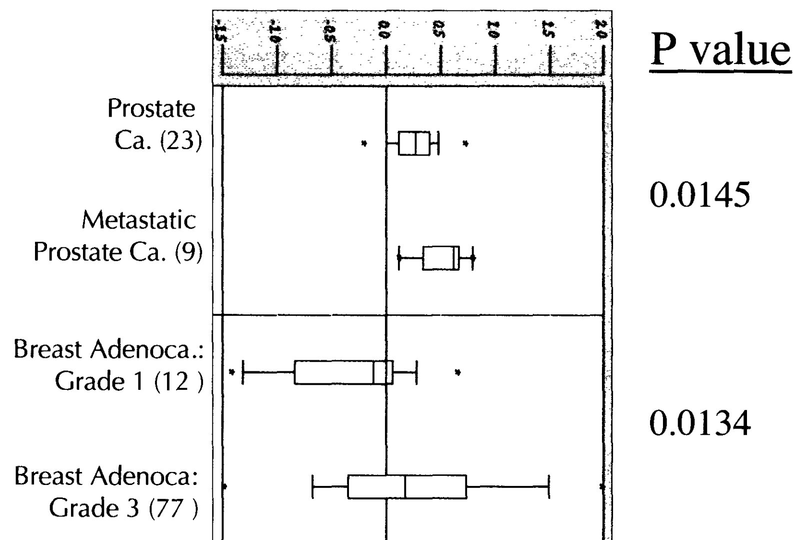


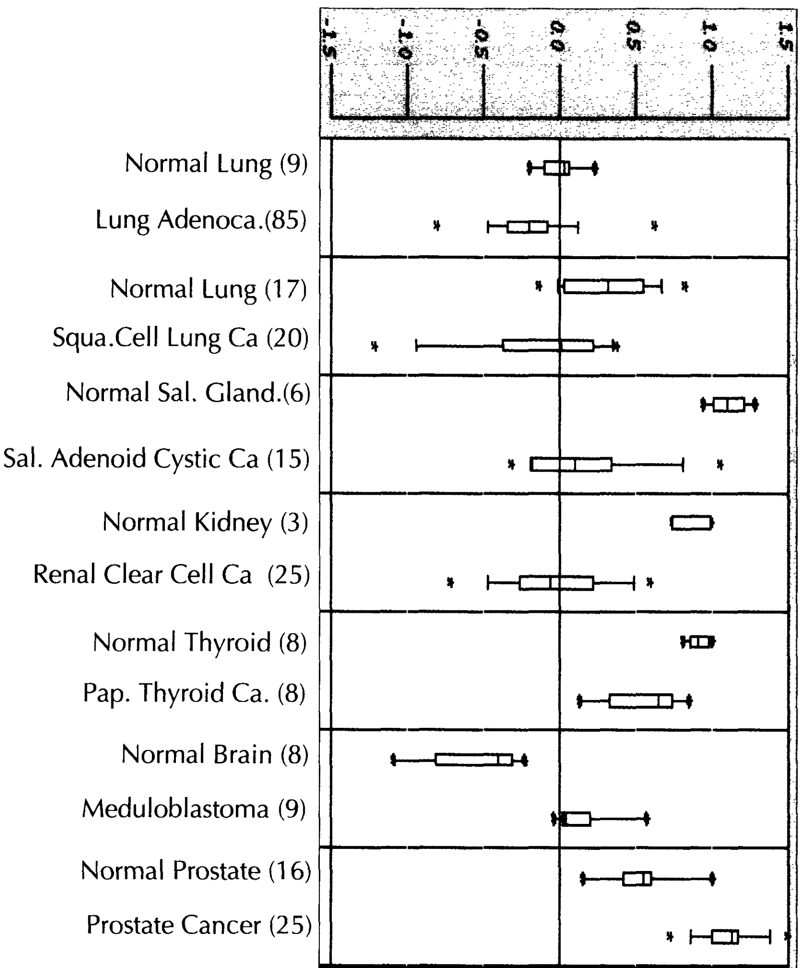
Figure 2-5: IQGAP1 expression profile analysis in human cancer as revealed by ONCOMINE.

A. IQGAP1 is significantly upregulated in several human cancers relative to normal tissue, including non-small cell lung adenocarcinoma ($P = 0.0031$ and $P = 0.001$), small cell lung carcinoma ($P = 0.0072$), prostate cancer ($P = 0.0001$ and 0.0003) and colon carcinoma ($P = 0.0087$).

B. IQGAP1 is significantly upregulated in advanced breast adenocarcinoma (grade 3 versus grade 1, $P = 0.0134$) and in metastatic prostate cancer compared to nonmetastatic prostate cancer ($P = 0.0145$). Only samples with statistically significant expression differences are shown. X-axis units are normalized expression values (standard deviations above or below the median per array.) The number of samples in each class is given in parentheses. "Adenoca." indicates adenocarcinoma. "Ca." indicates cancer.

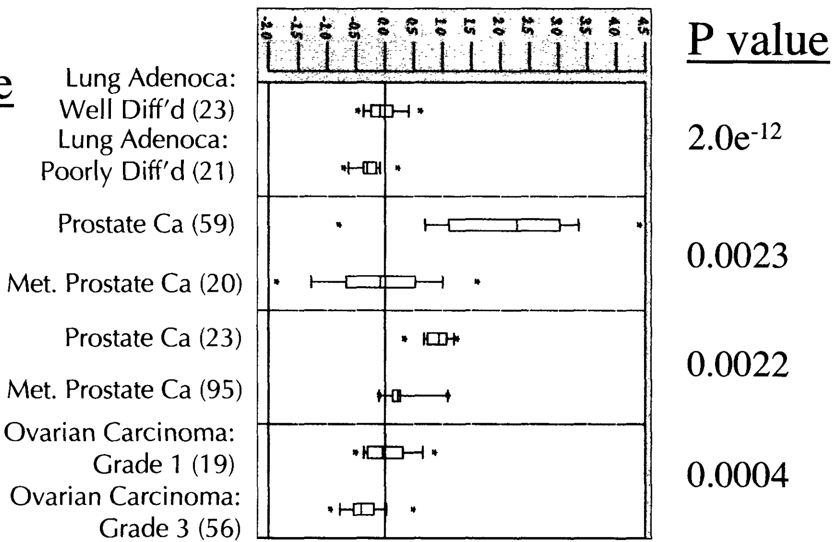
Figure 2-6

A.



P value
0.0008
0.0007
$7.0e^{-8}$
0.0002
0.0045
$2.3e^{-5}$
0.0001

B.

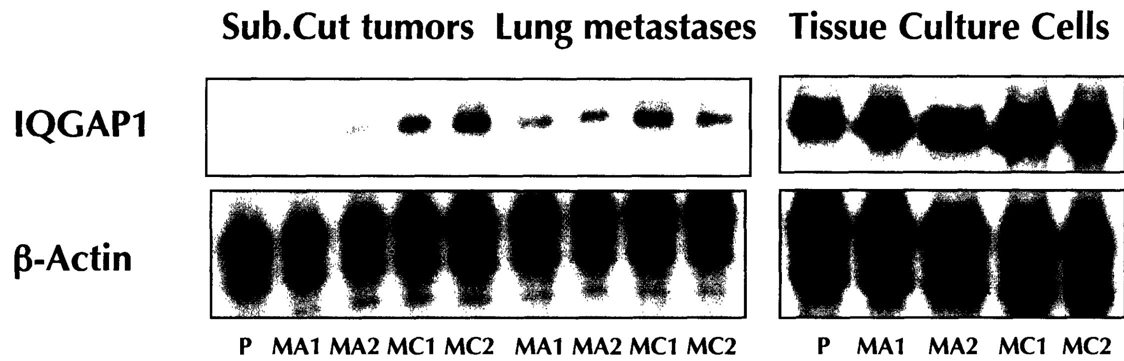


P value
$2.0e^{-12}$
0.0023
0.0022
0.0004

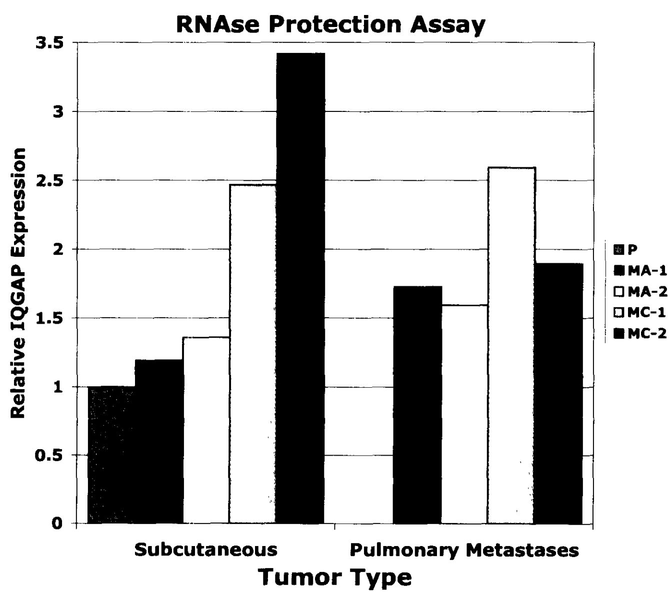
Figure 2-6: IQGAP2 expression profile analysis in human cancer as revealed by ONCOMINE.
A. IQGAP2 is significantly upregulated in prostate cancer ($P = 2.3e^{-5}$) and in medulloblastoma (P =0.0001) relative to normal tissue. IQGAP2 is also significantly downregulated in a number of other tumors, including lung (P=0.0008, P=0.0007), salivary gland ($P=7.0e^{-8}$), kidney (P=0.0002) and thyroid (P=0.0045).
B. IQGAP2 is significantly reduced in progressive and metastatic disease, including metastatic prostate cancer versus nonmetastatic ($P=2.0e^{-12}$, P=0.0023), poorly differentiated lung adenocarcinoma versus well differentiated (P=0.0022), and grade 3 ovarian carcinoma compared to grade 1 (P=0.0004). Only samples with statistically significant expression differences are shown. X-axis units are normalized expression values (standard deviations above or below the median per array.) The number of samples in each class is given in parentheses. "Adenoca." indicates adenocarcinoma. "Ca." indicates cancer.

Figure 2-7

A.



B.



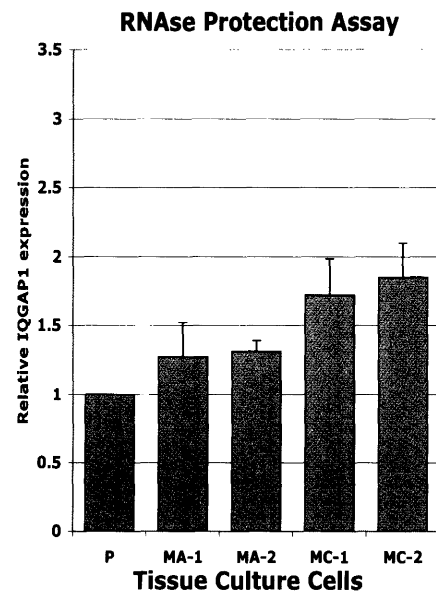


Figure 2-7: RNAse Protection analysis of IQGAP1 expression in metastatic melanomas.

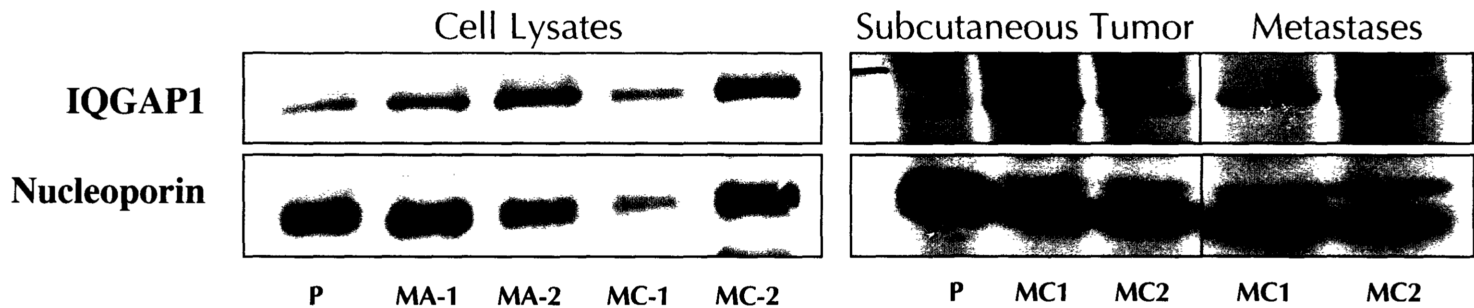
A. RNAse protection analysis of A375P cells grown either as a subcutaneous tumor or in tissue culture and from metastatic variants (MA-1, MA-2, MC-1, MC-2) grown as subcutaneous tumors, pulmonary metastases or in tissue culture. Protected fragments for IQGAP1 and β -actin probes are shown.

B. Densitometry was used to quantify the single IQGAP1 protected fragment and the multiple β-actin protected fragments from the *in vivo* samples. IQGAP1 expression data were standardized using the β -actin probe and are presented as fold expression compared with the parental tumor.

C. Densitometry was used to quantify the single IQGAP1 protected fragment and the multiple β-actin protected fragments from the *in vitro* samples. IQGAP1 expression data were standardized using the β -actin probe and are presented as fold expression compared with the parental cells.

Figure 2-8

A.



B.

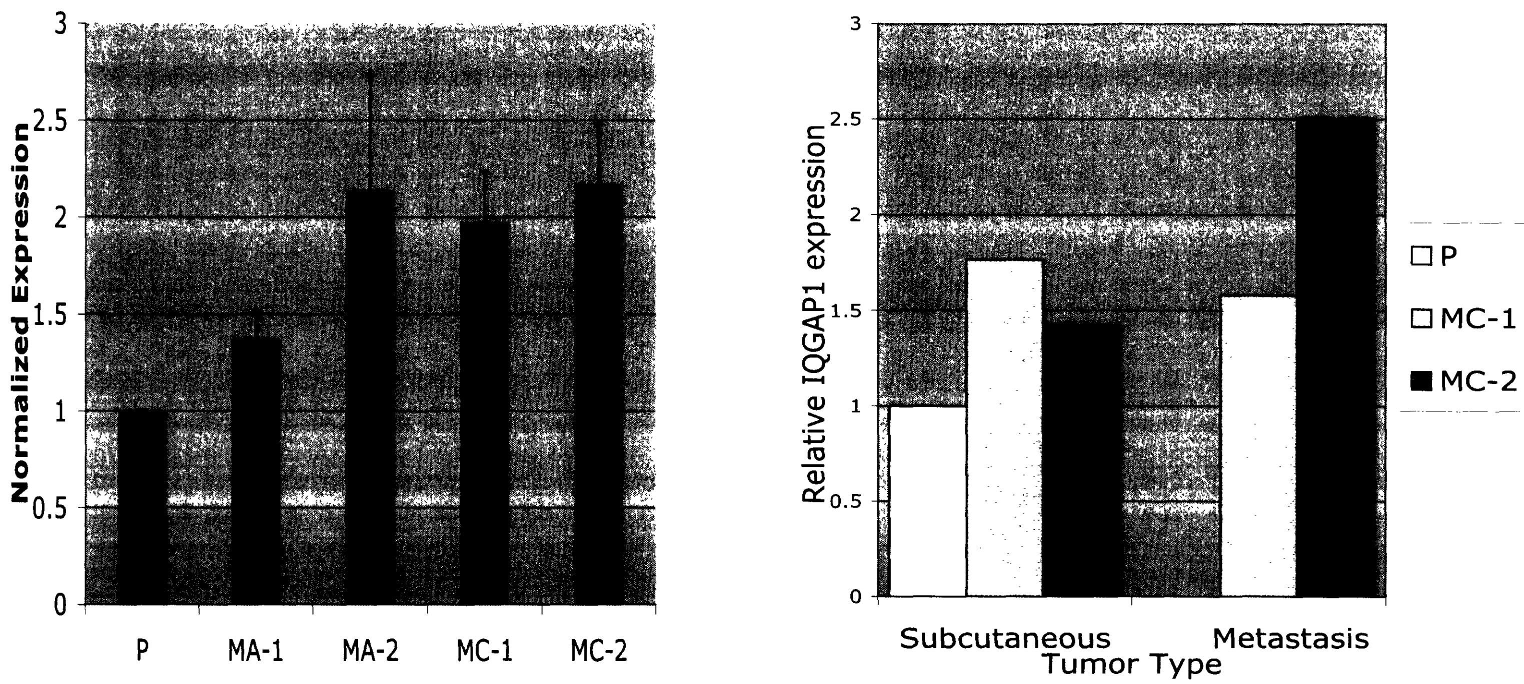
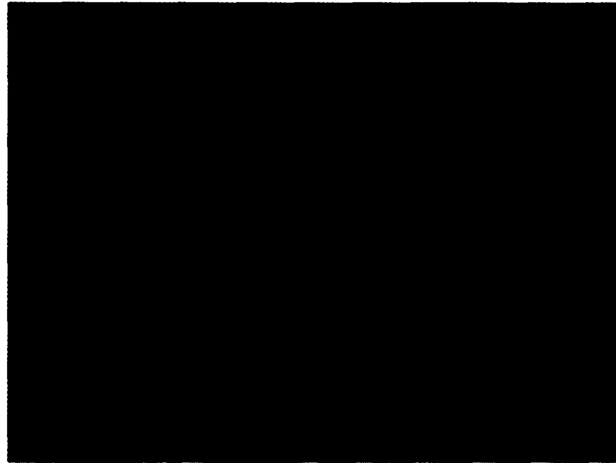


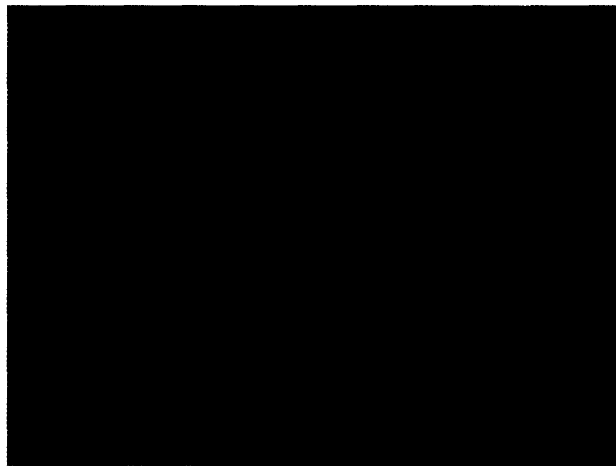
Figure 2-8: Western blot analysis of IQGAP1 expression in metastatic melanomas.
A. A375P cells were grown either as a subcutaneous tumor or in tissue culture and metastatic variants (MA-1, MA-2, MC-1, MC-2) were grown as subcutaneous tumors, pulmonary metastases or in tissue culture. Cell lysates of tumor samples (in RIPA) and tissue culture cells (in SDS) were prepared. Equal amounts of protein were loaded and electrophoresed on 7.5% SDS-PAGE gels and transferred to nitrocellulose. The blot was cut into strips and separately incubated with monoclonal antibodies against IQGAP1 and nucleoporin (a loading control).
B. Densitometry was used to quantify the IQGAP1 and nucleoporin bands. IQGAP1 expression data was standardized to nucleoporin and is presented as fold expression compared with the parental sample.

Figure 2-9

P



MA-2



MC-2

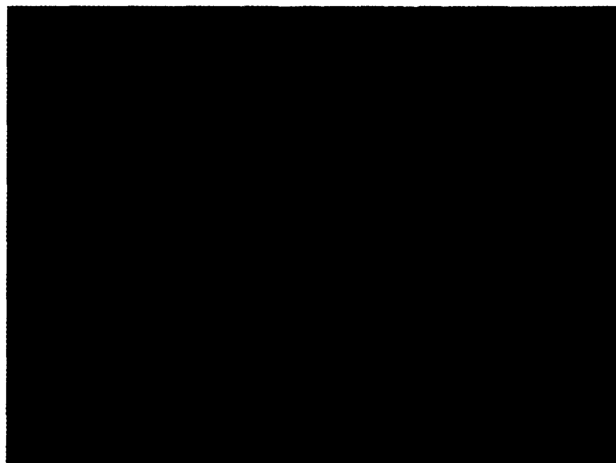
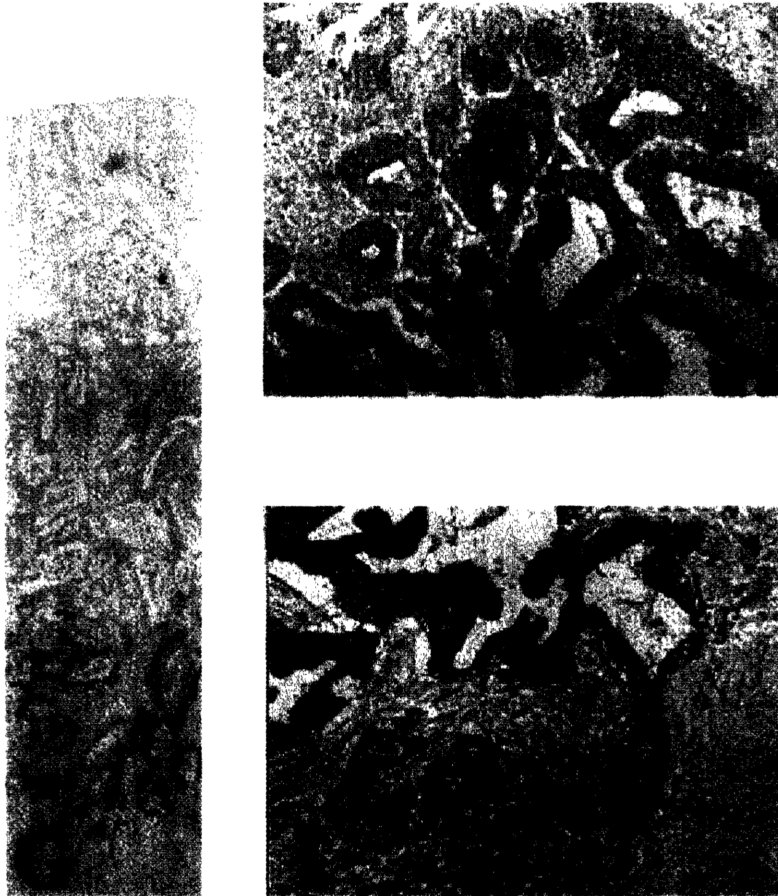


Figure 2-9: Immunofluorescence
Poorly (P) and highly metastatic (MA-2, MC-2) A375 melanoma cells were plated on glass coverslips for 16h at 37°C, then fixed, permeablized and stained for IQGAP1.

Figure 2-10



Nabeshima *et al.* Cancer Letters (2002) 176: 101-109

Figure 2-10: IQGAP1 expression in human colon carcinoma.
Reproduced from (Nabeshima *et al*, 2002), Figure 1.
G. A low-power view (x5) showing intense IQGAP1 staining at the deeper portion of the carcinoma that includes the invasion front.
H. High power view (x66) of upper portion in G.
I. High power view (x66) of invasion front in G.

Chapter 3

Functional Characterization of IQGAP1 in Metastasis

Introduction

The acquisition of a motile and invasive phenotype is an important step in the progression of metastasis. The process of invasion involves a number of discrete steps, including alterations in cell-cell and cell-substrate adhesion, remodeling of the extracellular matrix, organization of the actin cytoskeleton and an increase in cell motility. Cell movement across a two-dimensional substrate can be observed as a continuous, dynamic interplay of attachment at the cell front and de-adhesion at the cell rear, combined with a traction machinery that pulls the net cell body forward (Schmitz et al. 2000). Given that adhesion and detachment of a cell occur at opposite cell edges, the moving cell must both acquire and maintain spatial and functional asymmetry, a process called polarization. The asymmetry develops between two opposite cell edges; one becomes the leading edge, exhibiting lamellipodial or filopodial protrusions and the other becoming the rear edge, undergoing retraction (Manes et al. 2000).

The Rho GTPases have been implicated in a wide range of cellular processes, including cytoskeletal organization, cell adhesion to the substratum, cell polarity and transcriptional activation (Etienne-Manneville and Hall 2002). They are best known for their distinct effects on the actin cytoskeleton (Hall 1998). In classical studies with Swiss 3T3 fibroblasts, activation of Cdc42 led to the formation of filopodia, activation of Rac resulted in the formation of lamellipodia and membrane ruffling, and Rho activation caused the formation of stress fibers (Ridley and Hall 1992; Ridley et al. 1992).

Rho-family GTPases cycle between GDP-bound inactive and GTP-bound active conformations and have intrinsic GTPase activities. The nucleotide-bound state is determined by three classes of regulators: guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP for GTP; Rho GDP dissociation inhibitors (GDIs), which interact with GDP-bound Rho GTPases and inhibit the exchange of GDP for GTP; and GTPase activating proteins (GAPs), which enhance the intrinsic GTPase activities of Rho GTPases (Hall 1998). These regulators tightly regulate the Rho GTPases both spatially and temporally. Upon extracellular stimulation, such as by growth factors or

cell-adhesion molecules, Rho GTPases are thought to shuttle between the cytosol and specific membrane sites. In polarized cells, Rac1 and Cdc42 localize to the leading edge of cells (Etienne-Manneville and Hall 2001), whereas RhoA localizes mostly to the cytosol.

Additional experiments performed primarily in fibroblasts have shown that Cdc42, Rac and Rho cooperate to regulate the cytoskeletal changes required for the migratory behavior of cells (reviewed in (Schmitz et al. 2000; Etienne-Manneville and Hall 2002)). In brief, Cdc42 regulates the polarity of cell migration, whereas Rac regulates the formation of membrane protrusions at the leading edge of migrating cells, required for forward movement. RhoA is required for the generation of contractile force leading to rounding of the cell body. Rho family proteins also regulate microtubule polymerization involved in migration (Fukata et al. 2003).

When GTP-bound, the active Rho GTPase family members exert their specific effects on the actin cytoskeleton via a number of characteristic effector proteins. These downstream targets include protein kinases, lipid-modifying proteins and proteins involved in actin polymerization (Bishop and Hall 2000). These effectors are crucial for normal cellular responses and are targets for subversion during tumor progression. Given their importance in cell migration, invasion, and polarity, perturbation of the natural balance of these GTPases in a cell may ultimately lead to phenotypes of invasion and metastasis (Schmitz et al. 2000). Indeed, Rho family proteins, as well as their regulators, are degregulated in many tumor types and are capable of inducing a variety of cellular behaviors associated with tumor progression (Schmitz et al. 2000; Sahai and Marshall 2002a; Friedl and Wolf 2003).

In the previous chapter, I've presented evidence that enhanced expression of IQGAP1, a regulator and effector of GTP-bound Rac1 and Cdc42, positively correlates with metastasis. A further review of the IQGAP1 literature revealed that IQGAP1 functions at the intersection of many signaling pathways that could contribute to metastasis. The

mammalian IQGAP proteins contain several protein-protein interaction domains with which they bind directly to a number of interesting partners (see Figure 1). Starting from the amino-terminus, IQGAP1 binds to F-actin using a calponin homology domain (CHD) (Erickson et al. 1997; Joyal et al. 1997; Ho et al. 1999). The WW domain, a polyproline binding domain, has recently been shown to mediate ERK2 binding (Roy et al. 2004). The name IQGAP is derived from the following two domains: the IQ domain is a tandem repeat of four IQ motifs in human IQGAP1, which binds calmodulin (Hart et al. 1996; Joyal et al. 1997; Ho et al. 1999), myosin essential light chain (Weissbach et al. 1998), and S100B, a Zn^{+2}- and Ca^{+2}-binding protein (Mbele et al. 2002). The GAP-related domain (GRD) mediates the binding of IQGAP1 to active, GTP-bound Cdc42 and Rac1 GTPases (Hart et al. 1996; Kuroda et al. 1996; Joyal et al. 1997; Ho et al. 1999; Swart-Mataraza et al. 2002). The carboxyl-terminal domain (CTD) interacts with the microtubule plus-end binding protein Cytoplasmic Linker Protein-170 (CLIP-170) (Fukata et al. 2002a), and is also necessary for binding to E-cadherin (Kuroda et al. 1998; Li et al. 1999) and β-catenin (Briggs et al. 2002). By interacting with its various target proteins, IQGAP1 participates in signaling networks that regulate cell-cell adhesion and cytoskeletal rearrangements.

IQGAP1 can negatively regulate E-cadherin-mediated cell-cell adhesion. Overexpression of IQGAP1 and the translocation of endogenous IQGAP1 to cell-cell junctions, where it colocalizes with E-cadherin and β-catenin, coincides with decreased E-cadherin-mediated adhesion (Kuroda et al. 1998; Li et al. 1999). A direct interaction between IQGAP1 and β-catenin dissociates α-catenin from the adhesion complex, thus unlinking the adhesion complex from the actin cytoskeleton, resulting in a disruption of cell-cell adhesion (Kuroda et al. 1998).

IQGAP1 can also regulate the actin cytoskeleton both directly and indirectly. Through its CHD, IQGAP1 directly binds F-actin and has been shown to enhance actin cross-linking *in vitro* (Bashour et al. 1997; Erickson et al. 1997; Fukata et al. 1997). Both endogenous and ectopically expressed IQGAP1 colocalize with actin in lamellipodia and ruffling cell

membranes (Hart et al. 1996). IQGAP1 can also induce actin cross-linking downstream of activated Rac1 and Cdc42. Despite the family name, no GAP function has been demonstrated for IQGAP1. Instead, it inhibits the intrinsic GTPase activity of Cdc42 and has been shown to stabilize active Cdc42 *in vitro* (Brill et al. 1996; Hart et al. 1996; Ho et al. 1999). Experimentally overexpressing IQGAP1 in cells increases active Cdc42 and stimulates formation of filopodia (Swart-Mataraza et al. 2002). In addition, IQGAP1 has been shown recently to regulate the microtubule cytoskeleton. By binding CLIP-170, IQGAP1 captures microtubules at the leading edge of migrating fibroblasts, resulting in cell polarization (Fukata et al. 2002b).

Results from several studies suggest that IQGAP1 functions as a scaffolding protein that modulates crosstalk among diverse pathways. In fact, IQGAP1 can bind several proteins simultaneously. For example, the interactions with actin and CLIP-170 described above occur downstream of active Rac or Cdc42 signaling. A ternary complex of Cdc42, IQGAP1 and actin was immunoprecipitated using antibodies to Cdc42 and a similar complex of IQGAP1, Cdc42 or Rac, and CLIP-170 has been identified (Erickson et al. 1997; Fukata et al. 2002b). In addition, individual members of the multiprotein complexes are capable of altering the ability of IQGAP1 to bind to other proteins. Cdc42 has been reported to augment the F-actin cross-linking mediated by IQGAP1 *in vitro* and to enhance the association of IQGAP1 with CLIP-170 (Fukata et al. 1997; Fukata et al. 2002b). Conversely, activated Rac and Cdc42 counteract the negative effect of IQGAP1 on E-cadherin-mediated cell-cell adhesion by preventing it from interacting with β -catenin (Kuroda et al. 1998). The master IQGAP1 regulator, however, appears to be Ca^{+2}/calmodulin. Through its interaction with IQGAP1, Ca^{+2}/calmodulin prevented IQGAP1 from stabilizing active Cdc42 and from promoting actin cross-linking *in vitro* and modulated its effect on E-cadherin-mediated cell-cell adhesion in intact cells (Ho et al. 1999; Li et al. 1999; Abdel-Wanis and Kawahara 2001; Briggs et al. 2002; Mateer et al. 2002). The data suggest that IQGAP1 can act as a scaffolding protein that forms multiprotein complexes that are regulated by several target molecules. Therefore, IQGAP1 integrates several distinct signaling pathways, including cytoskeletal

architecture, Cdc42 and Rac signaling, Ca^{+2}/Calmodulin signaling and E-cadherin-mediated cell-cell adhesion.

To assess experimentally the role of IQGAP1 in metastasis, a dominant-negative approach was used to disrupt IQGAP1 activity in the highly metastatic A375 MC-1 and MC-2 melanoma cells. Two IQGAP1 dominant-negative mutants, ΔGRD and CT, were chosen for further analysis. The ΔGRD mutant, which lacks part of the GAP-related domain, has been shown to decrease substantially the amount of GTP-bound Cdc42 in cell lysates (Swart-Mataraza et al. 2002). Endogenous IQGAP1 inhibits the intrinsic GTPase activity of Cdc42 and Rac1, and stabilizes the GTPases in their active GTP-bound forms (Hart et al. 1996; Zhang et al. 1997a; Ho et al. 1999). In marked contrast, the ΔGRD mutant actually behaves like a "GAP" for Cdc42 by increasing its intrinsic GTPase activity, resulting in an increase in the amount of inactive, GDP-bound Cdc42 in cells (Swart-Mataraza et al. 2002). When HEK-293 cells were transiently transfected with full length IQGAP1, the pool of active Cdc42 increased. Co-transfection of equal amounts of full length IQGAP1 and ΔGRD abrogated the increase in active Cdc42 produced by wild-type IQGAP1. ΔGRD also prevented bradykinin-mediated activation of Cdc42, translocation of Cdc42 to the membrane fraction, and formation of filopodia. It induced severe morphological changes in MCF-7 cells and significantly reduced the motility of MDA-MB-231 cells in Transwell assays (Mataraza et al. 2003). Therefore, the ΔGRD mutant appears to disrupt Cdc42 activity that is normally promoted by endogenous IQGAP1. Importantly, this mutant had no effect on endogenous Rac1 activity.

The IQGAP1 carboxy-terminal fragment (CT) that contains amino acids 1503-1657 of full-length IQGAP1, is another putative dominant-negative mutant. The CT fragment contains the region that is necessary for direct binding of full-length IQGAP1 to E-cadherin, β-catenin and CLIP-170 (Kuroda et al. 1998; Fukata et al. 2002b). In addition, this mutant specifically and directly interacts with full-length IQGAP1 *in vitro* (Fukata et al. 2001). *In vivo*, the CT mutant disrupts a number of endogenous IQGAP1 functions.

For example, when MDCKII cells are treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) or hepatocyte growth factor/ scatter factor (HGF/SF), the cells dissociate from one another and ultimately scatter. Within ninety minutes of TPA treatment, IQGAP1-β-catenin complexes increase with a concomitant loss of α-catenin from cell-cell junctions (Fukata et al. 2001). When the CT mutant was expressed in MDCKII cells, however, it delocalized endogenous IQGAP1 from sites of cell-cell contact and inhibited the TPA-induced disappearance of endogenous α-catenin from sites of cell-cell contact (Fukata et al. 2001). Therefore, this mutant can disrupt IQGAP1 regulation of E-cadherin-mediated cell-cell adhesion.

The CT mutant can also disrupt IQGAP1 regulation of cell polarity. Downstream of active Rac1 and Cdc42 signaling, IQGAP1 binds directly to CLIP-170, resulting in the targeting of microtubules to the polarized leading edge. The carboxyl terminal domain of endogenous IQGAP1 is necessary for its interaction with CLIP-170. The CT mutant inhibited the interaction of full-length IQGAP1 with CLIP-170 *in vitro* by directly binding to CLIP-170 and forming a complex with microtubules (Fukata et al. 2002b). In addition, when the CT dominant-negative was microinjected into polarized fibroblasts, CLIP-170 was displaced from the tips of growing microtubules, and the microtubule array was altered. Therefore, this mutant can inhibit not only IQGAP1's effects on E-cadherin-mediated cell-cell adhesion, but also IQGAP1-regulated cytoskeletal rearrangements downstream of Rac1 and Cdc42 signaling.

In the previous chapter, we described the use of microarray technology to compare the global gene expression profiles of poorly and highly metastatic melanoma cells. A375 MC-1 and MC-2 cells were selected *in vivo* for their enhanced ability to metastasize and subsequently have been shown to express higher levels of IQGAP1 than the poorly metastatic melanoma cells from which they were derived (described in Chapter 2). These data and the available IQGAP1 literature convinced us that IQGAP1 was a candidate worthy of additional investigation. In this chapter, we describe a mutational approach to analyze the consequence of IQGAP1 disruption in metastasis. Here, we report

experimental evidence that suggests that IQGAP1 may play a functional role in metastasis of A375 melanoma cells. Furthermore, we show that IQGAP1 may serve to enhance the migratory and invasive potential of these cells. Therefore, IQGAP1 resides at the nexus of multiple signaling pathways that regulate cellular behaviors that can influence the progression of metastasis, including cytoskeletal regulation, motility, invasion and adhesion.

Materials and Methods

Construction of the pMICG retroviral vector

Several steps were required to construct the bicistronic retroviral vector MSCV-IRES-CD8GFP. This vector contains a fusion of the extracellular domain of murine CD8 and GFP downstream of an internal ribosomal entry site (IRES). First, the pBS-mCD8GFP vector (a kind gift from Liqun Luo, Stanford, CA) (Lee and Luo 1999) served as a template for PCR to generate a 1.4kb fragment containing the CD8GFP fusion with a 5' Sal1 restriction site and 3' **EcoR1** and Sal1 restriction sites. Forward Primer: 5'-ACGCGTCGACCTCGAGCAAAATGGCCTCACCGTTGACC and Reverse Primer: 5'-GCTATGGCCGACGTCGAC**GAATTC**TCTAGATTATTTGTATAGTTCATCC. This PCR fragment was subcloned into the pCR-BluntII-TOPO vector (described in Chapter 2.) The QuikChange Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA) was used to remove an internal EcoR1 restriction site in mCD8GFP. Essentially, two complementary oligonucleotides cgtgggacgagaagct**C**aattcgtcgaaactgttttctgcc and ggcagaaaacagtttcgacgaatt**G**agcttctcgtcccacg that contain the desired nucleotide mutation flanked by unmodified sequence are used as PCR "primers" to exchange the sequence with a degenerate codon. A PCR-BluntII-mCD8GFP Sal1 fragment that dropped out the mCD8-GFP fragment was subcloned into the Sal1 site downstream of the IRES in the pIRES vector (BD Biosciences Clontech, Palo Alto, CA). A pIRES-mCD8GFP EcoR1 fragment dropped out the IRES-mCD8GFP, which was then subcloned into the EcoR1-digested backbone of the pMiT vector (a kind gift from Phillippa Marrack, University of Colorado, CO) (Mitchell et al. 2001). The resulting bicistronic retroviral vector expressed the mCD8-GFP fusion just downstream of the IRES sequence. The pMICG vector also contained a Not1 restriction site 5' to the IRES that was used for subcloning of IQGAP1 constructs (see below). The sequences of all subcloned fragments were determined by diagnostic restriction digest analysis and DNA sequencing.

Construction of retroviral vectors expressing IQGAP1 constructs.

A two-step process was used to subclone IQGAP1 dominant-negative constructs IQGAP1ΔGRD (deletion of amino acids 1122-1324) (Sokol et al. 2001) and IQGAP1-CT (amino acids 1503-1657) (Fukata et al. 2001) into the pMICG or pMX-IRES-GFP (pMIG) (a kind gift from Harvey Lodish, MIT, MA) (Liu et al. 1997) retroviral vectors. First, PCR fragments containing the dominant-negative constructs and flanked with Not1 restriction sites were generated as follows: for IQGAP1ΔGRD, human IQGAPΔGRD in pcDNA3 vector (a kind gift from David Sacks, Brigham & Women's Hospital, MA) was used as a template and a 5029-bp fragment was synthesized using the primers,
Forward: 5'- AAGGAAAAAGCGGCCGCATGGAGCAGAAGC
Reverse: 5'- TTTTCCTTTTGCGGCCGCAATGCTTCCTTTGAACATGACTGTCAC.
Similarly human IQGAP1-CT in pEYFP-Mem (also a kind gift from David Sacks) was used as a template for synthesizing a 513-bp fragment with the primers
Forward: 5'- AAGGAAAAAGCGGCCGCCCGAACTAGTGAAACTGCAACAGAC
Reverse: 5'-TTTTCCTTTTGCGGCCGCCTTCATCCTTCTGGGCTGGCAGCAAACG.
An N-terminal myc tag was added to the IQGAP1-CT fragment by using the different forward primer: 5'-AAGGAAAAAGCGGCCGCCCACCATGGAGCAGAAGCTGA TCTCCGAGGAGGACCTGCTAGTGAAACTGCAACAGAC. Not1 restriction sites are underlined. The Not1-flanked PCR fragments were subcloned into the pCR-BluntII-TOPO vector (described in Chapter 2). pCR-BluntII- IQGAPΔGRD and pCR-BluntII-IQGAP1-CT vectors were digested with Not1 restriction enzyme and ligated into Not1-digested pMIG with Quick T4 DNA ligase (New England Biolabs, Beverly, MA). Similarly, pCR-BluntII-mycIQGAP-CT was digested with Not1 and ligated into the Not1 site of pMICG. Sequences of all inserts were determined by diagnostic restriction digest analysis and DNA sequencing.

Retroviral Infection of A375 Melanoma Cells

Phoenix cells (Kinsella and Nolan 1996) were grown in DMEM, supplemented with 10% fetal calf serum (FCS) and 2mM glutamine. Cells were cultured 1:10 every three days. To infect the *in vivo*-selected A375 metastatic melanoma cells with the pMIG, pMIG-

ΔGRD, pMIG-CT, pMICG and pMICG-mycCT vectors, viral particles were first packaged by transfecting the plasmids into the Phoenix retroviral producer cell line. On the night prior to transfection, $5x10^5$ Phoenix cells were plated in 6 cm dishes with 3 ml media. The next day, 25μM chloroquine was added to the cells five minutes prior to transfection. A 500μl DNA precipitate containing 15μg of plasmid diluted in H_2O and 50μl 2.5M $CaCl_2$ was vigorously bubbled into an equal volume of Hepes buffered saline solution (pH 7.05), and the solution was immediately added dropwise to the cells. The cells were incubated at 37°C for 24 hours. On day two, fresh medium was added to the Phoenix cells, and the dishes were then incubated at 33°C for 24 hours. Two days after transfection, the Phoenix cell medium (viral supernatant) was removed, sterilized through a 0.45μ filter and used to infect A375 metastatic melanoma cells that were plated ($6x10^5$ cells in a 6cm dish) one day earlier. For the infection, 1ml medium was removed and 5μg/μl polybrene added to each A375 plate. Then, 1ml of viral supernatant was added to each A375 dish, and the cells were placed on a rocking platform at 33°C. Twenty-four hours post infection, fresh medium was added to the A375 cells which were then incubated at 37°C. The A375 cells infected with pMIG vectors were expanded in cell culture for five days before performing Fluorescently Activated Cell Sorting (FACS) for GFP-positive cells. The sorted A375 cells were expanded in cell culture for two weeks, and the GFP-positive cells were then sorted into high (top 20%) and low (remaining 80%) GFP-expressing populations.

Alternatively, the A375 cells infected with the pMICG vectors were expanded in cell culture for five days before sorting the cells with anti-CD8 (murine) magnetic beads (Dynal Biotech Inc., Lake Success, NY). The desired amount of magnetic beads was washed three times in PBS +2% FBS with the aid of a Dynal Magnetic Particle Concentrator (MPC), and added to a confluent dish of pMICG-infected A375 cells at a concentration of 10^7 beads in 25μl. The cells were incubated at 4°C for 20 minutes with vigorous shaking every five minutes. Cells were then washed three times with PBS to remove excess beads, and trypsinized. CD8-positive cells were separated using the MPC and carefully washed one time with PBS. The magnetically sorted cells were expanded

in cell culture for one-two weeks before undergoing a second round of CD8 magnetic sorting.

In Vitro Migration and Invasion Assays

A375 human melanoma cells maintained in DMEM +10% FBS were plated in 6cm dishes ($4x10^5$ cells/dish) on Day 0, and incubated at 37°C. On Day 2, the medium was replaced with DMEM +1% FBS reduced-serum medium (RSM), and cells were incubated at 37°C for another 24 hours. The cells were trypsinized the next day; RSM containing 500μg/ml soybean trypsin inhibitor was used to stop the reaction. Cells were then washed in RSM two times, counted with a Coulter counter and resuspended at a concentration of 200,000 cells/ml in RSM. For migration assays, 250μl of cells were added to the upper chamber of an eight-micron Transwell (Corning Inc., Corning, NY). 750μl of DMEM+10% FCS was added to the bottom chamber, and cells were allowed to migrate for 21-24 hours at 37°C. Invasion assays were set up in the same way except that Matrigel invasion chambers (BD Biosciences Discovery Labware, Bedford, MA) were used and cells were allowed to invade at 37°C for 24 hours. Swabbing the top of the membrane to remove nonmigrating cells ended the assay. Transwells were then fixed in 4% PFA for ten minutes, washed in PBS and stained with 0.1% crystal violet. Migrating cells were quantified by counting five 10X fields per well. All assays were performed in triplicate.

Results

Stable Expression of IQGAP1 Dominant-Negative Mutants in A375 Metastatic Melanoma Cells by Retroviral Gene Transfer

IQGAP1 dominant-negative mutants were used to elucidate the role of IQGAP1 in metastasis (Figure 3-1). The ΔGRD mutant lacks amino acids 1122-1324 and disrupts endogenous IQGAP1 regulation of and by Cdc42 (Swart-Mataraza et al. 2002). The CT mutant contains amino acids 1503-1657 and inhibits endogenous IQGAP1 regulation of E-cadherin-mediated cell-cell adhesion and CLIP-170-mediated capture of microtubules (Fukata et al. 2001; Fukata et al. 2002b). Retroviral gene transfer studies were used to express the IQGAP1 dominant-negative mutants in the highly metastatic A375 MC-1 or MC-2 melanoma cells that express enhanced levels of IQGAP1 (described in Chapter 2). The CT and ΔGRD mutants were individually subcloned into the bicistronic retroviral vector pMX-IRES-GFP (pMIG), and then introduced into a retroviral packaging cell line. Retroviral particles were then used to infect MC-1 or MC-2 cells (Figure 3-2). MC-1 or MC-2 cells stably expressing high levels of the CT or ΔGRD mutants (designated MC1-CT, MC1-ΔGRD, MC2-ΔGRD) were selected using fluorescence-activated cell sorting (FACS) for GFP expression. After expansion in culture, the cells were subjected to FACS for a second time to isolate high- and low-GFP expressing populations (designated "hi" or "lo").

Endogenous IQGAP1 Expression in Metastatic Cells Expressing IQGAP1 Dominant-Negative Mutants

Because anti-IQGAP1 antibodies did not recognize the dominant-negative constructs cloned into pMIG, I assessed the success of retroviral gene transfer by Western blot analysis with an anti-GFP monoclonal antibody (Figure 3-3A). The results suggest that highly metastatic melanoma cells that stably and differentially express ΔGRD - or CT-expressing retrovirus were successfully generated. In addition, the retroviral transfer of dominant-negative IQGAP1 mutants did not alter the expression of full-length IQGAP1

in the highly metastatic melanoma cells (Figure 3-3B). These results show that neither the CT nor ΔGRD mutant interferes with endogenous IQGAP1 expression.

IQGAP1 Mutants Inhibit Metastasis of A375 Metastatic Melanoma Cells

The functional consequences of the expression of IQGAP1 mutants on metastasis were assessed using the *in vivo* experimental metastasis assay. A375 MC-1 and MC-2 cells stably expressing pMIG-CT or pMIG-ΔGRD retrovirus were introduced into nude mice by tail vein injection, and pulmonary metastases were quantified five to eight weeks later. MC-1 cells expressing the IQGAP1 CT mutant generated significantly fewer pulmonary metastases than MC-1 cells expressing control pMIG alone (p=0.0063) (Figure 3-4). Expression of the ΔGRD mutant slightly suppressed the generation of pulmonary metastases in both MC-1 and MC-2 metastatic melanoma cells, but the effects were not statistically significant. It is worth noting, however, that the experimental datasets for MC-1 and MC-2 cells expressing the ΔGRD mutant are small (n=3 and n=7, respectively), and the variance among the control data points is large. Therefore, additional *in vivo* assays are currently being performed with these cells to increase the statistical power of the analysis. The results to date demonstrate that an IQGAP1 mutant, CT, can significantly suppress experimental *in vivo* metastasis. Furthermore, these results suggest a functional role for IQGAP1 in metastatic progression, however, additional analyses are needed to demonstrate this experimentally (which I will discuss in detail below).

Endogenous IQGAP1 Expression in Pulmonary Metastases of A375 Melanoma Cells

I compared IQGAP1 expression in lung metastases from highly metastatic A375 MC-1 and MC-2 cells with that of A375 MC-1 and MC-2 cells stably expressing IQGAP1 mutants, and found that expression of endogenous IQGAP1 was similar in all samples (Figure 3-5). This result shows that endogenous IQGAP1 expression is not perturbed in

the rare cells within the heterogenous MC1-CT and MC2-ΔGRD populations that are capable of generating pulmonary metastases.

Stable Expression of myc-tagged CT Dominant-Negative Mutant in A375 Metastatic Melanoma Cells by Retroviral Gene Transfer

To expedite the investigation into the functional consequences of expression of IQGAP1 mutants on metastasis, a new retroviral vector was constructed. This bicistronic vector, pMX-IRES-CD8-GFP (pMICG), contains a fusion of the extracellular domain of murine CD8 and GFP, downstream of an internal ribosomal entry site (IRES). Because cells infected with pMICG express the extracellular domain of CD8, they can be magnetically sorted with anti-mCD8-coated magnetic beads. This quicker approach enhanced the throughput of our analysis and was a better method than sorting pMIG-infected cells by FACS. In addition, because the pMICG vector also expresses GFP, FACS could be used to sort pMICG-infected cells into high and low GFP-expressing populations, as described above for pMIG-infected cells.

As I mentioned above, the CT mutant was not recognized by available anti-IQGAP1 antibodies, which limited the analysis of its cellular behavior. Therefore, I added a myc tag to the amino-terminus of the CT construct and subcloned the tagged mutant into the pMICG retroviral vector. Tagging the CT mutant facilitated the direct analysis of its expression, molecular interactions and cellular localization in metastatic melanoma cells. The pMICG-mycCT construct was introduced into a retroviral packaging cell line and retroviral particles were used to infect the highly metastatic MC-1 or MC-2 cells. MC-1 or MC-2 cells stably expressing the myc-tagged CT mutant (designated MC1-mycCT or MC2-mycCT) were isolated by magnetic sorting with beads coated with anti-mCD8 antibody (Figure 3-6). After expansion in culture, the cells were magnetically sorted with anti-mCD8-coated beads for a second time to enrich the population of mycCT-expressing cells (designated "sort1" or "sort2").

A375 MC-1 and MC-2 cells stably expressing pMICG or pMICG-mycCT retrovirus were then introduced into nude mice by tail vein injection to again assess their functional consequences on metastasis. Five to eight weeks after mice were injected intravenously with retrovirus-infected cells, host mice were sacrificed and pulmonary metastases were quantified. MC-1 cells expressing the pMICG vector alone generated significantly fewer pulmonary metastases than MC-1 cells (P=0.042) (Figure 3-7). In addition, MC-2 cells expressing the pMICG vector alone also generated significantly fewer pulmonary metastases than MC-2 cells (P=0.025). Although several mice were injected with MC1-mycCT or MC2-mycCT cells, the results from the pMICG vector alone control experiments unfortunately precluded any future *in vivo* analyses of cells infected with this retrovirus. Therefore, although I attempted to address directly how the CT mutant imposes its negative effects on metastasis, my efforts were stopped short by the unexpected *in vivo* behavior of the pMICG retroviral vector.

Overexpression of IQGAP1 in A375 Poorly Metastatic Melanoma Cells by Retroviral Gene Transfer

I also addressed whether enhanced expression of IQGAP1 is sufficient for metastasis. Retroviral gene transfer was used to overexpress full-length IQGAP1 in the poorly metastatic A375 parental (P) cells and the *in vivo* experimental metastasis assay was used to analyze the metastatic capacity of these cells. Initial attempts to express full-length IQGAP1 from the pMIG retroviral vector were unsuccessful. However, a second approach was taken to express amino-terminal myc-tagged full-length IQGAP1 from the pMICG retroviral vector. The pMICG-mycIQGAP1 construct was introduced into a retroviral packaging cell line and retroviral particles were used to infect the poorly metastatic A375P cells. A375P cells stably expressing pMICG or pMICG-mycIQGAP were isolated by magnetic sorting with beads coated with anti-mCD8 antibody. After expansion in tissue culture, the cells were again magnetically sorted with anti-mCD8-coated beads to enrich the population of mycIQGAP1-expressing A375P cells (designated "P + mycIQ1"). Western blot analysis revealed that myc-tagged full-length

IQGAP1 was expressed in these cells, leading to an increase in total IQGAP1 expression (Figure 3-8).

The effect of enhanced IQGAP1 expression in A375P poorly metastatic melanoma cells on cellular proliferation was analyzed both *in vitro* and *in vivo.* Cell counting assays were performed to compare the *in vitro* growth rates of A375P cells stably expressing pMICG or pMICG-mycIQGAP1. All cells exhibited logarithmic growth and grew at similar rates (Figure 3-9A). In addition, *in vivo* growth rates were determined by growing these cells as subcutaneous tumors. A375P cells expressing pMICG alone formed significantly larger tumors than A375P cells not expressing the vector (Figure 3-9B). Once again, the *in vivo* behavior of cells expressing the pMICG vector alone was altered, and precluded future *in vivo* analyses of cells expressing IQGAP1 constructs from this retroviral vector. Therefore, the effect of enhanced IQGAP1 expression on the metastatic capacity of the A375P poorly metastatic melanoma cells could not be properly assessed in these assays.

IQGAP1 Mutants Disrupt neither Cellular Morphology nor Cell Proliferation

I have shown in Figure 3-4 that the IQGAP1 CT mutant severely inhibits *in vivo* metastasis of A375 metastatic melanoma cells. I next used *in vitro* assays to dissect further the functional consequences of IQGAP1 mutants. Several of these *in vitro* assays are used to model specific cellular behaviors that are required for individual steps in the progression to metastasis, including survival, proliferation, migration and invasion. First, the gross cellular morphology of A375 melanoma cells expressing the IQGAP1 mutants was analyzed. When cultured *in vitro* on tissue culture-treated dishes, all cells were slightly elongated, and formed phase-bright cell-cell junctions as they reached confluency (Figure 3-10). Polarized cells that extrude membrane ruffles were also seen. Neither the CT nor the ΔGRD mutant appeared to have any appreciable effect on gross cellular morphology in A375 MC-1 or MC-2 cells, respectively. It has been previously shown that the ΔGRD mutant severely alters the morphology of MCF-7 cells, yielding small,

round cells (Swart-Mataraza et al. 2002). The morphology of A375 metastatic melanoma cells, on the other hand, was not affected by the expression of the ΔGRD mutant under the conditions tested. Therefore, these results show that the effect of the ΔGRD mutant on cellular morphology was not as severe in A375 melanoma cells as was observed in MCF-7 cells.

The cellular proliferation of A375 metastatic melanoma cells expressing IQGAP1 mutants was then analyzed both *in vitro* and *in vivo*. Cell counting assays were performed to compare the *in vitro* growth rates of the MC-1 or MC-2 cells stably expressing CT, myc-CT or ΔGRD mutants. All cells exhibited logarithmic growth and grew at similar rates (Figure 3-11A). In addition, the *in vivo* growth rates of these cells were determined by growing them as subcutaneous tumors. Likewise, expression of the IQGAP1 mutants (CT, myc-CT, or ΔGRD) did not significantly alter *in vivo* cell growth (Figure 3-11B). These results show that neither the CT nor ΔGRD mutant perturbs cellular proliferation.

The IQGAP1 CT Mutant Inhibits *in vitro* Migration and Invasion

In vitro transwell migration assays and Matrigel invasion assays were also performed to investigate the functional consequences of expression of the CT mutant in additional specific cellular processes necessary for metastasis. Stable expression of the CT (or myc-CT) mutant in MC-1 cells was associated with a marked (10-fold) reduction in both *in vitro* migration and invasion (Figure 3-12). These results show that expression of the IQGAP1 CT mutant disrupts cellular migration and invasion, two important processes in the progression of metastasis. The ΔGRD mutant has also been shown previously to significantly reduce motility and invasiveness of cells *in vitro* (Mataraza et al. 2003). Together, these results suggest a functional role for IQGAP1 in the promotion of cell migration and invasion.

Discussion

In Chapter 2, we showed that there exists a strong positive correlation between IQGAP1 expression levels and metastatic potential both in metastatic melanoma cells derived in our laboratory and in human clinical samples. In this chapter, I describe an experimental approach to assess the functional role of IQGAP1 in metastasis. Two previously characterized IQGAP1 dominant-negative mutants that are known to disrupt various endogenous functions of IQGAP1 were utilized for the analysis. Retroviral gene transfer was used to generate A375 MC-1 or MC-2 metastatic melanoma cells that stably expressed either the CT or ΔGRD mutant. The metastatic capacity of these cells was then assessed using an *in vivo* experimental metastasis assay. As shown in Figure 3-4, expression of the CT mutant in MC-1 cells severely inhibited the ability of these cells to generate pulmonary metastases. These *in vivo* results support the association of IQGAP1 function with metastasis.

Expression of the ΔGRD mutant in MC-1 or MC-2 cells also reduced the ability of these cells to generate pulmonary metastases, although the results to date are not statistically significant. There are several possible reasons for this lack of statistical significance that are currently being addressed. First, the experimental sample sizes for MC-1 and MC-2 cells expressing ΔGRD were small (n=7 and n=3, respectively), and more animals are currently being analyzed to generate larger experimental datasets. Secondly, the variance within the control MC-1 and MC-2 datasets for *in vivo* metastasis was large, and may possibly be interfering with the clarity of the results of the experimental metastasis analyses. The data presented here for *in vivo* metastasis of MC-2 cells, in particular, are not representative of the "highly metastatic" behavior that was originally attributed to these cells, and are not in agreement with the results of other researchers in the lab. Tumor cells are by their very nature quite unstable, and it is possible that over the course of *in vitro* passaging, the cells have "gone off" in culture. For that reason, I have gone back to early-passage cells that have not been cultured extensively *in vitro* and remain true to their original "highly metastatic" character, and will begin additional *in vivo* analyses of IQGAP1 function in metastasis using these cells.

Another possible explanation for the phenotypic differences between the two IQGAP1 mutants may have to do with their expression levels in the A375 melanoma cells. Although the IQGAP1 ΔGRD mutant was previously shown to induce severe morphological changes in MCF-7 cells (Swart-Mataraza et al. 2002), A375 MC-1 or MC-2 melanoma cells stably expressing ΔGRD appeared grossly normal. Based on these data, it is possible that the ΔGRD mutant may not be eliciting as severe an effect in the retrovirally-infected A375 melanoma cells as it did in the transiently-transfected MCF-7 cells, perhaps because of differences in levels of ΔGRD expression. Although I indirectly measured the expression of the ΔGRD and CT mutants from the pMIG bicistronic retroviral vector via Western blot analysis of GFP expression, direct measurement of the expression levels of the IQGAP1 mutants in the metastatic cells should be performed in the future. I attempted to address this issue by adding an amino-terminal myc tag to the CT mutant, which I will discuss in more below.

The two IQGAP1 mutants may also differ in their functional "severity." The ΔGRD mutant was shown to disrupt the interaction between endogenous IQGAP1 and Cdc42 and to function as a 'GAP' for Cdc42, resulting in significantly less activated Cdc42 in MCF-7 cells (Swart-Mataraza et al. 2002). Importantly, this mutant did not disrupt the amount of active Rac1 in these cells. In contrast, the CT mutant has been shown to disrupt both the Rac1- and the Cdc42-dependent interaction between full-length IQGAP1 and CLIP-170, resulting in an altered microtubule array at the leading edge of migrating cells (Fukata et al. 2002b). Therefore, the phenotypic differences between A375 cells expressing either the ΔGRD or CT mutant might be due to differences in endogenous IQGAP1-dependent signaling downstream of Rac1 and Cdc42. Therefore, we need to address the functional consequences of the IQGAP1 mutants at the molecular level. PAK binding domain (PBD) pulldown assays can be used to measure the levels of active Cdc42 and Rac1 in these cells. In addition, the status of the microfilament and microtubule cytoskeletons, which are regulated in part by IQGAP1 signaling downstream of Rac1 and Cdc42, should be analyzed by immunofluorescence. These experiments will

address whether the phenotypic differences observed for A375 melanoma cells expressing CT or ΔGRD are due, in part, to differences in Rac1 and Cdc42 signaling.

In addition to its effect on IQGAP1 signaling downstream of active Rac1 or Cdc42, the CT mutant has also been shown to disrupt negative regulation of E-cadherin-mediated cell-cell adhesion by endogenous IQGAP1. Therefore, the CT mutant may elicit additional functional consequences compared with the ΔGRD mutant. One way to address this question is to analyze the integrity of cell-cell junctions in A375 cells expressing IQGAP1 mutants by using immunofluorescence to monitor the expression of cell-cell adhesion markers such as E-cadherin, β-catenin and α-catenin. In addition to these analyses, a more thorough investigation into the cellular and molecular consequences of CT expression is needed to understand better how this mutant specifically disrupts *in vivo* metastasis, and whether modulation of endogenous IQGAP1 function is necessary for this inhibition.

Although the CT mutant has previously been shown to be dominant negative for various IQGAP1 functions, we do not yet have a clear understanding of how this mutant is inhibiting metastasis in A375 melanoma cells. The CT mutant can directly bind to full-length IQGAP1 or CLIP-170 *in vitro* (Fukata et al. 2001; Fukata et al. 2002b), and has been shown to delocalize endogenous IQGAP1 from sites of cell-cell contact in MDCKII cells (Fukata et al. 2001). It has also been shown to disrupt the interaction between full-length IQGAP1 and CLIP-170, resulting in an altered microtubule array at the leading edge of migrating cells (Fukata et al. 2002b). These data suggest that CT might cause multiple functional effects by titrating full-length IQGAP1 away from its cognate binding partners, or by itself interacting with IQGAP1 target proteins. However, it is also possible that CT may elicit its effects by interacting with additional intracellular proteins, such as the closely related IQGAP2 protein. Therefore, we must continue our investigation by using immunofluorescence, immunoprecipitation, and Western blot analysis to assess the expression, cellular localization and molecular interactions of both endogenous IQGAP1 and the IQGAP1 mutants. I have shown by Western blot analysis

that expression of endogenous IQGAP1 is not significantly altered in MC-1 or MC-2 melanoma cells expressing CT or ΔGRD dominant-negative mutants. In the future, we will perform the assays mentioned above to analyze whether the interactions between endogenous IQGAP1 and its cognate binding partners are disrupted by expression of the dominant-negative mutants. These analyses may also identify novel interaction partners for the IQGAP1 mutants themselves.

I constructed the pMICG retroviral vector to expedite the cellular and molecular analysis of the IQGAP1 mutants. A375 cells that were infected with this bicistronic vector, which expressed a fusion of the extracellular domain of murine CD8 and GFP downstream of an internal ribosomal entry site, were magnetically sorted with anti-mCD8 beads. This approach was quicker and more convenient than FACS sorting, which was used for pMIG-infected cells. I used the pMICG retroviral vector to express the myc-tagged CT mutant in A375 MC-1 and MC-2 metastatic melanoma cells, and to express myc-tagged full-length IQGAP1 in A375P poorly metastatic melanoma cells. Unfortunately, I was unable to assess the *in vivo* behavior of cells expressing these IQGAP1 constructs because the pMICG vector alone altered cellular behavior. The incidence of lung metastasis in MC-1 and MC-2 cells expressing the extracellular domain of mCD8 on their cell surface (via pMICG infection) was significantly reduced compared with non-infected cells. This effect of pMICG expression on *in vivo* metastasis was not expected, and is poorly understood. Although these results suggest interesting functional consequences for modulating immune responses in metastatic progression, they do not add to our understanding of the role of IQGAP1 in metastasis, and have not been considered further. Therefore, to continue with the analysis at hand, a different expression vector must be used to stably express the myc-tagged CT mutant (and additional IQGAP1 mutants) in the highly metastatic A375 MC-1 and MC-2 melanoma cells and to stably overexpress full-length IQGAP1 in the poorly metastastic A375P melanoma cells. As I mentioned above, usage of the pMICG vector was expeditious for generating A375 cells that stably expressed IQGAP1 constructs because cells infected with this vector could be sorted magnetically. Perhaps a vector that expresses a different

extracellular marker that is not involved in the immune response could be constructed to continue these analyses. A vector that expresses both a drug-selectable marker and a marker for differential cell sorting (*i.e.* GFP) would also be convenient. It is clear that the analyses that were stopped short by the effects of pMICG need to be continued to address how IQGAP1 may be modulating *in vivo* metastasis.

The *in vivo* experimental metastasis assay measures the collective ability of tumor cells to survive in the circulation, arrest, invade and migrate out of the circulation, and grow in a distant organ. In order to assess the metastatic capacity of cells at each of these specific steps, *in vitro* assays are commonly used to model the individual processes necessary for the progression of metastasis. Several *in vitro* assays were used in this study to assess the role of IQGAP1 function in specific processes necessary for metastasis. Matrigel invasion assays revealed that expression of the IQGAP1 CT dominant-negative mutant reduced the *in vitro* invasion of MC-1 cells 10-fold compared with control cells. Similarly, transwell chemotaxis assays revealed that the CT mutant also severely inhibited the *in vitro* migration of MC-1 cells. These results suggest that a prometastatic activity of IQGAP1 may be required to promote cell migration and invasion. In addition, the results that IQGAP1 dominant-negative mutants did not affect the growth rate of melanoma cells, either *in vitro* or *in vivo* (grown as subcutaneous tumors), suggest that the suppression of pulmonary metastasis by the CT mutant was not due to a negative effect on cellular proliferation. Additional *in vitro* assays could be performed to analyze further the role of IQGAP1 at specific stages in metastatic progression, including soft agar colony assays and apoptosis assays to measure tumor cell anchorage-independent growth and survival.

As described above, I have shown that MC-1 melanoma cells that stably express the CT mutant exhibited significantly reduced migration and invasion *in vitro* compared to control cells. In addition, previous studies with MCF-7 cells have shown that cell motility and invasion were significantly decreased both by expression of the ΔGRD mutant and by knockdown of endogenous IQGAP1 using small interfering RNA (siRNA)

(Mataraza et al. 2003). This group also showed that overexpression of IQGAP1 in mammalian cells enhanced cell migration in a Cdc42- and Rac1-dependent manner. In combination, these results suggest that IQGAP1 functions to increase migration and invasion during metastatic progression. The results also suggest that the putative prometastatic activity of IQGAP1 may be mediated, in part, through active Rac1 or Cdc42. IQGAP1 is both a regulator and an effector of Rac1 and Cdc42. IQGAP1 binds to GTP-bound Rac1 and Cdc42 and keeps them in their active state by inhibiting their intrinsic GTPase activities (Brill et al. 1996; Hart et al. 1996; Ho et al. 1999). In turn, GTP-bound Cdc42 enhances IQGAP1-mediated cross-linking of F-actin and GTP-bound Rac1 and Cdc42 promote the interaction between IQGAP1 and CLIP-170, resulting in the capture of microtubules to special cortical spots at the leading edge of migrating cells (Bashour et al. 1997; Erickson et al. 1997; Fukata et al. 1997; Fukata et al. 2002b). Fukata *et al.* (2002) have shown that the CT mutant can disrupt the IQGAP1/CLIP-170-mediated capture of microtubules at the leading edge. I have shown in this study that the CT mutant significantly impairs *in vitro* migration and invasion as well as *in vivo* metastasis of metastatic A375 melanoma cells. In combination, these results suggest that IQGAP1 may promote metastasis by integrating Rac1 and Cdc42 signals to coordinate the microfilament and microtubule cytoskeleton at the leading edge of migrating cells. The capture and stabilization of microtubules near the cell cortex leads to a polarized cell morphology and promotes directional cell migration. Improper regulation of cell migration may promote the invasiveness of tumor cells, an important step in the progression of metastasis.

We have shown that enhanced IQGAP1 expression positively correlates with metastatic potential, and that expression of the IQGAP1 CT mutant in highly metastatic A375 melanoma cells reduces their metastatic potential. These results support the hypothesis that IQGAP1 may be required for metastatic progression. Furthermore, the use of two different dominant-negative IQGAP1 mutants that have already been shown to disrupt separate IQGAP1 interactions allows us to speculate about a possible role for IQGAP1 function in cytoskeletal regulation during metastatic progression. Our analyses, however,

do not yet directly demonstrate a causal role for IQGAP1 function in metastasis, and additional analyses will be needed to further address this hypothesis. As described above, a better understanding of the molecular interactions of the CT mutant is needed to clarify how this mutant inhibits metastasis, specifically whether modulation of IQGAP1 function is required for this inhibition. Further analyses of additional IQGAP1 mutants are also needed to address how specific IQGAP1 cellular activities may modulate metastasis, and I have discussed above how we are currently continuing the *in vivo* analysis of the ΔGRD mutant. Knockdown of IQGAP1 expression by RNAi has been previously shown to inhibit *in vitro* migration and invasion of MCF-7 cells (Mataraza et al. 2003), and we should similarly use siRNA to knock down IQGAP1 expression in the highly metastatic A375 melanoma cells. By analyzing the metastatic capacity of these cells with the *in vivo* experimental metastasis assay, we could directly assess the role of IQGAP1 function on metastasis. In addition, we have recently received IQGAP1$^{-/-}$ mice from Andre Bernards (MGH Cancer Center, Charlestown, MA). These mice, which are viable and fertile, can be interbred with endogenous mouse models of tumorigenesis, specifically those models that exhibit frequent distant metastases, to assess the role of IQGAP1 function in all stages of tumorigenesis (discussed further in Chapter 4).

IQGAP1 functions at the intersection of several distinct signaling pathways that can influence metastatic progression. The results described in this thesis support the hypothesis that IQGAP1 may play a causal role in metastasis. In addition, the analyses described above will be performed to continue the investigation into how IQGAP1 might function to promote cell polarization, migration, and invasion, and thus ultimately define the role of IQGAP1 in metastasis.

Figure 3-1

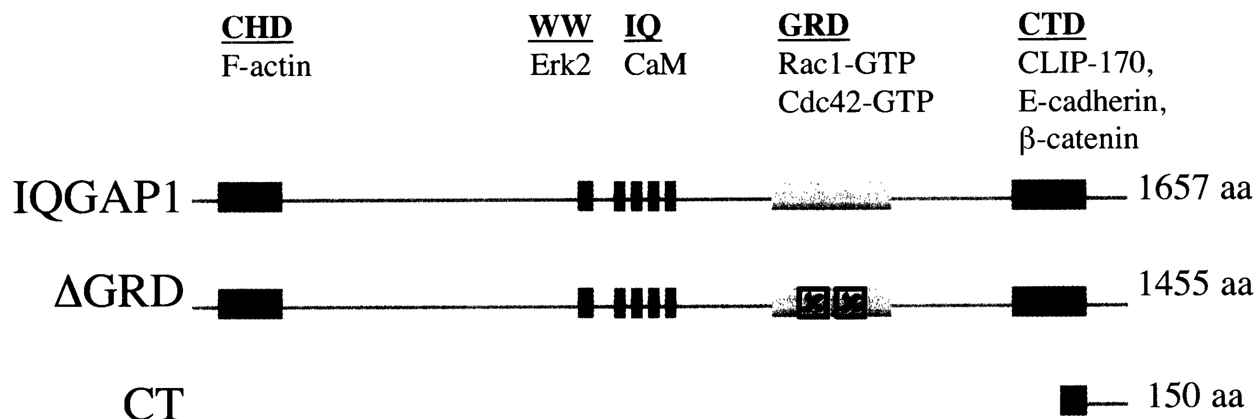


Figure 3-1: Domain structure of full-length IQGAP1 and dominant-negative mutants.
Schematic diagram of full-length IQGAP1 adapted from the Pfam database. The ΔGRD mutant is missing amino acids 1122-1324. The CT mutant contains the carboxy- terminal amino acids 1503-1657. Abbreviations include: CHD, calponin homology domain; WW, poly-proline-binding domain; IQ, calmodulin-binding motif; GRD, Ras GTPase-activating protein (GAP)-related domain; CTD, carboxy-terminal domain; ERK, extracellular signal-regulated kinase; CaM, calmodulin; CLIP-170, cytoplasmic linker protein 170.

Figure 3-2

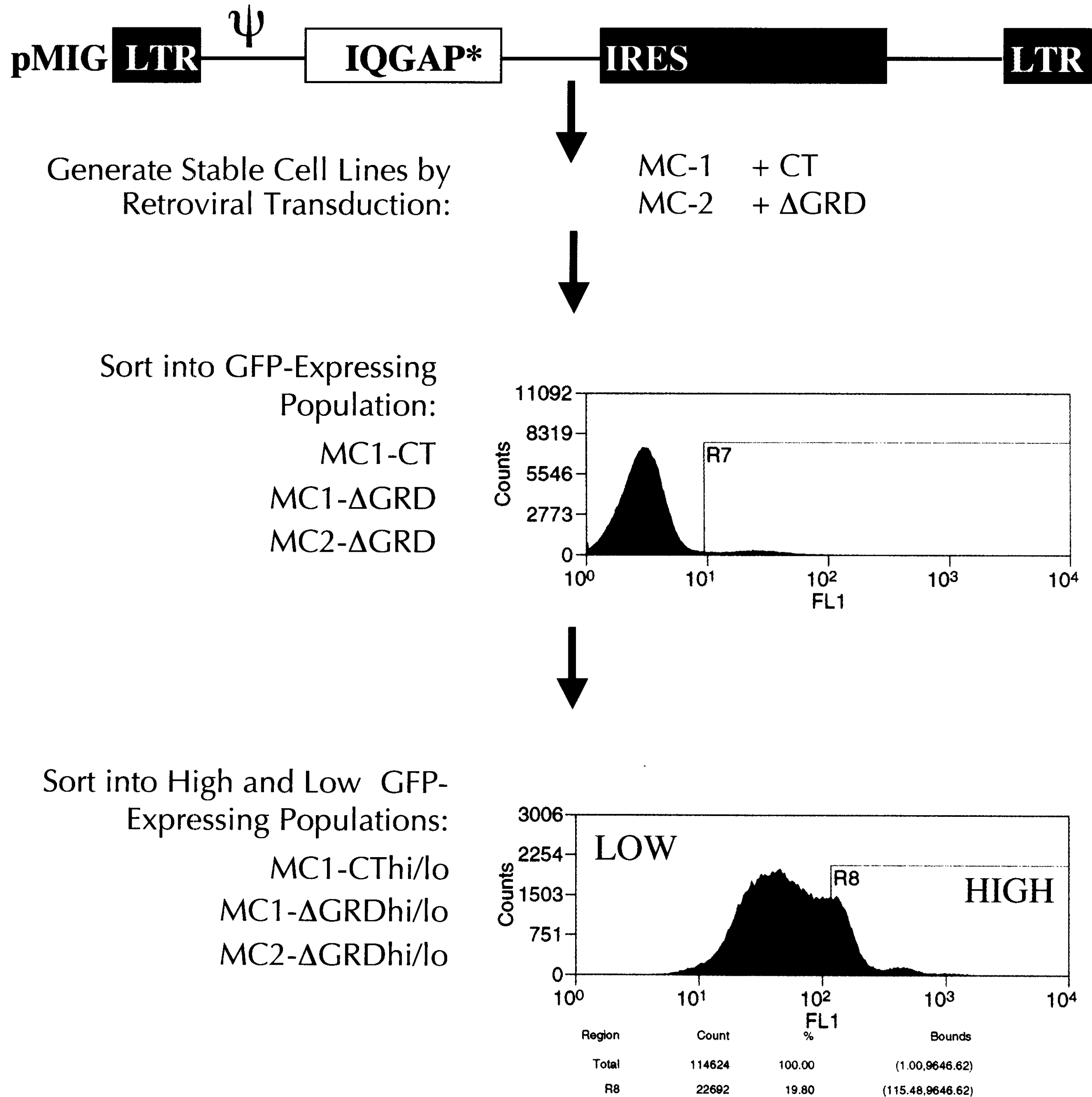


Figure 3-2: Generation of highly metastatic cell lines that express IQGAP1 dominant-negative mutants by pMIG retroviral gene transfer.
Schematic diagram of pMX-IRES-GFP (pMIG) bicistronic retroviral vector. IQGAP1 dominant-negative mutants, CT and ΔGRD, were cloned into pMIG upstream of IRES-GFP. A375 MC-1 and MC-2 cells were infected with CT- or ΔGRD-expressing retrovirus and GFP-positive cells were sorted by FACS. Stable retrovirus-expressing cells were expanded in tissue culture and FACS-sorted for a second time into high- and low-GFP expressing populations.

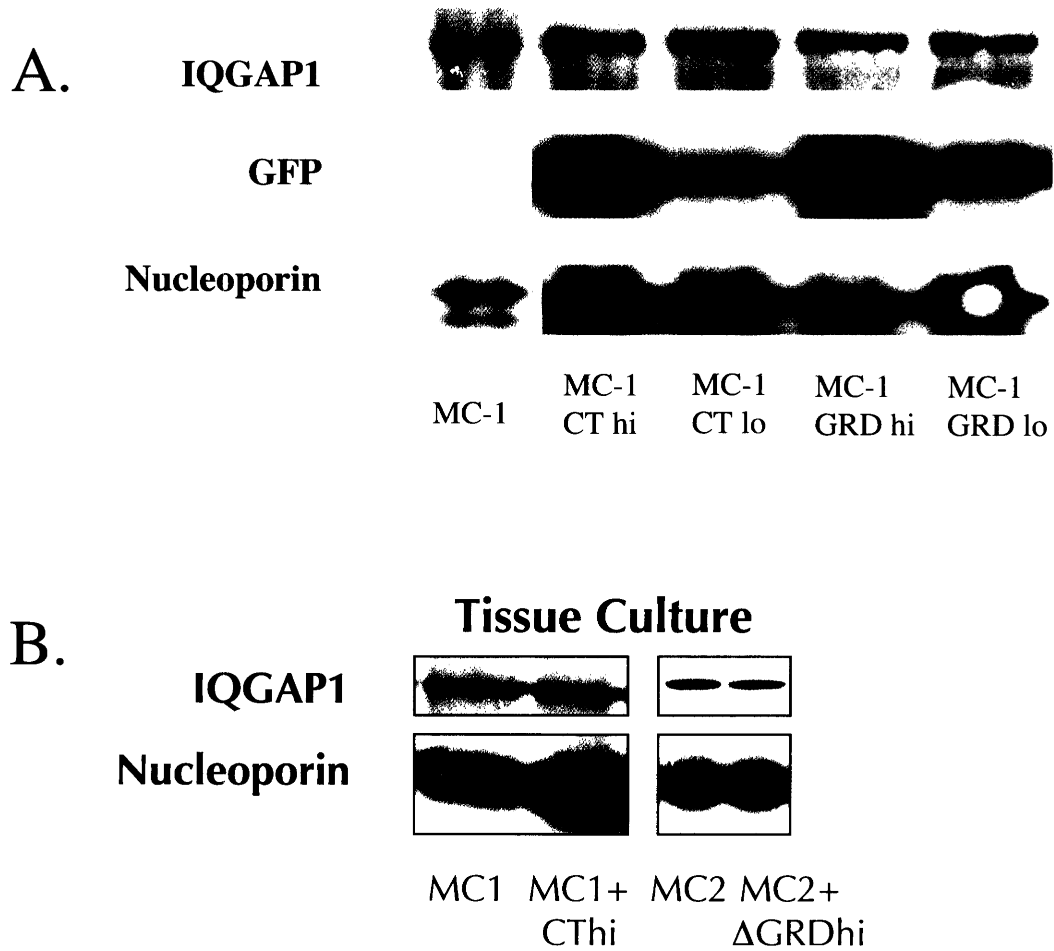


Figure 3-3: Western blot analysis of GFP expression in MC cells infected with pMIG retrovirus.

A. MC-1 cells infected with pMIG-CT or pMIG-ΔGRD were sorted by FACS into high and low GFP-expressing populations, and SDS lysates were prepared from cells expanded in tissue culture. Equal amounts of protein were loaded and electrophoresed on 7.5% SDS-PAGE gels and transferred to nitrocellulose. The blot was cut into strips and separately incubated with monoclonal antibodies against IQGAP1, GFP and nucleoporin (a loading control).

B. Expression of endogenous IQGAP1 is not affected in A375 MC-1 or MC-2 cells expressing CT or ΔGRD mutants, respectively. SDS lysates were prepared from cells grown in tissue culture. Equal amounts of protein were loaded and electrophoresed on 7.5% SDS-PAGE gels and transferred to nitrocellulose. The blot was cut into strips and separately incubated with monoclonal antibodies against IQGAP1 and nucleoporin (a loading control).

Figure 3-4A

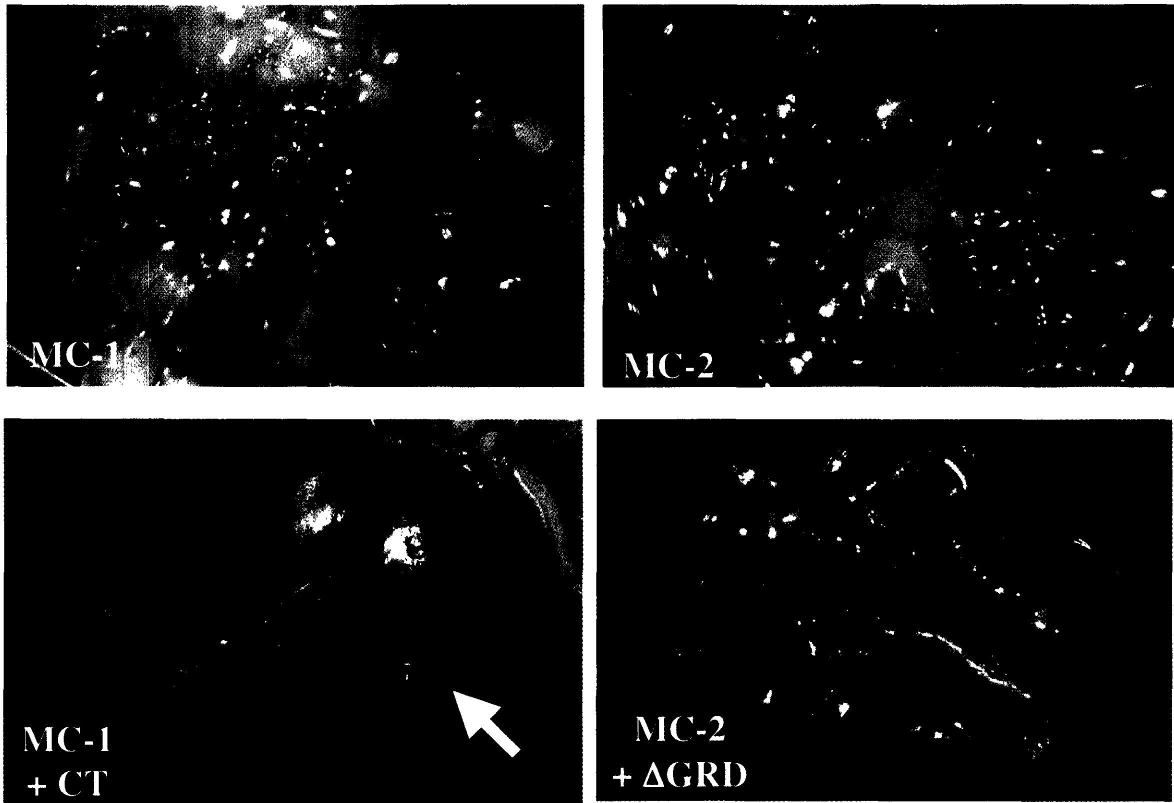


Figure 3-4B

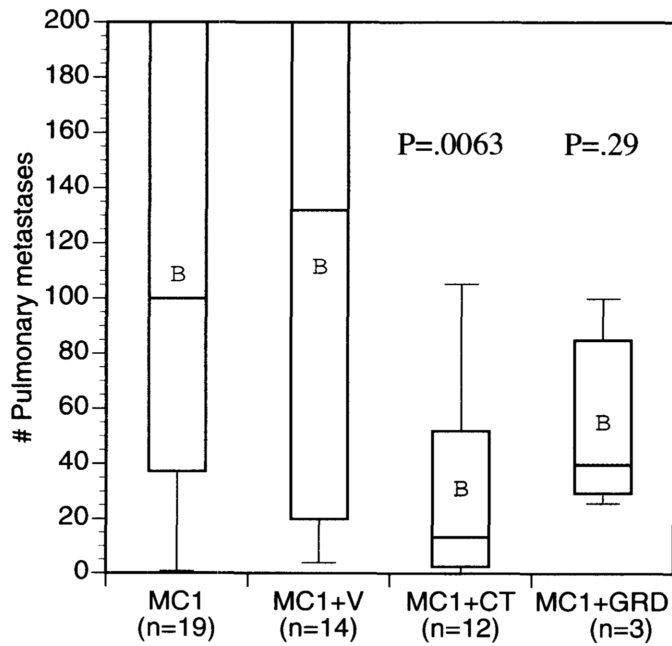


Figure 3-4C

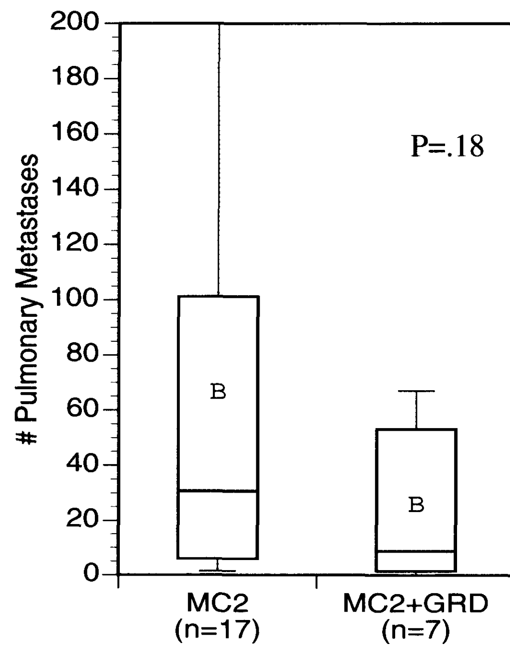


Figure 3-4: Pulmonary metastases in host mice injected with A375 human melanoma cells expressing IQGAP1 mutants.

A. A375 MC-1 cells expressing pMIG vector only, pMIG-CT, or pMIG-ΔGRD and A375 MC-2 expressing pMIG-ΔGRD were tested in the experimental metastasis assay. Host mice were injected with highly metastatic A375 melanoma cells +/- IQGAP1 mutants and sacrificed five-eight weeks later. Representative pictures of dissected lungs with metastatic nodules are shown.

B. Quantification of pulmonary metastases in host mice injected with A375 MC-1 cells expressing pMIG-CT or pMIG-ΔGRD. Macroscopic pulmonary metastases were quantified by visualization under a dissecting microscope. MC1-CT cells were significantly less metastatic than MC-1 cells expressing pMIG vector only control, as determined by the unpaired Student's t-test (*P = .0063). The reduction of metastasis in MC1-ΔGRD cells was not statistically significant (P = 0.29), however the experimental sample was small.

C. Quantification of pulmonary metastases in host mice injected with A375 MC-2 cells expressing pMIG-ΔGRD. Host mice were injected with highly metastatic A375 melanoma cells +/- IQGAP1 mutants and sacrificed five-eight weeks later. Macroscopic pulmonary metastases were quantified by visualization under a dissecting microscope. The reduction of metastasis in MC2-ΔGRD cells was not statistically significant (P= 0.18), but again, the experimental sample was small.

Figure 3-5

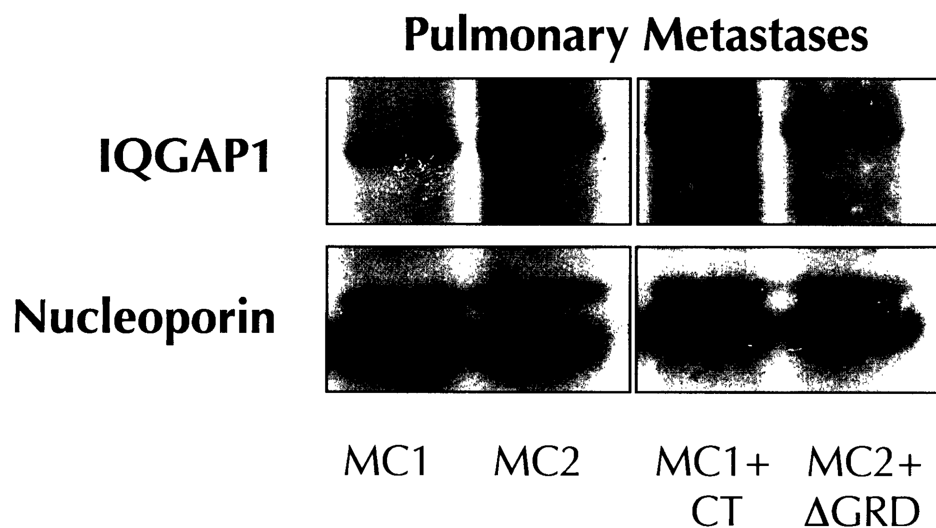


Figure 3-5: Western blot analysis of endogenous IQGAP1 expression in metastatic melanomas.
A. MC-1, MC1-CT, MC-2 and MC2-ΔGRD cells were used in the experimental metastasis assay. Cell lysates of pulmonary metastases (in RIPA) were prepared. Equal amounts of protein were loaded and electrophoresed on 7.5% SDS-PAGE gels and transferred to nitrocellulose. The blot was cut into strips and separately incubated with monoclonal antibodies against IQGAP1 and nucleoporin (a loading control).

Figure 3-6

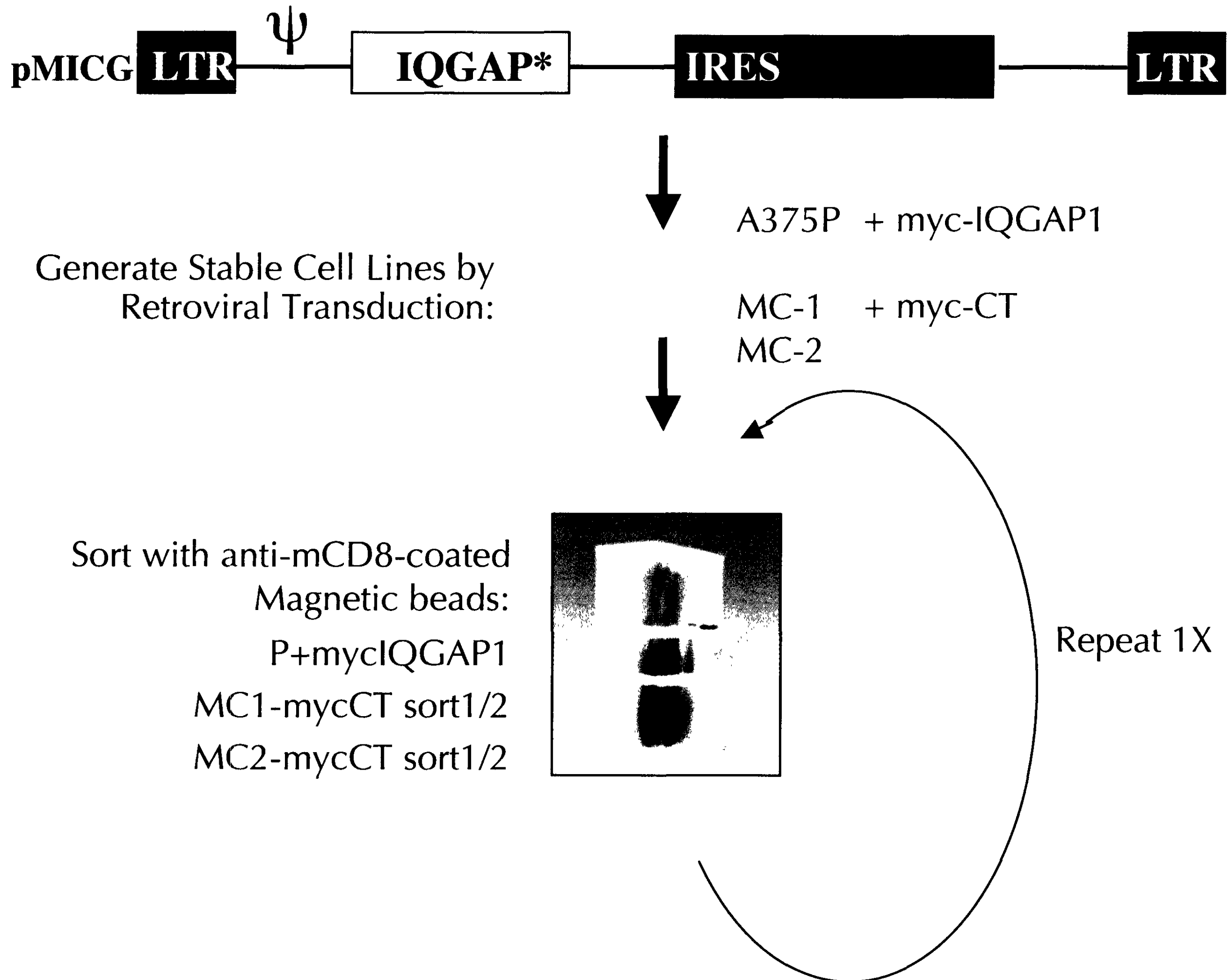


Figure 3-6: Generation of highly metastatic cell lines that express IQGAP1 dominant-negative mutant by pMICG retroviral gene transfer.
Schematic diagram of pMX-IRES-mCD8GFP (pMICG) bicistronic retroviral vector. N-terminal myc-tagged full-length IQGAP1 and CT dominant-negative mutant were individually cloned into pMICG upstream of IRES-mCD8GFP. A375P cells were infected with mycIQGAP1 retrovirus and A375 MC-1 and MC-2 cells were infected with mycCT-expressing retrovirus, and CD8-positive cells were magnetically sorted with anti-mCD8-coated magnetic beads. Stable retrovirus-expressing cells were expanded in tissue culture and magnetically-sorted for a second time to enrich CD8 expressing populations.

Figure 3-7

In vivo Metastasis Assay

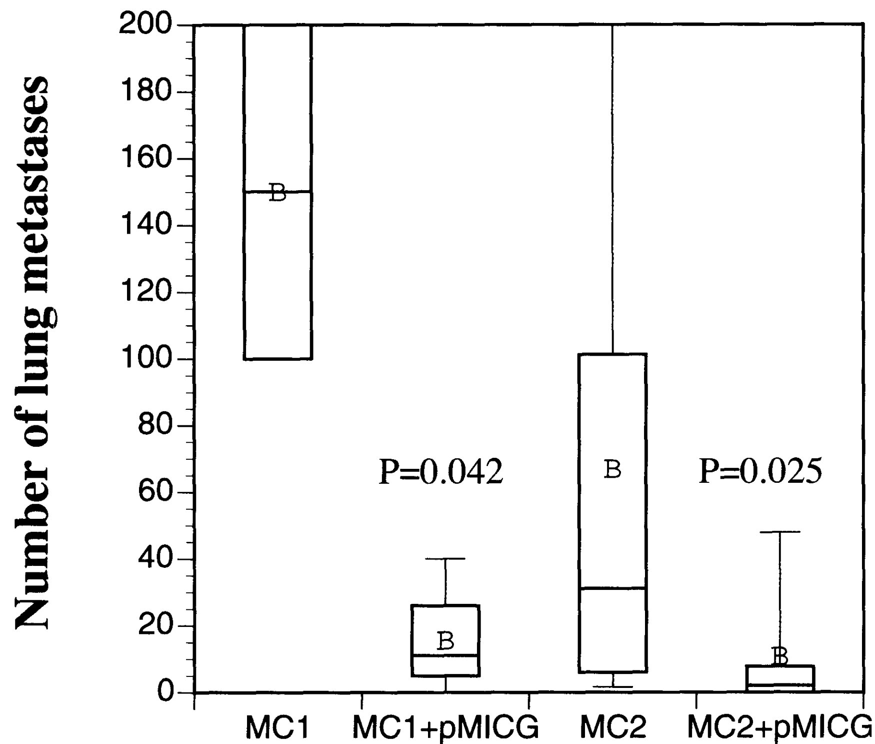


Figure 3-7: Quantification of pulmonary metastases in host mice injected with A375 human melanoma cells.
Host mice were injected with highly metastatic A375 MC-1 or MC-2 melanoma cells expressing pMICG or pMICG-mycCT and sacrificed five-eight weeks later. Macroscopic pulmonary metastases were quantified by visualization under a dissecting microscope.

Figure 3-8A

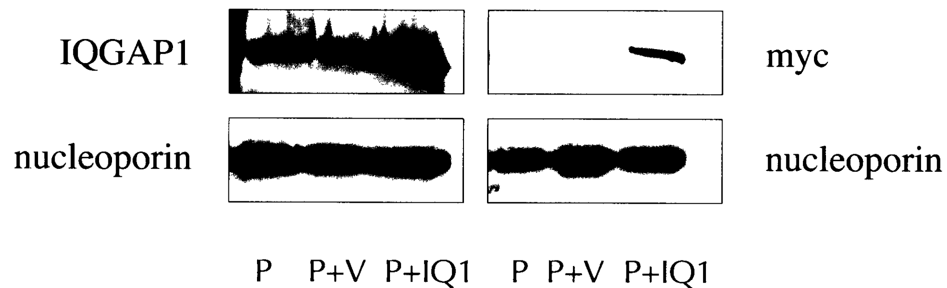


Figure 3-8B

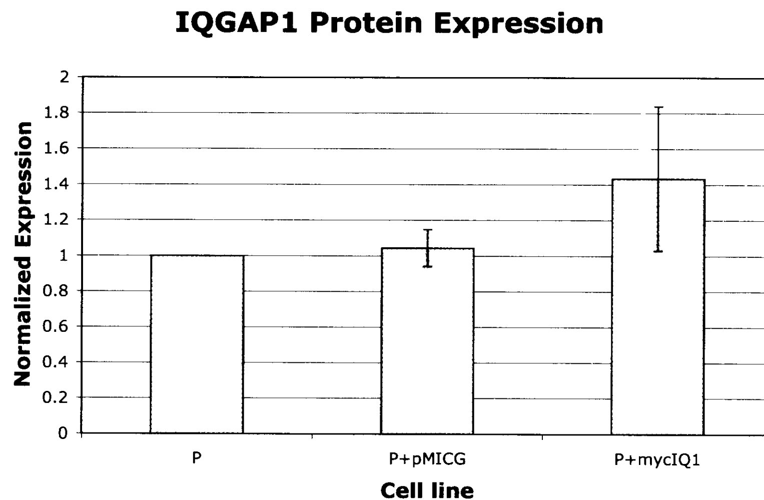


Figure 3-8: Western blot analysis of endogenous and ectopic IQGAP1 expression.
A. A375P cells infected with pMICG or pMICG-mycIQGAP1 were magnetically sorted with anti-mCD8-coated beads, and SDS lysates were prepared from cells expanded in tissue culture. Equal amounts of protein were loaded and electrophoresed on 7.5% SDS-PAGE gels and transferred to nitrocellulose. The blot was cut into strips and separately incubated with monoclonal antibodies against IQGAP1 or c-myc and nucleoporin (a loading control).
B. Densitometry was used to quantify IQGAP1 and nucleoporin bands. IQGAP1 expression data was standardized to nucleoporin and is presented as fold expression compared with the parental sample.

Figure 3-9A

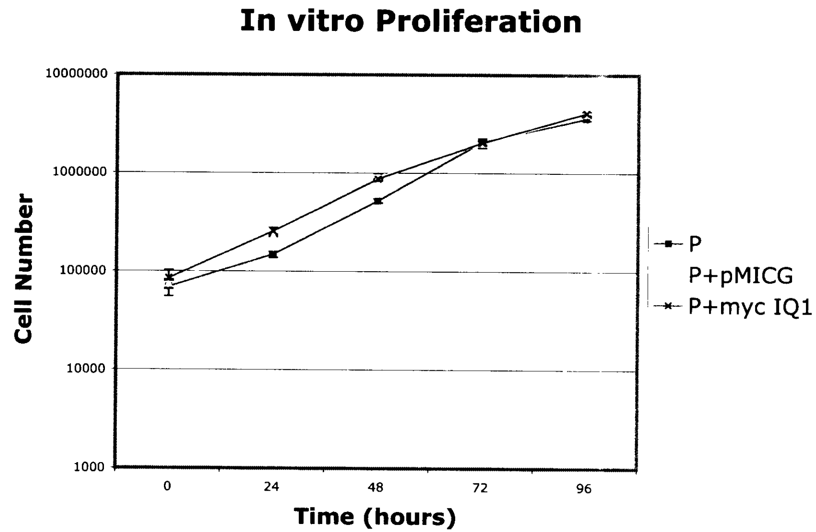


Figure 3-9B

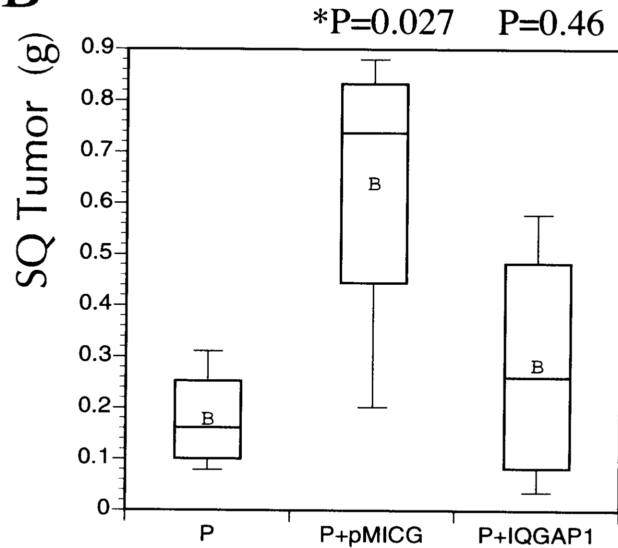


Figure 3-9: Cell proliferation of poorly metastatic melanoma cells expressing full-length IQGAP1

A. A375 P cells expressing pMICG or pMICG-mycIQGAP1 were plated at $5x10^4$ cells on Day 0. Cells were trypsinized and counted on days 1-5. Results are expressed as absolute cell numbers +/- SD (n=3).

B. A375 cells ($2.5x10^5$) were injected into the rear flank of nude mice. Mice were sacrificed five to six weeks later, and tumors were removed and weighed. Results are represented as box and whisker plots. Differences in tumor weight are statistically significant, as determined by an unpaired Student's t test.

Figure 3-10

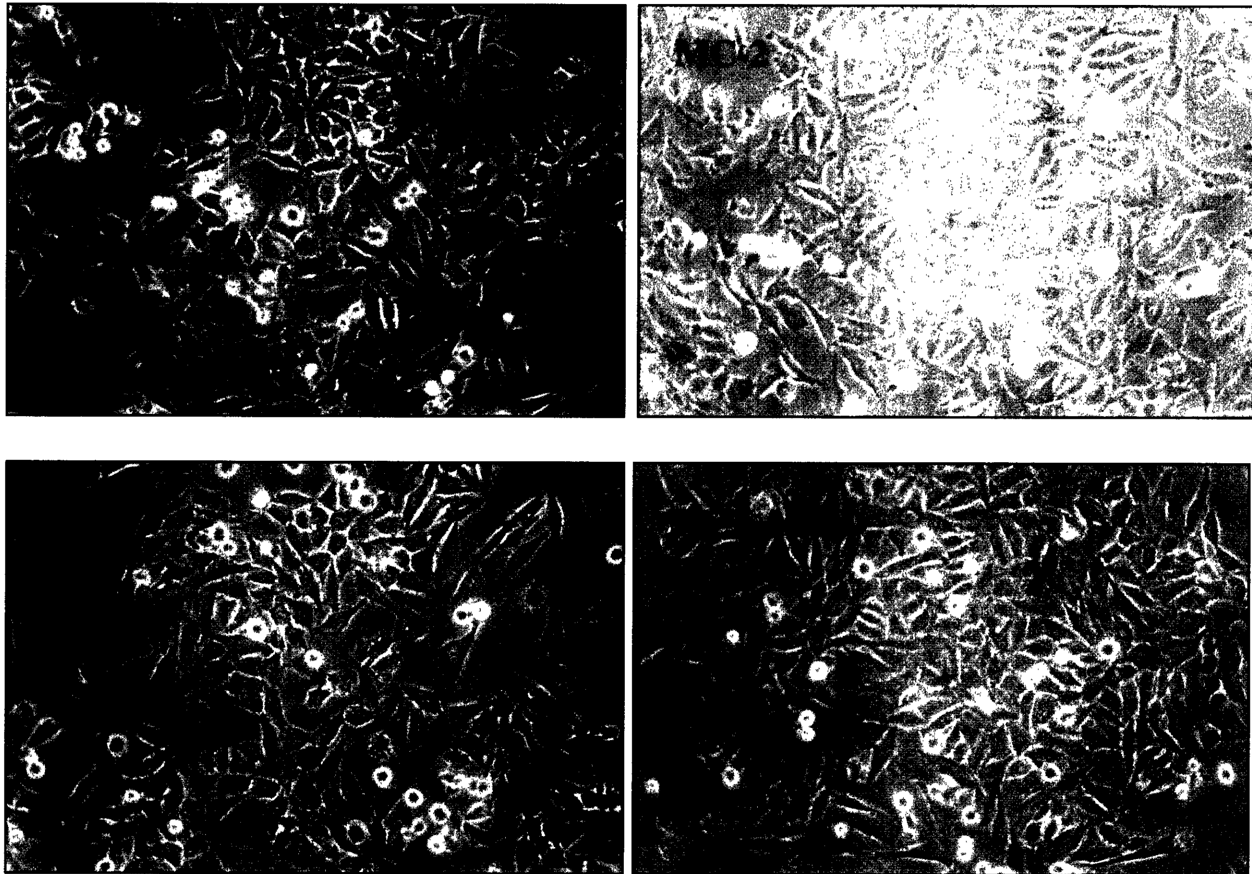


Figure 3-10: Gross morphology of highly metastatic melanoma cells expressing IQGAP1 dominant negative mutants.
Phase contrast microscopy of MC-1, MC1-CT, MC-2 and MC2-ΔGRD cells. Both populations of cells exhibit phase bright cell-cell junctions and membrane ruffles at the leading edge.

Figure 3-11A

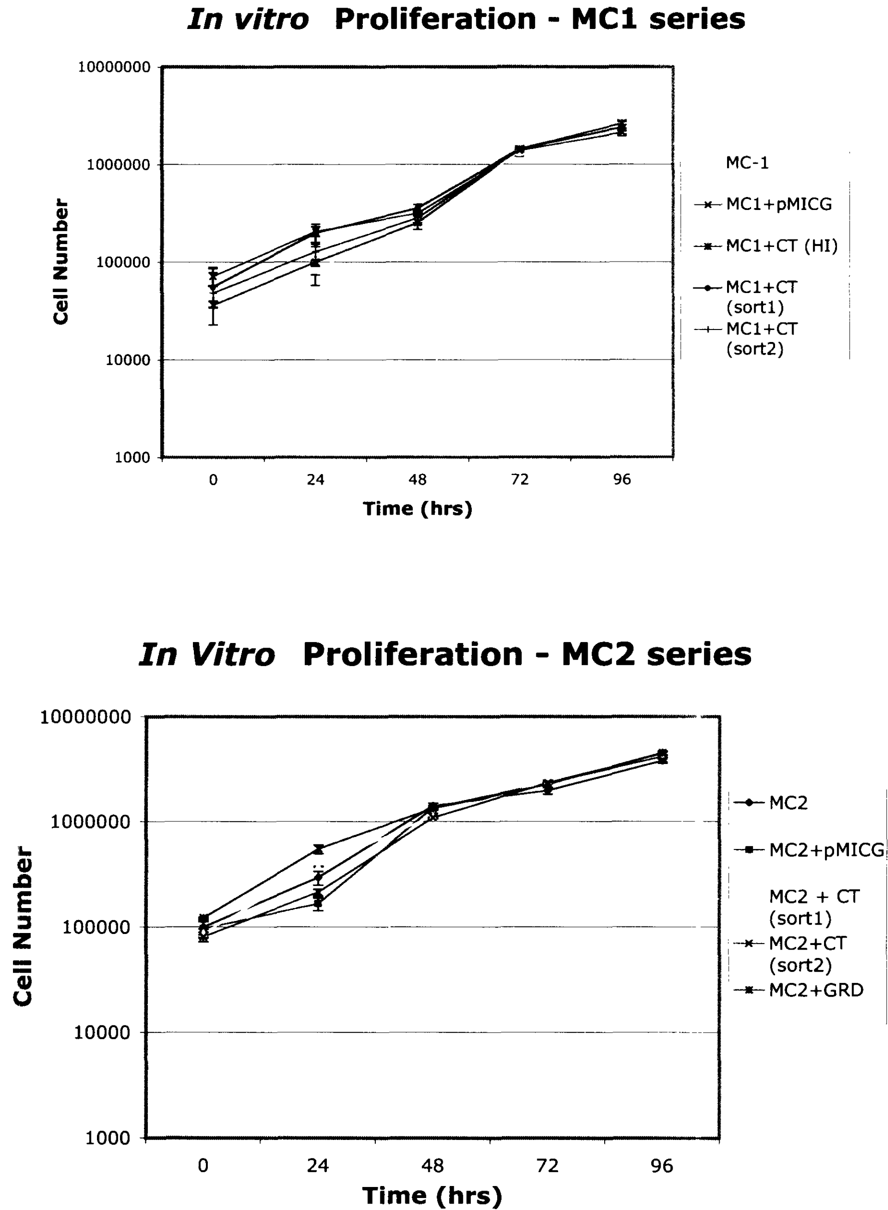


Figure 3-11: Cell proliferation of highly metastatic melanoma cells expressing IQGAP1 dominant-negative mutants.
A. A375 MC-1 or MC-2 cells expressing pMIG, pMIG-CT, pMIG-ΔGRD, pMICG or pMICG-mycCT were plated at $5x10^4$ cells on Day 0. Cells were trypsinized and counted on days 1-5. Results are expressed as absolute cell numbers per dish +/- SD (n=3).

Figure 3-11B

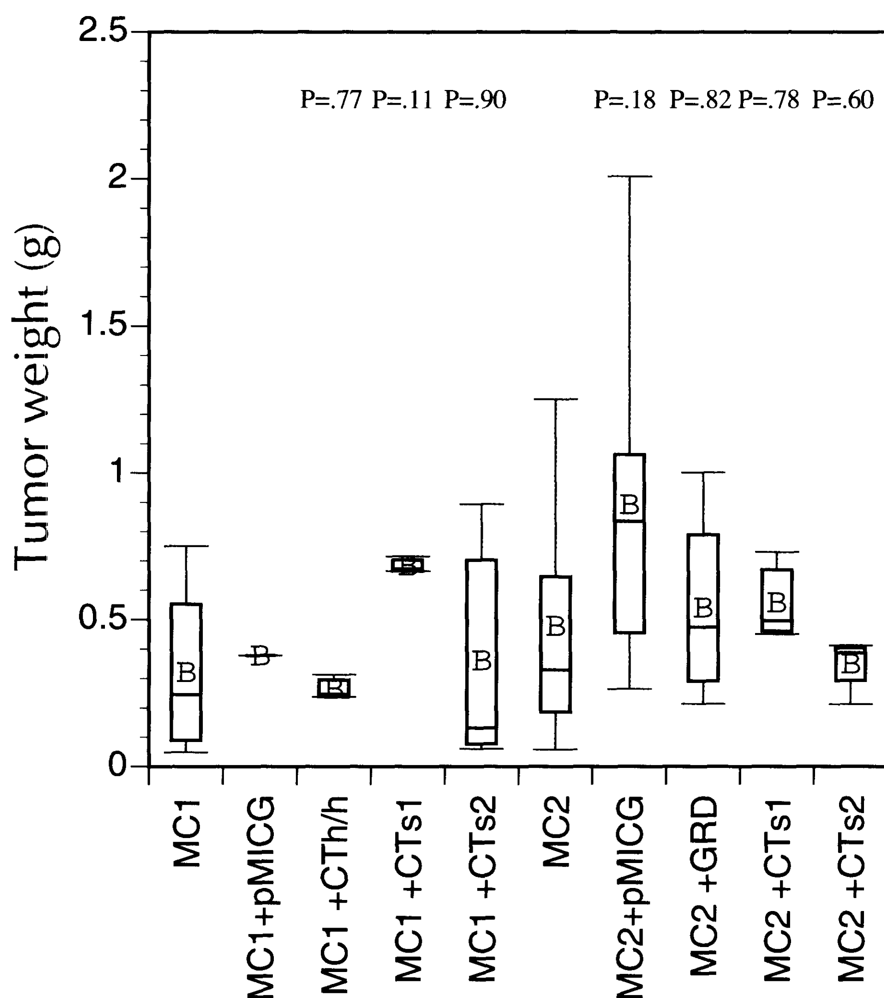


Figure 3-11: Cell proliferation of highly metastatic melanoma cells expressing IQGAP1 dominant-negative mutants.
B. A375 cells ($2.5x10^5$) were injected into the rear flank of nude mice. Mice were sacrificed five to six weeks later, and tumors were removed and weighed. Results are represented as box and whisker plots. The top, middle and bottom of the box corresponds to the 75th percentile (top quartile), 50th percentile (median) and 25th percentile (bottom quartile), respectively. The whiskers extend from the 10th percentile (bottom decile) to the 90th percentile (top decile.) The mean is represented by the letter "B". Differences in tumor weight were not statistically significant, as determined by an unpaired Student's t test.

Figure 3-12A

In Vitro Invasion

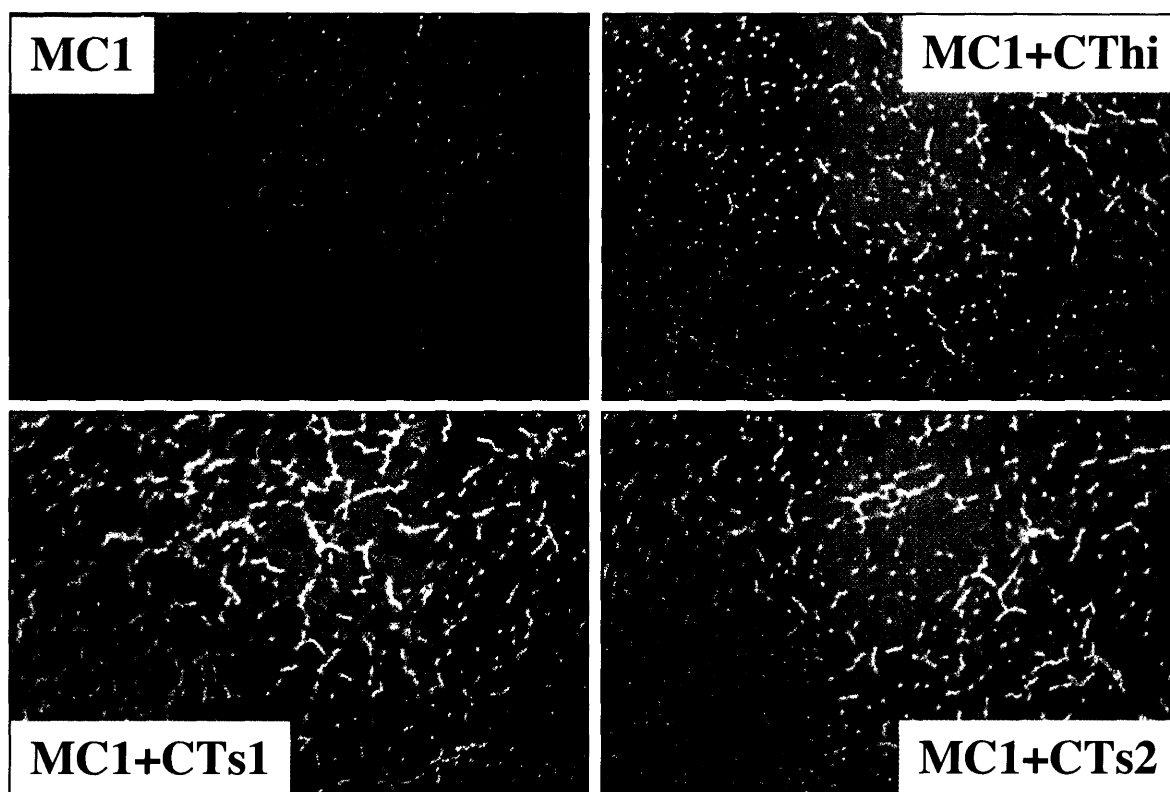


Figure 3-12B

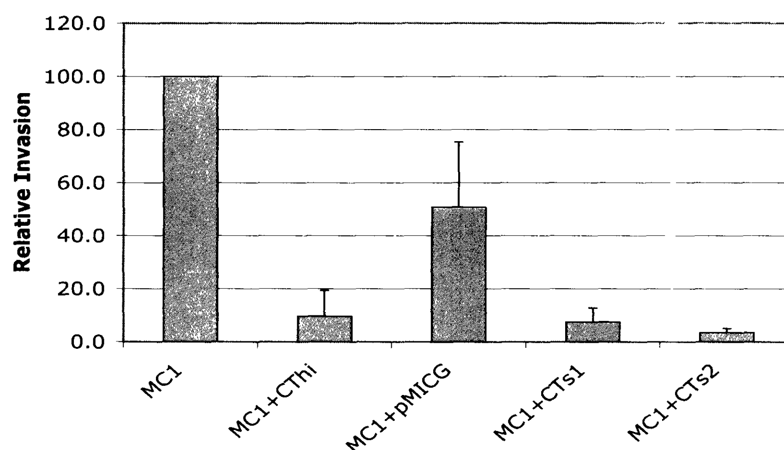
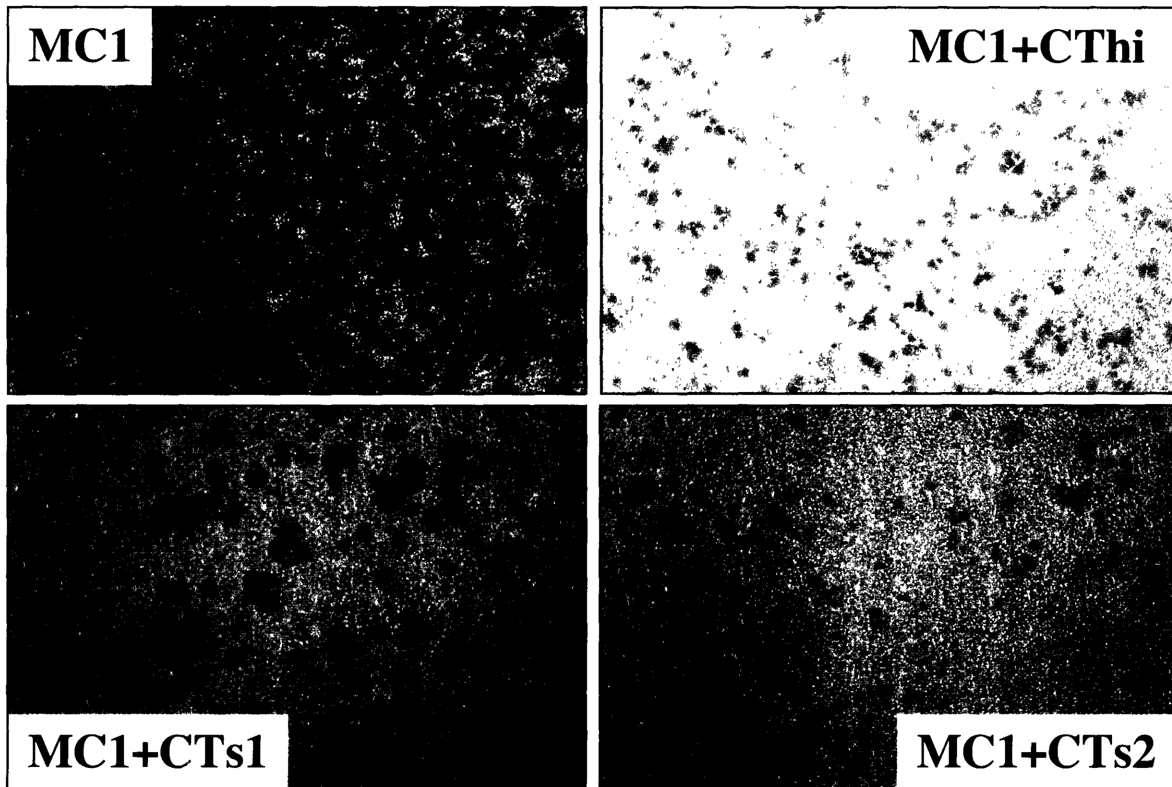


Figure 3-12: *In vitro* migration and invasion of highly metastatic melanoma cells expressing IQGAP1 dominant-negative mutants.
A. MC-1 cells expressing pMIG, pMIG-CT, pMICG or pMICG-mycCT were allowed to migrate through Transwell pores or invade through Matrigel inserts for 21 or 24 hours, respectively. Migratory and invasive cells were fixed in 4% para-formaldehyde and stained with 0.1% crystal violet.
B. Invasive cells were quantified by counting five microscope fields per assay using a 10X objective. At least six wells were assayed per cell line. Data are expressed relative to the migration of MC-1 cells and represent the mean +/- s.d.

Figure 3-12A



Chapter 4

Conclusions and Future Directions

Metastasis, the spread and relentless growth of tumor cells from the primary neoplasm to distant organs, is an insidious disease that is responsible for 90% of human cancer-related deaths (Hanahan and Weinberg 2000). Little progress has been made in treating metastatic disease over the past several decades because our understanding of systemic cancer progression is remarkably poor. For new diagnostic and therapeutic strategies to be developed, a deeper understanding of the systemic, cellular and molecular pathogenesis of metastasis is needed.

The advent of DNA microarray expression analysis has transformed the field of metastasis research and unveiled a wealth of new information relevant to the question of metastatic progression. These genome-wide expression analyses allow a nearly complete molecular picture of the cellular events that lead to metastasis. Recently, several groups have used microarrays to analyze the expression profiles of various human tumors in the hope of identifying molecular signatures of "good" and "bad" prognosis that can then be used to predict the clinical outcome of individuals with cancer at diagnosis (Perou et al. 2000; Ramaswamy et al. 2001; Sorlie et al. 2001; van 't Veer et al. 2002; Kang et al. 2003). Although these kinds of clinical prognosticators are still in their infancy, they have the potential to be extremely powerful clinical tools.

DNA microarray analyses are also changing the way that metastasis is studied in the laboratory. For several decades, techniques have been available to study and derive cell lines with different metastatic potential (Fidler 1986). These cell lines have been useful in identifying genes and gene products that both enhance and suppress metastatic potential. In recent years, the confluence of experimentally-derived cell lines and post-genomic analysis of these cell lines have led to many new and exciting developments in cancer biology. The difficulty in these times seems to be that there is too much data, making the process of picking targets for further validation an artful one. Regardless, microarray expression analyses have provided a wealth of data for cancer biologists to explore.

Several genes have recently been shown to play a role in metastasis as a direct result of microarray analyses. Our own lab, for example, using the expression analysis previously described in Chapter 2, has recently shown that RhoC, a gene whose expression was enhanced in highly metastatic melanoma cells compared to poorly metastatic melanoma cells, is essential for metastasis (Clark et al. 2000a). The overexpression of RhoC in poorly metastatic melanoma cells enhanced metastasis while the expression of a dominant-negative Rho construct in highly metastatic cells inhibited metastasis. In fact, proteins that associate with the Rho family of small GTPases are beginning to emerge as key regulators of metastasis. Although several effectors and regulators of Rho-family GTPases have previously been implicated in malignant progression *in vitro*, many fewer have been validated *in vivo*. For example, the ERM proteins (Ezrin, Radixin, and Moesin) and Merlin are closely related members of the band 4.1 superfamily of proteins that, when activated, interact with both membrane proteins and the actin cytoskeleton (Bretscher et al. 2002). Rho-dependent phosphorylation activates ERM proteins, which, in turn can regulate Rho. By organizing membrane-cytoskeleton-associated complexes and creating specialized membrane domains, the ERM proteins regulate cellular activities such as survival, adhesion and migration/invasion, all of which are important during tumor development and progression (Bretscher et al. 2002; McClatchey 2003). Two recent papers provide strong evidence that Ezrin promotes tumor metastasis (Khanna et al. 2004; Yu et al. 2004). They established by microarray analysis that high levels of Ezrin expression are linked to metastatic behavior in different types of tumors from diverse species and went on to experimentally validate a prometastatic function for Ezrin.

These studies further validate our microarray expression data that several genes that modulate the cell cytoskeleton are important in metastastic progression. We have shown that one of these genes, IQGAP1, positively correlates with metastasis in a number of independently derived highly metastatic melanoma cells. In addition, we surveyed the ONCOMINE cancer microarray database and found that IQGAP1 expression was enhanced in other examples of progressive disease, namely advanced breast carcinoma and metastatic prostate cancer. We then used a mutational approach to analyze the

functional role of IQGAP1 in metastasis. We found that the CT dominant-negative mutant severely suppressed the generation of pulmonary metastases in metastatic human melanoma cells. The ΔGRD mutant, which may be a less inhibitory mutant, also suppressed the generation of pulmonary metastases, but to a less severe degree. We found that IQGAP1 mutants can also inhibit the invasive and migratory potential of melanoma cells. Since IQGAP1 is such a large protein with so many binding partners, functional disruption by different dominant-negatives was useful for allowing us to narrow down the specific activity of IQGAP1 that is necessary for metastasis. This insight would not have been possible if we used a blunt disruption approach such as RNAi. The differences in metastatic capacity that were observed between the ΔGRD mutant and the CT mutant, as well as their effects on *in vitro* proliferation and migration allow us to speculate that IQGAP1 promotes metastasis through its interactions with the filamentous actin cytoskeleton and the microtubule cytoskeleton via CLIP-170. In addition, these results suggest the first demonstration that a scaffolding protein that links the microfilament cytoskeleton to the microtubule cytoskeleton may play a role in metastasis.

Recent studies have further identified members of the Rho family of GTPases as key intermediates in transmitting signals to cortical factors that mediate capture of dynamic microtubules at specific sites. The finding that IQGAP1 binds to microtubules (MTs) via CLIP-170 downstream of Rac1/Cdc42 signaling suggests that it behaves as a 'bridging protein' that can directly link the microtubule tip protein and the actin cytoskeleton. I propose that this function of IQGAP1 is necessary for cell polarization and directed cell migration, processes that are important for the progression of metastasis. I also propose that the disruption of this "bridging" function results in an altered microtubule array and a subsequent loss of directed cell migration, thus suppressing the invasive and metastatic phenotype.

Polarized cell migration is a tightly regulated process that occurs in tissue development, chemotaxis, and wound healing (Fukata et al. 2003). Malignant cells acquire changes in

their ability to migrate and invade in order to metastasize. Cells migrate directionally in response to a variety of signals, including gradients of growth factors, chemokines or extracellular matrix proteins (Lauffenburger and Horwitz 1996). When a cell responds to a migration stimulus, it polarizes by extending protrusions like membrane ruffles and filopodia in the direction of migration.

Establishing and maintaining cell polarity in response to extracellular stimuli appear to be mediated by a set of interlinked positive feedback loops involving Rho family GTPases, phosphoinositide 3-kinases (PI3Ks), integrins, microtubules, and vesicular transport (Ridley et al. 2003). Rho family GTPases are implicated in cell polarization in several cell types, including fibroblasts, macrophages and astrocytes (Allen et al. 1998; Nobes and Hall 1999; Etienne-Manneville and Hall 2001, 2003). Recent intensive analyses have started to clarify how Rho family GTPases reorganize microtubules through their effectors to regulate cell polarity (Fukata et al. 2003).

Cdc42 is active toward the front of migrating cells and both inhibition and global activation of Cdc42 can disrupt the directionality of migration (Etienne-Manneville and Hall 2002; Itoh et al. 2002). Cdc42 can also affect polarity by localizing the microtubule-organizing center (MTOC) and Golgi apparatus in front of the nucleus, oriented toward the leading edge. Cdc42-induced MTOC orientation may contribute to polarized migration by facilitating microtubule growth into the lamella and microtubule-mediated delivery of Golgi-derived vesicles to the leading edge, providing membrane and associated proteins needed for forward protrusion (Etienne-Manneville and Hall 2002; Rodriguez et al. 2003).

Cdc42 appears to exert its effects on MTOC position mainly through a pathway involving the Cdc42 effector PAR6, which exists in a complex with PAR3 and an atypical kinase C (aPKC) (Hawkins et al. 1995). Etienne-Manneville and Hall have shown that Cdc42 regulates cell polarity by acting on Par6, PKCζ (an atypical protein kinase C [aPKC]), glycogen synthase kinase 3β (GSK-3β) and the tumor suppressor adenomatous polyposis

coli (APC) in the migration of primary astrocytes during wound healing (Etienne-Manneville and Hall 2001, 2003).

APC accumulates at the ends of MTs that extend into actively migrating regions and bundles (i.e. stabilizes) MTs (Nathke et al. 1996; Zumbrunn et al. 2001). Phosphorylation by GSK-3β decreases APC-induced MT bundling (Zumbrunn et al. 2001). Etienne-Manneville and Hall have shown that these two important signaling pathways (Cdc42 -> Par6 -> aPKC -> and GSK-3β -> APC -> MTs) link together. Cdc42 activates PKCζ through Par6, leading to phosphorylation and inactivation of GSK-3β at the leading edge of migrating astrocytes. Inactivated GSK3-β allows APC to stabilize MTs at the leading edge (Etienne-Manneville and Hall 2003). Inhibition of any one of the components (Cdc42, Par6, aPKC, GSK-3β, or APC) blocks the reorientation of the MTOC toward the direction of migration. Because the inactivation of GSK-3β lasts for at least 12hours, it is possible that the Cdc42 -> Par6 -> aPKC -> GSK3β-APC pathway plays a role in maintaining microtubule arrays and thus, cell polarity.

RhoA was also found to regulate the formation of a subset of stabilized MTs in the leading edge of migrating fibroblasts through its effector mDia1 (Cook et al. 1998). Rho appeared to regulate MT capture at the cell cortex, as time-lapse movies showed that Rho activation induced a subset of MTs near the leading edge to pause for long periods without affecting parameters of dynamic instability. MTs stabilized by mDia1 did not grow or shrink and are thought to be capped on their plus ends to give them long term (>1 hour) stability (Infante et al. 2000).

mDia1 is a member of the formin family, a relatively new class of actin nucleators that direct assembly of straight filaments (Pruyne et al. 2002; Sagot et al. 2002). They are large multidomain proteins that are required for cytokinesis and maintenance of cell polarity (Wasserman 1998). These cytoskeleton-organizing proteins direct assembly of actin structures, such as stress fibers, and also regulate microtubule stability in eukaryotic cells. The active form of mDia1 induces longitudinally aligned MTs in parallel to F-actin

bundles along the long axis of the cell (Yayoshi-Yamamoto et al. 2000). A second member of the family, mDia2, may also be important in regulating microtubule dynamics, since it interacts with activated Cdc42 at the MTOC (Gasteier et al. 2003). A number of cellular processes have been shown to be dependent on formins, including the formation of adherens junctions between epithelial cells, microtubule-independent movement of meiotic chromosomes, cell migration, movement of early endosomes along actin filaments and formation of filopodia, microspikes and lamellipodia (Leader et al. 2002; Sahai and Marshall 2002a, 2002b; Gasman et al. 2003; Koka et al. 2003; Peng et al. 2003).

Therefore, Rho-family GTPases have begun to emerge as key regulators of MTs, and play a pivotal role in stabilizing and maintaining MTs. Very little is known, however, about how the Rho-family GTPases initially capture MTs at the cortex. The interaction between IQGAP1 and CLIP-170 has been previously described in Chapter Three. Briefly, activated Rac1/Cdc42 forms a tripartite complex with IQGAP1 and CLIP-170 and enhances the interaction of IQGAP1 with CLIP-170 (Fukata et al. 2002b). Live-cell imaging showed that these interactions resulted in the transient stabilization of MT at the base of the leading edge and filopodia, respectively. The observed transient pause that lasted only for intervals up to two minutes may serve to concentrate dynamic MTs in areas of active remodeling, such as the leading edge of migrating cells. This raises the possibility that activated Rac1 and Cdc42 could provide docking sites for MT plus ends near the cortex through IQGAP1 and CLIP-170 and could reinforce cell polarization by establishing a polarized MT array. In support of this, expression of a putative constitutively active mutant of IQGAP1, which is defective in Rac1/Cdc42 binding and bypasses the stimulatory effect of Rac1/Cdc42 on the CLIP-170-IQGAP1 interaction, induces multiple leading edges (Fukata et al. 2002b). On the basis of these results, several groups have proposed that Rac1/Cdc42 marks special cortical sports where the IQGAP1 and CLIP-170 complex is targeted, leading to polarized MT arrays and cell polarization (Gundersen 2002; Fukata et al. 2003).

Listed above are three different pathways through which Rho-family GTPases capture and stabilize microtubules through their effectors near the cell cortex, leading to polarized cell morphology and directional cell migration. It is possible that these individual pathways might function in a hierarchical way to promote the polarized phenotype. For example, the complex composed of Rac1/Cdc42-IQGAP1-CLIP-170-MTs might be involved in the initial attachment of the MTs to the cortical regions, while the other complexes (RhoA-mDia-MTs and Cdc42-Par6-aPKC-GSK3beta-APC) might come in to stabilize and maintain the MTs at the cortical regions, respectively. Clearly much more needs to be elucidated about the compositions of and crosstalk between these GTPase-regulated polarization complexes. I will speculate that not only may IQGAP1 play a role in metastatic progression, but that the proteins listed above may also play a functional role in the progression to an invasive or metastatic phenotype by participating in pathways to target MTs to the cell cortex.

In summary, the functional analysis of the ΔGRD and CT IQGAP1 mutants suggest that IQGAP1 may promote metastasis by coordinating the MT and MF cytoskeleton at the leading edge of migrating cells and that this function may be required for polarization and directional cell migration. However, the CT mutant may also be affecting other aspects of endogenous IQGAP1 function that are essential for metastasis. To analyze the specific interactions that are regulated by CT, a wider array of IQGAP1 mutants may be needed. One way to identify these mutants is via the yeast "split hybrid system." This variation of the standard yeast two-hybrid assay utilizes a two-component reporter that positively reports disruptions of protein-protein interactions (Shih et al. 1996). Analyzing the CT mutant in the split two-hybrid system might be useful for delineating specific binding sites for individual binding partners or identifying mutations in this region that specifically disrupt IQGAP1 interactions with CLIP-170, E-cadherin or β-catenin.

IQGAP1 knockout mice exhibit gastric hyperplasia and dysplasia, but have no obvious defects in cell migration (Li et al. 2000). We have generated Murine Embyronic

Fibroblasts (MEFs) from IQGAP1-null mice and have started to analyze specific IQGAP1 functions *in vitro* with them. In addition to the ΔGRD and CT dominant-negative mutants that were described in Chapter 3, we are also analyzing a putative constitutively active (CA) mutant and a point mutant that no longer binds F-actin. The CA mutant is defective in Rac1/Cdc42 binding and bypasses the stimulatory effect of Rac1/Cdc42 on the CLIP-170-IQGAP1 interaction, thus inducing multiple leading edges when expressed in fibroblasts. This phenotype is similar to that seen when macrophages are treated with MT-destabilizing drugs, suggesting that this mutant causes mispolarization of the cell and mistargets MTs to the cell cortex. We are currently beginning experiments to assess how MEFs that express different IQGAP1 mutants either singly or in combination polarize in response to various extracellular stimuli.

We have shown a potentially positive role for IQGAP1 function in *in vivo* metastasis, whereas other groups have shown that loss of endogenous IQGAP1 by siRNA leads to a reduction of motility and invasion *in vitro* (Mataraza et al. 2003). As previously noted, IQGAP1 knockout mice get late-onset gastric hyperplasia and dysplasia (Li et al. 2000). By crossing IQGAP$^{-/-}$ mice to transgenic models of metastasis, we can analyze the role of IQGAP1 in spontaneous metastatic progression within physiological conditions. We showed in Chapter 2 that IQGAP1 expression was significantly enhanced in human progressive breast adenocarcinoma and metastatic prostate cancers. Therefore, the transgenic mouse models of these cancers may be an obvious start for analyzing IQGAP1 function in a spontaneous metastasis assay. Transgenic mice that carry the polyoma middle T oncogene under the transcriptional control of the mouse mammary tumor virus promoter/enhancer (MMTV-PyV-mT), for example, have been shown to be excellent models of human breast cancer progression. In MMTV-PyV-mT mice, four distinctly identifiable stages of tumor progression from premalignant stages to malignant stages occur in a single primary tumor focus and this malignant transition is followed by a high frequency of distant metastasis to the lung (Guy et al. 1992). Interbreeding IQGAP1 null mice with this transgenic model of mammary tumorigenesis and metastasis will allow us to specifically assess the function of IQGAP1 in disease progression under physiological

conditions, and may potentially lead to a greater understanding of the multi-stage nature of tumorigenesis.

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