THE ROLE OF R-RAS IN ARF6 ACTIVATION DOWNSTREAM OF THE HGF-C-MET SIGNAL TRANSDUCTION PATHWAY

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ABSTRACT

Epithelial cells grow as sheets that function as a selectively permeable barrier that is sealed together with an extensive network of junctions. Under normal circumstances, this network forces the cells to be non-motile. However, it is possible for these cells to become motile during embryonic morphogenesis and the metastasis of epithelial cancers. This movement is prompted by a variety of growth factors such as HGF that can cause epithelial cell line scattering and motility *in vitro*. The binding of HGF to its receptor c-Met has the ability to stimulate the activation of PI3-kinase and the small GTPase Ras. Ras and other subfamily members such as R-ras have been known to play a role in proliferation and cell survival as well as cell migration and morphology. The small GTPase Arf6, which regulates actin cytoskeletal organization and can be activated by HGF, has also been associated with, and is required for, epithelial cell movement. While it is known that R-Ras and Arf6 are both required for epithelial motility, the signaling pathway linking these components to cell shape changes remains unclear. Therefore, we hypothesize that R-ras and Arf6 are downstream of the HGF-cMet signal transduction pathway to regulate epithelial cell migration.

The involvement of the Ras related protein, R-Ras, within this pathway was initially investigated via a morphological study. R-ras activity was altered using either dominant negative or constitutively active mutants and cell morphology and migration was monitored. A reduction in R-ras activity inhibited cell spreading and scattering after stimulation with HGF while constant R-ras activity led to cell scattering without further addition of HGF, as had been previously shown. The migration promoted by active R-ras
was inhibited by the addition of SecinH3, a cytohesin inhibitor. These observations were further supported by western blot analysis of Arf6 activation in the mutant expressing cells. A decrease in R-ras activity reduced Arf6 activation in the presence of HGF. The constant activation of R-ras also caused a decrease in Arf6 activation in the presence of HGF. Together, this data shows that R-Ras is downstream of the HGF-c-Met signal transduction pathway and has the ability to alter cell shape and migration.
**TABLE OF CONTENTS**

List of Figures .............................................................................................................. vii
List of Abbreviations ................................................................................................. ix
Acknowledgments ........................................................................................................ x

CHAPTER 1: INTRODUCTION

Epithelial Cell Growth and Structure ................................................................... 1
Cell Migration ............................................................................................................. 2
HGF and c-Met Receptor ......................................................................................... 5
HGF and MDCK Cells ............................................................................................... 6
The Small GTPase Ras ............................................................................................... 7
The Ras Isoforms ........................................................................................................ 9
The Ras Family GTPase Related-Ras (R-ras) .......................................................... 10
The Small GTPase Arf6 ............................................................................................ 11
The Arf6 GEFS .......................................................................................................... 12
Connecting R-ras and Arf6 ...................................................................................... 15
CHAPTER 2: MATERIALS AND METHODS

Cell Culture and Transfection Method................................................................. 19

Antibodies, Plasmids, and Reagents........................................................................ 19

Immunofluorescence.................................................................................................. 20

Arf6 Pulldown Assay................................................................................................. 20

CHAPTER 3: The involvement of R-Ras in the HGF-induced Arf6 activation signal transduction pathway........................................................................................................ 22

Experiment 1: Use of T43N R-ras mutant.............................................................. 23

Experiment 2: Use of the G38V R-ras mutant......................................................... 25
CHAPTER 4: The morphological effects of R-Ras on cellular motility........................................... 28

Morphological Study Part 1- Effect of T43N R-ras................................................................. 29

Morphological Study Part 2- Effect of G38V R-ras............................................................. 34

Inhibition of G38V-induced migration by SecinH3............................................................... 35

CHAPTER 5: DISCUSSION........................................................................................................... 38

REFERENCES............................................................................................................................ 46
List of Figures

Chapter 1: Introduction

1.1: Epithelial Cell Migration on a 2-D substrate
1.2: Introduction to GTPases
1.3: Arf6 Guanine Nucleotide Exchange Factors (GEFs)

Chapter 3: The involvement of R-Ras in the HGF-induced Arf6 activation signal transduction pathway

3.1: The proposed signaling pathway leading from HGF to active Arf6
3.2A: Dominant negative R-Ras (T43N) decreases Arf6 activation
3.2B: Dominant negative R-Ras (T43N) decreases Arf6 activation
3.3A: Constitutively active R-ras (G38V) increases Arf6 activation in the presence of HGF
3.3B: Constitutively active R-ras (G38V) increases Arf6 activation in the presence of HGF

Chapter 4: The morphological effects of R-Ras on cellular motility

4.1A: Dominant negative R-Ras (T43N) inhibits cell spreading and scattering in MDCK cells (0 hours)
4.1B: Dominant negative R-Ras (T43N) inhibits cell spreading and scattering in MDCK cells (4 hours)
4.1C: Dominant negative R-Ras (T43N) inhibits cell spreading and scattering in MDCK cells (18 hours)

4.2: Dominant negative R-ras (T43N) inhibits typical cell migration in the presence of HGF

4.3: Dominant negative R-ras (T43N) inhibits cell scattering after 18 hours of HGF incubation

4.4: Constitutively active R-ras (G38V) can induce cell spreading and scattering in the absence of HGF

4.5: The cytohesin inhibitor, SecinH3, reduces cell migration induced by constitutively active R-ras.

Chapter 5: Discussion

5.1: Newly proposed pathway leading from HGF to Arf6 activation
List of Abbreviations

EMT- Epithelial to Mesenchymal transition
GTPase- Guanosine Triphosphatase
HGF/SF- Hepatoocyte Growth Factor/ Scatter Factor
PI3-K- Phosphotidylinositide 3-kinase
MDCK- Madin Darby Canine Kidney cells
GTP- Guanosine triphosphate
GDP- Guanosine diphosphate
GEF- Guanine nucleotide exchange factor
GAP- GTPase activating protein
Arf6- ADP-ribsoylation factor 6
ARNO- ARF nucleotide binding site opener
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CHAPTER 1 - INTRODUCTION

Epithelial Cell Growth and Structure

Epithelial cells grow as sheets connected through a system of cell-to-cell and cell-to-matrix junctions. These sheets act as a selectively permeable barrier, separating the compartments of the body. This highly organized barrier allows for the polarization of the cell and therefore transport between compartments (Balkovetz 1998). Occluding and adhering junctions are two main types of adhesive elements responsible for this tissue architecture. Occluding junctions, such as tight junctions, form cell-to-cell contacts and create the selectively permeable barrier. Adhering junctions, such as cadherins and integrins, form contacts between adjacent cells and the extracellular matrix, which is crucial in maintaining tissue integrity (Gumbiner 1996).

This type of structure and organization usually leaves the epithelial cells immobile. However, during embryonic morphogenesis, tissue repair and regeneration cells can become motile. Embryonic development is coordinated by cell migration where collectively migrating cells form the embryo and subsequent tissues and organs (Ridley, Schwartz et al. 2003). For mature epithelia to become motile, the sheet of cells must be triggered to move by a chemotactic factor such as hepatocyte growth factor. These factors bind through cell surface receptors and tranduce signals, which induce actin cytoskeleton rearrangements, activate vesicular transport and promote re-polarization.
2D Cell Migration

The earliest stages of epithelial cell migration can be seen in a developing embryo during compaction of the blastula. For the formation of more complex body structures, epithelial cells must transition from their restrained phenotype to one that has an enhanced migratory capacity and increased invasiveness such as that of the mesenchymal cell. These two cell phenotypes are a hallmark of normal development and allow for a highly dynamic cellular environment. The conversion between epithelial and mesenchymal cells is a process called Epithelial to Mesenchymal Transition (EMT) (Kalluri and Weinberg 2009). EMT starts with the dissolution of tight, adherens, and gap junctions. This allows for the mixing of apical and basolateral membrane components and the degradation of the basement membrane. Next, cell surface proteins such as E-cadherin and epithelial-specific integrins are replaced by N-cadherin and a more transient type of integrin specific to mesenchymal migration. This dissolves connections with neighboring cells and the basement membrane. Eventually, the cell will take on a spindle like shape and forego its cuboidal, epithelial shape. The upregulation of N-cadherin and extracellular component binding integrins drives the cell to migrate in the direction of extracellular cues and away from the epithelial layer in which it was derived (Kalluri and Weinberg 2009; Micalizzi, Farabaugh et al. 2010).

The first step in single cell migration, which occurs after the completion of full EMT, is the formation of a membrane protrusion at the leading edge (Rorth 2009). This fingerlike protrusion is pushed forward by polymerized actin, which has been rearranged by activated small GTPases Rac and Cdc42. The protrusion can be a large, broad lamellipodia
or a spike-like filopodia. Next, the protrusion will adhere to the extracellular matrix for stability. The newly formed adhesions serve as footing for the cell as it moves forward and eventually mature into focal adhesions. Another small GTPase, Rho, then regulates the translocation of the cell. Acto-myosin filaments are assembled and contract, propelling the cell body in the direction of movement. Finally, the trailing end of the cell will retract by disassembling cell-substrate adhesive structures and the migration cycle can continue (Ridley, Schwartz et al. 2003; Yamazaki, Kurisu et al. 2005). This process can be seen in Figure 1.1.
Figure 1.1: Epithelial Cell Migration on a 2-D substrate. Migration consists of four consecutive processes: protrusion, adhesion, translocation, and retraction. All four are required for progressive cell movement (adapted from Yamazaki, Kurisu et al. 2005).

EMT provides the flexibility to transition between cell types, which is required for single cell migration. However, it is not always necessary to undergo a full epithelial to mesenchymal transition to migrate. Epithelial plasticity requires some disruption of the epithelial tissues fixed position and the adoption of some mesenchymal properties. An example of a process that does not require a full epithelial to mesenchymal transition is wound healing (Micalizzi, Farabaugh et al. 2010). This is a type of collective cell migration
in which the cells on either edge of an injury migrate towards each other in order to close the open wound. Collective cell migration is advantageous in this situation because it allows the tissue to stay continuous during the remodeling process and helps retain the tissue integrity (Rorth 2009). Because the cells are migrating as a collective group, they maintain cell-cell contacts and do not experience a full EMT. However, the leading edge cells display mesenchymal properties such as developing a cell protrusion, which exhibits directional cell movement. The leading edge cells also lose apical-basal polarity but manage to maintain the basic epithelial phenotype and completely retain this once the wound is closed (Micalizzi, Farabaugh et al. 2010).

Though the process of cell migration and EMT are critical during development and wound healing, properties of each have been connected to cancer metastasis. While the process of metastatic migration is not as well-defined, many human cancers have the basic features of EMT and inappropriately express regulators of this cellular transition and cell motility. Cancerous cells have the ability to reactivate epithelial developmental programs and manipulate environmental cues to increase invasion and migration, which will lead to metastatic spread (Micalizzi, Farabaugh et al. 2010).

**HGF and c-Met receptor**

A known regulator of epithelial plasticity and particularly potent epithelial motogen is hepatocyte growth factor or scattering factor (HGF/SF) (Birchmeier, Birchmeier et al. 2003). This polypeptide growth factor can not only induce cell motility but can stimulate
mitogenesis and the development of tissues (Balkovetz, Pollack et al. 1997). Synthesized as a single chain, which is largely inactive, the protein is later converted proteolytically into a 2-chain, active heterodimer. The amino-terminal fragment of this molecule, NK1, is the main receptor-binding site and causes receptor dimerization. c-Met, the only receptor for HGF, is a receptor tyrosine kinase that functions as a heterodimer (Birchmeier, Birchmeier et al. 2003). This transmembrane receptor is comprised of an α chain that is exposed at the cell surface and β chain that spans the plasma membrane. Both chains are required for HGF binding which in turn causes dimerization and the autophosphorylation of the receptor on tyrosines 1349 and 1356 (Stella and Comoglio 1999). Once phosphorylated, both sites are able to bind substrates such as the Grb2 adaptor for SOS, the Gab1 multiadaptor protein, phosphotidylinositol 3-kinase (PI3-K) and others. These protein transducers contain Src-homology 2 domain binding sites and can mediate interaction between downstream effectors. There are two major cascades activated following c-Met activation: the PI3-K and Ras/MAP kinase pathways. Coupling its p85 subunit to the c-Met receptor activates the PI3-K pathway and the Grb-2 adaptor protein links c-Met to SOS to activate the Ras/MAP kinase pathway (Zhang and VandeWoude 2003).

**HGF and MDCK cells**

c-Met activation stimulated by HGF binding can induce several different motogenic and morphogenic events, particularly in the Madin-Darby canine kidney (MDCK) epithelial cell line. When treated with HGF, cells in culture will experience a disruption in
intracellular junctions, take on a fibroblast morphology, and scatter away from colonies hence the name ‘scatter factor’ (Stoker 1989). MDCK cells grown within a collagen matrix will also undergo cell shape changes when treated with HGF. Not only will MDCK cells become more invasive in the presence of HGF but they will also form branching tubules (Weidner, Behrens et al. 1990; Montesano, Matsumoto et al. 1991). Research has also shown that both PI3-K and MAP kinase must be activated for HGF to induce scattering and branching tubulogenesis in MDCK cells. (Khawaja, Lehmann et al. 1998; Potempa and Ridley 1998). Their responsiveness to HGF in addition to the ability to form a continuous, polarized monolayer in culture are characteristics that make MDCK cells a convenient model in the study of epithelial motility.

**The Small GTPase Ras**

A key component in the activation of the MAP kinase and PI3-K signaling pathways is Ras. Ras is a small guanosine triphosphatase (GTPase), which acts as a molecular switch, cycling between an active and inactive form (Figure 1.2). While in the active form, Ras is bound to GTP and was prompted into this state by regulatory proteins called guanine-nucleotide exchange factors (GEFs). This protein facilitates the exchange between GDP and GTP. It is in the active form that Ras can interact with downstream effectors (Wennerberg, Rossman et al. 2005). For cycling to continue, a GTPase activating protein (GAP) must act on the GTPase and help to increase the rate of GTP hydrolysis. This will allow the hydrolysis of GTP to GDP and promote the formation of the inactive form. Another
important biochemical feature of Ras is its lipid modification. The c-terminal end of Ras is terminated with a CAAX motif, which is a sequence that determines the type of interactions in different membrane compartments (Brown and Sacks 2009).

![Diagram of GTPase cycle](image)

Figure 1.2: **Introduction to GTPases.** Small GTPases, such as Ras and Arf6, are proteins that cycle between an active and inactive state. During their active state, the protein is bound to GTP. During their inactive state, the protein is bound to GDP. Once in the active state, the GTP bound protein is free to interact with and activate downstream effectors.

The best characterized and most studied Ras signaling pathway is the MEK/ERK cascade. Ras-GTP binds to and recruits Raf kinase to the plasma membrane. Raf kinase becomes fully active and phosphorylates MEK1/2 protein kinase. This kinase then
phosphorylates and activates ERK1/2 protein, which is translocated to the nucleus. Activated ERK will phosphorylate transcription factors and consequently promote proliferation and differentiation (Wennerberg, Rossman et al. 2005).

In addition to Ras’ traditionally observed roles, it also plays a part in cell migration. Once activated, Ras can regulate adhesion molecules and cytoskeletal rearrangements that control migration and invasion. It has been shown that inhibition of Ras can prevent HGF-induced cell scattering and addition of activated Ras can induce cell spreading and actin reorganization (Ridley, Comoglio et al. 1995). This data demonstrates that Ras is essential in epithelial motility and more recent studies have implicated distinct Ras isoforms responsible for these effects.

**Ras Isoforms**

There are three different Ras isoforms: H-Ras, K-Ras, and N-Ras. These molecules have almost complete sequence identity in the N-terminal region, which contains the effector interaction sites and nucleotide binding regions. The hypervariable region (HVR), located at the C-terminal end, is the only area of sequence divergence (Omerovic, Laude et al. 2007). Though these isoforms use a common set of effectors and activators, they generate distinct outputs and have individual roles in the cell. Initial isoform knockout studies revealed that the isoforms are expressed in specific tissues and have different roles during development. It has also been found that the isoforms can be localized to different
microdomains in the plasma membrane because of additional, distinct HVR modifications (Rajalingam, Schreck et al. 2007).

These differences allow the isoforms to display functional diversity during cell migration. Each isoform has an effect on cellular motility; For example, it has been shown that H-Ras can promote proliferation and downregulate integrin affinity (Zhang, Vuori et al. 1996)(Kwong, Wozniak et al. 2003). K-ras has the ability to regulate the PI3-K/AKT signaling pathways while N-ras can regulate the MEK/ERK1 cascade responses and neither can substitute the other (Liao, Planchon et al. 2006).

**The Ras Family GTPase Related-Ras (R-ras)**

There is also another member of the Ras superfamily, R-ras, that has a role in cell motility. R-ras is 55% identical to and shares many common downstream effectors such as Raf1 and PI3-Kinase with the Ras isoforms; however, it acts in opposition to all three. Unlike H-, K-, and N-ras, R-ras can inhibit cell proliferation, promote cell spreading and adhesion, induce cell scattering and focal adhesion formation, and upregulate integrin affinity (Zhang, Vuori et al. 1996; Khawaja, Lehmann et al. 1998; Kwong, Wozniak et al. 2003; Goldfinger, Ptak et al. 2006). In order to perform these tasks, R-ras is targeted to the plasma membrane where it can co-localize with Arf6, a small GTPase involved in membrane trafficking, and β1 integrins. R-ras can then help to localize and group integrins to specific areas of the plasma membrane via its effector binding loop and control their endocytosis (Oertli, Han et al. 2000). This allows R-ras to regulate the recycling of integrins
and modulate cell adhesion and spreading (Furuhjelm and Peranen 2003; Conklin, Ada-
Nguema et al. 2010).

R-ras can accomplish its unique cellular functions with the help of an R-ras specific effector named RLIP76. This protein was found using a proteomic screen, which was used to isolate Ras-binding proteins (Goldfinger, Ptak et al. 2007). The study reveals that RLIP76 binds to only activated R-ras but not a dominant-negative version of R-ras, demonstrating that RLIP76 binds to R-ras in a GTP-dependent manner. RLIP76 is also required for R-ras induced cell spreading and appears to be a downstream effector of R-ras (Goldfinger, Ptak et al. 2006).

It has been found that cell spreading and migration can be stimulated by activated R-ras, which in turn can activate the Rho GTPase, Rac1 (Holly, Larson et al. 2005). Through its localization to the plasma membrane, Rac1 has the capacity to alter the actin cytoskeleton and produce changes to the cells leading edge such as lamellipodia formation (Heasman and Ridley 2008). Though this may be cell type specific, it has been found that R-ras and its effector RLIP76 are required for adhesion-induced Rac activation and directional cell migration (Wozniak, Kwong et al. 2005; Goldfinger, Ptak et al. 2006).

The small GTPase Arf6

Another important regulator of Rac activity is the small GTPase Arf6. The ADP-ribosylation factor proteins are also members of the Ras superfamily and are divided into three classes. Class I Arfs include Arf1, Arf2, and Arf3. This class is more than 96%
identical and regulates the assembly of coat complexes on vesicles involved in the secretory pathway. Class II Arfs include Arf4 and Arf5, which have a role in early golgi transport (D'Souza-Schorey and Chavrier 2006). Arf6 is the sole member of class III and can influence membrane trafficking and recycling and actin cytoskeletal changes at the plasma membrane. Early research showed that Arf6 is required for the conversion to a motile cell (Palacios, Price et al. 2001). It is now known that Arf6 can also regulate cell-cell adhesion by initiating a downregulation of Rac1 activity and promoting endocytosis of E-cadherin to disassemble adherens junctions during the early stages of migration. As the migration process moves forward, Arf6 is able to mediate endosomal recycling and recruitment of Rac1 to the plasma membrane, which induces modifications of the actin cytoskeleton and influences the formation of leading edge projections such as lamellipodia (Schweitzer, Sedgwick et al. 2010). Goldfinger et al. have shown that Rac localization and activation mediated by Arf6 is controlled by RLIP76 in that a knockdown of RLIP76 blocks adhesion-induced Arf6 activation.

The Arf6 GEFS

The cellular functions attributed to Arf6 are obviously complex and involve multiple sites of action suggesting that the protein is activated and inactivated in several areas places at the plasma membrane (Donaldson 2003; Schweitzer, Sedgwick et al. 2010). In order for Arf6 to become active, it requires a guanine nucleotide exchange factor (GEF), which stimulates GTP loading. Eukaryotic Arf GEFS can be divided in five different families
based on domain similarities and organization. These five families include GBF/BIG, cytohesins, EFA6, BRAGs, and Fbox (Figure 1.3). Each family of GEFs contain the Sec7 domain which is the central catalytic domain 200 amino acids long that is responsible for nucleotide exchange. Of the five families, Arf6 is known to interact with three of them: EFA6, BRAGs, and cytohesins.

Exchange factor for Arf6 (EFA6) was the first Arf6-specific GEF identified. The general structure of the EFA6 family is a Sec7 domain, a PH domain responsible for plasma membrane localization, and a coiled-coil domain, which is involved in actin cytoskeletal remodeling (Franco, Peters et al. 1999). The expression of EFA6 is accompanied by the reorganization of cortical actin, forming membrane ruffles via the activation of Rac1. These results indicate that EFA6 is coordinating membrane and actin remodeling by catalyzing the exchange of GDP for GTP therein activating Arf6 and Rac1 (Franco, Peters et al. 1999). EFA6 also plays a role in cell polarity development, however, both the Arf6-specific nucleotide exchange and coiled-coil domain are necessary in tight junction stabilization (Luton, Klein et al. 2004).

Brefeldin-resistant Arf GEFs (BRAG) are best characterized by the presence of an IQ-domain, which like the Sec7 domain, is catalytic in nature and is adjacent to the PH and coiled-coil domain (Casanova 2007). Of the BRAG family, BRAG2/Arf GEP_{100} has been found to preferentially increase the nucleotide exchange of Arf6 and partially colocalize with this and endosomal protein EEA1 at the plasma membrane. The depletion of Arf-GEP_{100} increases B1 integrin levels on the cell surface and effects cell adhesion. These findings show that Arf-GEP100 has a function at the cell periphery by regulating Arf6
activation, which can control endocytosis and integrin internalization (Someya, Sata et al. 2001; Dunphy, Moravec et al. 2006).

The ARNO/cytohesin family contain the catalytic Sec7 homology domain, the pleckstrin homology domain that allows for membrane targeting, and the N-terminal coiled-coil domain (Casanova 2007). Cytohesin-2 and -3 are ubiquitously expressed while cytohesins-1 and -4 are found primarily in specific cell types. These ArfGEFS can be found mainly at the plasma membrane and could be recruited to this location through several different models. One study suggests that cytohesins can be recruited via an interaction with phosphoinositides and inositol phospholipids in response to PI3-K signaling (Klarlund, Guilherme et al. 1997). However, there is another model that suggests Arf6 binding is responsible for ARNO membrane recruitment. The interaction between ARNO and Arf6 occurs at the PH domain and allows active Arf6 to recruit ARNO to the plasma membrane (Cohen, Honda et al. 2007).

Unlike the EFA and BRAG families, the cytohesins are rather promiscuous in their substrate binding. Although it appears that Arf6 and ARNO colocalize at the plasma membrane suggesting Arf6 is ARNO’s primary substrate, Arf1 also has regulating roles at the plasma membrane and potentially interacts with ARNO (Santy 2001). An overexpression of ARNO can overcome defects in cell spreading and this rescue can be reversed by the reduction in Arf1 or Arf6 activity implicating both GTPases in connection to ARNO (Goldfinger, Ptak et al. 2006). Therefore, ARNO may activate Arf1 in a subsequent GTPase cascade implying that Arf6 can be both an effector and a substrate of the cytohesins (Casanova 2007; Cohen, Honda et al. 2007).
Figure 1.3: **Arf6 Guanine Nucleotide Exchange Factors (GEFs)**. The cytohesins, EFA6, and BRAG family are able to exchange a guanine nucleotide to activate the small GTPase Arf6. All of these families contain the catalytic Sec7 domain used during guanine exchange (adapted from Casanova 2007).

**Connecting R-ras to Arf6**

This family of cytohesins, specifically ARNO, has also been implicated in epithelial cell migration. ARNO has been shown to have the ability to alter the actin cytoskeleton and change cell shape. ARNO overexpression induces cell scattering in MDCK cells, which
mimics scattering induced by HGF (Santy 2001). RLIP76 also has an effect on the Arf-GEF ARNO. The two proteins physically associate, which leads to Rac localization and subsequent cell spreading. The knockdown of either RLIP76 or ARNO was sufficient enough to block cell spreading and lamellipodia formation. Therefore it appears that RLIP76 is the key linker of R-ras with adhesion-induced Arf6 and Rac1 activation (Goldfinger, Ptak et al. 2006).

**Hypothesis**

While it is known that Ras and ARF6 are both required for epithelial motility, the signaling pathway linking these components to cell shape changes remains unclear. Hepatocyte growth factor/Scatter factor is a potent motogen, which can cause cell spreading and scattering in MDCK cells. The small GTPase R-ras is able to mimic this spreading and scattering as well as induce branching tubulogenesis when the activated form is expressed in this same cell line implicating it as a possible downstream effector of Met. R-ras also has a role in adhesion-induced activation of Arf6, a GTPase that is also downstream of c-Met; however, the two GTPases have never been linked via a pathway involving HGF. Therefore, I hypothesize that R-Ras activation stimulated by HGF will promote Arf6 activation downstream of c-Met and alter the cell’s shape and morphology.
Aims of Study

1. Determine R-ras involvement in the HGF-induced Arf6 activation signal transduction pathway.
2. Observe the morphological cell shape changes stimulated by R-ras in the presence and absence of HGF.

Specific Aim 1

Using biochemical methods, I would like to show that R-ras has a part in the Arf6 activation pathway. In order to show this I will use different mutants of R-ras to inhibit or amplify the GTPase’s activity and then examine the Arf6 activation response to each. This will demonstrate if R-ras, the downstream effector activated by HGF, can stimulate Arf6, establishing the role of the two GTPases in the cell motility signal transduction pathway.
Specific Aim 2

It has been found that Ras, specifically R-ras, can alter cell shape and morphology in response to HGF (Khawaja, Lehmann et al. 1998). This R-ras induced cell migration was reproduced to support the findings in Specific Aim 1, showing that R-ras has a regulatory role in normal HGF-induced scattering. Using the mutants of R-ras described in specific aim 1, I will show how they each affect MDCK cell morphology in the presence and absence of HGF.
CHAPTER 2 - MATERIALS AND METHODS

Cell culture and Transfection Method
The T23 line of MDCK cells were maintained in DMEM supplemented with penicillin, streptomycin, and fungizone, and 10% fetal bovine serum. Cells were cultured at 37°C in a 5% CO₂ incubator. Transient transfection of this cell line was done using the Neon transfection system (Invitrogen) according to the manufacturers instructions. The 1400 30 1 setting was used to perform the electroporation. The amount of DNA used was as follows: 5 µg of T43N and G38V R-ras, 4 µg of HA-Arf6, and 2 µg of pcDNA3 (control plasmid). The amount of cells used per 6 cm plate were as follows: 1X10⁶ control cells and 2X10⁶ T43N R-ras cells per Arf6 pulldown experiment, 1X10⁶ control and G38V R-ras cells per Arf6 pulldown experiment, 1X10⁶ for all transfections during immunofluorescence up to 4 hours and 7.5X10⁵ control, T43N and G38V R-ras cells for 18 hour immunofluorescence. After transfection, cells were allowed to recover for 18 hours.

Antibodies, Plasmids and Reagents
The following antibodies were used for western blotting and immunofluorescence: rabbit anti-Arf6 (a gift from Jim Casanova, University of Virginia), mouse anti-HA (Covance), mouse anti-myc 9E10 (Covance), and horseradish peroxidase goat anti-rabbit and goat anti-mouse as secondary antibodies (Invitrogen). Rhodamine-conjugated Phalloidin and Alexa 488 conjugated anti-mouse antibodies were also used during immunofluorescence
and purchased from Covance. Recombinant Human HGF was obtained from Peprotech. SecinH3 was obtained from Calbiochem. HA-Arf6 was a gift from Jim Casanova. Myc-G38V and myc-T43N R-Ras were gifts from Larry Goldfinger, University of California, San Diego. T43N R-Ras was subcloned into the pCXN2 vector using EcoR1 and Xba1 sites. The control vector used in all experiments was pcDNA3.

**Immunofluorescence**

MDCK cells were transfected and plated on a glass coverslips coated with 30 µg/ml fibronectin. Cells were allowed to recover for 18 hours and were then incubated in the presence or absence of 60ng/ml HGF. Cells transfected with T34N R-Ras were incubated with HGF for 0, 4, and 18 hours. Cells transfected with G38V R-Ras were incubated in the absence of HGF or presence of 30 µM SecinH3 for up to 32 hours. After incubation, cells were fixed with 4% paraformaldehyde in PBS, blocked with PBS containing 10% normal goat serum and 0.2% saponin, and stained with mouse anti-myc antibody followed by staining with alexa 488 conjugated anti mouse antibody and Rhodamine-conjugated phalloidin. The cells were observed with an Olympus 1X 81 microscope and Hamamatsu Orca Camera. The software used was Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO).

**Arf6 Pulldown Assay**

MDCK cells were incubated in the presence or absence of 30ng/ml HGF for 5-6 hours and lysed at 4°C in 0.65ml of 200mM NaCl, 50mM Tris, pH 7.5, 10mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5% glycerol, 1mM DTT, and protease inhibitors.
Lysates were cleared by centrifugation at 14,000 rpm for 2 minutes at 4°C in the presence of CL-4B Sepharose beads. Arf6-GTP was isolated using glutathione S-transferase (GST)-GGA3 beads. 0.5 ml of the cleared lysate was incubated with the 40 µg of the GGA3 beads for 30 minutes at 4°C. The results of this assay were analyzed by western blotting. Quatification of the blots was done using ImageJ. Levels of active Arf6 were normalized to the total amount of Arf6 in the starting lysate. Normalized levels of active Arf6 in R-ras expressing cells in the presence of HGF was then divided by normalized levels of active Arf6 in the absence of HGF in control cells. Differences in Arf6 activation were analyzed for significance using a paired t test.
CHAPTER 3: The involvement of R-Ras in the HGF-induced Arf6 activation signal transduction pathway.

Epithelial cell migration is triggered by growth factors such as HGF. Once HGF is bound to its cell surface receptor c-Met, a signal transduction pathway is initiated. The binding of HGF and subsequent c-Met activation causes the activation of downstream effectors such as Ras and PI3-K. These interactions can trigger actin cytoskeleton rearrangements, loss of cell-to-cell contacts and vesicular transport via the activation of small GTPases, such as Arf6. A member of the Ras superfamily, R-ras, also has a part in cell migration. While it is 55% identical to Ras and interacts with many of the same downstream effectors, R-ras has some unique functions and effectors different from the traditional form of Ras. R-ras can have an effect on cell spreading in MDCK cells in that activated R-ras can induce scattering and tubulogenesis suggesting it is an important component of the normal cellular response downstream of c-Met. All of these results point to the possibility of R-ras and Arf6 acting together in the HGF signal transduction pathway. At present, the two have not been linked via HGF. Figure 3.1 shows the proposed pathway. Therefore, the role of R-ras in the activation of Arf6 via HGF will be examined. In order to determine R-ras’ involvement, different mutants were used: T43N, the dominant negative form of R-ras, and G38V, the constitutively active form of R-ras.
Figure 3.1: The proposed signaling pathway leading from HGF to active Arf6. The pathway proposed shows that c-Met could activate Ras, which, through a protein scaffold and the Arf6 GEF ARNO, activates Arf6 leading to epithelial cell movement.

EXPERIMENT ONE: USE OF THE T43N R-RAS MUTANT

In the first experiment, dominant negative T43N R-ras was used to dramatically decrease R-ras activity and potentially disrupt the proposed pathway. The dominant negative mutant is one that is constantly in the inactive, GDP-bound form. This means that
the expressed mutant will not be able to cycle to the active, GTP-bound state. The mutant GTPase hinders endogenous R-ras activation by sequestering the GEFs needed to continue cycling between the active and inactive state. Though this does dramatically reduce R-ras activity, it is not a complete knockdown and some residual endogenous R-ras activation exists. MDCK cells were transiently transfected with T43N R-ras and incubated in the presence or absence of HGF for 6 hours. The earliest signs of robust Arf6 activation in control cells can be seen after 6 hours of incubation in HGF (Figure 3.2A, B). The cells transfected with R-ras showed markedly reduced Arf6 activation after incubation with HGF (Figure 3.2A, B). Therefore, these results imply that R-ras plays a role in HGF-induced Arf6 activation.

![Figure 3.2A: Dominant negative R-Ras (T43N) decreases Arf6 activation.](image)

MDCK cells were transfected with myc-T43N R-Ras or pcDNA3 (empty vector). Transfected cells were allowed to recover for 18 hours and incubated in the presence or absence of 60 ng/ml HGF.
for 6 hours. Arf6 activity was assessed using the GST-GGA3 pulldown assay, which isolates only active Arf6. The results of this assay were analyzed via western blot detecting endogenous Arf6 and Myc. 3.2A shows a representative gel from a pulldown experiment quantified in 2B.

Figure 3.2B: Dominant negative R-Ras (T43N) decreases Arf6 activation. Activation of Arf6 in MDCK cells expressing T43N R-Ras or PCDNA3 (empty vector) in the presence (+) or absence (-) of HGF. Data shown is mean ± standard error of 5 independent experiments.
EXPERIMENT TWO: USE OF THE G38V R-RAS MUTANT

The second experiment used a constitutively active form of R-ras, G38V, in order to further examine the role of R-ras in the proposed signal transduction pathway. This form of R-ras is constantly in the GTP-bound state. MDCK cells were co-transfected with G38V and HA-Arf6 in order to observe strong Arf6 activation. These cells were incubated in the presence or absence of HGF for 5 hours. Control cells showed strong activation of HA-Arf6 after 5 hours of HGF incubation. The G38V expressing cells in the presence of HGF showed a decrease in HA-Arf6 activation when compared to the HGF+ control. The fact that Arf6 activation is not increased similarly to the control cells in the presence of HGF could be due to the downregulation of pathway components as a result of R-ras hyperactivation. Additional trials in the presence and absence of HGF in G38V R-ras expressing cells are necessary to fully verify if there is a significant increase in Arf6 activation. These results along with the G38V R-ras morphological study presented in Chapter 4 allude to the conclusion that R-ras has a role in the HGF-induced Arf6 activation signal transduction pathway.
Figure 3.3A: **Constitutively active R-ras (G38V) decreases Arf6 activation in the presence of HGF.** MDCK cells were transfected with either myc-G38V or pcDNA3 (empty vector). Transfected were allowed to recover for 18 hours and incubated in the presence (+) or absence (-) of 30 ng/ml HGF for 5 hours. Arf6 activity was assessed using the GST-GGA3 pulldown assay, which isolates only active Arf6. The results of this assay were analyzed via western blot targeting HA-Arf6 and Myc. 3.3A shows a representative gel from a pulldown experiment quantified in 3.3B.
Figure 3.3B: Constitutively Active R-ras (G38V) decreases Arf6 activation in the presence of HGF. Quantification of western blots show in 3.3A. Shown is the activation of Arf6 in MDCK cells expressing G38V R-ras or pcDNA3 (empty vector) in the presence (+) and absence (-) of HGF. Data shown is mean ± standard error of 5 independent experiments.
CHAPTER 4: The morphological effects of R-Ras on cellular motility.

Epithelial cell motility is characterized by an epithelial-to-mesenchymal transition. This allows the cell to move from a rigid, fixed structure to a motile, more invasive cell phenotype. During this process, the cell will dissolve its network of junctions, lose polarization, and take on a spindle like shape. Once EMT is completed, the cell can form leading edge protrusions, adhere to surrounding ECM that will act as traction for the moving cell, translocate the cell body in the direction of cell movement, and retract the tail end to accomplish coordinated motility. The small Ras-related GTPase, R-ras, has been implicated in this process of cell spreading and migration. R-ras can promote cell adhesion by regulating integrin function and focal adhesion formation (Kwong, Wozniak et al. 2003). Leading edge membrane protrusions formed by actin cytoskeletal rearrangements are also regulated by R-ras and its control over Rac1 and Rho GTPases (Wozniak, Kwong et al. 2005; Ada-Nguema, Xenias et al. 2006). Activated R-ras can also induce scattering and tubulogenesis in MDCK cells in the same manner as HGF-induced scattering in the same cell line (Khawaja, Lehmann et al. 1998). Therefore, I wanted to observe the morphological changes induced by R-ras in MDCK cells in the presence or absence of HGF.
Morphological Study Part 1- Effect of T43N R-ras

Initially, MDCK cells were transfected with T43N dominant negative R-ras to determine whether a defect in cell migration would occur. The transfected cells were then incubated in the presence or absence of HGF for 0, 4, or 18 hours. This allowed for the MDCK morphology changes to be observed over time as compared to the control cells. After 0 hours, both the control and T43N transfected cells showed no migration attempt and remained stationary in small, confined islands (Figure 4.1A).

After 4 hours in the presence of HGF, control cells show moderate cell spreading and the beginnings of cell scattering. The cells appear flattened and have formed leading edge projections away from the grouping of cells indicative of cell spreading. Some cells have even begun scattering in which they have completely separated and migrated away from other cells. However, T43N R-ras transfected cells show very little cell spreading and no cell scattering after 4 hours of incubation with HGF. The majority of the cells remain part of the cellular island while few have formed flattened, front-end projections. Not only are these forward projections scarce but they also appear abnormal when compared to the control (Figure 4.1B).

After 18 hours in the presence of HGF, control cells showed almost complete cell scattering. Of these cells, 65% were completely scattered (Figure 4.3). Cells were no longer confined to islands and were migrating away from one another. The T43N transfected cells, however, showed only a limited amount of cell spreading, though more abundant that after 4 hours of incubation with HGF (Figure 4.1C). The spreading observed in this group did appear more pronounced in that the front-end projections had migrated further away.
from cell groupings (Figure 4.2). Only 5% of the T43N cells also showed scattering, even after 18 hours of incubation with HGF (Figure 4.3). Therefore, a decrease in R-ras activity impairs the cells normal migratory function and alters the cells morphology and shape during this process.

Figure 4.1A: Dominant negative R-Ras (T43N) inhibits cell spreading and scattering in MDCK cells (0 hours). MDCK cells were transfected with T43N r-ras or pcDNA3 and plated on fibronectin-coated coverslips. Cells were then incubated in the absence of HGF and prepared for immunofluorescence through fixing and staining with mouse anti-myc antibody followed by Alexa 488 conjugated anti-mouse antibody and Rhodamine phalloidin.
Figure 4.1B: Dominant negative R-Ras (T43N) inhibits cell spreading and scattering in MDCK cells (4 Hours). MDCK cells were transfected with T43N R-ras or pcDNA3 and plated on fibronectin-coated coverslips. Cells were allowed to recover for 18 hours and then incubated in the presence of HGF for 4 hours. Immunofluorescence was done through fixing and staining with mouse anti-myc followed by Alexa 488 conjugated anti-mouse antibody and Rhodamine phalloidin.
Figure 4.1C: **Dominant negative R-Ras (T43N) inhibits cell spreading and scattering in MDCK cells (18 hours).** MDCK cells were transfected with T43N R-ras or pcDNA3 and plated on fibronectin-coated coverslips. Cells were allowed to recover for 18 hours and then incubated in the presence of HGF for 18 hours. Immunofluorescence was done through fixing and staining with mouse anti-myc followed by Alexa 488 conjugated anti-mouse antibody and Rhodamine phalloidin.
Figure 4.2: **Dominant negative R-ras (T43N) inhibits typical cell migration in the presence of HGF.** Displayed are four representative pictures of cells expressing T43N R-ras. Their ability to migrate, even in the presence of HGF for 18 hours, is impaired compared to the control cells. They exhibit delayed cell spreading and inhibited cell scattering.
Figure 4.3: **Dominant negative R-ras (T43N) inhibits cell scattering after 18 hours of HGF incubation.** MDCK cells were transfected with T43N R-ras or pcDNA3 (control) and were incubated in the presence of HGF for 18 hours. The percent scattered cells was quantified as the number of scattered cells divided by the total amount of cells. A scattered cell is defined as a cell that has formed a tail end process pointing away from the direction of movement, which is a hallmark of a scattered cell. Data shown is mean ± standard error of 3 independent experiments.
**Morphological Study Part 2- Effect of G38V R-ras**

Next, MDCK cells were transfected with the constitutively active R-ras to determine if cell spreading and scattering could be induced in the absence of HGF. Cells were transfected with G38V R-ras and incubated in the absence of HGF for 32 hours. After 32 hours, transfected cells had begun to scatter when compared to the control (Figure 4.4). This result implies that R-ras is a part of the normal functioning cell migration pathway and once activated can induce cell spreading and scattering.

The G38V R-ras cells were also incubated in the presence of SecinH3, a cytohesin inhibitor, to test cytohesin involvement in cell movement downstream of R-ras activation. In the absence of secinH3, cells are able to spread and scatter easily after 32 hours. In the presence of secinH3, it appears that cells are able to spread but experience inhibited cell scattering. Figure 4.5 shows the secinH3 treated cells forming leading edge lamellipodia but are still maintaining cell island contacts, even after 32 hours. The disruption in normal cell migration shows that cytohesins have a function downstream of R-ras activation.
Figure 4.4: **Constitutively active R-ras (G38V) can induce cell spreading and scattering in the absence of HGF.** MDCK cells were transfected with G38V R-ras or pcDNA3 and plated on fibronectin-coated coverslips. Transfected cells were allowed to recover for 32 hours and were prepared for Immunofluorescence. Cells were stained with mouse anti-myc followed by Alexa 488 conjugated anti-mouse antibody and Rhodamine phalloidin.
Figure 4.5: The cytohesin inhibitor, SecinH3, reduces cell migration induced by constitutively active R-ras. MDCK cells were transfected with G38V R-ras and plated on fibronectin-coated coverslips. Transfected cells were allowed to recover for 12 hours and were then incubated with 30 µm SecinH3 for 24 hours. Preparation for immunofluorescence was as follows: fixing and staining with mouse anti-myc followed by Alexa 488 conjugated anti-mouse antibody and Rhodamine palloidin.
Chapter 5: Discussion

In 2007, cancer was the 2nd leading cause of death in the United States causing 23.2% of all deaths (Jemal, Siegel et al. 2010). The most common types of cancer are of the lung, stomach, breast, colon/rectum, and uterine cervix, which are all cancers of the epithelia or carcinomas that account for 90% of human cancers (Alberts, Lewis et al. 2002). If these cancers progress to a stage of metastasis, chance for survival significantly decreases and is responsible for 90% of cancer deaths (Christofori 2006). Metastasis is a process in which the primary tumor will leave its initial site, invade surrounding tissue, enter the blood stream, and travel to a secondary site. While cell migration is critical for cancer cell metastasis, it is also necessary for embryonic development and wound healing. Therefore, some of the basic underlying molecular mechanisms involved are common to both normal and cancerous cell movement (Yamazaki, Kurisu et al. 2005). Germline and somatic mutations and gene amplifications in cell migration components can lead to cell transformation, which causes a normally migrating cell to become invasive and metastatic. For example, an increase in c-Met activity are frequently found in various cancer types and are considered key initiators of invasive growth and tumor progression (Christofori 2006). The HGF-c-Met receptor-signaling pathway is also important for typical epithelial cell movement and change in cell morphology in a normally functioning cell. Therefore, an understanding of cell migration in non-neoplastic cells will lead to help in understanding invasion and metastasis in cancerous cells and could uncover potential therapeutic targets.

A number of potential targets are members of the Ras superfamily of GTPases, which are commonly mutated in malignancies. Several proteins of this family that are
frequently mutated in human cancers and leukemias are the isoforms N-ras, K-ras and H-ras. Besides these 3 Ras proteins, another Ras subfamily member, R-ras, has also been implicated in transformation, invasion, and metastasis of several different cancer types (Ehrhardt, Ehrhardt et al. 2002). R-ras has been implicated in malignant transformation of fibroblasts, initiating migration and invasion in breast cancer cells, and promoting metastasis in cervical cancer epithelial cells (Cox, Brtva et al. 1994; Keely, Rusyn et al. 1999; Mora, Rosales et al. 2007). Until recently, little was known about R-ras’ downstream effectors and distinct cellular functions. It has been uncovered that R-ras has a role in cell migration and morphology through integrin regulation and endocytosis. The exact mechanism of how R-ras is promoting transformation and subsequent metastasis is relatively unknown (Gawecka, Griffiths et al. 2010). Though some progress has been made in this field, more study of R-ras and its function in normal cell migration is necessary to understand how this process has become aberrant in cancerous cells.

As well as controlling integrin endocytosis and activation, R-ras has a function in promoting cell adhesion and spreading during migration by coupling with PI3-K and its own unique effector RLIP76. This allows for downstream Rac activation through the activation of Arf6 GTPase and its GEF ARNO. Subsequent actin cytoskeletal rearrangements result from this activation and produce leading edge protrusions and progressive cell movement. Though we know that all of these components are involved in cell motility, the signaling pathway linking them together is unknown.
Biochemical Study of R-ras and its effect on Arf6 Activation

This study has connected R-ras to Arf6 activation via the HGF-c-Met signal transduction pathway in epithelial cell migration. When the activity of R-ras was reduced using a dominant negative mutant, the activation of endogenous Arf6 was reduced in the presence of HGF while Arf6 activation was promoted in the control MDCK cells in the presence of HGF. These results lead us to believe that R-ras is downstream of the c-Met receptor tyrosine kinase.

To further substantiate this result, the involvement of cytohesins in this pathway must be explored. The cytohesin family, more specifically cytohesin 2/ARNO, has been found to be necessary for β1 integrin recycling therein possibly connecting R-ras, however, the two have never been directly linked in cell migration (Oh and Santy 2010). To test this biochemically, ARNO activity would need to be decreased or eliminated and Arf6 activation in the presence of HGF would be examined via a pulldown assay and western blot. We hypothesize that, after the reduction of ARNO activity, Arf6 activation would be decreased in the presence of HGF. This result would support ARNO’s involvement in the HGF-c-Met signaling pathway.

Also, previous data shows that cytohesins are downstream of R-ras through the interaction between ARNO and the R-ras effector RLIP76 (Goldfinger, Ptak et al. 2006). However, this interaction is weak and does not appear to be direct. We propose that this binding intermediate is CNK3, a scaffolding protein that has the ability to interact with the coil-coiled domain of ARNO to produce Arf6 activation and subsequent Rac activation. Though the interaction between ARNO and RLIP76 was discovered in an adhesion-induced
migration assay and could be the result of increased integrin signaling, this remains to be confirmed. Therefore, an investigation of the interaction between RLIP76 and CNK3 is necessary to fully establish the connection between R-ras with ARNO downstream of c-Met.

Also examined in this study was the effect of the constitutively active form of R-ras on Arf6 activation. Surprisingly, a decrease in Arf6 activation could be seen in the presence of HGF. As a result of the hyperactivation of R-ras, pathway components such as essential GEFs or recruitment proteins could have been downregulated over the course of the 32-hour experiment timeline, reducing Arf6 activation in the presence of HGF to less than that of the control. Further experiments examining expression and localization of R-ras and other effectors involved in the pathway are needed in order to confirm that R-ras is a component of the HGF-c-Met signaling pathway. Figure 5.1 shows the new proposed pathway containing R-ras and other potential components downstream of c-Met.
Figure 5.1: **Newly proposed signaling pathway leading from HGF to Arf6 activation.**

The new proposed pathway shows HGF activating R-ras and subsequently activating Arf6 through a series of interactions that involve the R-ras specific effector RLIP76, the scaffolding protein CNK3 and the Arf6 GEF ARNO.
Morphological Study of R-ras’ Involvement in Cell Spreading and Scattering

The morphological study was done to observe the effects R-ras can have on the cell’s ability to spread and scatter when treated with the epithelial motogen HGF. Not only did R-ras have an effect on Arf6 activation but it also affected their motility. First, the MDCK cells were transfected with dominant negative R-ras and incubated in the presence or absence of HGF for 0, 4, or 18 hours. The control cells grouped in an island at 0 hours spread away from each other starting at the group edges and finished by scattering and completely separating by 18 hours. The cells expressing dominant negative T43N R-ras, however, had begun very little scattering and experienced limited spreading even after 18 hours of HGF incubation. Only 5% of the T43N expressing cells had begun scattering after 18 hours of HGF exposure compared to the 65% cell scattering seen in the control cells. The T43N cells that were successful in spreading were located along the outer most edge of an island, which was similar to initial control cell movement.

The defects in spreading and scattering of the cells expressing dominant negative R-ras could not only be a result of blocking the HGF- c-Met signaling pathway but also from a lack of sufficient integrin signaling. Integrins are cell surface receptors that bind both the cell’s cytoskeleton and components of the extracellular matrix such as fibronectin, collagen, and laminin. This essential link allows integrins to relay signals from these ECM attachments to promote actin cytoskeletal rearrangements and activate intracellular signaling called outside-in signaling. Conversely, integrins can also be activated by intracellular triggers in a process called inside-out signaling where the integrin will experience an allosteric rearrangement of its extracellular domains and increases ligand
binding affinity (Kinbara, Goldfinger et al. 2003). Therefore, the activation of integrins and induction of signaling is bi-directional and highly regulated.

Cell-ECM adhesion is a key event in epithelial migration. Once a cell adheres to the extracellular matrix, integrin signaling can result in the induction of N-cadherin expression and supports the stimulation of EMT (Yilmaz and Christofori 2009). This permits the cell to take on mesenchymal-like properties and begin the formation of leading edge projections such as lamellipodia, which are anchored to the ECM via integrin contacts. Scattering and increased motility can be promoted by enhanced integrin adhesion. This is, however, not the only type of adhesion necessary to induce scattering supporting the notion that epithelial migration is a complex process with many important regulators (de Rooij, Kerstens et al. 2005).

One regulator of the bi-directional integrin activation and signaling and consequent migration is the GTPase R-ras. This G protein was first shown to regulate integrin function by increasing integrin affinity (Zhang, Vuori et al. 1996). It was later found that expression of activated R-ras could stimulate integrin-dependent migration in breast cancer cells by signaling through the integrin alpha cytoplasmic domain (Keely, Rusyn et al. 1999). It has also been suggested that a primary mechanism for driving tumor metastasis is enhanced endocytotic trafficking of integrins therein linking R-ras to these processes (Gawecka, Griffiths et al. 2010).

As a result of these studies, it was discovered that R-ras can act as an activator of integrins and also be activated downstream of integrin-mediated adhesion. These roles were discovered in the absence of HGF or other migration stimulants. However, it has been found that HGF can upregulate integrin-mediated adhesion suggesting that integrin
adhesion and the HGF-c-Met signaling pathway could be working in concert to promote motility. This could explain the hindered, but somewhat functional cell spreading observed in the T43N R-ras morphological study. The T43N expressing cells form lamellipodia from the edges of islands where integrin contacts and signaling would be the most abundant. This integrin signaling combined with residual endogenous R-ras activation via HGF could be sufficient to promote some cell spreading but not cell scattering. In order to induce scattering, this combination of signals would need to generate a higher degree of R-ras activity in order for the cell to continue to move forward. An investigation of integrin activity and localization after the depletion of R-ras would need to be conducted in order to test this hypothesis.

A morphological study of constitutively active R-ras’ effect on cell migration was also conducted. MDCK cells were transfected with G38V R-ras and were allowed to recover for at least 32 hours. Even in the absence of HGF, cell spreading and scattering was induced. Though HGF was absent from the G38V R-ras samples, the movement induced by constant R-ras activation mimicked the cell scattering stimulated by HGF implying that R-ras acts as a key regulator of cell migration downstream of c-Met.

Cells transfected with G38V R-ras were also incubated in the presence of secinH3, a cytohesin inhibitor. It was found that cell migration induced by the constant activation of R-ras was disrupted in the presence of secinH3. The cells were able to spread normally but were unable to continue migrating and scatter. These defects in cell migration were similar to those observed in the T43N R-ras expressing cells in the presence of HGF where lamellipodia are formed from the edges of cell groupings but the cells are unable to migrate any further. This somewhat active migration and lamellipodia formation also support the
theory that integrin signaling in addition to signaling downstream of Met may be necessary to promote migration. Therefore, this implies that in order to achieve full, efficient migration, both pathways must be intact and functional. Again, further investigation of integrin signaling in combination with HGF-c-Met signal transduction will be needed to confirm this conclusion.

This experiment also sheds light on the connection of cytohesins with R-ras. A member of the cytohesin family, ARNO, is a known GEF of Arf6, which this research has shown is downstream of R-ras and Met. However, R-ras and ARNO have never been directly linked. This study shows that cytohesins function downstream of R-ras to help promote cell migration, though western blot analysis examining Arf6 activation after cytohesin depletion will be necessary to fully verify this claim. In theory, secinH3 would block Arf6 activation indicating that cytohesins are downstream of R-ras.

Future morphological studies would include time-lapse imaging of cells expressing the different mutants of R-ras. This would provide a more in-depth look at exactly how the cells are migrating by examining different parameters such as migration paths, speed of migration, and localization of R-ras during movement.
References:


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