Spring 1-1-2012

Characterization of the Role of MEK1 Kinase Activity in TGF-Beta Induced Collective Cell Migration

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CHARACTERIZATION OF THE ROLE OF MEK1 KINASE ACTIVITY IN TGFβ INDUCED COLLECTIVE CELL MIGRATION

by

DOUGLAS ANDREW CHAPNICK

B.A., Colgate University, 2004

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Chemistry and Biochemistry
2012
This thesis entitled:
Characterization of the Role of MEK1 Kinase Activity in TGFβ Induced Collective Cell Migration
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has been approved for the Department of Chemistry and Biochemistry

_______________________________________
Xuedong Liu

_______________________________________
Robert Kuchta

Date__________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
ABSTRACT

Chapnick, Douglas Andrew (Ph.D., Biochemistry)

Characterization of the Role of MEK1 Kinase Activity in TGFβ Induced Collective Cell Migration

Thesis directed by Professor Xuedong Liu

The migration of individual cells and collective groups of cells has been repeatedly shown to be an important cellular behavior throughout normal biology and disease progression. Although a significant amount of research has been conducted to understand how an individual cell achieves migration, relatively little is known about how groups of cells coordinate their efforts to migrate collectively. Several protein cytokines, including Transforming Growth Factor Beta (TGFβ), have been identified that can be globally administered to cells in vitro, but yield highly directional collective cell migration. In this thesis, I detail my efforts in elucidating how TGFβ stimulation of the human keratinocyte cell line, HaCaT, results in directional and collective cell migration.

I developed an in vitro assay and computational analysis program, named Pathfinder, to accurately quantify the angular movements of individual cells in time-lapse microscopy videos. This innovative approach allowed me to measure the degree to which individual cells turn as they migrate and the degree to which neighboring cells adopt the same migration direction. Using my newly developed tools to measure cellular migration, I found that TGFβ stimulation of HaCaT cells...
indirectly activates MEK1 kinase activity, which is modulated by ligand induced cellular spreading. The resulting MEK1 kinase activity is required and sufficient to elicit collective cell migration in a group of physically interacting cells. I also showed that such collective cell migration is likely the result of the ability of MEK1 kinase activity to suppress turning behavior in individual cells, which contributes to the spontaneous induction of collective migration behavior in a group of cells. My findings contribute to the TGFβ field by providing an explanation for how MEK1 kinase can be activated by TGFβ receptor activation. In addition, my findings contribute to the collective cell migration field by detailing the important role of MEK1 activity in cellular steering, and providing an improved simple model for how collective migration can arise spontaneously.
ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Xuedong Liu, whose guidance over the years has significantly shaped my approach to science. I am especially grateful for him teaching me the value of balancing deconstructive criticism with constructive criticism. In addition, I have learned from him that obstacles are merely challenges, and not something to be discouraged by. I would also like to thank my thesis committee members Dr. Robert Kuchta, Dr. Natalie Ahn, Dr. Amy Palmer, and Dr. Kristi Anseth for their advice and guidance over the years. I am very grateful to have conducted my PhD at University of Colorado, as I have had the pleasure of interacting with countless people who have become my friends and have provided me with stimulating discussions and advice both inside of science and beyond. Tom Cheung, Eric Witze, Kristen Barthel, Dave Clarke, Kasey Couts, Theresa Nahreini, and Dana Ungemannova all have put in their time to laugh with me about serious things and complain about trivial ones. Particularly, Tom Cheung has been integral in helping me deconstruct incredibly complicated experimental observations and life choices. Recently, a new batch of scientists has additionally aided me in a similar way, and I am equally grateful to Gilson Sanchez, Eric Gunther, Ted Kee, and Zipei Feng for the discussions and time shared with me. I would also like to thank Jeremy Jacobsen, whose
programming skills were an indispensible component to the research that I have conducted for my thesis.

Working towards a PhD for seven years is not a walk in the park, and could not be done without the help of my friends outside of science. Peter Marcus, Tim Warnecke, Autumn York, J’aime Manion, Eryc Klein, Kyla Sand, Chris and Farrell Hall, Brian and Brianna Kearney, Glenn Pezzulo, Marc Mosello, Mike Persky, Aaron and Vanessa Pence and Andrew Thorpy were all instrumental in reminding me that life is not just science. Peter taught me to dust myself off when I get knocked down, and Andrew taught me that a successful adventure means you get knocked down.

My family also deserves extreme recognition, as they have shaped my personality and helped me through life from the beginning. My parents, Michael and Lucille Chapnick, fostered my interest in both science and art ever since I can remember. They also put up with the consequences of raising a moody scientist, which means there will always be hundreds of projects and animals strewn about the house. My siblings, Alistair Chapnick, Suzanne Chapnick, Joyce Phillips, and Julie Chapnick, and my siblings in-laws, Elyse Chapnick and Jason Phillips all helped positively influence my life. Alistair taught me compassion for all living things. Suzanne taught me to stand up for what I believe in. Joyce taught me to talk about my feelings, at least once in a while. Julie has always watched over and helped me when I needed it, whether that was when we were growing up, going to college near each other, or more recently.
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CHAPTER I

INTRODUCTION

Cellular Migration in Biology

Throughout each our lives, cells migrate within our bodies, propelling in some manner every single biological process. From embryo implantation [1] to cancer progression [2-5], individual cells are guided by biochemical and physical stimuli [6] that are interpreted by an extremely complex intracellular signal transduction network of proteins [1, 3, 4, 7-9]. Additionally, such stimuli can come from neighboring cells, resulting in local coordination amongst groups of cells in a process termed “collective migration” [10-13]. Currently, the cell migration field has identified a plethora of proteins and biochemical interactions that comprise the cell motility network, but still has a relatively large amount of information to gather before the dynamic flow of signal transduction is understood to the point that directional motility behavior can be predicted. In this chapter, I will describe how cell migration propels biological processes, the current models for individual and collective cell migration, and how Transforming Growth Factor Beta (TGFβ) and Mitogen Activated Protein Kinase Kinase 1 (MEK1) have been implicated in the regulation of cellular migration. Our ultimate challenge in the field of cell migration is to understand the structure and dynamics of the cell motility protein-protein interaction network for individual cells and groups of cells, so
that we may one day be able to completely and efficiently manipulate cell migration in order to treat disease and improve the standard of living.

**Individual Cell Migration**

Individual cells migrate through a cyclical process of front end extension and rear end retraction, which ultimately propels the cell forward [14]. A migrating cell exhibits morphological polarization, where the front and rear ends do not qualitatively have the same structure when examined under a bright-field microscope. The front end of a migrating cell is described either as a lamellipodia, when it appears as a broad flat sheet, or a filopodia, when it displays abundant spike-like projections [15]. In contrast, the rear of a cell frequently appears to be a relatively long tubular structure [1]. Such asymmetric cellular structure is supported by an internal asymmetric actin cytoskeleton, which is shaped and maintained by polarized biochemical activities that control actin polymerization and depolymerization, in a spatiotemporal manner [1, 14, 16-21]. Large multiprotein transmembrane cellular adhesion complexes (often referred to as focal adhesions) physically connect the intracellular actin cytoskeleton to the extracellular matrix upon which a cell migrates, resulting in cellular traction [22]. These adhesion complexes are spatiotemporally regulated in a similar manner to that of the actin cytoskeleton [22-25]. Thus, a critical principle of our current model for how an individual cell migrates relies on the coordination of the dynamic regulation of both the actin cytoskeleton and the adhesion complexes throughout a cell.
**Actin Structure Regulates Front End Protrusion**

At the front end of a cell, whether a lamellipodia or multiple filopodia will form is determined by the type of actin cytoskeleton structure that supports the plasma membrane. In lamellipodia, the structure of the actin network is highly branched [15]. This branching is mediated by Arp2/3 binding to the end and internal locations of an actin filament, resulting in the nucleation of an actin filament “branch”[15, 26]. The whole branched network is further strengthened by filament crosslinking via filamin A and α-actinin [15]. In contrast to lamellipodia, filopodia maintain long parallel bundles of actin that form as the result of the activation of Ena/VASP proteins, which suppress actin capping and Arp2/3 activity [15]. Filopodia bundles are crosslinked through the protein fascin, which increases the stability of the filopodia structure [27].

The strength of the actin network in both types of front end structures is an important quality that allows for front end protrusion, as the actin network must push the plasma membrane forward during this process [1]. However, the force to push the plasma membrane forward is thought to be generated differently between these two structures. In lamellipodia, the force comes from the release of stored elastic energy as actin filaments transition from a bent formation to a straight formation [26]. In filopodia, the force comes directly and entirely through actin “tread milling”, where filaments merely extend the membrane outwards by the addition of actin monomers to the barbed end (sub-membrane) and removal of actin monomers at the pointed
end (away from the membrane) [15]. The identification of two types of front end protrusion mechanisms suggests that there is at least two distinct modes of individual cell motility mechanisms.

The Creation and Destruction of Adhesion Complexes

As the front end of a cell protrudes, new cellular adhesion complexes are formed upon contact of the protruding membrane with the extracellular matrix [22]. Exactly how these multiprotein complexes assemble upon contact is less understood than their composition and disassembly. Integrin transmembrane receptors within adhesion complexes are the proteins that mediate direct contact with the extracellular matrix [28-31]. The linkage between actin filaments and integrin receptors is achieved through the proteins Talin, Tensin, Paxillin, Zyxin and Vinculin, which assemble in a ECM context dependent manner [32, 33]. As a cell migrates forward, adhesion complexes remain stable, resulting in the localization of older adhesions complexes towards the rear of the cell [1]. The formation of adhesion complexes at the front of the cell is regulated by Rho GTPase activity [34]. However, the mechanistic determinant of exactly where an adhesion complex forms is not fully understood.

Disassembly of adhesion complexes is also incompletely understood, but there appears to be three main components to the mechanism of disassembly. First, Focal Adhesion Kinase (FAK), Src and Rho GTPase activities all lead to increased turnover of adhesion complexes [35, 36]. However, it is not yet clear how these disassembly events are spatially activated at some, but not all,
adhesion complexes in the cell. Second, the physical tension of actin fibers due to actinomyosin contraction at the rear end of the cell has also been shown to be sufficient to “tear” the adhesion complex apart [14]. Lastly, such tension at the rear end is sufficient to activate stretch-activated calcium channels, which leads to Calpain mediate proteolytic cleavage of adhesion proteins [37]. The extreme complexity of biochemical activities at adhesion sites and the diverse context dependent regulation of adhesion complexes has made the studying of these molecular structures difficult. Significant progress on this front will require improved assays that can determine the composition of adhesion complexes, over time, in live cells, at single site resolution.

**Determinants of Cellular Front End Identity**

Front and rear end identities in a cell are determined by the subcellular polarization of Rac activity [1]. Rac activity is activated by adhesion complex formation at the front end [38], and suppressed by Rho activity elsewhere in the cell [21, 39]. At least one Rho subfamily GTPase, CDC42, is seemingly always recruited to the front of a migrating cell via the polarization of Rac activity [17]. The downstream effectors of Rac/CDC42 activation are the WASP/WAVE family of Arp2/3 protein activators [16, 40, 41], which explains how Rac activation stimulates lamellipodia formation but not how Rac activation stimulates filopodia formation. How the latter is achieved remains a mystery in the field.

CDC42 localization to the front end of a cell is required for maintenance of cell polarity and directional migration [17, 18], and the restriction of
CDC42 activity to this location prevents lamellipodia formation at other locations in the cell [42]. Although the migration field has made significant advances in describing the molecular events in a polarized cell, it has not been able to fully elucidate the initial molecular or physical determinant for front end identity. However, a recent publication has presented a new theory that states that the front end of a cell exhibits high membrane tension, compared to the rest of the cell [6]. This asymmetric membrane tension can be perturbed in capillary pipet based experiments, which results in the disruption of CDC42 asymmetry in the cell. Thus, it appears that high membrane tension might be the absolute initial response of the cell to its environment, which subsequently recruits proteins that are observed to be restricted to the front end through an unknown molecular mechanism. Future studies that measure and manipulate membrane tension will be needed before such a theory is fully accepted by the migration field.

**Cellular Density Sensing**

Although the biochemical mechanisms behind cellular mechano-sensing of their environment are still unclear, the ability of cells to sense neighboring cell is not in question. This first described observation of this cellular migration behavior, in 1953, showed that chick heart fibroblasts reduced their migration speed when in close contact with neighboring cells [43]. Such behavior was termed “contact inhibition of locomotion”, and was used to explain how wound healing is initiated. The process of contact inhibition of locomotion became even more interesting to researchers when it was
discovered that malignant transformed cells displayed a reduced ability to respond in this manner to neighboring cells [44-46]. However, several decades passed with little progress in understanding this mechanism, due to the lack of molecular biology and imaging techniques that are required to address this problem [47]. More recently, such techniques have been developed, and more progress has been made on understanding contact dependent cellular migration. For instance, cellular contacts have been repeatedly shown to govern cellular polarity and directional migration [48-50]. These recent studies have marked a drastic change in the focus of investigations at the border of the cellular communication and the cellular migration fields, as investigations now seek to understand how cellular communication activates migration, rather than inhibits migration. This cellular contact dependent coordinated migration behavior of groups of cells is referred to as “collective migration”.

**Defining Collective Migration**

In the cell migration field, one definition for collective migration that has been proposed has three parts: cells are physically linked by strong cell-cell interactions, cells migrate in the same direction, and the underlying ECM is modified upon migration of the group [3]. In conflict, another definition has been set forth that proposes that collective migration is the condition in which neighboring cells physically interact and migrate in the same direction [11]. I favor the second definition for two reasons. First, in the biological example of the rostral migratory stream, neural precursor cells migrate together from the
subventricular zone to the olfactory bulb [51, 52]. Although these cells display relatively weak cell-cell interactions [11], their shared migration direction is clearly observable. As a result, the requirement for strong cell-cell interactions does not seem justified in the definition of collective migration. In any case, a clear metric for measuring cell-cell contact strength has not been presented in the field, and would be required for identifying collective migration according to the first definition. Additionally, since ECM modifications occur even in the case of individual cell migration [1, 3, 7], ECM modification should not be considered in the definition of collective cell migration, as it is a quality of all cell migration. Thus, collective migration is more accurately defined as physically linked cells that migrate in the same direction.

**Collective Cellular Migration in Biology**

An extreme number of biological processes in nature depend upon the collective migration of neighboring cells [51, 53-57]. In slime molds, individual cells organize and migrate in a slug formation in order to achieve collective translocation of a group of cells that can originate from independent spores [9, 58]. In vertebrates, during the gastrulation phase of embryogenesis, sheets of cells migrate collectively in order to achieve the invagination of the developing embryo [59]. Similarly, epithelial cells collectively migrate in sheets during embryonic eyelid closure in mice [57]. Additionally, sheets and globular groups of cells have been observed to migrate collectively during cancer metastasis [3, 47]. The advantage of such collective behavior is that
During migration, tissue organization remains intact, and migration is highly directional [3, 4, 11].

In cancer biology, collective migration aids tissue invasion in the absence of epithelial to mesenchymal transition (EMT), which is the known mechanism by which cells display loss of cell-cell contacts [60, 61]. In this case, the therapeutic application of studying collective migration aims to hinder such migration. A theoretical way in which cancer progression is aided by collective migration is that individual cells with low proliferative and migratory capacity at the site of origin can be brought into another tissue in which they would have high proliferative and migratory capacity. In this manner, the cells that govern the migration speed and the direction of the collective group are not necessarily the same cells that perpetuate cancer progression upon metastasis. Since the directional guidance of groups of cells during collective migration is recognized as critical for both development and cancer progression, it is not surprising that this subject is currently at the forefront of the collective migration field.

**Leader and Follower Cells in Collective Migration**

During collective migration, not all cells must actively contribute to the motility of the group [10, 62, 63]. In some cases, a small subset of cells (termed leader cells) is thought to guide the direction of “follower” cells, such that the entire group appears to actively migrate in a single well-defined direction. The cellular characteristic that identifies a leader cell from a follower cell has been proposed to be that leader cell polarity orients towards
the migration of the collective group, while follower cell polarity does not [62]. However, there is currently only one truly quantitative investigation in the literature of leader/follower cell behavior, where the direction and speed of each cell within a group were determined as a function of time throughout in vitro migrating endothelial sheets [63]. In this investigation, FGF treated endothelial sheets composed of HUVEC cells display lamellipodia formation in the leading edge cells (those at the wound edge), but not in cells farther back in the sheet. As a result, leader cells were identified based on their content of lamellipodia, rather than according to the definition presented in the field, above. This discrepancy illustrates the lack of a consensus definition of leader cell identity, and the requirement of future studies in order to determine exactly what the necessary characteristics of leader cells are. Nevertheless, using experimental observations and mathematical modeling, the investigators concluded that these leader cells pull the follower cells forward and govern the direction of the collective group through adhesive cell-cell contact forces. Although this study has significantly contributed to our understanding that only a few cells can efficiently guide a group of cells in collective migration, there are many unanswered questions. For instance, can a filopodia containing cell be substituted for a leader cell with lamellipodia? Can all cells actively and collectively migrate as the result of other ligand stimulations? If so, how do cells without lamellipodia migrate actively in the same direction? Can cells migrate collectively in large groups in the absence of a wound? If so, how is the direction determined for the group? These answer to these questions are not currently known.
TGFβ Signaling and Cellular Migration

TGFβ has been implicated in vivo in wound healing [64-66], where upon injury to skin, TGFβ secretion coincides with the early stages of tissue repair [67]. Additionally, TGFβ mediates the activation of cell migration during embryonic eyelid closure and dorsal closure, both of which are developmental processes that involve the collective migration of epithelial sheets of cells [56, 57]. Furthermore, TGFβ signaling has been shown to promote the invasive behavior of transformed cells during breast cancer metastasis, where transient Smad activation leads to an increased metastatic potential of individual cells, but not groups of cells [68]. However, the role of TGFβ signaling in cancer related processes that depend upon collective cell migration has not been clearly defined.

Signal Transduction in the TGFβ Pathway

TGFβ signals through both the canonical and the non-canonical pathways. In the canonical pathway, ligand binding to the TGFβ type II receptor (TβRII) leads to kinase activation and hetero-oligomerization of TβRII with the type I receptor (TβRI) [69]. Trans-phosphorylation of TβRI by TβII leads to the activation of TβRI Ser/Thr kinase activity, ultimately leading to phosphorylation of receptor regulated Smad proteins (R-Smads), Smad2 and Smad3, at their respective C-terminal SSXS motifs [70]. Phosphorylated R-Smads undergo homo and hetero oligomerization with Smad4 [71-73], resulting in a decrease in the nuclear export rate and the subsequent nuclear
accumulation of the Smad proteins [74]. Upon nuclear accumulation, the Smad proteins act as transcription factors, which regulate changes in the cellular transcriptome to elicit the appropriate cellular response [69]. Non-cannonical signaling is less well understood, where ligand stimulation leads to the activation of several MAP Kinase (MAPK) proteins, including ERK1/2, P38 and JNK, in a cell type specific manner [57, 75-81]. Activation of MAPKs has been shown in at least one case to be achieved through Ras activation by SHC and TRAF6, which interact directly with TβRI [80]. Although the non-canonical TGFβ pathway is required for ligand induced cellular migration [57], the mechanism by which ligand stimulation leads to collective and directed cellular migration remains unknown.

MEK1 and Cellular Migration

MEK1 kinase activity has been implicated in the regulation of cellular migration as a regulator of actin polymerization and as a potential collective migration guidance cue. MEK1 regulation of actin polymerization has been shown to occur through the Ras-MEK1 pathway’s ability to inhibit LIM Kinase in 3 dimensional in vitro studies [82]. LIM kinase phosphorylates and inactivates the actin remodeling protein cofilin, whose main function is to sever actin filaments at internal locations [82-85]. Cofilin mediated severing of actin filaments is recognized to be an important step in the generation of membrane protrusions, particularly in lamellipodia formation [86]. Thus, MEK1 kinase activity is required for the membrane protrusion step in the process of cellular migration.
Additionally, MEK1 activity has been shown to regulate the ability of CDC42 to activate JNK kinase [87]. Although MEK1 has been shown to regulate CDC42, and CDC42 has been shown to regulate cellular polarity, MEK1 has not been directly shown to regulate CDC42’s ability to mediate cellular polarity [17, 87]. As a result, the full understanding of the mechanism by which MEK1 regulates cellular migration requires further investigation.

In addition to regulating actin remodeling in individual cells, MEK1 kinase activity has been proposed to be involved in the directional guidance of collectively migrating cells [8, 11, 88]. Such guidance is thought to be achieved through the asymmetric distribution of cells with high MEK1 activity throughout a collectively migrating group of cells. This asymmetry has been shown in migrating epithelial sheets and 3 dimensional spheroids [89-91]. In all of these cases, the direction of collective migration correlates with the orientation of MEK1 activity in the collective group. However, it is not known how such asymmetric MEK1 activity effectively guides a collectively migrating group of cells, nor is it known exactly how such asymmetry occurs [11]. In addition, no investigation has addressed whether spatial constraint of MEK1 activity causes different collective migration compared to uniform MEK1 activation. Since malignant tumors are frequently shown to have increased MEK1 activity, there is a clear need for future studies to investigate the role of MEK1 in both individual cell migration and collective cell migration in order to better understand the process of cancer metastasis.
The Motivation for Improved Computational Tools for Measuring Cell Migration

Cellular migration is an important process in cancer progression, development, tissue repair, and immune response [2, 5, 88, 92-95]. As a result, a plethora of research has been performed to identify the molecular mechanisms behind how individual cells achieve migration, as well as how neighboring cells migrate cooperatively in collective migration (reviewed in [7, 9, 14] and [11], respectively). Primarily, this research has been conducted through the tracking of cells in a two dimensional cell culture model system using time-lapse fluorescence microscopy, a task that has proven to be a challenge without the use of an automated cell tracking software. Although such motility programs exist (Table 2.1), they differ in the parameters they report, largely ignore the parameters of migration direction and migration persistence, and lack the high throughput capability of analyzing multiple videos. In order to provide a tool with such capabilities to the cell migration field, we have developed an automated high throughput cell tracking software, named...
Table 2.1: The Pathfinder Cell Motility Program (PFP) Incorporates Assessments of Cellular Parameters

<table>
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<tr>
<th>Parameter</th>
<th>Persistence</th>
<th>Direction</th>
<th>Speed</th>
<th>Position</th>
<th>Website URL</th>
<th>Name</th>
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<td>✓</td>
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<tr>
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<td>✓</td>
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<tr>
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</tr>
<tr>
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<td>✓</td>
</tr>
<tr>
<td>Cell Tracker</td>
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<tr>
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</tr>
</tbody>
</table>

Note: The table represents the assessment of cell motility parameters using the Pathfinder Cell Motility Program (PFP). Each parameter is evaluated for its impact on cell behavior and movement, as indicated by the ✓ symbol.
Pathfinder, which is capable of measuring and reporting cellular speed, cellular direction and migration persistence of fluorescently labeled cells.

**Cellular Migration Persistence Measurements**

The migration persistence of cells (the ability of cells to migrate continually in one direction) is the result of a cyclical process of front end extension and rear retraction, which involves the anterior to posterior polarization of actin dynamics throughout a cell [1, 19, 26, 96]. Such actin dynamics are governed by the polarization of multiple biochemical activities within a cell, including Rac and CDC42 GTPases at the front end [1, 17, 18, 20, 97, 98], which regulate where in a cell actin is polymerized, through their effector proteins WASP and WAVE [16]. At the sides and rear of the cell, Rho GTPases suppress the activity of Rac, which in turn prevents actin polymerization at these locations [21, 39]. These actin regulating proteins are themselves regulated by complex signaling networks that can accept input signals from extracellular sources, including cytokines, matrix composition, and physical stimuli [5, 6, 99]. Alterations in migration persistence presumably reflect the changing polarity of actin regulating proteins, where cells that constantly reestablish the relative location of actin regulating proteins migrate with poor persistence, and turn more frequently. However, it is not entirely clear how cells alter polarity during cellular migration [1]. Nevertheless, the quantitative measurement of the persistent behavior of cells is likely to be a required technique to understand how cells actively respond to their surroundings.

Although all of the cellular motility programs that are available report the speeds of cells, only one published program allows for the measurement of migration persistence by reporting the persistence time, or the estimated time that a cell commits to a well defined direction (Table 2.1). We discuss the short comings of the
The method of measuring persistence time, as well as how we use an alternative parameter, termed the angle of deflection, which reports the instantaneous change in direction of a cell. The method of calculating the angle of deflection of a cell overcomes the limitations of persistence time measurements and can accurately report when a population of cells responds to an environmental stimulus by altering cellular migration persistence. Thus, our improved method aims to significantly contribute to the cell migration field by providing a robust means for instantaneous observation of migration persistence throughout a large population, as well as for individual cells.

**The Need for Quantitative Methods for Measuring Collective Cell Migration**

In addition to investigations of individual cell migration, investigations into the mechanisms behind collective cell migration are becoming more abundant in the cell migration literature. Collective migration is defined as the ability of physically interacting cells to adopt a common migration direction [10, 11]. The collective migration of cells is an important process in cancer progression, development and wound repair [8, 51, 53, 54, 56, 57, 66]. Such collective behavior results largely from each cell responding to the environmental stimuli of neighboring cells, in addition to non-cell environmental stimuli [1, 10, 11]. Although collective migration is recognized as important for many biological processes, high throughput computational tools to measure collective migration have not been made readily available to researchers. For this reason, our Pathfinder software was developed with the capability to report the directions of each cell in each frame of a time-lapse microscopy video. This parameter, termed the angle of trajectory, allows researchers
to determine whether neighboring cells share a common migration direction, which is an accepted required feature of collective migration [3, 4, 10, 11].

The Pathfinder Software’s Contribution to the Field of Cellular Migration

Although technological advances in fluorescence microscopy continue to propel the cell migration field forward, the current limitation for researchers is not rooted in data acquisition, but rather in data analysis. Our newly developed high throughput cell migration software overcomes this limitation, as it provides the means to quantify turning behavior in individual cells and to quantify collective behavior in groups of cells, both with time resolution. As a result, this novel software is likely to dramatically contribute to the cell migration field’s ability to investigate not only the molecular mechanisms behind cellular migration, but also how cells sense and respond to their neighbors.

Methods

Fluorescent Labeling of Cells, Cell Culture and Cellular Imaging

Stable transgenic HaCaT and MDA-MB-231 cell lines were fluorescently labeled via retroviral mediated gene transfer of mCherry-Histone H2B using the pRex-mCherry-H2B plasmid. For all experiments, cells were cultured in DMEM lacking phenolphthalein red and supplemented with 2 mM L-glutamine, 100 Units/mL penicillin and 100 µg/mL streptomycin. For low density assays, cells were plated at an average density of 300 cells/mm² for both HaCaT and MDA-MB-231 cells. For confluent monolayer experiments, HaCaT cells were plated at an average density of 1000 cells/mm². For Epithelial sheets assays, HaCaT cells were plated at an average
density of 1200 cells/mm² for 3 hours at 37 °C, after which half of partially adherent
cells were manually removed using a 200 µl pipet tip. An ImageXpress MicroXL
high throughput wide-field fluorescence microscope was used for imaging
experiments at 37 °C and 5 % CO₂. All microscopy videos were acquired with a
frame rate of one frame every seven minutes, in the mCherry fluorescence channel.
The accompanying MetaXpress software was used to compile video files from time-
lapse images for each well of a 96 well plate.

**Programming, Input Parameters, and Output for the Pathfinder Software**

The Pathfinder software was written in the Java programming language. User
specification is required for cell size (pixels), minimum track length, the interval of
frames for desired calculations (Frame _ – Frame _), Percentage of pixels that
represent cells, and the directory path for the folder containing .avi files. The output
for each video file is a single Excel spreadsheet (Figure 2.3).

**Calculation of Migration Parameters, Persistence Time and Nearest Neighbor
Analyses**

Cellular speed was calculated as the displacement of a cell (pixels) over 1 frame.
Conversion to µm/hour is determined by the following equation:

\[
Conversion \ Factor = \frac{(\text{Camera Pixel Size} \times \text{Binning Factor})}{(\text{Magnification Factor} \times \text{Frame Rate})}
\]

The Angle of Trajectory was calculated from the following discontinuous equations:

\[
\text{if} (dx > 0, dy > 0), \text{then } \theta_{\text{trajectory}} = 360 - \arctan\left(\frac{dy}{dx}\right)
\]

\[
\text{if} (dx > 0, dy < 0), \text{then } \theta_{\text{trajectory}} = -\arctan\left(\frac{dy}{dx}\right)
\]

\[
\text{if} (dx < 0, dy > 0), \text{then } \theta_{\text{trajectory}} = 180 - \arctan\left(\frac{dy}{dx}\right)
\]

\[
\text{if} (dx < 0, dy < 0), \text{then } \theta_{\text{trajectory}} = 180 - \arctan\left(\frac{dy}{dx}\right)
\]
Angle of Deflection was calculated from the following discontinuous equations:

\[
\text{if} (\theta_{\text{deflection}} \geq 180), \text{then } \theta_{\text{deflection}} = (\theta_{\text{trajectory} t=n+1} - \theta_{\text{trajectory} t=n}) - 360
\]

\[
\text{if} (\theta_{\text{deflection}} \leq -180), \text{then } \theta_{\text{deflection}} = (\theta_{\text{trajectory} t=n+1} - \theta_{\text{trajectory} t=n}) + 360
\]

Persistence measurements were performed using both the “Cell Motility Project” program and a modified in-house MatLab based program developed by Dr. Douglas Lauffenburger (MIT). Results display calculations from the latter program.

Nearest neighbor calculations were done in Excel using an inter-centroid distance matrix of (all cells) x (all cells) for each frame. Nearest neighbors were defined as cells within whose centroids are within 100 µm of each other.

**Overlapping Intervals Suppresses Noise in Directional Cellular Behavior**

The Pathfinder software is unique from other software in that it calculates angular information about individual cells. However, such angular information requires that the determination of a cell’s position be relatively noise free. When we closely examine the tracks of individual cells, we find that tracks exhibit slight vibration on the short timescale (7 minutes), such that a cell that migrates relatively straight does not display a perfectly straight track. As a result, we use overlapping intervals for our calculations of angular information in order to suppress the effects of such vibration on angular calculations. The schematic diagram in Figure 2.1A illustrates how overlapping intervals aid in the reduction of noise in the calculation of
cellular speed, direction, and persistence. Presented is the path of a single hypothetical cell that travels from positions 1 to 6. When calculating the trajectory of the movement from position 1 to 2, the resulting vector does not accurately represent the underlying trajectory of the cell over time. However, as the calculation is repeated in the same manner for a change in position from 1 to increasing successive positions, the resulting vectors quickly to converge on the underlying trajectory of the cell. Each interval represents the cellular behavior in a video that has a frame rate that is the (acquisition frame rate) x (the interval size). For instance, if the interval size is 3 frames, then calculations are conducted on frames 1, 4, 7, 10, and so forth. When successive intervals are combined, with a single frame shift from one interval to the next, the resulting data provides a time dependent parameter that has greatly suppressed noise. Figure 2.1B and C show the effect of increasing interval size on the time dependent average absolute angle of deflection trend (B) and the average standard deviation of the absolute angle of deflection trend (C) for HaCaT cells treated with TGF. With an interval size of 1 frame, both the average (B) and the error (C) of the angle deflection measurements are extremely noisy. With increasing interval size, such noise is suppressed, where an interval size of greater than 2 does not yield significant additional suppression of noise. For all cellular experiments detailed in this investigation, an interval size of 3 frames was used. This method of overlapping intervals was applied to all measurements, with the exception of persistence time measurements.
Results

The Overview and Capabilities of the Pathfinder Software

The Pathfinder software was developed to allow researchers to easily analyze large data sets of time-lapse fluorescence microscopy videos of motile cells. Since cellular tracking is already a well-established technique, our software implements a previously validated tracking algorithm (“Particle Tracker”) developed by Sbalzarini et al. to detect each fluorescently labeled nuclei in each frame (Figure 2.2A), as well as to assemble such positional information into cellular tracks (Figure 2.2B), as described in their publication [100]. Since cellular positions alone are of little use to researchers in the cell migration field, we developed an analysis algorithm to transform the previous Particle Tracker output into an excel spreadsheet that displays calculations of the speed, the persistence, and the direction of individual cells, as well as the average values for a population of cells (Figure 2.2B and Figure 2.3). In addition, Pathfinder is capable of running batch parallel processing of unlimited .avi files, allowing for automated and high throughput data processing of fluorescent time-lapse microscopy videos.

Using the Average Absolute Angle of Deflection to Measure Cellular Persistence

In order to provide a means for high throughput calculation of cellular migration persistence, we used a non-traditional, but direct, approach of calculating the angle of deflection for each cell at each time. Figure 2.2B illustrates how the angle of deflection measures migration persistence. The diagram represents a single cell, whose position is measured at three successive time points (1, 2, and 3, respectively). As the cell travels from 1 to 2 it maps out a line representing the trajectory of the cell between these two times. Similarly, as the cell travels from 2 to 3, another line is
Comparison of Migration Persistence Methods

A. Live Cell Time-Lapse Microscopy
B. Data Analysis
C. Unbiased Cellular Tracking
D. Quantitative Analysis
Figure 2.2 Angular Measurements of Cellular Migration Can Reveal Cellular Behavior. A) The Pathfinder program converts time-lapse microscopy videos of fluorescent HaCaT cells (left) to cellular tracks (right). B) The Pathfinder program uses the positional information of cellular tracks to calculate speed (top), migration persistence through the absolute angle of deflection (bottom), and the migration direction relative to a well-defined axis orientation in the field of view (right). C) Cells do not prefer to turn right or left in either the presence (right) or absence (left) of EGF stimulation. A binned histogram of percent of cells versus percent of right turns is normal and centered around 50 percent. D) A comparison of persistence time calculations and average absolute angle of deflection methods for measuring migration persistence yields identical trends in both the presence and absence of either TGF-Beta or EGF for MDA-MB-231 cells and HaCaT cells.
Additionally, Palmblad reports a squared histogram of parallel cell traces for 14 directions (11) the percentage of cells running greater than 90 degrees (12) and the average absolute angle of deflection (13) for the population of cells. Palmblad reports the mean (6) dependent change in the average displacement (10) the average angle of deflection (7) and a mean squared displacement (8). Mean squared displacements can be used to calculate the persistence time for a cell (2). A cell is assigned an x (3) and y (4) coordinate, and displacement from the last frame is used to calculate the angle of deflection (6) to an angle of 360 degrees 

| Frame | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |

**Population Statistics**

<table>
<thead>
<tr>
<th>Event</th>
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**Individual Cell Measurements**

| Event | N | T | T | T | T | T | T | T | T | T | T | T | T | T | T | T |
formed. The angle of deflection is the angle between these two lines, where a clockwise turn has a positive value, and a counterclockwise turn has a negative value. Using this calculation, each cell at each time can be assigned an angle of deflection, such that the sampling of many cells at a single time point can provide an accurate measurement of how straight cells are migrating within the population. A decrease in the average absolute value of the angle of deflection for a population of cells (\(|\theta_{\text{deflection}}|\)) reflects an increase in the migration persistence. We use the absolute value of the angle of deflection for describing the persistence of migration in large populations, rather than maintaining the sign of the angle of deflection, because cells do not display a bias in which direction they prefer to turn (Figure 2.2C).

Although we measure migration persistence using the average absolute angle of deflection, the measurement of migration persistence is currently conducted in the cell migration field through the determination of persistence time, which is calculated using data fitting of time dependent mean squared displacement trends to Equation 1, where \(MSD\) is the mean squared displacement of the cell, \(n_d\) is the number of dimensions in which cells are migrating, \(S^2\) represents the squared speed of the cell, \(P\) represents the persistence time of a cell, and \(t\) is the time.

\[
MSD = n_dS^2Pt\left[1 - \frac{P}{t}(1 - e^{-tP})\right]
\]  

Equation 1

Persistence time is used to measure migration persistence because local changes in mean squared displacements trends are likely to be associated with changes in the direction of cellular migration, provided that speed is taken into consideration. A key difference between our approach and the persistence time approach is that persistence time measurements focus on how long a cell maintains a direction, while the average
absolute angle of deflection measurements focus on the degree to which cells in a population turn in each frame. Thus, persistence time measurements reflect behavior over a specified interval of time (usually 2-4 hours), while the average absolute angle of deflection measurements reflect the relatively instantaneous behavior of cells.

We compared our technique of measuring migration persistence to the method of measuring persistence time by examining the time-lapse microscopy videos of two cell lines, MDA-MB-231 and HaCaT, stably expressing fluorescent nuclear markers and treated with no ligand, TGFβ or EGF. When such videos of HaCaT cells (24-26 hours post ligand stimulation) are analyzed by the two methods, both techniques lead to the same conclusions; TGFβ and EGF stimulation cause increased migration persistence, where EGF has an impact of higher magnitude than that of TGFβ (Figure 2.2D, right). Both methods also agree when the same analysis is applied to MDA-MB-231 cells, where only TGFβ has a low magnitude effect on migration persistence (Figure 2.2D, left). Thus, these techniques yield the same results under these experimental conditions. Taken together, this data shows that these two techniques both accurately measure migration persistence in motile cells.

The Shortcomings of Measuring Persistence Time

Although persistence time calculations are often used in motility investigations, they are computationally expensive due to the data fitting technique that they employ, require uniform track lengths, and are sensitive to the interval of time being examined. The largest flaw of the persistence time method is that it is sensitive to the interval of time being investigated. This phenomena has been described by others, where cells are observed to migrate with poor persistence on long time scales (>6 hours) [101]. To illustrate this point, we repeated our persistence time and average absolute angle of deflection calculations over a 9 hour timescale. Although the
average absolute angle of deflection technique reveals an almost identical trend, the
trend for persistence time calculations is extremely different (Figure 2.4A). In this
case, ligand treatments are apparently causing decreasing migration persistence, and
all persistence times have unrealistic magnitudes (>>1000 minutes). Thus,
persistence time measurements are sensitive to the interval of time pre-determined by
the researcher, requiring a complicated method for determining the reliable interval
of time that yields accurate results. The dependency of persistence time calculations
on time interval size means that comparisons between cells are only valid if track
lengths are identical. In high throughput analysis of cellular migration, this would
lead to truncation of data, such that all cells that are unsuccessfully tracked
throughout a given time are discarded. This truncation is not necessary when using
the average absolute angle of deflection method, as each angle of deflection is
calculated for a cell on a single frame basis. The sensitivity to time interval size
makes the persistence time method an inferior choice for time dependent analysis of
migration persistence in time-lapse videos.

We further analyzed the technique of persistence time measurement by plotting
the trend of $R^2$ values from data fitting versus the calculated persistence time. This
trend shows a clear relationship between these two parameters, where low persistence
times are assigned to mean squared displacement trends that are poorly fit (Figure
2.4B). This suggests that the accuracy of persistence time calculations for poorly
persistent cells is low. For this reason, we compared the persistence times with the
average absolute angle of deflection values for cells from the summation of our
experiments, discussed below, in which data fitting was successful ($R^2 > 0.9$). Such
comparison reveals that the two parameters are proportional to each other (Figure
2.4C). The relationship between these two parameters was further shown to be linear
between the inverse of persistence time and the square of the average absolute angle
Figure 2.4 Extended Comparison of Migration Persistence Time Calculations and Average Absolute Angle of Deflection Calculations. A) Persistence time calculations report unrealistically high migration persistence times and misleading ligand dependent trends upon the increasing of sampling time to 9 hours for both MDA-MB231 and HaCaT cells (left two). Alternatively, the average absolute angle of deflection method is largely insensitive to increases in sampling time interval size (right two). B) In persistence time calculations over the duration of 2 hours, data fitting success correlates with persistence time magnitude. C) For cells with successful data fitting in the persistence time method ($R^2>0.9$), persistence time measurements and average absolute angle of deflection measurements correlate with each other on a single cell basis. Averaging of absolute angle of deflection measurements was conducted over 2 hours, consisting of approximately 17 time points. D) The same cells were used to show that the square of the average absolute angle of deflection correlates directly with the inverse persistence time for each cell. E) Cells with poor data fitting in persistence time measurements are unlikely to report accurate measurements of migration persistence, as the square of the average absolute angle of deflection does not correlate directly with the inverse persistence time for each cell. The cells used for B,C,D, and E represent random sampling of cells from a combined dataset of untreated, TGF-beta, and EGF treated HaCaT cells.
of deflection ($R^2 > 0.72$) (Figure 2.4D). However, the same relationship does not exist for cells that exhibited a poor data fitting score for persistence time calculations ($R^2 < 0.9$) (Figure 2.4E). Taken together, these data show that the persistence time calculation is likely to be largely qualitative, and not quantitative, for cells in which data fitting was unsuccessful.

In light of the three aforementioned shortcomings of persistence time measurements, we chose the angle of deflection method for our computational motility program, which produces identical results to that of persistence time measurements on short timescales (2 Hours), is insensitive to interval size, reports migration persistence nearly instantaneously, and is relatively computationally inexpensive.

Measuring Time Dependent Changes in Migration Persistence and Speed

Upon mere qualitative assessment of cellular tracks, cellular behavior is difficult to deduce for large populations of cells. For example, when we examine MDA-MB-231 and HaCaT cell migration in response to either TGFβ or EGF by looking at the tracks of cells between 0 and 35 hours post ligand stimulation, MDA-MB-231 cells appear to not change behavior in response to ligand treatments and HaCaT cells appear to respond in the same manner upon TGFβ and EGF stimulation (Figure 2.5A and B). However, MDA-MB-231 cells do in fact respond to ligand stimulations and HaCaT cells do in fact respond differently to TGFβ and EGF stimulation, which is elaborate upon below. Thus, only through rigorous quantitation can large populations be characterized for their cellular behavior.

Using our quantitative approach to measuring cellular migration, we were able to determine that either TGFβ or EGF causes MDA-MB-231 cells to migrate faster, but has almost no effect on how persistently cell migrate. Only ligand stimulation of
Figure 2.5 Measuring Individual Cellular Behavior with the Speed and Migration Persistence Reveals Cell Type and Ligand Specific Cellular Migration Behavior. Cellular tracks of low density cells are displayed for treatments of either Mock, TGF-Beta or EGF for MDA-MB-231 cells (A) or HaCaT cells (B). Calibration bars represent 150 µm. C) Neither TGF-Beta nor EGF stimulation affects migration persistence in MDA-MB-231cells (top). In contrast, both treatments affect cellular speed, but with different induction kinetics (bottom). D) In HaCaT cells, both ligand treatments affect migration persistence and cellular speed (top and bottom, respectively). However, EGF stimulates migration persistence with earlier kinetics than that of TGF-Beta (top), and EGF is a poor stimulator of migration speed (bottom, right).
TGFβ causes a statistically significant \( (p = 0.003) \), but extremely small change in magnitude, decrease in migration persistence, as evident by a slight elevation in the average absolute angle of deflection of cells (Figure 2.5C, top left). Both TGFβ and EGF treatments yield an increase in the average speed of these cells, where EGF response is early (approximately 1 hour after treatment) and TGFβ response is late (approximately 10 hours) (Figure 2.5C, bottom). Thus, ligand stimulations cause MDA-MB-231 cells to speed up, rather than migrate more persistently.

In contrast to MDA-MB-231 cells, both speed and migration persistence are activated by TGFβ and EGF treatment in HaCaT cells, where the effects of EGF appear early (approximately 1 hour) and the effects of TGFβ appear late (approximately 10 hours) (Figure 2.5D). Taken together, these data illustrate the importance of time resolution in measuring cell migration responses to environmental stimuli, as well as the importance of measuring both the speed and the persistence of cells in order to characterize the full behavior of cell migration.

**Quantifying Collective Migration with Nearest Neighbor Analyses of Angular Measurements**

The increasing interest in the collective behavior of cells in the cell migration field prompted us to integrate the capability of characterizing collective migration into the Pathfinder software. We introduced the parameter of “the angle of trajectory” to provide a tool for researchers to determine if a group of cells is migrating collectively or independently. For this parameter, each cell is assigned an angle that ranges from 0 to 359 degrees relative to a well-defined set of axes in the field of view. Figure 2.2B (right) illustrates the orientation of these axes in the field of view,
and provides an example of a cell that is migrating in the direction of 45 degrees. In a similar manner to the case of angle of deflection, each cell gets such an assignment for each time, providing time resolution of cellular direction. In order to demonstrate how the angle of deflection calculation can be used to characterize collective migration, we examined HaCaT and MDA-MB-231 cells in confluent monolayers in response to EGF stimulation. Manual inspection of the cellular tracks of these monolayers suggests that neighboring cells migrate in a similar direction in a ligand dependent manner in HaCaT cells (Figure 2.6A, top). This behavior is not observed for MDA-MB-231 cells (Figure 2.6A, bottom). Since collective migration is defined as the ability of cells to adopt a common migration direction, we quantified the average standard deviation of the angle of trajectory, also referred to as the “paired random migration index” (PRMI $\Theta_{\text{trajectory}}$), amongst pairs of nearest neighboring cells at 24 hours post ligand stimulation. An increase in this quantity indicates that nearest neighboring cells are migrating in increasingly different directions, meaning collective migration is decreasing. We excluded pairs of neighbors in which one cell migrates with a direction of 0-90 degrees and another cell migrates in a direction of 270-360 degrees, as these pairs would have a falsely high standard deviation due to the discontinuous transition between 360 degrees and 1 degrees. In agreement with our qualitative observations of cellular tracks, nearest neighboring HaCaT cells display a lower PRMI $\Theta_{\text{trajectory}}$ in response to EGF stimulation (Figure 2.6B, top left). We compared nearest neighbor behavior to random pairing behavior in order to identify when shared behavior of cells is global versus local. If random pairing does not change the magnitude of the PRMI $\Theta_{\text{trajectory}}$, then the shared behavior is entirely global. In contrast, when such magnitudes are affected by random pairing, the phenomena is local. Upon random pairing of HaCaT cells, a similar trend is observed for the PRMI $\Theta_{\text{trajectory}}$, but the magnitudes increase for both mock treatment and EGF
Figure 2.6 Angular Measurements Can be Used to Quantify Collective Migration Behavior. A) Cellular tracks of confluent monolayers of HaCaT (top) and MDA-MB-231 cells (bottom) in the presence and absence of EGF stimulation. Calibration bar represents 150 µm. B) Confluent monolayers of HaCaT cells in the presence and absence of EGF stimulation were quantified for their collective migration behavior by calculating the average standard deviation of either the angle of trajectory (also called the paired random migration index (PRMI Θ_{trajectory}))(top, left) or the angle of deflection (PRMI Θ_{deflection}) (top, right) amongst nearest neighboring cells. Random pairing was used to determine whether the observed behavior was local or global amongst the population. The same quantification was conducted for MDA-MB-231 cells (bottom). C) Cellular tracks of epithelial sheets of HaCaT cells in the presence and absence of EGF stimulation. Calibration bar represents 150 µm. D) Inspection of the spatial distribution of collective migration behavior reveals that EGF stimulation elicits collective migration uniformly throughout an epithelial sheet, when either the PRMI Θ_{trajectory} (left) or the PRMI Θ_{deflection} (right) is used to calculate collective behavior.
treatment (Figure 2.6B, top left). Thus, HaCaT cells exhibit local collective migration in the absence of ligand stimulation, which becomes activated in response to EGF.

Using the same technique on MDA-MB-231 cells, these cells display a statistically significant, but low magnitude, increase in the \( \text{PRMI} \Theta_{\text{Trajectory}} \) amongst nearest neighbors in response to EGF, suggesting that ligand stimulation of these cells causes neighboring cells to exhibit slight repulsion, and migrate more in opposing directions upon EGF stimulation (Figure 2.6B, bottom left). Random pairing of MDA-MB-231 cells led to an increase in the magnitude of the \( \text{PRMI} \Theta_{\text{Trajectory}} \), revealing that the collective migration of these cells is a local phenomenon (Figure 2.6B, bottom left).

Whether or not the apparent collective migration behavior of neighboring MDA-MB-231 cells in the absence of ligand stimulation constitutes collective migration according to the accepted definition will require further investigation into the requirement of cellular junctions in this process. However, it is worth noting that the neighboring cells do in fact have physical contact with each other (Figure 2.7), albeit for short timescales (data not shown).

Although cooperation amongst nearest neighbors in the angle of trajectory is a recognized necessity for collective migration, we investigated whether the cooperation amongst nearest neighbors in the angle of deflection is also characteristic of collective migration. We repeated our nearest neighbor analysis for the angle of deflection measurements by calculating the \( \text{PRMI} \Theta_{\text{Deflection}} \), which revealed that HaCaT cells display local cooperation in migration persistence in the presence of EGF. Upon EGF stimulation, random pairing has little effect on the average standard deviation amongst nearest neighbors of the angle of deflection, suggesting that persistent migration is globally exhibited by these cells (Figure 2.6B, top right). Thus, the angle of deflection cannot
Figure 2.7: MDA-MB-231 cells maintain physical contact with their nearest neighboring cell, reveals their ability to respond to EGF. This microscopic observation highlights the importance of cell-cell interactions in tissue homeostasis.

Physical contact with each other reveals MDA-MB-231 cells' ability to respond to EGF.
be used to calculate collective migration in the PRMI calculation, because cellular migration persistence is a common feature amongst distant cells under these circumstances. MDA-MB-231 cells do not exhibit changes in the magnitude of the PRMI $\Theta_{\text{deflection}}$ upon random pairing in either the presence or absence of EGF, suggesting that migration persistence and collective behavior are mutually exclusive in these cells (Figure 2.6B, bottom right). Taken together, these data demonstrate our ability to quantify the collective migration behavior of cells by measuring the average standard deviation of the angles of trajectory ($\text{PRMI } \Theta_{\text{trajectory}}$) amongst nearest neighbors. Such techniques open the door for future experiments to be conducted in which the molecular mechanisms behind exactly how nearest neighbors coordinate their migration behavior is further elucidated.

**Using Nearest Neighbor Analyses of Angular Measurements to Characterize Collective Migration in Epithelial Sheets**

In order to determine the versatility of our method of collective migration quantification, we repeated our experiments on HaCaT cells using EGF stimulation, but under the condition in which cells were arranged into epithelial sheets (Figure 2.6C). We asked whether or not ligand stimulation affects all cells equally in an epithelial sheet, and found that indeed the collective behavior response to ligand stimulation does not depend upon the distance from the leading edge of the epithelial sheet. Cells display a uniform collective migration response to EGF stimulation throughout the sheet, as we observed a uniform reduction in the PRMI $\Theta_{\text{trajectory}}$ amongst nearest neighbors (Figure 2.6D, left). In agreement with our experiments on confluent monolayers, ligand stimulation also leads to global reduction in the PRMI $\Theta_{\text{deflection}}$ (Figure 2.6D, right). Since both confluent monolayers (Figure 2.6B, top) and
epithelial sheets (Figure 2.6D) of HaCaT cells respond similarly to EGF stimulation in terms of collective migration behavior, we conclude that the presence of a wound plays no role in whether or not these cells migrate collectively, a phenomena that has not been shown in the literature. Thus, our technique of quantitatively characterizing collective migration has already revealed a new feature of collective migration for the field.

**Discussion**

**The Pathfinder Software Provides Novel Useful Tools For Studying Individual and Collective Cell Migration**

Our Pathfinder software is the first high throughput automated cell migration software to introduce the use of angular parameters for the measurement of cell migration behavior. Our parameter of the angle of deflection has proven useful for characterizing the migration persistence of cells in two dimensional fluorescence microscopy experiments. In addition to helping researchers characterize the molecular mechanisms behind migration persistence, this parameter can be used to identify the differential temporal responses of cells to distinct ligand stimulations, and can provide insight into the molecular mechanisms behind each ligand response. Furthermore, the available cell migration software has been entirely devoid of tools that can be used to readily characterize collective migration. As a result, investigations of collective migration have been more appropriately described as qualitative rather than quantitative. The Pathfinder software provides the first high throughput tool to quantitatively characterize collective cell migration using the “Paired Random Migration Index” \( \text{PRMI}_{\text{trajectory}} \) amongst nearest neighbors with near single frame resolution. This tool is likely to propel the collective migration
field forward, as it can provide a means for accurately measuring the degree to which
cells are migrating collectively. In conclusion, our Pathfinder software provides
novel techniques for characterizing cellular migration in a high throughput platform,
which are likely to aid the cell migration field in its investigation of the molecular
mechanisms behind individual and collective migration.
CHAPTER III

Transforming Growth Factor-Beta Induced Collective Cell Migration
Through MEK1 Mediated Suppression of Cellular Turning

INTRODUCTION

Cellular motility is an important process that mediates embryonic development, organogenesis, immune response, metastasis, wound healing and tissue maintenance [2, 3, 14, 66, 92, 93, 95]. The migration of individual cells in response to chemotactic signals has been extensively studied in mammalian and Dictyostelium model systems and is driven by a dynamic, polarized and coordinated process of actin polymerization and depolymerization, in concert with focal adhesion assembly/disassembly and actinomyosin contractions [7, 9, 19, 22, 26, 102, 103]. However, many processes in development and cancer progression depend not only on the motility of individual cells, but also on the collective guidance of groups of cells [11, 51, 53-57]. During cancer metastasis, the invasive behavior of tumors frequently relies on the collective migration of globular groups, strands, or sheets of cells [3, 47]. In all of these cases, groups of physically linked cells migrate coordinately in the same direction, where individual cell migration contributes to the directed translocation of the group. Strong
evidence has been presented that, in most cases, collective migration is guided by leader cells at the front edge of the group, which display elevated actin dynamics in response to external signals from the extracellular matrix or from the secretion of cytokines [11, 104-108]. Alternatively, collective migration can be driven by the pushing of cells in the rear, as has been proposed for the process of branching in the mammary gland [109]. However, it is not clear how a group of actively migrating cells, or all leader cells, achieves collective migration.

MEK1 kinase activity has been implicated in the regulation of cellular migration as a regulator of both actin polymerization. Primarily, this has been shown to occur through the Ras-MEK1 pathway’s ability to inhibit LIM Kinase in 3 dimensional in vitro studies [82]. LIM kinase phosphorylates and inactivates the actin remodeling protein cofilin, whose main function is to sever actin filaments at internal locations [82-85]. Cofilin mediated severing of actin filaments is recognized to be an important step in the generation of membrane protrusions, particularly in lamellipodia formation [86]. Thus, MEK1 kinase activity is required for the membrane protrusion step in the process of cellular migration. Additionally, MEK1 activity has been shown to regulate the ability of CDC42 to activate JNK kinase [87]. Although MEK1 has been shown to regulate CDC42, and CDC42 has been shown to regulate cellular polarity, MEK1 has not been directly shown to regulate CDC42’s ability to regulate cellular polarity [17, 87]. As a result, the full understanding of the mechanism by which MEK1 regulates cellular migration requires further investigation.
In addition to regulating actin remodeling in individual cells, MEK1 kinase activity has been proposed to be involved in the directional guidance of collectively migrating cells [8, 11, 88]. Such guidance is thought to be achieved through the asymmetric distribution of cells with high MEK1 activity throughout a collectively migrating group of cells. This asymmetry has been shown in migrating epithelial sheets and 3 dimensional spheroids [89-91]. In all of these cases, the direction of collective migration correlates with the orientation of MEK1 activity in the collective group. However, it is not known how such asymmetric MEK1 activity effectively guides a collectively migrating group of cells, nor is it known exactly how such asymmetry occurs [11]. In addition, no investigation has addressed whether spatial constraint of MEK1 activity causes different collective migration compared to uniform MEK1 activation. Since malignant tumors frequently have increased MEK1 activity, there is a clear need for future studies to further investigate the role of MEK1 in both individual cell migration and collective cell migration.

In this study, we show that MEK1 activation by TGFβ stimulation is an indirect result of ligand induced cellular spreading, which is likely driven by ligand dependent weakening of cellular junctions. Such spreading is inhibited by high cellular density, and asymmetric cellular density in epithelial sheets leads to confinement of both spreading and MEK1 activity to the leading edge of ligand stimulated sheets. Additionally, we show that MEK1 activation in a group of physically interacting cells is sufficient to induce collective migration. Thus, the spatial constraint of MEK1 activity to the leading edge of epithelial sheets limits ligand dependent collective migration behavior to the
leading edge of an epithelial sheet. At the level of individual cells, MEK1 activation suppresses cellular turning, suggesting that increased migration persistence in individual cells may be essential for collective migration to occur. Our study provides novel insight into the mechanism of collective migration, as it shows that cellular spreading, migration persistence, and collective migration are cellular behaviors that are linked through the kinase activity of MEK1.

Methods

Cell Culture and Experimental Platforms

HaCaT cells were cultured in DMEM supplemented with 2 mM L-glutamine and 100 Units/mL penicillin and 100 µg/mL streptomycin under 5% CO2 at 37 °C. For sheet migration studies, 48,000 cells (1500 cells/mm²) were seeded using 100 µl of media into each well of a 96 well plate. After 3 hours half of the cells (on one side of the well) were removed using a 200 µl pipette tip. Non-adherent cells were removed and media was exchanged for 100 µl of supplemented DMEM lacking phenol red. For the colony assay format, 7,000 cells were added using 90 µl of media to each well 24 hours prior to the addition of 10µl of fresh media supplemented with or without the appropriate ligand/inhibitor to reach the target concentration desired. In the cell density experiments, cells were seeded 24 hours prior to treatment such that the following number of cells per well were present after 24 hours of settling: 9,600 (300 cells/mm²), 14,400 (450 cells/mm²), 19,200 (600...
cells/mm²), 28,800 (900 cells/mm²), and 38,400 (1200 cells/mm²). Cellular densities of 300, 450, and 600 cells/mm² result in cell colonies, while cellular densities of 900 and 1200 cells/mm² results in confluent monolayers. 1 mg/ml Doxycycline was used to activate constitutively active MEK1 in Tet-CA cells. In hybrid sheet migration assays, 48,000 cells were seeded into each well. After 3 hours, half of HaCaT H2B GFP cells were removed, similar to the procedure for sheets. 48,000 HaCaT H2B mCherry cells were added to each well. After 3 hours, all cells were trypsinized for 2 minutes at 20°C, and a 200 µl pipet tip was used to remove desired cells. Figure 3.1 illustrates this process. Images displaying the mCherry channel for hybrid sheets are the result of the subtraction of the mKO channel from the raw mCherry channel, using ImageJ, as mKO is the reporter for viral infection of Tet-control and Tet-CA constructs.

**Construction of Stable Cell lines**

Transgenic N-terminally tagged H2B mCherry cell lines were generated using murine leukemia virus mediate gene transfer, where 293T cells were simultaneously transfected with MuLV packaging vector, pHCMV-VSV-g, and pRex-H2B-mCherry. Viral supernatant was used to infect HaCaT cells, supplemented with 10 µg/ml polybrene. HaCaT H2B GFP Cells were made in an identical procedure with the pRex-H2B-GFP plasmid. HaCaT Tet-Control and Tet-CA H2B GFP cells were similarly generated using lentiviral mediated gene transfer, where 293T cells were simultaneously transfected with pMDLg, pHCMV-VSV-g, pREV and CSIV-TRE-RfA-CMV-KT, or CSIV-TRE-CA-
was made with a 200 nL pipet tip (Step 4a or 4b).

3) After 3 hours, cells were hypotysonized for 2 minutes to disrupt cell-cell contacts, after which a wound area
Step 2) WT cells were then added to construct a hybrid monolayer with a confluent band of TET-CA cells (Step
monolayer (Step 1) After 3 hours, we removed all but a confluent band of TET-CA cells with a 200 nL pipet
epithelial sheets of WT and TET-CA cells, we used a 4 step procedure. We placed TET-CA cells into a confluent

Figure 3.1 A schematic diagram of Hydroxyapatite Sheet Construction. In order to construct hybrid

Step 4b

Step 4a

Step 3

Step 2

Step 1

Hacat WT

Hacat TET-CA
MEK1-RfA-CMV-KT plasmids (gifts of Hiroyuki Miyoshi, RIKEN BioResource Center, Japan). Constitutive active MKK1 is the R4F mutation (a gift of Dr. Natalie Ahn, University of Colorado), and was cloned into CSIV-TRE-RfA-CMV-KT using gateway cloning (Invitrogen).

Live Cell Imaging

Data acquisition of mCherry timelapse videos was performed using either a Cellomics ArrayscanVTI fitted with a live cell chamber and Cellomics ArrayScan HCS Software (Thermo) or an ImageXpress MicroXL and MetaXpress Software (Molecular Devices). Time-lapse Imaging was done at 7 minute intervals for 35 Hours.

Cellular Tracking and Data Analysis

Cellular Tracking and Data Analysis were performed using the Pathfinder software, described in Chapter II. Quantification of cellular density and average standard deviations in cellular density was performed using densitometry in ImageJ and Microsoft Excel for calculations [110]. Single cell evaluation of total fluorescence intensity and cellular footprint size was performed using the MetaXpress software.

Statistical Analysis

The error bars in all experiments represent the standard error of the mean (SEM), where the number of observations is 3, representing experiments done
in triplicate. \( P \) values for all plots were generated from a two tailed t-test analysis using data from three replicate trials.

**Western blotting**

The following antibodies and dilutions (v/v) were used 1:250 pSmad2 (Cell Signaling #3108), 1:10000 Smad2/3 (Santa Cruz Biotechnology SC-133098), 1:5000 Ezrin (Sigma E8897), 1:3000 ppERK1/2 (Sigma M9692), 1:3000 ERK2 (Santa Cruz Biotechnology SC-154), 1:1000 pMEK (Cell Signaling 9154), 1:1000 MEK (Cell Signaling), 1:3000 Lamin A/C (SC-376248).

**Immunofluorescence**

Cells grown in 96 well plates were fixed with -20 \(^\circ\)C 100 % MeOH and Incubated at 4 \(^\circ\)C overnight (Endogenous) or 3.7 % PFA for 5 minutes (fluorescent protein containing samples). Fixed cells were rehydrated in Phosphate Buffered Saline (PBS) and blocked with 0.5 % BSA in 50 mM Tris-HCl supplemented with 0.05 % Tween-20 for 1 hour. Primary antibody was substituted for BSA at a dilution of 1:200 for 3 hours at room temperature. Secondary antibodies were used at a dilution of 1:200 for 1 hour at room temperature. The antibodies used were Smad3 (SC-8332), ppERK1/2 (Sigma M7802), Alexa555 Goat anti-Rabbit (Molecular Probes), and Alexa488 Goat anti-Mouse (Molecular Probes).
Results

TGF-β Promotes Collective Cell Migration in Epithelial Sheets

Collective cell migration and TGF-β signaling have been independently shown to be important processes for tissue invasion during cancer metastasis, prompting us to investigate whether these two processes are related to each other. We developed a live cell experimental platform that allows for the accurate real time tracking of individual cells via the introduction of a fluorescent nuclear marker into the spontaneously immortalized HaCaT human keratinocyte cell line (HaCaT H2B mCherry Cells) (Discussed in Chapter 2). Since HaCaT cells display tight junctions (Figure 3.2A) and epithelial sheets of HaCaT cells display increased forward migration into a wound area upon TGF-β stimulation (Figure 3.2B and C, and Figure 3.3A), we investigated whether such ligand induced sheet migration constitutes collective migration. We characterized collective migration behavior by measuring whether cells within epithelial sheets display a uniform migration direction, and by measuring the PRMI_{\text{Trajectory}} amongst pairs of nearest neighboring cells (as discussed in Chapter 2). Using these two parameters, we found that these epithelial sheets display collective migration in the absence of ligand stimulation, and that this collective migration is enhanced upon ligand stimulation. The migration directions that are sampled by the population are diverse in the absence of TGF-β (Figure 3.3B, top), and ligand stimulation causes such sampling to have a narrow distribution centered around 270
Figure 3.2: Hacat cells physically contact each other during epithelial sheet migration. A) Hacat cells maintain cellular junctions while in epithelial sheet, as E-cadherin staining is present at the junctions between each cell and its neighbor. B) Epithelial sheets migrate towards the wound edge in TGF-Beta 2 simulation. Images are of cellular junctions, with blue indicating E-cadherin protein localization.

Simulation of Hacat migration and epithelial sheet migration upon TGF-Beta 2 stimulation increases the migration speed of epithelial sheets.
Figure 3.3 Epithelial Sheets of Hacat Cells Migrate Collectively Upon TGF-Beta Stimulation. A) Cellular Tracks of HaCaT H2B mCherry cells reveal straight, parallel paths of cells upon TGF-Beta stimulation. B) Cells within 1400 µm of the leading edge of sheets display increased uniformity in the migration directions that are sampled upon ligand stimulation. C) The PRMI $\Theta_{\text{trajectory}}$ was used to measure collective migration behavior between nearest neighbors and random neighbors. Random directions were substituted for angles of trajectory in nearest neighbor calculations to measure the maximum value of PRMI $\Theta_{\text{trajectory}}$, which is attributed to completely random migration behavior.
degrees, which is in the direction of the wound (Figure 3.3B, bottom). In addition, the average standard deviation of the angle of trajectory (PRMI $\Theta_{\text{trajectory}}$) of nearest neighboring cells is lower compared to that of random pairing, suggesting that local collective migration is occurring even in the absence of ligand (Figure 3.3C). Upon TGF-$\beta$ stimulation, the PRMI $\Theta_{\text{trajectory}}$ amongst nearest neighbors decreases relative to that of untreated cells, suggesting that ligand stimulation activates collective migration behavior (Figure 3.3C). Upon random pairing, the magnitude of this quantity rises, which means that the collective migration behavior of cells occurs stronger at the local level than the global level. As a control, we introduced random theoretical values for the angle of trajectory into both datasets, which yielded the same magnitude of approximately 85 degrees for the PRMI $\Theta_{\text{trajectory}}$ for both untreated cells and TGF-$\beta$ treated cells, which represents the maximum value attributed to randomly migrating pairs of cells (Figure 3.3C). Taken together, this data informs us that collective migration is enhanced in epithelial sheets of HaCaT cells upon ligand stimulation.

**Epithelial Sheets Display Asymmetric Non-canonical TGF-$\beta$ Signaling**

Previous studies on sheet migration have shown that epithelial sheets display wound-induced activation of ERK1/2 [89, 90]. Since MEK1 is both the kinase that phosphorylates Erk1/2 and known to be activated by the non-canonical TGF-$\beta$ pathway, we investigated whether TGF-$\beta$ treatment results in the activation of MEK1 activity in migrating sheets of HaCaT cells. Using
immunofluorescence to determine the ppErk levels at single cell resolution, we found that epithelial sheets display elevated ppErk levels at the leading edge in response to ligand stimulation (Figure 3.4A). When the same epithelial sheets are examined for Smad3 subcellular localization, TGF-β stimulation leads to the enrichment of nuclear Smad3 uniformly across the epithelial sheet (Figure 3.4B), suggesting that the cells distal from the leading edge have active TGF-β receptor signaling, but do not respond to ligand stimulation in terms of MEK1 activation.

**MEK1 Signaling is Required for Ligand Induced Collective Migration**

Using the MEK1 inhibitor CI-1040, we found that MEK1 activity is required for TGF-β dependent collective migration in epithelial sheets. Upon exposure of sheets to a CI-1040, the ligand induced spatial constraint of MEK1 activity disappears (Figure 3.5A). MEK1 inhibition also increases the diversity of angles of trajectory throughout the population and increases the PRMI θ_{trajectory} amongst nearest neighbors (Figure 3.5B), both of which indicate that MEK1 inhibition is sufficient to prevent ligand dependent activation of collective migration.

**Asymmetric MEK1 Activity and Uniform MEK1 Activity Both are Sufficient to Induce Collective Migration in the Absence of TGF-β Stimulation.**

Although asymmetric MEK1 signaling has been shown to occur in both two dimensional sheets of cells and three dimensional cellular spheroids, the
Figure 3.4 Epithelial Sheets Display Asymmetric Non-Canonical TGF-Beta Signaling. A) Phospho-Erk1/2 staining by immunofluorescence in the presence and absence of ligand stimulation reveals that MEK1 activity is localized to the leading edge of epithelial sheets at 24 hours post ligand treatment. B) The same procedure reveals that Smad3 nuclear localization is ligand dependent, but spatially uniform.
Figure 3.5 MEK1 Activity is Required for Collective Migration in Epithelial Sheets. Chemical inhibition of MEK1 by CI-1040 inhibits MEK1 activation in epithelial sheets (A), and ligand induced collective migration behavior (B).
Figure 3.6 Hybrid Epithelial Sheets Allow for Spatial Control of MEK1 Activation. Hybrid epithelial sheets of WT and Tet-CA cells were constructed to limit DOX dependent MEK1 activation to the leading edge (A), an internal region (B), or uniformly throughout (C) epithelial sheets.
Figure 3.7 Characterization of Hybrid Sheet Collective Migration Behavior. Localization of MEK1 activity in the absence of ligand stimulation can activate collective migration behavior (A). Movement of MEK1 activity to an internal location is not sufficient to elicit collective migration towards the wound area (B). Uniform Activation of MEK1 activity in epithelial sheets is sufficient to elicit collective migration (C). Dotted boxes indicate the region that was used to measure collective migration behavior.
requirement for such asymmetry in cellular migration has not been directly investigated [89-91]. To address the how important asymmetry is for the collective behavior of epithelial sheets, we constructed hybrid sheets using HaCaT H2B mCherry cells (WT) and HaCaT H2B GFP cells with a tetracycline inducible promoter driven constitutively active MEK1 kinase (Tet-CA), such that we could accurately control where in the collective group of cells MEK1 activity was stimulated. By placing Tet-CA cells at the leading edge of an epithelial sheet, we were able to stimulate MEK1 activity only at the leading edge in a doxycycline (DOX) dependent manner (Figure 3.6A). Similarly, Tet-CA cells could be move to an internal location, which allowed for DOX dependent control of MEK1 activity at an internal region of the sheet (Figure 3.6B). Lastly, we were able to uniformly activate MEK1 activity throughout a sheet in a DOX dependent manner by constructing sheets entirely made of Tet-CA cells (Figure 3.6C). We used these sheets to answer whether MEK1 activity must be at the leading edge and whether MEK1 activity must be restricted to the leading edge. When Tet-CA cells were restricted to the leading edge, DOX treatment yields near identical collective migration behavior, as compared to TGF-β treated WT cells (Figure 3.7A and Figure 3.3B and C). Thus, activation of MEK1 activity at the leading edge is sufficient to drive collective migration throughout the epithelial sheet. Surprisingly, movement of Tet-CA cells to an internal location completely abolishes DOX dependent collective migration between the leading edge and the band of Tet-CA cells, suggesting that MEK1 activation at an internal location cannot push forward the cells in front of them (Figure 3.7B). Lastly,
when sheets entirely composed of Tet-CA cells are stimulated with DOX, we see a similar collective migration behavior as compared to both TGF-β stimulated WT cells and hybrid sheets with Tet-CA cells at the leading edge (Figure 3.7C). Thus, Mek1 activation must be localized to the leading edge of an epithelial sheet in order for collective migration to occur, but need not be restricted only to this location.

**MEK1 Activity is Sufficient to Induce Collective Migration in the Absence of a Wound**

Since collective migration has been primarily quantitatively investigated using *in vitro* wound healing assays [63], we asked whether MEK1 induced collective migration required the presence of a wound, and found that it does not. We repeated our hybrid sheet experiment with Tet-CA cells in an internal band, but did not introduce the presence of a wound. This hybrid monolayer of cells exhibits collective migration behavior upon DOX stimulation, where a portion of Tet-CA cells migrate collectively upwards, and a portion of Tet-CA cells migrate collectively downwards, such that the hybrid sheet becomes increasingly dense at a central location in the band of Tet-CA cells (Figure 3.8A). Although this behavior is complex, MEK1 activation in this scenario does lead to collective migration in the absence of a wound. To support this claim, we repeated the experiment with monolayers entirely composed of Tet-CA cells. These monolayers also display collective migration behavior in the absence of a wound (Figure 3.8B). Thus, induction of collective migration in a group of physically interacting cells seems to be a function of whether or not
Figure 3.8 MEK1 Activation in Epithelial Monolayers Elicits Collective Migration Behavior. A) Hybrid epithelial monolayers exhibit collective migration behavior upon DOX stimulation. The outer dotted box indicates the region used to construct the cellular direction histogram, while the inner dotted box indicates the region used to measure collective migration behavior. B) In monolayers that are entirely composed of Tet-CA cells, DOX stimulation also yields collective migration.
MEK1 activity is high, and need not be limited to conditions in which a wound is present.

**MEK1 activity and Collective Migration Behavior Propagate Backwards in a Migrating Epithelial Sheet**

Upon closer inspection of HaCaT epithelial sheets, we found that between 15 hours and 35 hours post TGF-β stimulation, MEK1 activity is observed increasingly farther away from the leading edge (Figure 3.9). As our previous experiments showed that the location of collective migration is a function of the location of high MEK1 activity throughout a collective group of cells, we investigated whether the collective migration behavior of cells also propagates towards the rear of an epithelial sheet over time. Indeed, collective migration behavior does propagate towards the rear, shown through a propagating time dependent decrease in the PRMI_\(\theta\)Trajectory amongst nearest neighboring cells (Figure 3.10). These data in conjunction with our MEK inhibitor and hybrid sheet/monolayer experiments provide strong evidence that TGF-β induced collective migration behavior is likely to be entirely through ligand dependent MEK1 activation.

**Low Cellular Density Promotes MEK1 Activity**

Although the spatial constraint of MEK1 activity throughout an epithelial sheet was shown to serve only to limit the size of a collectively migrating group of cells, we investigated how such spatial constraint arises in order to better understand how MEK1 is activated by TGF-β stimulation. We found
Figure 3.9 Ligand Dependent MEK1 Activation in Epithelial Sheets Propagates Backwards as Migration Proceeds. Epithelial sheets with and without ligand stimulation were fixed at 15, 24 and 35 hours, and assayed for their MEK1 activity levels by immunofluorescence of ppErk1/2. Between 15 and 35 hours, ligand treated sheets display MEK1 activation that propagates towards the rear of the sheet (bottom). The relative intensity of epithelial sheets was normalized to the intensity at the rear (1800 µm from the leading edge) of the sheet.
Figure 3: Collective migration behavior propagates backwards in epithelial sheets as migration proceeds. Collective migration behavior as a function of distance from the leading edge of epithelial sheets was measured using the PRMI. The trajectory of nearest neighboring cells at 15, 24, and 35 hours post ligand stimulation. The PRMI trajectory of nearest neighboring cells at 15, 24, and 35 hours post ligand stimulation.
that while MEK1 activity propagates towards the rear of a migrating epithelial sheet, low cellular density also propagates towards the rear in a time and ligand dependent manner (Figure 3.11). This suggests that cellular density may play some role in the activation of MEK1. To test this hypothesis, we plated HaCaT cells at increasing cellular density and measured the ppErk1/2 levels and found that these two parameters correlate indirectly (Figure 3.12A). Additionally, we measured the average cellular footprint area for these cells, which correlates indirectly with both cellular density and ppErk1/2 levels (Figure 3.12A and B). Thus, cells that are more heavily spread in either the presence or absence of ligand stimulation have increased MEK1 activity. Additionally, MEK1 activation in the absence of ligand stimulation is not sufficient to induce cellular spreading (Figure 3.13). Since TGF-β stimulation activates simultaneous cellular spreading and MEK1 activity (Figure 3.12A), and MEK1 is insufficient to induce cellular spreading (Figure 3.13), we propose that ligand dependent MEK1 activation is an indirect result of ligand induced cellular spreading.

**Cellular Spreading is Likely to Result From TGF-β Dependent Weakening of Cellular Junctions**

In order to better understand how TGF-β stimulation leads to cellular spreading and subsequent MEK1 activation, we investigated the role of ligand stimulation in cellular junction regulation by examining the spatial distribution of E-Cadherin using immunofluorescence. TGF-β stimulation results in structural alterations of cellular junctions, such that cellular
Figure 3.11 Low Cellular Density Propagates Towards The Rear of Epithelial Sheets as Migration Proceeds. Ligand stimulation of epithelial sheets results in decreased cellular density at the leading edge, which extends towards the rear of the sheet, over time. The relative intensity of epithelial sheets was normalized to the intensity at the rear (1800 µm from the leading edge) of the sheet.
Figure 3.12 Cellular Density Negatively Regulates Cellular Area and Cellular MEK1 Activity. A) HaCaT cells were plated at increasing cellular density, and assayed for cellular footprint area, ppErk1/2, pMek1, and pSmad2 levels in both the presence and absence of ligand stimulation. B) Cellular density and cellular footprint area correlate indirectly in both the presence and absence of ligand stimulation.
junctions appear to consist of highly irregular spike like projections, compared to the smooth perimeter structure observed in untreated cells (Figure 3.14). Although a significant number of experiments are necessary to determine whether and how alterations in cellular junctions mediate cellular spreading, alterations in cellular junctions may be the primary cause for ligand induced cellular spreading, and therefore MEK1 activation. Alternatively, cellular spreading could drive changes in cellular junctions, and future experiments are needed to determine the relationship between these two processes.

**Cellular Spreading is Not Required For Collective Migration**

In addition to discovering that cellular density can negatively regulate both cellular spreading and MEK1 activity, we also discovered that MEK1 activity plays an important role in density homeostasis throughout a group of collectively migrating cells. Upon closer examination of epithelial sheets of DOX treated Tet-CA cells, we found that universal MEK1 activation results in heterogeneous cellular density throughout a sheet, independent of ligand stimulation (Figure 3.15). Not only do Tet-CA sheets not spread during their collective migration, but also they exhibit extremely high cell density in local areas that are migrating collectively (Figures 3.13, 3.15 and 3.7C). Although the mechanism behind how exogenous MEK1 activation leads to poor density homeostasis is entirely not understood, this phenomena illustrates one very important point: collective migration is not the mere result of cellular spreading within a partially confined boundary. In other words, cells do not need to spread in order for collective migration to occur.
Figure 3.4: TGF-Beta Simulation Affects Cellular Architecture.

E-cadherin staining by immunofluorescence was used to investigate the architecture of cellular junctions in the presence and absence of ligand.

Cells under investigation showed jagged cellular junctions compared to untreated conditions.
Figure 3.15 MEK1 Activity is Required for Maintenance of Cellular Density in Epithelial Sheets.
Epithelial sheets of Tet-CA cells were stimulated with TGF-Beta, DOX or Both treatments, and assayed for the variance in cellular density as a function of distance from the leading edge at 24 hours post stimulation.
TGF-β and MEK1 Suppress Cellular Turning Independent of Collective Migration

Exactly how MEK1 activation leads to collective migration is not currently known in the collective migration field. As a result, we investigated the effect of elevated MEK1 activity on individual cell migration in a scenario in which cells are physically connected, but collective migration does not occur. When HaCaT cells are plated into low density cell colonies, TGF-β stimulation results in poorer collective cell migration, but increased individual cell speed (Figure 3.16A and B). Thus, this scenario is a condition in which ligand activation causes stimulation of individual cell migration. Using this experimental setup, we found that TGF-β stimulation causes suppression of cellular migration persistence that follows identical kinetics to that of MEK1 activation (Figure 3.16C). This ligand effect on migration persistence occurs through MEK1 activation, as this effect can be blocked in a dose dependent manner using chemical inhibition of MEK1 activity (Figure 3.17A) and can be activated by exogenous expression of a constitutively active MEK1 (Figure 3.17B). Thus, TGF-β causes suppression of cellular turning through MEK1 activation.

Since increasing cellular density caused inhibition of MEK1 activity, we tested whether increasing cellular density was able to block the ligand dependent activation of cellular migration persistence. Indeed, cells at a higher density exhibit suppressed cellular turning, independent of ligand stimulation.
Figure 3.16 HaCaT Cells in Low Density Colonies Display Ligand Dependent Activation of Migration Persistence but not Collective Migration. A) HaCaT cell colonies exhibit motility upon ligand stimulation, where cells within colonies expand away from each other. B) Such colonies display decrease collective migration behavior upon ligand stimulation, but increased individual cellular speed (C). D) TGF-Beta stimulation of HaCaT cell colonies results in suppression of cellular turning with similar kinetics to MEK1 activation.
A.

- No Treatment
- 100 nM TGF-β 0 (1×)
- 500 nM TGF-β 0 (1×)
- 100 nM TGF-β 0 (5×)

B. Hacat TERT-Cells

Low cell density colonies
(300 Cell/mm²)

- CI-1040 (600 nM 24 H)
- TGF-β 0 (100 nM 24 H)
- Doxycycline Treatment (5 µ)

- 0
- 3
- 6
- 9
- 12
- 15
- 18
- 24
- 24

('99 Detection (°) (Degrees)')
Figure 3.17 MEK1 Activation is Sufficient and Required for Suppression of Cellular Turning. A) HaCaT cell colonies were treated with increasing concentration of the MEK1 inhibitor CI-1040, which results in inhibition of MEK1 activity and ligand dependent suppression of cellular turning, in a dose dependent manner. B) Tet-CA cells in low density colonies display DOX dependent suppression of cellular turning that correlates with MEK1 activation.
Similarly, epithelial sheets, which display asymmetric cellular density (Figure 3.11) exhibit cellular migration persistence measurements that correlate indirectly with cellular density (Figure 3.18B). We further determined that exogenous expression of a constitutively active MEK1 at high cellular density is sufficient to overcome the effect of high cell density on cellular migration persistence, as these cells exhibit insensitivity to cellular density, and migrate with high persistence (Figure 3.19A and B). Taken together, this data suggests that the primary role of MEK1 in ligand dependent activation of cellular migration is to inhibit cellular turning, resulting in increased migration persistence.

Discussion

Cancer metastasis has been shown to occur through the invasive migration of either single cells or collective groups of cells into healthy tissues [3, 10, 47]. Although TGF-β has been implicated in the metastasis of individual cells, the role of TGF-β in the collective migration during this process is poorly understood [68]. We show that TGF-β stimulation elicits collective migration in epithelial sheets of HaCaT cells through MEK1 activation. MEK1 activation in these cells is likely to be the indirect result of ligand dependent cellular spreading. We are currently investigating the mechanism by which cellular spreading regulates MEK1 activity. At the level of individual cells, TGF-β stimulation promotes increased cellular migration persistence through
Figure 3.18 Cellular Density Correlates Directly with Cellular Turning Behavior. A) HaCaT cells were plated at increasing cellular densities and assayed for their turning behavior. B) Epithelial sheets display ligand dependent asymmetry in cellular turning behavior at 24 hours post ligand stimulation, where cells towards the leading edge turn less than those at the anterior of the sheet.
Treatment, TEL-CA cells exhibit suppressed cellular humming in both the presence and absence of ligand stimulation.

Upon DOX treatment, the presence and absence of DOX treatment and in TEL-CA cells in the absence of DOX treatment, high cell density inhibits ligand dependent suppression of cellular humming in TEL-control cells in the absence of DOX treatment. TEL-control cells were used as a negative control to demonstrate if DOX exposure expression of constitutively active MEKI in TEL-CA cells leads to suppression of cellular humming even when the experiment is repeated.

**A.** 3T3 J2 Exogenous MEKI Activation at High Cell Density Can Restore Mitigation Resistance.

**B.** 3T3 J2 Exogenous MEKI Activation at Low Cell Density Monolayers.

**C.** 3T3 J2 Exogenous MEKI Activation at Low Cell Density Colloidal.
MEK1 activation, which may serve as a fundamental driving force for spontaneous collective migration.

**TGF-β Activation of MEK1 Through Ligand Dependent Modulation of Cellular Density**

The activation of MAP Kinase proteins in the non-canonical TGF-β pathway is largely cell type specific [81]. Here, we show that TGF-β induced MEK1 activation is an indirect effect of ligand stimulation. TGF-β treatment causes induction of cellular spreading, which correlates strongly with MEK1 activity (Figure 3.12A and B). Although ligand stimulation causes cellular spreading (Figure 3.11 and 3.12), MEK1 activation in the absence of TGF-β does not (Figure 3.13). Thus, we conclude that MEK1 activation likely occurs downstream of cellular spreading, albeit future experiments are needed to show whether cellular spreading alone is sufficient to drive activation of MEK1. Such indirect effects of TGF-β are likely to explain why non-canonical TGF-β pathway activation is largely cell type specific, and why MEK1 activation is delayed (>10 hours) in HaCaT cells (Figure 3.16C).

We propose that MEK1 activity is a central component in the mechanism by which individual cells sense the surrounding cellular density. As cellular density increases in a population of cells, cellular spreading and MEK1 decreases (Figure 3.12B). This density sensing role of MEK1 explains why epithelial sheets display asymmetric MEK1 activity upon TGF-β stimulation (Figure 3.4A, 3.5A and 3.9), as these sheets display asymmetric cellular density (Figure 3.11). However, we do not currently understand the
biochemical mechanism by which MEK1 activity is modulated by cellular density. As a result we are pursuing experiments in which we perturb cellular junction integrity through TGF-β treatment, RNAi depletion of Alpha-Catenin (a known regulator of cellular junction integrity [63]) and exogenous expression of K- and H-Ras, while at the same time monitoring either Ras subcellular localization or MEK1 activity in live cells. As Ras is known to be a regulator of cellular junctions and to regulate MEK1 activity (reviewed in Chapter 4 and [81]), it serves as an ideal candidate for further investigation into our observed density sensing mechanism. We hypothesize that TGF-β stimulation ultimately leads to Ras-mediated MEK1 activation, which can be blocked by the additional activation of cellular junction integrity at high cell density. Although the mechanism of MEK1 regulation by cellular density requires further investigation, our observation that cellular density is a regulator of MEK1 activity is a novel contribution to the cell motility field that can explain the historic finding that cells inhibit migration in response to high cell density [43, 44, 46].

**Spatial Constraint of MEK1 Activity is Not a Guidance Cue for Collective Migration**

Although MEK1 activity has been proposed to be important for the guidance of collectively migrating cells, the mechanism by which such guidance occurs is thought to rely on asymmetric MEK1 activity throughout a group of cells [8, 10, 11, 111]. We show that such asymmetric MEK1 activity throughout an epithelial sheet does form in response to TGF-β stimulation
(Figure 3.4A, 3.5A and 9), but this asymmetry does not greatly impact the guidance of epithelial sheets, as sheets with spatially constrained and uniform MEK1 activity both migrate equally well towards the wound area (Figure 6A and C, and Figure 7A and C). In similar monolayers with uniform MEK1 activation, collective migration appears guided in that cells collectively migrate in a well-defined direction, but no spatial constraint of MEK1 exists throughout the group (Figure 3.8B). We propose that the low resistance of paths that lead into the wound serve as the dominant guidance cue for collective migration, in agreement with other investigations [3, 11]. This principle can also explain our peculiar observation that epithelial monolayers with spatially constrained MEK1 activity seem to exhibit collective migration towards MEK1 activity, in the absence of the wound (Figure 3.8A). In this case, we propose that the path of least resistance for a cell with high MEK1 activity is to migrate towards another migrating cell, and away from a non-motile cell. In this manner, cells with high MEK1 activity always orient their migration away from non-motile cells, creating the appearance that collective migration is guided by spatial constraint of MEK1 activity. Based on our experiments, MEK1 seems to directly control whether or not collective migration occurs, rather than in what direction it travels (discussed below). Therefore, guidance of individual cells in a collectively migrating group is likely to be determined primarily by where physical resistance is encountered, such that cells migrate away from resistance.
MEK1 Activation Suppresses Cellular Turning

Our data suggest that one role of MEK1 in collective migration is to suppress cellular turning. We found that ligand activation of MEK1 and exogenous activation of MEK1 are sufficient to cause increased migration persistence, which is causally linked to suppression of cellular turning (Figure 3.16C and 3.17B). Inhibition of MEK1 activity through chemical inhibitors or the indirect effects of cellular density was sufficient to inhibit migration persistence and activate cellular turning (Figure 3.17A, 3.18 and 3.19). Clearly, the ability of an individual cell to travel in a straight path is a function of the level of MEK1 activity that it maintains. Although MEK1 activity has been reported to affect actin treadmilling [82], it is not clear how actin treadmilling could govern the migration polarity of a cell. The likely explanation for this effect of MEK1 activity is that MEK1 activity is important for maintaining cell polarity during migration. We are currently investigating the biochemical mechanism behind MEK1 dependent suppression of cellular turning. Nevertheless, our experiments show that MEK1 activity is required for individual cells to migrate with high persistence and for groups of cells to migrate collectively.

Migration Persistence is a Required Feature of Collective Migration

Even though we are still currently investigating the mechanism by which MEK1 activation causes suppression of cellular turning, this phenomenon could explain why MEK1 activation leads to collective migration in certain circumstances, such as in epithelial sheets and monolayers (Figures 3.3C,
We propose that individual cellular migration persistence is a cause of, rather than a consequence of, collective migration. To support our claim, we found that colonies of HaCaT cells do not migrate collectively, but do exhibit increased migration persistence upon MEK1 activation (Figure 3.16A and B). In this case, cells within a colony appear to follow the lowest resistance path, which is away from the colony’s center. As a result, the expanding colony does not display collective migration. When the same cells are arranged in an epithelial sheet, the lowest resistance path for all cells is likely to lead into the wound. In this case, independently persistent cells are likely guided by resistance into parallel paths. Thus, collective migration may be a spontaneous result of persistently migrating cells that are presented with only a single low resistance path. The implications of such a model is that collective migration is not as “collective” as previously described, where the polarization of one cell elicits the polarization of the cell behind it in the group [3, 4, 112]. Instead, our model emphasizes that the combination of migration persistence and guidance by resistance for each individual cell can completely explain the collective migration that we observe, where the importance of cellular junctions is much lower than previously thought.

**Novel Insights into MEK1 Inhibition in Chemotherapy**

Although MEK1 inhibitors are likely to effectively suppress cancer metastasis by blocking the persistent migration behavior of both individual cells and groups of collectively migrating cells, this chemotherapeutic
approach of complete MEK1 inhibition will require strong delivery of high doses of MEK1 inhibitors to the tumor site. Our data suggests that low doses of MEK1 inhibitors might be more effective in preventing metastasis. In Figures 3.8 and 3.15, we showed that local MEK activation distal from a wound leads to increasing cellular density. In the hybrid monolayer experiment in Figure 3.8A, we noted that DOX dependent activation of MEK1 in the internal band of Tet-CA cells led to the contraction of the region with locally high MEK1 activity. We proposed that in this case, MEK1 activation leads to persistent migration in these cells, which are guided away from border cells with low MEK1/migration persistence because cells are following the lowest resistant path. However, this experiment provides supporting evidence that an effective chemotherapeutic approach might be to inhibit MEK1 around a tumor, rather than inside a tumor. In this way, cellular migration within a tumor can be used to a therapeutic advantage. If MEK1 can beselectively inhibited around a tumor, cellular migration inside a tumor could drive tumor density to increase. The resulting dense tumor will not only be more easily detected by MRI scanning techniques, but also will experience local depletion of nutrients that are required for survival. Although dense tumors are likely to be harder to kill with conventional chemotherapeutics, clinical studies may show that the local depletion of nutrients in such tumors is sufficient to drive nonspecific cell death alone. This novel approach of treating tumor progression by using cell migration as a weapon against cancer seems attractive and warrants further investigation.
CHAPTER IV

Partners in Crime: The TGFβ and MAPK Pathways in Cancer Progression

INTRODUCTION

A cell must acquire several key characteristics in order to become invasively cancerous: proliferation, resistance to apoptosis, evasion of anti-growth signals and immune destruction, as well as increased cellular motility [113, 114]. The TGFβ and Ras-MAPK pathways have each been implicated in all of the cellular processes that a cancer cell must exploit on the path to malignancy. However, it is becoming increasingly clear that these pathways interact, such that the resulting signal crosstalk contributes largely to the acquisition of many of the key characteristics of a cancer cell.

The TGFβ signaling pathway regulates differentiation, migration, and death during normal development. Mutations to the TGFβ signaling pathway are commonly seen in many genetic diseases and cancers [115-117]. For example, early lesions in colorectal and pancreatic cancers frequently include mutations of the cell surface TGFβ receptors or Smad transcription factors. However, the role of the TGFβ ligand in cancer progression has been somewhat puzzling and paradoxical due to its multiple, often opposing,
effects on cell growth. This TGFβ paradox is best exemplified by the fact that TGFβ has been shown to act not only as a tumor suppressor, but also as a promoter of tumor growth and metastasis [118, 119].

Ras-MAPK signaling has been linked to fundamental cell processes such as differentiation, migration and proliferation [120, 121]. The GTPase Ras and the Ras-MAPK cell surface receptors that initiate the intracellular signaling pathway (EGFR, FGFR, etc.) are often mutated in colorectal and pancreatic cancer, and these mutations lead to a constitutively active Ras-MAPK pathway [122]. Once commandeered, MAPK transcription factor substrates promote unchecked cellular proliferation, leading to tumor initiation. This review will examine the parallels between and intersections of these two pathways, with emphasis placed on their relevance in colorectal and pancreatic cancers. We will explore how the interactions between these pathways contribute to the physiological changes displayed by cancer cells, and how these interactions are modulated throughout tumor progression and metastasis.

The canonical Ras-MAPK pathway

The Ras-MAPK (Mitogen Activated Protein Kinase) pathway begins with growth factor binding to transmembrane Receptor Tyrosine Kinases (RTKs) (Figure 4.1). Growth factor binding initiates homodimerization and auto-phosphorylation in trans at specific tyrosines of the RTKs [122, 123]. Growth factor receptor-bound protein 2 (Grb2) is then recruited to the cytosolic portion of the RTKs via a Src homology 2 (SH2) domain which
Figure 4.1 A Schematic Diagram of Both Non-Canonical and Canonical TGF-Beta Signaling. In the canonical pathway, TGF-Beta receptor activation leads to Smad2/3 phosphorylation and nuclear accumulation. In the non-canonical pathway, receptor activation leads to indirect activation of the Ras-MEK pathway.
binds to phosphotyrosines. Grb2 then recruits a guanine exchange factor, Son of Sevenless (SOS), via an SH3 domain and the GTPase Ras [122, 123]. Ras, a protein that was originally identified as the transforming component in oncogenic viruses, is then post-translationally modified with an isoprenyl group that localizes it to the plasma membrane [114, 124]. The GTPase activity of Ras is enhanced by the GTPase Activating Protein (GAP). Ras recruits and activates the MAPK kinase kinase (MAPKKK) Raf which initiates a phosphorylation cascade from Raf to MEK (MAPKK) which finally phosphorylates ERK (MAPK, Extracellular Regulated Kinase). Phosphorylated ERK then translocates to the nucleus where it phosphorylates transcription factors important for proliferation and differentiation [122, 123].

The regulation of the activity and the specificity of MAPKs is critical for proper function and accurate transduction of extracellular signals. ERK is deactivated by MAPK phosphatases, resulting in a pathway that can terminate signaling in the absence of upstream stimuli [125]. The binding sites on ERK, auxiliary to the substrate recognition motif in the active site of ERK, confer the specificity for substrates [126]. Recent studies indicate that scaffolding proteins may play an important role in the timing and the amplitude of Ras-MAPK signaling by regulating the location of MAPK components [127]. The scaffold either sequesters them to the cytosol and delays a response or facilitates a faster phosphorylation cascade through the spatial concentration of the kinases (Figure 4.1), as has been shown for the scaffolding protein KSR (Kinase Suppressor of Ras) [127, 128].
The canonical TGFβ pathway

TGF-β signals through two receptor types, the TGF-β type I and type II receptors (TβRI and TβRII, respectively) [69]. Both receptors are Ser/Thr kinases. While the type II receptor is constitutively active, the type I receptor is inactive in the absence of ligand[129]. TGF-β can bind the type II receptor independent of the type I receptor [130]. Upon binding of the ligand, TGF-β induces oligomeric receptor complex formation which enables the type II receptor to phosphorylate the type I receptor in the GS domain and consequently cause a conformational change and release of inhibitory molecules such as FKBP12[129, 131]. Activated TβRII recruits and phosphorylates Smad2 and Smad3 (R-Smads), a process that is aided by SARA (Smad Anchor for Receptor Activation)[132, 133]. Phosphorylation of the R-Smads causes a conformational change such that the R-Smad is released from SARA [132]. Phosphorylated R-Smad binds Smad4 forming a R-Smad/Smad4 complex, as well as forming homo-oligomeric complexes, which translocate to the nucleus and directly interact with DNA, resulting in the recruitment of coactivators or chromatin remodeling components [134]. The recruitment of coactivators, corepressors and chromatin remodeling components determines the ultimate Smad dependent cellular response to the ligand stimulation [134]. TGF-β regulates the transcript levels of approximately 100-300 genes in various cell types (Figure 4.1) [1-3].
The Non-canonical TGFβ pathway

Activation of Smads by TGF-β is invariant in most cell types and therefore this pathway is known as the canonical TGF-β pathway. Beside activation of Smads, TGF-β also modulates the activity of several other signaling pathways in a ligand and receptor dependent manner (e.g. MAP kinase (MAPK) pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways [80, 135, 136]). Unlike the canonical pathway, modulation of these pathways by TGF-β is often cell type specific and context dependent [137]. They are collectively referred to as non-Smad or noncanonical TGF-β signaling pathways. Although canonical TGF-β signaling through Smads dominated the field of TGF-β research over the last decade, there has been increasing attention paid to the noncanonical TGF-β signaling, particularly in the context of cell migration and epithelial-mesenchymal transition (EMT). In mammalian cells, all three MAPKs are activated by TGF-β (Figure 4.1) (Each MAPK has different isoforms and, for simplicity, they are generically referred as Erk, JNK and p38). However, the kinetics of these activations vary with cell types and culture conditions [75, 78, 80, 135, 136]. The upstream signal transducers to MAPKs are likely small GTPases (Ras, Rac, RhoA and Cdc42) [75, 80, 135, 136]. Their activities are frequently regulated by TGF-β through diverse mechanisms. Here, we will focus on the Ras-MAPK pathways. For detailed discussions on the various noncanonical or non-Smad TGF-β pathways, please refer to several excellent reviews on this topic [80, 135, 136, 138].
It has been known for some time that TGF-β rapidly activates Erk MAP kinases through Ras, but the magnitude of the resulting activation of Erk1/2 is much lower compared the activation by RTKs [78]. One mechanism that has been identified involves phosphorylation of ShcA by the TGFβ receptor complex on the tyrosine residues of ShcA. Similar to the RTK signaling, ShcA phoshotyrosines recruit the Grb2/SOS/Ras complex which subsequently trigger the activation of the Ras-MAPK pathway (Box 1, Figure 4.1) [139]. The biological significance of intrinsic activation of Ras-MAPK by TGFβ in normal cellular process is still poorly understood. Gene expression profiling studies implicate that TGF-β-stimulated Erk activation is involved in the modulating of a subset of genes that figure prominently in cell motility and cell-matrix interaction [140]. Some of the genes are often associated with epithelial to mesenchymal transition (EMT), an indispensable mechanism for producing mesenchymal cells, tissues and organs during normal development [141-143]. Thus, the intrinsic, relatively low, activation of Ras-MAPK is likely crucial for specific induction of genes regulating EMT and cellular motility.

**Signaling Crosstalk Between the Ras-MAPK and TGFβ pathways**

The integration of Ras-MAPK and TGFβ pathways can occur via balanced activation of both canonical and noncanonical TGFβ pathways by TGFβ alone, through simultaneous stimulation of TGFβ and ligands of other signaling pathways that modulate the activity of Ras-MAPK, or through crosstalk via interactions between the intracellular effector proteins distinct to
each of the two pathways. In the two lattermost mechanisms, there are two common points of integration of the Ras-MAPK and TGFβ pathways: 1) phosphorylation of coactivators of R-Smads by Erks, 2) phosphorylation of Smad2/3 by Erks in the linker region. Most of the TGFβ signaling responses are cell context dependent [137]. The cell type specific responses are in part due to Smad interaction with cell type specific transcription factors [134]. For example, activated Smad3 binds its cognate sites with Oct4 in embryonic stem cells (ESCs), MyoD in myotubes and PU.1 in pro-B cells [134]. The association between activated Smad2/3 and transcriptional cofactors can be regulated by the Ras-MAPK pathway. For example, Ras-MAPK activity has been shown to regulate the interaction of p53 and Smad2/3. In this mechanism, FGF signals through Ras-MAPK to regulate p53 phosphorylation at its N-terminus (Ser6 and Ser9) by CK1ε/δ [144]. Such phosphorylation of p53 enables its interaction with activated Smad2/3 to regulate target genes that are important for TGFβ cytostatic program [144]. Similarly, Src-activated Epidermal Growth Factor Receptor (EGFR) signals through Ras-MAPK to engender phosphorylation of the E-box binding transcription factor USF and facilitate its interaction with activated Smad2/3 to regulate PAI-1 gene expression [145]. Thus, the RTK activated Ras-MAPK pathway converges with the canonical TGFβ pathway at chromatin in order to regulate gene expression.

Ras-MAPK has also been shown to phosphorylate R-Smads auxiliary to the activation regions[146, 147], sequestering them to the cytoplasm to attenuate TGFβ signaling [147]. However, the opposite effect of activated
Ras-MAPK on Smad activity has also been reported [148-150]. A possible explanation for the contradictory observations is that the levels of Ras activity could dictate the outcome of the experiment [151]. In a majority of human cancers carrying oncogenic Ras mutations, the activity rather than the levels of Ras expression is elevated. Thus, the levels of Ras activity translate into the levels of MAPK activity, ultimately shaping the TGFβ response via crosstalk between R-Smads and the Ras-MAPK pathway.

The linker region between Mad Homology Domains 1 and 2 (MH1 and MH2) have been shown to be phosphorylated by GSK-3 and ERK1/2. Phosphorylation of Ser204 on Smad3 via GSK-3 kinase activity results in decreased affinity between Smad3 and CREB binding protein, suggesting that the functional significance of the linker region phosphorylation status is to modulate transcription factor activity of SMAD proteins, rather than to regulate the nucleocytoplasmic localization of Smad proteins[152]. Consistent with this theory, the linker region phosphorylation of Smad2 and Smad3 by ERK1/2 at positions Ser240/Ser245/Ser250 and Ser204/Ser208/Ser215, respectively, has been shown to enhance Smad transcriptional activity of the Type I and Type III Collagen gene [148, 153]. Additionally, Small C-terminal Phosphatase (SCPs) in the nucleus have been shown to dephosphorylate the linker region phosphorylation sites in Smad2, confirming a primarily nuclear role of the linker region phosphorylation [154]. Such dephosphorylation by SCPs results in activation of Smad dependent transcriptional regulation of exogenous luciferase reporters[154]. Thus, conflicting results about the functional consequences of linker region phosphorylation suggest that not
only is Smad2/3 transcriptional activity regulated in a cell type specific manner, but also perhaps in a single cell type discrete genes are positively or negatively regulated by linker region phosphorylation in a chromatin context dependent manner. Taken together, the current and previously reported data strongly suggests that different cellular responses to TGFβ stimulation in different cell types and cellular contexts largely results from the status of MAPK activity at the time of ligand stimulation.

**Systems Features of MAPK and TGFβ pathways**

Even though the signaling of the TGFβ and Ras-MAPK pathways initiates upon ligand binding at the plasma membrane, the modes of signal transmission are quite different [155, 156]. The Ras-MAPK pathway features a cascade of sequential kinase phosphorylations/activations from RAF to ERK (Figure 4.1). This type of pathway architecture can lead to significant amplification of the original upstream receptor/ligand binding signal. The RTKs also activate other signaling pathways such as PLCγ, PI3K or Src. Some of these signaling pathways can feed into Ras-MAPK and produce positive feedback loops. There are also multiple well characterized negative feedback loops in the Ras-MAPK pathway that cause desensitization or dampening of the signal [157]. The presence of these feedback loops have been shown to produce switch-like (i.e. bistable) responses to receptor activation, allowing for a tight threshold of cellular response during development [158, 159].
Unlike the Ras-MAPK pathway, the TGFβ signal relay system is relatively short. There are no apparent signal amplification steps in the signaling cascade subsequent to the receptor phosphorylation of R-Smads. The constant shuttling of Smads in and out of the nucleus allows the Smads to constantly monitor receptor activity [156]. This type of arrangement results in a more linear response to ligand binding. However, a more recent study reveals that TGFβ depletion during signaling is important to produce threshold-like input/output response to receptor activation [160]. Clearly, there is also nonlinearity in the TGFβ pathway.

Systems analysis of biological signaling pathways suggests that signaling pathways interact with one another and the final biological response is shaped by interaction between pathways [159]. Since there are multiple integration points between the Ras-MAPK and TGFβ pathways, it is not a surprise that the integration of these two pathways produces quite complex biological outcomes, depending on the cellular context. The challenge is how to precisely determine the behaviors of the interacting pathways and how to manipulate the end-point biological responses in normal and cancer cells.

Lesions in the Ras-MAPK and TGFβ pathways in Cancers

Colorectal and pancreatic cancers are, respectively, the fourth and fifth causes of cancer deaths [161]. More than one third of colorectal cancer patients develop metastasis. Of these cancers, about 35% are the result of genetic predisposition due to inherited lesions in the TGFβ pathway [162]. Additionally, mutations that inactivate the key signal
components, including receptors and Smads, are frequently observed within the tumor. Loss of function mutations in TβRII have been found in a majority of colorectal and gastric carcinomas with microsatellite instability (MSI) [163, 164]. In microsatellite stable colon cancer cell lines, missense mutations are identified in ~15% of cases [165]. Inactivating mutations of TβRII occurs at low frequency in pancreatic and biliary carcinomas [166] but relatively high in ovarian cancers with wild type TβRII [167]. In one example of TGFβ pathway lesion, TGFβRI*I6A, the TGFβRI is missing three alanines from the signal sequence. TGFβRI*I6A was previously associated with hereditary cancers based on analysis of a limited numbers of cases [168, 169]. Recent work using a much larger number of case studies suggests that there is no increased hereditary risk of colon cancer associated with this mutation [170]. However, it is still possible that TGFβRI*I6A does convey increased risk in specific patient populations or in somatic mutations [170].

Inactivation of TGF-β signaling also occurs by disabling Smad proteins. SMAD4 was originally identified as a tumor-suppressor gene lost in pancreatic cancers called DPC4 (deleted in pancreatic cancer 4) [171]. Subsequent studies revealed that deletion of Smad4 also occurs in 16–25% of colorectal cancer [172]. Germline mutations of SMAD4 are associated with juvenile polyposis and hamartomatous polyposis [173, 174]. Inactivating mutations of Smad2 have been identified in ~6% of colorectal cancers while loss of function mutations of Smad3 are rare in colorectal cancers [172, 175].
Escaping the TGFβ growth constraint is one of the hallmarks of tumor cells [114]. Tumor cells explore different mechanisms to achieve this. Despite the fact that inactivating mutations in the canonical TGFβ pathways in colorectal and pancreatic tumors are wide spread, cells isolated from other tumor types preserve the functionality of the canonical TGFβ signaling components and achieve TGFβ growth resistance by altering other aspects of the pathways [117]. Thus, oncogenic activation can also interdict TGFβ growth inhibitory responses by modifying the activity of downstream elements such as cell cycle inhibitors or transcriptional cofactors of Smad transcription factor activity.

Throughout tumor progression, the TGFβ ligand becomes highly expressed and actually promotes proliferation, particularly in the case of prostate cancer [176]. This may be due to its ability to stimulate MAPK pathways [177]. High levels of TGFβ are correlated with increased angiogenesis, as the tumor recruits blood vessels to allow for greater growth by inducing greater TGFβ dependent VEGF expression [178]. In the clinic, patients with high levels of TGFβ have decreased survival, likely due to aggressive tumor progression and metastasis [179]. In addition to over secretion of the TGFβ ligand, deregulation of the intracellular pathway can sensitize cells to an unchanging amount of TGFβ ligand. For example, in situ cell culture RNAi knockdown of Phosphatase and Tensin Homolog (PTEN), a TGFβR phosphatase, resulted in the increased TGFβ dependent invasion potential of cells [180]. Recent studies also suggest that TGFβ is involved, in some cell types, in directing metastatic cells to specific locations.
such as bone [181, 182]. The cellular response to TGFβ in tumors clearly plays a role in tumor metastasis, even though there is not strong evidence that R-Smads mediate this role.

The Ras-MAPK pathway is an important component of many cancerous cells. The majority of cancer associated lesions to the Ras-MAPK pathway result in constitutive activation of the pathway [124]. Mutations that have been identified are located early in the pathway and include overexpression of RTKs, activating mutations to RTKs, sustained expression of activating autocrine or paracrine ligands, and mutations to Ras and Raf [114, 124]. Temporally, these lesions occur early in tumorigenesis and are sustained for the life of the cancer (Figure 4.2). Ras mutations have been found in 30% of all cancers, 90% of pancreatic cancers and 50% of colon cancers [124]. B-Raf mutations have been found in 65% of melanomas, 45% of papillary thyroid cancers and 36% of ovarian cancer [183-185]. In summary, a large portion of human tumors have a constitutively active Ras-MAPK pathway and have acquired resistance to TGFβ induced cell cycle arrest. Thus, the role of TGFβ in cancer progression seems to be largely through lesions that stimulate the non-cannonical signaling, and are aided by inactivating lesions in the canonical pathway, which are associated with cell-cycle arrest and the apoptotic response.
MAPK activity

TCF7 activity

Healthy cells

Late lesions: cells begin to exhibit hallmarks of cancer

Early lesions: and metastasis
Figure 4.2 A Schematic Diagram of the Role of TGF-Beta and MAPK Activities in Cancer Progression. TGF-beta activity is elevated in the late stages of cancer progression, while MAPK activity is elevated in the early stages of cancer progression.
Crosstalk between TGFβ and Ras-MAPK Signaling Pathways drives EMT

Crosstalk between the TGFβ and Ras-MAPK pathways appears to be required for tumor metastasis, likely through the role of crosstalk in epithelial-mesenchymal transition (EMT). TGFβ induced EMT, which is a fundamental mechanism that drives metastasis in vivo or invasion in vitro, requires constant TGFβ signaling to become a stable phenotype [186, 187]. The connection between EMT and metastasis is exemplified through the finding that fibroblast cells that undergo EMT are more conducive to promote tumor invasion [188]. EMT is marked by changes to the actin cytoskeleton, loss of cell polarity, and migration/invasion [143, 189]. Acting alone, neither pathway is successful in permanently converting from an epithelial to a mesenchymal phenotype [149]. Long-term expression and cooperation of TGFβ and Ras- MAPK causes full EMT through induction of cytokine feedback loops that include upregulating TGFβ autocrine signaling [186]. Recently, a new mechanism by which TGFβ induces EMT has been introduced, where TGFβ induces isoform switching of fibroblast growth factor (FGF) receptors [188]. Prolonged treatment of TGFβ results in increased FGFR1IIIc, which is the mesenchymal isoform, and decreased expression of the epithelial isoforms of FGF receptors. FGF-2 but not FGF-7 signals through FGFR1IIIc[188]. The switch in FGF-2 receptor expression enables cells to respond to FGF-2 to activate Ras-MAPK. Furthermore, epithelial cells treated with both TGFβ and FGF-2 undergo EMT instead of epithelial to epithelial–myofibroblastic transition (EMyoT). Thus, the
crosstalk between the Ras-MAPK and TGFβ pathways may be harnessed to promote tumor growth through EMT.

**Conclusions and Future Directions**

Colorectal and pancreatic cancers account for a large portion of cancer incidence and fatalities in the United States. Thus, they are important models for the study of the complicated roles of Ras-MAPK and TGFβ pathways. As more is learned about these pathways in normal and cancerous cells, it becomes increasingly clear that crosstalk between the distinct pathway components are important for TGFβ signal interpretation in a cell type and cell context dependent manner. Future studies are required to address the interdependency of these pathways, paying close attention to the connection between the cellular context in a study, the level of contribution of the Ras-MAPK pathway to the TGFβ response, and the ultimate cellular response to TGFβ ligand stimulation.

A consequence of this emerging theme of crosstalk may be the reevaluation of cancer therapeutic strategies. Currently, Ras and Raf have been the hot targets in the Ras-MAPK pathway for anticancer drugs. Inhibition of isoprenylation of Ras seemed promising. However, it appears that the enzymes that catalyze this reaction are somewhat promiscuous and off-target effects have limited the successful development of inhibitors of Ras. Other Ras-MAPK components such as Raf, MEK and ERK are also being investigated and are proving more successful drug targets [190]. One drug targeting Raf has been approved by
the FDA for treatment of renal cell carcinoma and a handful of other drugs are in Phase I/II/III trials that target Raf and MEK [190]. TGFβ’s involvement in promoting a metastatic phenotype in aggressive cancers makes it a highly sought after pathway to target for anticancer therapies. The obvious targets of choice are the TGFβ ligand itself, the ligand-binding surface on TGFβRI/II and betaglycan [191]. As a result, a humanized anti-TGFβ monoclonal antibody (GC1008) is in Phase I/II clinical trials. Additionally, soluble TGFβRII and betaglycan recombinant receptors, which compete for extracellular TGFβ, as well as inhibitors TGFβRI kinase activity have shown encouraging anti-metastatic results [192]. As might be the case for many kinase inhibitors, long term administration of a single agent may select for more aggressive drug resistant tumor variants, as has been demonstrated for LY2109761, a TβRI/TβRII kinase inhibitor in a mouse skin model [193]. Considering the synergistic effects of TGFβ and MAPK pathways in tumorigenesis it is logical to try a combinatorial approach in cancer treatments, where simultaneous partial inhibition of the Ras-MAPK and TGFβ pathways, yields synergistic inhibition of TGFβ ligand induced metastasis, while having minimal impact on other aspects of TGFβ and Ras-
MAPK biology.
CHAPTER V

Dynamics of TGF-β/Smad Signaling

INTRODUCTION

The Basics of Transforming Growth Factor-β Signaling

TGF-β is the prototypical ligand of the TGF-β superfamily, which signal through receptor serine/threonine kinases. The superfamily is subdivided into two branches: (1) the TGF-β/Activin branch and (2) the Bone Morphogenetic Protein (BMP)/Growth and Differentiation Factor (GDF) branch. Each branch is further divided into subgroups based on sequence similarity [69]. The TGF-β/Activin branch includes TGF-β, Activin, Inhibin, Nodal, and Lefty ligands. The BMP/GDF branch includes BMP, GDF, and Mullerian Inhibitory Substance (MIS) ligands. This review will focus on the quantitative analyses of TGF-β signaling, which is the most studied ligand in terms of basic signal transduction mechanisms. There are also substantial quantitative studies of BMP signaling, especially in the context of morphogen gradient formation and interpretation which have been covered in several excellent reviews [194-197].

TGF-β is expressed in most cell types and is translated into a proprotein that is proteolytically cleaved into a noncovalently linked mature TGF-β and latency-associated protein (LAP) [198, 199]. The active TGF-β ligand is a 25
kDa dimer, covalently linked by a disulfide bond between cysteine residues from each monomeric peptide. When bound to LAP, TGF-β cannot bind to its receptors, resulting in a ligand that lacks bioavailability. Its bioavailability is further restricted by binding to another protein called Latent TGF-β Binding Protein (LTBP). The LAP-TGF-β complex is bound by LTBP during the secretion process [198]. LTBP binds the extracellular matrix and sequesters LAP-TGF-β in vivo [198]. Various proteases cleave LAP and LTBP to liberate the bioactive TGF-β [8]. Bioactive TGF-β can bind various non-receptor cell surface proteins such as decorin, biglycan, and betaglycan, which also serve to regulate its bioavailability, most likely through the enrichment of TGF-β at the plasma membrane [198, 199]. Therefore, multiple mechanisms serve to regulate the bioavailability of TGF-β in vivo.

Once bioavailable TGF-β reaches the surface of the target cell, it binds a homodimer of TGF-β type II receptors (TβRII) [69]. The TGF-β-TβRII complex provides a structural interface that facilitates stable complex formation with a homodimer of the TGF-β type I receptor (TβRI) [132]. Therefore, the active ligand-receptor complex is a heterotetrameric complex consisting of a dimer of TGF-β and homodimers of both TβRII and TβRI. Within the active receptor complex, the TβRII, which is a constitutively active kinase, undergoes autophosphorylation, as well as catalyzes transphosphorylation of the TβRI [132]. Transphosphorylation of the TβRI activates kinase activity. In the TGF-β pathway, Smad2 and Smad3 are receptor-regulated effector proteins (R-Smads), which are phosphorylated by
the activated TβRI at a C-terminal SSXS motif, resulting in R-Smad nuclear accumulation [132].

TGF-β signaling amplitude and duration can be regulated through the control of receptor trafficking. The ligand bound activated receptor complex is internalized via endocytosis [200]. Internalization of cell surface receptors can occur through either clathrin-mediated or caveolae-mediated endocytosis [201]. Through the clathrin pathway, activated ligand-receptor complexes are brought into early endosomes which are enriched with scaffold proteins such as SARA and Hrs [200]. The proximity of the activated receptor complex and scaffold proteins enhances the phosphorylation of Smad2/3 and their affinity towards Smad4, ultimately activating the nuclear accumulation of Smad4 (also known as the co-Smad) [202]. Therefore, clathrin-mediated endocytosis may promote TGF-β signaling by providing a platform for R-Smad phosphorylation and formation of active Smad signaling complexes. Ligand-bound receptor complexes in the early endosomes are further sorted to late endosomes, where TGF-β and the receptors are separated. Some of the unbound receptors can be recycled to the plasma membrane, while others are degraded, along with TGF-β, upon fusion with the lysosomes [201]. Since TGF-β is not recycled, internalization of TGF-β by endocytosis is the primary means of removing active TGF-β from the cell surface, and lysosomal degradation is the primary means of termination of TGF-β signaling [203, 204].

Activation of TGF-β receptors initiates both Smad-dependent and Smad-independent signaling events [135, 136, 205]. Since the majority of the
quantitative studies of TGF-β signaling have focused on Smad-dependent events, we will focus our discussion on the dynamics of the canonical pathway (Smad-dependent). In unstimulated cells, Smads constitutively shuttle between the cytoplasm and nucleus. Upon ligand stimulation, the Smads accumulate in the nucleus as the R-Smad/Co-Smad complex formation leads to a decrease in their nuclear export rate [155, 206, 207]. The Smad complex binds DNA in conjunction with other transcription factors and interacts with the general transcription machinery to regulate the expression of approximately 100-300 target genes [202]. Phosphatase(s) such as PPM1A can deactivate phospho-R-Smads, resulting in the disassembly of the Smad complex and providing a means for negative regulation of TGF-β signaling in the nucleus [155]. Therefore, the intracellular Smad signaling module is a dynamic circuit for ligand sensing.

**Mathematical modeling of the TGF-β signaling pathway**

Conventional cell signaling studies have largely focused on understanding the identity and the functions of the individual parts of each pathway. It is now realized that cell behaviors are not only shaped by the identity of the individual system components, but also by the weighted interactions of components that act together as a system. The systems biology approach using mathematical models has been proven as a powerful tool in studying such complex networks [208]. Mathematical modeling is helpful in predicting emergent cell behaviors and uncovering how the dynamic interactions of
signal transducers lead to context dependent cellular responses [209]. Several 
mathematical models have been established for the canonical TGF-β/Smad 
signaling [204, 206, 210-217]. These mathematical models provide 
quantitative analyses of TGF-β signaling dynamics, leading to a better 
understanding of the role of feedback mechanisms in the ultimate fate of 
context dependent signaling responses.

The most common way to describe TGF-β signaling dynamics is through a 
set of deterministic ordinary differential equations (ODEs), assuming the 
signaling molecules are well-mixed or homogenous in each compartment 
[208]. The ODEs represent the change of each signaling molecule over time. 
In order to perform simulations and predictions (modeling), it is necessary to 
know the values for two types of model parameters: the initial conditions of 
the pathway that correspond to the concentrations of the signaling molecules 
at time 0 (before TGF-β stimulation) and the rate constants that characterizes 
the signaling reactions. The principal molecular components of TGF-β 
signaling have been identified, yet relatively little is known about the 
quantitative values of particular components’ abundance and rate constants. 
The lack of experimental data on initial conditions and rate constants is 
currently one of the bottlenecks for developing high-quality predictive 
mathematical models for signaling networks.

Different approaches have been applied to estimate the model parameters. 
The initial conditions of the signaling network can be determined by the 
absolute abundance of the signaling proteins and the volume of the signaling 
compartment. It is possible to estimate the absolute abundance of a signaling
protein when the recombinant protein is available. For example, the absolute abundance of Smad2 protein per cell can be quantified by immunoblotting with a standard curve of recombinant tagged-Smad2 [218]. Cell volume is sometimes roughly estimated from cell diameter or it can be measured more accurately by confocal microscopy [219]. On the other hand, direct measurement of the rate constants for different signaling reactions is still experimentally challenging. In order to estimate the unknown model parameters, optimization algorithms are applied to find the most feasible parameters that make model simulations fit the experimental datasets as close as possible [220, 221]. During the past few years, some quantitative data has been experimentally measured for the TGF-β signaling pathway, aiding the modeling efforts for this network [201, 204, 207, 213]. Figure 1 summarizes our current knowledge about the model parameters for the canonical TGF-β/Smad signaling network.

**Quantitative analysis of signaling responses to different TGF-β stimulations**

The cellular responses to TGF-β superfamily ligands depend on the quantity to which the cells are exposed. In development, TGF-β superfamily members form morphogen gradients to determine the fates of cells [194]. Cells read the TGF-β concentration with high precision, as they can distinguish subtle differences in the concentration gradients and orchestrate different cell fates [222]. One of the best examples is the responses of
embryonic Xenopus cells to activin, in which five distinct cell responses or fates are observed by varying activin doses [222]. However, the mechanism by which cells are able to accurately decode the concentration of bioavailable TGF-β and elicit a corresponding cellular response remains largely unknown.

The Smad signaling response correlates with TGF-β molecules per cell rather than the concentration of TGF-β

An early study with mathematical models has shown that cell density affects signaling dynamics in response to the same concentration of ligand [223]. Through modeling analyses of receptor trafficking networks, Zi et al. showed that with the same concentration of ligand stimulation, cells have distinct signaling durations that depend upon cell density, where signaling persists longer when cell density is decreased [223]. Additionally, the model analyses indicate that the dose–response curve of signaling is shifted to the right as the cell density is increased, suggesting that increasing cell density allows for insensitivity to lower doses of ligand. Thus, the key parameter for successful experimental design cannot be “concentration of ligand”, but rather must be “molecules of ligand per cell”, which takes into account the number of cells in the experiment. Further model simulations indicate that signaling responses are regulated by the ratio of ligand to cell surface receptor number [223]. In cell culture experiments, individual cells are likely to express different amounts of receptors at cell surface. Thus, when all cells are exposed to the same amount of ligand, the ratio of ligand to cell surface receptor
number in each cell will be different, which might cause heterogeneous signaling responses at single cell level.

To provide additional evidence that cellular responses to ligand occur in terms of “molecules per cell” rather than by the concentration of the ligand, Clarke et al. investigated how cells transduce TGF-β doses into variable kinetic profiles of Smad2 phosphorylation at C-terminal motif (P-Smad2) by quantitative experimental assays [203]. Clarke et al. measured P-Smad2 levels in a two-level factorial experiment by varying four experimental parameters (TGF-β concentration, cell number at seeding, plate type, and medium volume). When the P-Smad2 data is plotted versus TGF-β concentration, large variations are observed for the same TGF-β concentration among different experimental setups. In contrast, the variation of P-Smad2 levels is significantly reduced if these levels are plotted versus the number of TGF-β molecules per cell. This result implies that the ligand dose expressed as TGF-β molecules per cell is a better predictor of P-Smad2 levels, which is in agreement with early modeling studies about the impact of cell density on signaling response.

Ligand depletion in the TGF-β network provide additional complexity and increases the difficulty of predicting the time dependent signaling responses. For example, the number of TGF-β molecules per cell in the media changes substantially with time because the cells deplete TGF-β from the surrounding medium. It was shown that TβRII defective, but not TβRI defective, cell lines lost their ability to deplete TGF-β from the media [203]. Thus, TβRII helps to shape the Smad signal amplitude and duration by constitutively depleting
extracellular TGF-β. TGF-β depletion most likely occurs through TβRII-mediated endocytosis. However, direct evidence that supports this notion remains to be presented in the literature. In this aspect, TGF-β degradation shares many similarities to EGF or TGF-α [224, 225] in that ligand-induced endocytosis does not merely serve as a mechanism for “down-regulation” of signaling, but also provides a mechanism whereby the receptor can continuously track the changes in the secretion of TGF-β by nearby cells. It should be noted that most studies of TGF-β signaling assume that TGF-β concentration in the medium is sufficient to describe the input variable (potency of ligand). Consequently, most modeling studies have assumed a constant level of TGF-β during signaling over time. However, in cell based experiments TGF-β concentration in the medium changes substantially with time, especially for low doses of TGF-β. Therefore, the assumption that TGF-β concentration is constant in medium might be appropriate for high doses of TGF-β, but it is invalid for low doses of TGF-β.

**TGF-β dose-responses are time dependent**

Earlier experimental and modeling analyses showed that Smad signal amplitude is gradually increased with the increments of TGF-β doses [211, 212, 226]. This leads to an important question about how cells convert continuous TGF-β doses into discrete or binary cellular fate decisions. Since most of modeling studies in the TGF-β field do not account for the TGF-β input variable by assuming constant TGF-β concentration in medium, this
omission often results in unreliable predictions of Smad signaling kinetics in response to variable doses of TGF-β stimulation [204]. Recently, we developed an improved mathematical model to describe the dynamics of Smad signaling in response to TGF-β [204]. The model was composed of TGF-β receptor trafficking, Smad phosphorylation and Smad nucleocytoplasmic shuttling. More importantly, it took into account the dynamics of TGF-β depletion. Model parameters were estimated by model fitting to several time course datasets, which include variables of TGF-β depletion in the medium, Smad2 nuclear localization and P-Smad2 dynamics. The model was further tested for its ability to predict the P-Smad2 signaling upon pulsation of TGF-β stimulation. With this data-calibrated model, novel predictions were made through model simulations. It was shown that TGF-β signaling responses display different sensitivities to ligand doses at different time scales [204]. In this study, modeling simulations and experimental results show that while short-term P-Smad2 is graded, long-term P-Smad2 response is switch-like to changes in TGF-β doses (Figure 2). In the short-term graded response, P-Smad2 signal gradually increases with rising increments of TGF-β dose. In the long-term switch-like response, a small change of TGF-β dose within a certain range results in a large change in P-Smad2 response. Furthermore, a switch-like response is observed for TGF-β induced long-term gene expression and growth inhibitory responses. Additional model perturbation experiments predicted that the switch-like, long-term P-Smad2 response is mainly affected by the parameters related to the ligand depletion. This prediction was experimentally confirmed [204].
The TGF-β pathway is insensitive to high frequency noise

While extensive studies have focused on signaling responses to continuous TGF-β stimulations, little is known about how cells respond to short pulses of TGF-β stimulations. Taking advantage of model simulations, Zi et al. have shown that short-term TGF-β pulse stimulation results in transient P-Smad2, whereas serial pulses result in sustained P-Smad2, similar to that seen with continuous stimulation [204]. To generate a sustained response, the gap between repeated pulse stimulation is ~30 min. This result suggests that with a strong TGF-β stimulation, the pre-bound receptors are capable of sustaining the signaling response for half of an hour and tiding it to the next stimulus. Incidentally, this optimal gap period is approximately the time to reach maximum Smad2 phosphorylation and nuclear accumulation of Smad2 after TGF-β stimulation [204]. Therefore, there is a time-delay in the TGF-β signaling system, which may be attributed to ligand-bound receptor endocytosis or Smad nucleocytoplasmic shuttling dynamics. Because of this built-in time-delay, the TGF-β signaling system can filter high frequency changes (short time pulsations) in ligand stimulations. Thus, combining mathematical modeling with guided experiments enables new discoveries in systems properties of TGF-β signaling that would have been difficult to reveal using the traditional biochemical approaches alone. The functional significance of pulses of TGF-β has yet to be shown in vivo, but is theoretically occurring within tissues, where the extracellular volume and
local secretion of TGF-β is extremely small in magnitude, resulting in a largely noisy extracellular level of TGF-β. Such noise would then be dampened by the delayed TGF-β response that has been observed in cell culture models.

**Quantitative analysis of transient and sustained signaling responses**

The duration of signaling responses can be critical for alerting cell fate decisions in response to growth factor. Previous work with PC12 cells showed that epidermal growth factor (EGF) induces transient activation of extracellular signal-regulated kinase (ERK) and results in cell proliferation, while sustained ERK activation triggered by nerve growth factor (NGF) leads to cell differentiation [227, 228]. Although some of these effects could be due to non-ERK dependent responses to the differing ligands, these experiments propose an interesting notion, where the duration of ligand stimulation can determine the prevailing cellular response.

In the case of the TGF-β signaling network, the duration of Smad signaling response seems to be context dependent. Experimental studies have shown that keratinocyte epithelial cells have sustained phospho-Smad responses to TGF-β, while some fibroblast and tumor cells display transient Smad activation [229, 230]. It was hypothesized that sustained TGF-β signaling may be required for growth inhibition, while transient signaling may cause the resistance to anti-proliferative effects of TGF-β in certain tumor cells [229]. However, the exact mechanism underlying the variation of Smad
signal duration in different cell types remains to be elucidated. Here we summarize the time dependent changes in the cellular responses to TGF-β.

**Sequestration of TGF-β receptors by endocytosis**

TGF-β signaling is initiated by the binding of TGF-β to TβRI and TβRII. The activation of the ligand-receptor complex is a relatively fast step in the TGF-β signal transduction pathway. An early study from Wrana et al. shows that the phosphorylation of TβRI in the receptor complex peaks at about 2 minutes after TGF-β stimulation [231]. The signal is relayed to the activation of Smad proteins, which arrive at their maximal levels in about 30-60 minutes. The time delay between ligand receptor complex and R-Smad activation may be due to intermediate processes, including receptor endocytosis, the recruitment of Smads to receptor complex and Smad activation. After 30-60 minutes, the phosphorylation of Smads correlates with the degree of TGF-β-receptor complex level, which might be due continuous nucleocytoplasmic shuttling of the Smads, but this shuttling fails to explain why there is a prominent delay following receptor activation and prior to Smad phosphorylation [74, 213].

Although it has been shown that two main types of endocytosis mediate the internalization of TGF-β receptors, clathrin-dependent and clathrin-independent [200, 232], different lines of experimental evidence display discrepancies in the requirement of receptor internalization for Smad phosphorylation. Using potassium depletion, which interferes with clathrin-dependent internalization of receptors, pSmad2 levels are reduced in Mv1Lu
cells [201, 233]. On the other hand, potassium depletion or mutant dynamin (K44A) in L17-TβRI cells reveals that receptor trafficking is entirely dispensable for TGF-β signaling to occur [200, 234]. Even though there is little debate about whether the TGF-β receptors undergo endocytosis, the precise role of receptor endocytosis in signaling remains controversial [200]. Several lines of evidence support a positive role of endocytosis on R-Smad phosphorylation [201, 233, 235, 236], while there are several reports describing Smad activation immediately at the cell surface without need of receptor endocytosis [234, 237, 238]. The disagreement among these studies could be attributed to different cell types and experimental systems. Despite the variable effects of receptor endocytosis on Smad phosphorylation, activation of non-Smad signaling pathways by TGF-β appears to require receptor internalization [238, 239].

To understand the role of receptor endocytosis on TGF-β signaling, mathematical models were established by focusing on TGF-β receptor endocytosis. Vilar et al. developed a concise model for TGF-β receptor trafficking and the ligand-receptor interactions. This model predicts that the duration of signaling activity is determined by the ratio of the constitutive to the ligand-induced degradation rate of the receptors, termed the “constitutive-to-induced degradation ratio” (CIR). Model analyses suggest that signal activity is transient with a low CIR, while sustained signal response is observed with a high CIR. The model has some assumptions including: (1) TGF-β signaling activity is proportional to the level of ligand-receptor complexes in the internalized endosomes, and (2) Ligand-receptor complexes
between type I and type II receptors have the same constitutive degradation rate. In addition, the model lumps the processes including non-clathrin dependent internalization, recycling and the degradation of the receptors into one reaction as “the ligand-induced receptor degradation from plasma membrane”. Thus, CIR defined in Vilar’s model is not directly determined by the ratio of the reaction rate constants for constitutive and ligand-induced degradations. Instead, it refers to the balance of the overall effect of the combined processes of the two branches of receptor degradation machinery.

Subsequently, a mathematical model developed by Zi and Klipp includes two major types of TGF-β receptor endocytosis, Smad activation and Smad nucleocytoplasmic shuttling [217]. Similar as Vilar’s model, the extended model assumes that Smad activation is proportional to the ligand-receptor complex in early endosomes. The model simulations using different sets of parameter values suggest that Smad activation is regulated by the balance between clathrin dependent endocytosis (\( k_{EE} \)) and caveolar/lipid-raft mediated (clathrin-independent) endocytosis (\( k_{Cave} \)). If clathrin-dependent internalization is dominant (\( k_{EE} \gg k_{Cave} \)), Smad activation becomes a sustained response. On the other hand, if clathrin-independent endocytosis is overwhelming (\( k_{Cave} \gg k_{EE} \)), Smad activation displays a transient response. Interestingly, the simulation results suggest that changing the balance between the two branches of endocytosis has relatively little effect on the early Smad signal, and has larger effect in reshaping long term Smad activity. This hypothesis might explain the variations among experimental observations about the impact of inhibiting TGF-β receptor endocytosis on TGF-β
signaling responses, where the different model systems may have different ratio of clathrin-dependent to clathrin-independent endocytosis. Thus, in a cell line with similar rates of clathrin-dependent and clathrin-independent endocytosis, an inhibition of clathrin-dependent will make clathrin-independent endocytosis (ligand-induced receptor degradation) dominant and result in a reduction in Smad activity. In other cell types, if the clathrin-dependent endocytosis is overwhelmed by clathrin-independent endocytosis, inhibiting clathrin-dependent internalization will not shift the balance between these two endocytosis branches and should result in little effect on the perturbation of signaling responses. Moreover, the inhibitors of endocytosis used in previous experimental studies may not be very specific and could have some off target effects. In the future, a systems biology approach can be useful to clarify this issue by combining mathematical models with quantitative experimental data of receptors and Smad kinetics.

**Quantitative analysis of the dynamics of Smad nuclear import and export**

A prominent feature of TGF-β signaling is the continuous shuttling of Smads in and out the nucleus, in both treated and untreated cells [74, 240]. Ligand-induced nuclear accumulation of R-Smad and Co-Smad is relatively slow and peaks at ~45 min after TGF-β exposure. Ligand-induced Smad shuttling dynamics is considered to be a prevailing mechanism for Smads to continuously monitor receptor activity [74, 240]. Different mechanisms have been invoked to account for Smad nucleocytoplasmic shuttling dynamics.
Mathematical modeling analysis has been instrumental in differentiating various hypotheses [155]. Clarke et al. [206] was the first to publish a kinetic model of Smad signaling dynamics. The simple kinetic model includes R-Smad phosphorylation/dephosphorylation, heterodimerization with Smad4, and nucleocytoplasmic shuttling steps. Analyzing the existing data with this model led to several novel hypotheses for Smad nuclear accumulation during TGF-β signaling. Through parameter sensitivity analysis, Clarke et al. posited that (1) the balance between the rates of R-Smad phosphorylation in the cytoplasm and phospho-R-Smad dephosphorylation in the nucleus determines Smad nuclear accumulation. (2) Smad homo- or hetero-oligomerization could protect the phospho-R-Smads from rapid dephosphorylation and therefore promote Smad nuclear accumulation. (3) Nuclear retention factors alone are insufficient for induction of Smad nuclear accumulation. Schmierer et al. investigated the relationship between receptor activation and Smad shuttling dynamics using a combined mathematical modeling and systematic experiment approach [213]. Two competing models were developed based on different assumptions of the Smad import mechanism. The retention/enhanced complex import (RECI) model assumes Smad complexes to be imported at a faster rate than the monomeric species, while the retention only (RO) model sets identical import rates for both Smad complexes and monomeric species but only allows nuclear retention of complexes. The two competing models were simultaneously fit to four sets of kinetic empirical data. The result of comparative analysis shows that RECI is clearly a more accurate model. Moreover, the RECI model also shows excellent agreement with fluorescence
recovery after photobleaching (FRAP) data of GFP-Smad2 that were not used to construct the model. The FRAP experiment was used to infer the shuttling of GFP-Smad2 between nucleus and cytoplasmic compartments. Therefore, quantitative analysis of Smad shuttling led to the notion that the Smad complex must have a faster nuclear import rate and that the phosphatase(s) that deactivate R-Smads likely resides in the nucleus. A novel insight that comes out of the modeling analysis is that time-delayed coupling between receptor activity and Smad nuclear accumulation could function as a low-pass filter to dampen the noise in receptor activity [213, 241].

**Negative feedbacks in TGF-β signaling**

Negative feedback in a signal transduction cascade is one of the major mechanisms for desensitization of sustained ligand stimulation and generation of transient, sometimes oscillating signaling outputs [242]. Even though transient or oscillatory responses associated with sustained TGF-β exposure are not ubiquitous, potential negative feedback motifs have been identified. The most well characterized example is Smad7, a TGF-β-inducible early response gene [243, 244]. Smad7 antagonizes TGF-β signaling through multiple mechanisms, both in the cytosol and the nucleus. Since Smad7 can bind the TGF-β receptors, but lacks the SXSS motif commonly found in R-Smads, it has been proposed that Smad7 is likely to be a pseudo substrate and a competitive inhibitor of R-Smads [243, 244]. Owing to its ability to form stable complexes with the TGF-β receptors and PP1 phosphatase [245] or the
ubiquitination E3 ligase Smurf1 and Smurf2 [246], Smad7 serves as an adapter to mediate inactivation of TGF-β receptors through dephosphorylation or ubiquitin-proteasomal degradation. Besides targeting receptors for inactivation, Smad7 also appears to be involved in repressing TGF-β-dependent transcription through either disruption of R-Smad/Co-Smad/DNA complexes or recruitment of histone deacetylases [247]. Regardless of the exact biochemical mechanisms of Smad7, the induction of Smad7 by TGF-β and its function as an antagonist for TGF-β signaling are very compelling. A key question is, “what is the strength of this feedback and to what degree does it impact TGF-β signal transduction.” Melke at al. analyzed a more comprehensive model of TGF-β/Smad signaling in endothelial cells that included canonical Smad signaling. They found that negative feedback through Smad7 was important for terminating signaling and for conferring global robustness to the TGF-β pathway [212]. TMEPAI is another antagonist of TGF-β signaling and is transcriptionally induced by ligand exposure [248]. TMEPAI possesses both transmembrane and Smad interacting domain (SIM). The inhibition of TGF-β signaling can be attributed to sequestration of both unphosphorylation and phosphorylated R-Smads from interacting with the receptors or Smad4 [248]. While inhibition of TGF-β signaling by Smad7 and TMEPAI predominantly occurs in the early step of signaling propagation, the negative feedback loop that involves SnoN appears to target the downstream signaling cascade at the chromatin level. SnoN and its relative Ski are both transcriptional co-repressors of the Smad signaling complex [249, 250]. In the early phase of TGF-β stimulation, SnoN is destabilized by association with
activated R-Smads and ubiquitin E3 ligases such as Arkadia [251-254]. Degradation of SnoN/Ski unleashes the full activity of the Smad complex, resulting in the transcriptional activation of target genes. SnoN is one of the TGF-β inducible genes and the elevated SnoN restrains the activity of Smad complexes [251]. Therefore, there are at least three negative feedback loops associated with TGF-β signaling. All of these are initiated by transcriptional induction of the antagonist, although spatial and temporal variations exist among them.

**Are there oscillations in TGF-β signaling responses?**

Oscillations of signaling responses have been observed in some pathways with negative feedbacks, for example, NF-κB, p53 and Erk systems [225, 255, 256]. Modeling analyses have shown that biochemical oscillations can occur if four general requirements are satisfied: negative feedback, time delay, nonlinearity of the reaction kinetics, and tightly controlled timescales of opposing chemical reactions [257]. Since different negative feedbacks have been proposed for regulating TGF-β signaling [258], theoretically, it is possible to generate oscillating responses in the TGF-β network. Recent modeling analyses by extensive sampling of parameter space show that oscillating Smad signaling can appear by fine-tuning only a few parameters related to negative feedback (e.g. Smad7) [210, 216]. However, it is still an open question about whether there are oscillations in TGF-β network because no oscillations of TGF-β signaling have yet been observed in cells. One
reason could be that the negative feedback regulations of TGF-β network may not be strong enough at the endogenous level. Moreover, the time-delay between negative feedback and Smad activation might not be coupled in a proper time scale at in vivo conditions. Last but not least, cell signaling often results in heterogeneous responses within single cells. Averaging signaling dynamics at cell population levels can mask dynamic signaling mechanisms within individual cells [259]. Oscillation responses may appear upon investigation at the single cell level in a variety of cell lines and culture conditions.

**Modeling cell context specific TGF-β signaling**

It has been long recognized that the cellular responses triggered by TGF-β are often cell type specific and stimulation specific [260]. For example, TGF-β is a potent inhibitor of normal epithelial cell proliferation but acts to stimulate fibroblast growth [260]. Even in the same cell type, TGF-β can produce opposite proliferation effects depending on the presence of other growth factors. In the presence of PDGF, TGF-β stimulates growth of Myc-1 cells, and in the presence of EGF, TGF-β inhibits growth of the exact same cells [260]. The exact molecular mechanisms underlying these contradictory cellular responses remain largely elusive. One possible explanation to account for these observations is that the pathways activated by TGF-β vary among different cell types and are restrained by cross-talk with other signaling
pathways. Even though TGF-β signals through Smad2 and Smad3 in most cell types through ALK-5, in endothelial and hepatic stellate cells TGF-β induces phosphorylation of both Smad1/5 and Smad2 via ALK-1 and ALK-5, respectively [212, 226, 261]. Based on this evidence, Melke et al. developed a mathematical model for the TGF-β pathway in endothelial cells, taking parallel activation of ALK1 and ALK5 into consideration. Their model recapitulates the kinetics of the experimental data and correctly predicts the behavior in experiments where the system is perturbed [212]. This study highlights a need to develop mathematical modeling tailored to a specific biological context in order to understand the multifunctional nature of TGF-β signaling.

**Outlook and concluding remarks**

TGF-β signaling is spatiotemporally regulated in at least three compartments (extracellular, cytosol and nucleus). Quantitative analysis of the TGF-β signaling pathway is still very much in its infancy. At present, modeling efforts have been focused on the canonical TGF-β signaling cascade. As TGF-β signals through both canonical and noncanonical pathways, it is imperative to develop mathematical models that comprise both pathways in order to accurately predict overall TGF-β biology. Since the noncanonical pathway is often cell type dependent and operates in the context of other signaling networks, it will be a major challenge to develop such models. Another challenge is to understand the quantitative coupling between

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Smad signaling kinetics and gene expression profiles. Most of the mathematical models developed so far assume that the dynamics of Smad2 and Smad3 phosphorylation is similar and indistinguishable. However, the biological functions of Smad2 and Smad3 are clearly different, based on mouse knockout studies and DNA microarray analysis [262, 263]. The abundance of Smad2 and Smad3 varies significantly in different cell types and the ratio of Smad2 and Smad3 influences the resulting cellular response to TGF-β stimulation [206, 264]. Future modeling efforts need to consider how to model the R-Smads separately. Finally, given the emerging role of TGF-β type III receptor in modulating TGF-β signaling [265], it would be interesting to quantitatively assess their contributions to the signaling dynamics. As more quantitative TGF-β signaling data becomes readily accessible, we anticipate that the innovative systems biology approach to study TGF-β/Smad signaling will fundamentally advance our knowledge in this major signaling network.
CHAPTER VI

Analysis of Ligand Dependent Nuclear Accumulation of Smads in TGF-beta Signaling

ABSTRACT

The growth inhibition of dividing cells and most of the transcriptional responses upon TGF-beta treatment depend on the Smad2, Smad3, and Smad4 transcription factors. These proteins shuttle continuously between the cytoplasm and the nucleus, transmitting the ligand status of the TGF-beta receptors to the nuclear transcription machinery. In the absence of TGF-beta ligand, Smads 2/3/4 reside predominantly in the cytoplasm. Following ligand binding to the TGF-beta receptors, the dynamic equilibrium of shuttling Smads 2/3/4 shifts towards a predominantly nuclear state, where a high concentration of these transcription factors drives transcriptional activation and repression of genes required for proper cellular response. Here, we describe live-cell imaging and immunofluorescence microscopy methods for tracking Smads subcellular localization in response to TGF-beta and leptomycin B treatment. In addition, a method of fractionating nuclear and cytoplasmic proteins used to confirm the imaging results was presented. Our results support the notion that the R-Smad shuttling mechanism is distinct from Co-Smad.
INTRODUCTION

Transforming Growth Factor Beta (TGF-beta) is a cytokine that can cause several distinct cellular responses, including growth inhibition, apoptosis, and differentiation (For Review [69]). The active form of the TGF-beta ligand binds as a dimer to the TGF-beta receptors on the plasma membrane of target cells, thus carrying a signal from one cell to another (For Review on ligand-receptor interactions:[132]). Type I and Type II TGF-beta receptors (TBRI and TBRII, respectively) are serine/threonine kinases that transmit the signal across the plasma membrane [69]. TGF-beta binding to TBRII induces hetero-oligomerization of TBRII with TBRI [69]. The receptor oligomerization initiates a protein phosphorylation cascade, eventually propagating the signal into the nucleus. This cascade begins when TBRII phosphorylates TBRI, resulting in activation of TBRI’s kinase activity [69]. Active TBRI then phosphorylates receptor regulated Smad proteins also known as R-Smads (Smad2 and Smad3) [70]. These phosphorylated Smad2/3 proteins homooligomerize into protein complexes, as well as hetero-oligomerize with Smad4 or Co-Smad [72, 73]. Oligomerization of Smads 2/3/4 correlates with nuclear accumulation of these proteins [71]. Smads 2/3/4 are transcription factors that activate the transcription of p21 and p15, which cause cell cycle arrest in the G1 phase of the cell cycle, as well as repress transcription of growth promoting genes such as c-myc and CDC25A [69]. It is through transcriptional regulation of these genes, and possibly others yet to be identified, that Smad nuclear accumulation leads to cytostatic responses.
Nucleocytoplasmic shuttling of signaling proteins is a common theme shared by many cellular signaling pathways. Smads are constantly cycling in and out of nucleus even in the absence of ligand stimulation [74, 266]. The Smad nuclear export rates exceed their import rates in the basal state consequently both Smad2/Smad3 and Smad4 are predominantly localized to the cytosol [207, 267]. Ligand stimulation decreases the export rates of Smad4 without significantly affecting the import rates resulting in nuclear accumulation of Smad4 [207]. Similar mechanism may also account for R-Smad nuclear accumulation although nuclear import and export mechanism of R-Smad and Smad4 appears to be distinct. Earlier studies have shown that Smad3 but not Smad2 or Smad4 can directly interact with importin β1 and interaction may be important for nuclear translocation of Smad3 [268-270]. Subsequent studies suggest that nuclear import of Smad2 and Smad3 can also occur through direct binding of Smad2/3 to nucleoporins Nup214 and Nup153 [266]. Thus, both importin β-dependent and importin β-independent pathways are involved in trafficking R-Smad into nucleus. For shuttling R-Smad out of the nucleus, exportin 4 has been implicated as the export factor for Smad3 and most likely for Smad2 as well [271]. Unlike R-Smad, nuclear import of Smad4 likely relies on importin 7/8 or importin alpha [270, 272] while Smad4 nuclear export occurs by binding to the nuclear export factor CRM1 as treatment of the specific small molecule inhibitor Leptomycin B (LMB), which targets CRM1, is sufficient to drive nuclear accumulation of Smad4 but not Smad2 or Smad3 [273-275]. Two nuclear export signals have been identified in Smad4 and mutation of these signals causes Smad4 to
exclusively localize to nucleus [269, 273, 274]. Despite all these advances, a number of outstanding questions still remain unanswered. For example, how is the phosphorylated R-Smad induced by TGF-beta treatment translocated to the nucleus? Is the import rate for the phosphorylated Smad2/3 higher than the unphosphorylated Smad2/3? How Smad homo- or heteroligomerization regulates Smad nuclear accumulation? Can the rate of Smad import or export be re-adjusted intrinsically or in response to signaling cross-talk? Here we described some of the key methods that can be used to determine the trafficking mechanisms of Smad in the mammalian system.

**MATERIALS**

**Cell Culture**

1. Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO, Invitrogen)
2. DMEM lacking phenol red (GIBCO, Invitrogen 31053-036)
3. GlutaMAX L-Glutamine Supplement (GIBCO, Invitrogen 35050-061)
4. Dulbecco’s Phosphate Buffered Saline (D-PBS) (GIBCO, Invitrogen 14190-136)
5. 100 X Penicillin G Solution (Solid Penicillin G from Sigma in distilled water to 10,000 U/ml)
6. Streptomycin Sulfate solution (Solid streptomycin sulfate from Sigma in distilled water to 10,000 U/ml)

**Live Cell Treatment**

1. Leptomycin B, 500 µg in Absolute Ethanol (LC Laboratories L-6100)
2. Transforming Growth Factor Beta1 (TGF-beta1) (R and D Systems 240-B-010)

**Cellular Fractionation**

1. Hypotonic Lysis Buffer (10 mM Tris Base HCl, 10 mM KCl, 1.5 mM MgCl₂, 1 mM Sodium Orthovanadate, 0.2 mM phenylmethanesulphonylfluoride, 1mM DTT)

2. RIPA buffer (150 mM NaCl, 1 % v/v Triton X 100, 1 % w/v sodium deoxycholic acid, 0.1 % w/v sodium dodecyl sulfate, 25 mM Tris Base HCl, 1 mM ethylenediaminetetraacetic acid, 0.2 mM phenylmethanesulphonylfluoride, 1 mM Sodium Orthovanadate, 1 mM DTT, 25 mM B-glycerophosphate, 25 mM NaF)

3. Salt Balancing Solution (10 % v/v Triton X 100, 1 M NaCl, 100 mM B-glycerophosphate, 100 mM NaF)

4. Cell Lifter (Costar, Corning 3008)

5. Dounce Homogenizer with 1 ml volume (Wheaton)

6. Ponceau S Staining Solution (0.5 g Ponceau S Dye, 5 % Glacial Acetic Acid to 100 ml with deionized water)

7. Protogel 30 % Acrylamide/Bisacrylamide Solution (National Diagnostics, EC-890)

8. Protogel 4 X Resolving Gel Buffer (National Diagnostics, EC-892)

9. Protogel Stacking Gel Buffer (National Diagnostics, EC-893)

10. 10 % Sodium Dodecyl Sulfate Solution (10% w/v SDS in distilled water)

11. Mouse anti-Smad1/2/3 antibody (Santa Cruz Biotechnology SC-7960)
12. Mouse anti-Lamin A/C antibody (Santa Cruz Biotechnology SC-7292)
13. Mouse anti-betaActin antibody (AbCam Ab8226)
14. Horse Radish Peroxidase conjugated Sheep anti-mouse antibody (GE Healthcare NA931-1ML)
15. SuperSignal West Dura Extended Duration Substrate chemiluminescence kit (Pierce Biotechnology 34076)
16. Protran Nitrocellulose Membrane (Whatman 104024 BA83)
17. Whatman Chromatography Paper (Whatman 3030-917)
18. Semi-dry transfer apparatus (Hoefer TE70)
19. BCA protein assay kit (Thermo 23225)
20. Spectra broad ranged multicolor protein ladder (Fermentas SM184)
21. Powerwave X Scanning Spectrophotometer Plate Reader (Bio-Tek)
22. 4 X SDS-Gel Loading Buffer (to Make 10 ml: 8 mg Bromophenol Blue, 1 ml 0.5 M EDTA, 40 mM DTT, 4 ml 100% glycerol, 0.8 g SDS, 2 mL 1 M Tris Base pH 6.8, to 10 ml with deionized water)
23. Transfer Buffer (5.8 g Glycine, 11.6 g Tris Base, 0.72 g Sodium Dodecyl Sulfate, 400 ml Methanol, to 2 L with deionized water)
24. Tris Buffered Saline supplemented with Tween-20 detergent (TBS-t) (8.8 g NaCl, 0.2 g KCl, 3 g Tris Base, 1 ml Tween 20, pH 7.4 to 1L with deionized water)
25. Western Blot Film (ISC BioExpress F-9023-8X10)

Immunofluorescence
1. Poly-D-Lysine Hydrobromide Solution (1 mg/ml Poly-D-Lysine Hydrobromide (Sigma P7405-5MG), 23.5 mM Sodium Tetraborate, 50 mM Boric Acid, pH to 8.5)

2. Round Glass Coverslips (Fisherbrand 12-546 18CIR-2)

3. Glass Slides (VWR 48312-003)

4. Rabbit anti-Smad2 antibody (Zymed Laboratories 51-1300)

5. Mouse anti-Smad4 antibody (Santa Cruz Biotechnology SC 7966)

6. Goat anti-Mouse Alexafluor488 conjugated antibody (Molecular Probes, Invitrogen A11001)

7. Goat anti-Rabbit AlexaFluor555 conjugated antibody (Molecular Probes, Invitrogen A21428)

8. Normal Goat Serum (Invitrogen PCN5000)

9. 3.7 % Paraformaldehyde solution (Made from dilution of 16 % solution in deionized water, Electron Microscopy Sciences 15710)

10. 10 mg/ml Hoescht 33258 Solution in deionized water (made from solid, Invitrogen H21491)

11. Clear Nail Polish (Sally Hansen ‘Hard as Nails’ 2103)

Live Cell Imaging

1. GFP-Smad4 HaCaT Cells was created by retroviral mediated gene transfer. Briefly, pMX-GFP-Smad4, a retroviral expression vector described previously [275], was transfected into the amphotrophic packaging cell line φNX 293T cells using Mirus (Mirus Bio, Madison, WI). Infection and selection of GFP positive stable cell lines using FACS sorting were performed as described [276]. Similar procedure was used to create YFP-Smad2 HaCaT Cells except the expression vector used was pREX-YFP-Smad2-IRES-Hygromycin.

2. Glass Bottom 35 mm Petridishes (Mat Tek Corporation P35G-0-7-C)

METHODS

Cellular fractionation of cytoplasm and nuclear proteins

1. Using the methods of cellular fractionation and western blot analysis, we find that both TGF-beta and Leptomycin B treatment of cells is sufficient to increase the fraction of total cellular Smad4 protein located in the nucleus. This is not observed to be the case for either Smad2 or Smad3, where only TGF-beta treatment was sufficient to increase the fraction of total cellular Smad2/3 protein in the nucleus (Figure 6.1 D).

2. 4 X 10 cm tissue culture Petri dishes were seeded with 10 ml and 2 x 10⁶ adherent HaCaT cells and allowed to grow for 24 hours at 37 °C in a 5 % CO₂ atmosphere (See Note 1) Two plates were treated for 30 minutes with 20 ng/ml LMB prior to addition of 100 pM TGF-beta1 to one of these
Figure 6.1 Measuring Endogenous Smad Nuclear Accumulation. A) Nuclear accumulation of Smad2 and 4 is measured by immunofluorescence and quantified by densitometry in HaCaT cells as a function of either ligand stimulation or leptomycin B treatment (B and C). D) Alternatively, cellular fractionation and western blotting can be used to quantify the relative accumulation of Smads in the nucleus.
plates and to one of two plates not treated with LMB. Cells were treated with TGF-beta1 for one hour. Total volume of media in all plates is 10 ml.

3. Each 10 cm plate containing 2 x 10⁶ adherent HaCaT cells are rinsed (See Note 2) one time with 10 ml 4 °C Dulbecco’s Phosphate Buffered Saline (D-PBS). D-PBS is removed by titling the plate vertically at an 80 degree angle in a bucket full with ice for one minute and aspirating all liquid from the bottom corner of the plate using a vacuum trap and a glass Pasteur pipette. 10 ml of 4 °C hypotonic lysis buffer is then added to the plate, which is then incubated horizontally on ice for 15 minutes. During this incubation, cells will swell, providing an easier lysis in the following steps (See Note 3) All hypotonic lysis buffer is removed in the same manner as stated above. 70 µl of 4 °C hypotonic lysis buffer supplemented with 0.4 % TX100 is added to each plate. With the plate tilted at an 80 degree angle in a bucket of ice, cells are scraped using a cell lifter, and all cells are pushed towards the pooling liquid in the bottom end of the tilted plate.

4. Scraped cells in lysis buffer are briefly homogenized by pipetting up and down the pooled liquid in the plate, being careful not to introduce air bubbles (See Note 4). The mixture of buffer and cells is then transferred to a 1.7 ml Eppendorf tube on ice, and incubated at 4 °C for 15 minutes.

5. Using a 200 µl pipette, cells are further homogenized by pipetting up and down without introducing air bubbles. Cells and buffer are transferred to a clean 4 °C 1 ml Dounce homogenizer, and the ‘loose fit’ plunger is raised and lowered 60 times. The plunger is removed and the liquid is allowed to collect in the bottom of the homogenizer for 30 seconds. 1-2 µl of liquid
homogenate is removed, and placed on a microscope slide and a cover-slip is applied to prevent evaporation. The homogenate is confirmed to be composed of nuclei, which look wrinkled oval and dark, and debris, which is the result of plasma membrane breakage. There should be no presence of in-tact cells at this point. However, if there are still 5-10% unbroken cells one may apply the plunger again and homogenize the cells 40-60 more times before proceeding to step 5 (See Notes 5 and 6).

6. Homogenate is transferred to a fresh 4 °C 1.7 ml eppendorf tube and spun for 5 minutes at 800 rcf and 4 °C. A small pellet forms at the end of the tube, and the supernatant contains plasma membrane components and cytoplasm contents. Most of the supernatant is removed by pipetting and labeled as cytoplasmic fraction, and care is taken not to disturb the pellet (See Note 7). Cytoplasmic fractions are balanced for salt, detergent, and phosphatase inhibitors by addition of 20 µl salt balancing buffer.

7. The last bit of supernatant is removed and discarded. The nuclear pellet is rinsed one time by addition of 100 µl of 4 °C hypotonic buffer lacking 0.4% TX100. The tube containing hypotonic wash buffer and nuclei is then spun for 5 minutes at 800 rcf and 4 °C (See Note 8). All liquid contents of the tube are removed by aspiration, being careful not to disturb the pellet or to scrape the inner walls of the tube. The nuclei are then lysed completely by addition of 70 µl of RIPA buffer followed by gentle flicking and inverting of the tube. These tubes are then labeled as nuclear fractions.

8. Cytoplasmic and nuclear fractions are rotated for 45 minutes at 4 °C.
9. Fractions are then spun for 10 minutes at 13,200 rcf and 4 °C. Supernatants are transferred to new labeled tubes and stored on ice (See Note 9).

Determining Protein Concentration and Performing Western Blot Analysis

1. Protein concentration is determined using a BCA assay kit, according to the manufacturer’s instructions. Briefly, serial dilutions of 2.0 mg/ml Bovine Serum Albumin (BSA) stock solution in a 1:10 dilution of RIPA solution:distilled water are made to produce 1.0 mg/ml, 0.5 mg/ml, 0.25 mg/ml standards, while a blank is made from 1:10 dilution of RIPA solution:distilled water alone. A 2 µl aliquot of each fraction is removed to make a 1:10 dilution of each unknown sample in distilled water, yielding a total of 20 µl of diluted unknown sample.

2. 5 µl of each unknown and each standard are mixed with 100 µl of 1 X BCA Solution (50:1 mixture of solutions A and B, from the BCA kit), each in 1 well of a clear 96 well polyethylene plate. Plates are completely sealed to prevent evaporation using parafilm tape, and incubated at 37 °C for 30 minutes.

3. The 96 well plate is read at 562 nm for absorbance using a 96 well plate scanning spectrophotometer. Using Excel, a spreadsheet is constructed to determine the mathematical relationship of BSA concentration to absorbance for the standards, and this relationship is used to determine the
concentration of the proteins in each unknown sample. The total yield in
the cytoplasmic fraction is 150 µg in 100 µl, while the total yield in the
nuclear fraction is 100 µg in 75 µl.

4. For each cytoplasmic fraction, 50 µg of total protein is prepared for
loading into a single well, while 33.3 µg of total protein is prepared for
loading into a single well for nuclear fractions (See Note 10). Each sample
for loading into an SDS-PAGE gel is mixed with 7 µl of 4 X SDS-loading
buffer and incubated at 95 °C for 5 minutes. Tubes are inverted and liquid
contents are briefly spun at 5000 rcf for 30 seconds. In this experiment, 2
identical sample sets are used to make two identical gels.

5. A 1.5 mm, 12 % polyacrylamide mini-gel polymerized with a 10 well
comb (manufactured using SDS-PAGE Protogel reagents from National
Diagnostics according to the manufacturer’s instruction) is loaded with
samples and 5 µl Spectra protein ladder (Fermentas) and 10 µl of SDS-
loading buffer is added to any spare/empty wells prior to application of
current. Each gel is run for 1 hour at 35 mA (190 V), at which time the
bromophenol blue dye in the SDS-loading buffer runs just out of the
bottom of the gel.

6. Each gel is transferred in a semi dry western blot horizontal transfer unit,
using a sandwich from cathode (bottom piece) to anode (top piece) with
the following scheme: 3 pieces of chromatography paper, 1 piece of
nitrocellulose paper, SDS-PAGE gel containing samples, 3 pieces of
chromatography paper. This sandwich is assembled under 50 ml of
transfer buffer, and removed as a sandwich and placed in the transfer
apparatus such that the gel is above the membrane, as proteins will be
deposited on the nitrocellulose membrane as they move down towards the
cathode. The sandwich is then lightly ironed with a 10 ml glass test tube to
ensure no air bubbles are trapped between the membrane and the gel. For
each sandwich containing one gel, for 1.5 hours at 45 mA or 7 V is
applied to the apparatus, which is assembled as indicated by the
manufacturer.

7. Nitrocellulose paper is removed from the apparatus following transfer of
proteins, and stained with 10 ml of Ponceau S Staining solution at room
temperature for 1 minute. Staining solution is removed and the membrane
is rinsed 5 times with 20 ml of distilled water to remove non-specific
Ponceau S stain. Each cytoplasmic lane should have even and bright red
staining. In contrast, far less staining should be present for each nuclear
sample. At this point, one can make a reasonable assessment of the purity
of the nuclear fractions by seeing whether abundant protein bands in the
cytoplasmic fraction lanes are shared in the nuclear fraction lanes.

8. Each membrane is blocked with 10 ml of 3 % (w/v) non-fat dry milk in
TBS-t at room temperature for 45 minutes.

9. Blocking buffer is completely removed and discarded prior to the addition
of 5 ml of 1:500 mouse anti-Smad1/2/3 in 3 % (w/v) non-fat dry milk in
TBS-t to one membrane, while another solution of 1:1000 mouse anti-
Smad4 in 3 % (w/v) non-fat dry milk in TBS-t is added to the other
membrane.
10. Each blot is incubated with primary antibody solution for 3 hours at room temperature, on a table top rocker.

11. Membranes are washed 2 times for 2 minutes with 10 ml of TBS-t.

12. Each membrane is then incubated with 3 ml of 1:2000 anti-mouse HRP conjugated secondary antibody solution in 3 % (w/v) non-fat dry milk in TBS-t for 50 minutes at room temperature on a table top rocker.

13. Each membrane is rinsed with 10 ml of TBS-t, and subsequently washed three times with 15 ml of TBS-t for 8 minutes each wash.

14. Membranes are removed from wash buffer and allowed to drip for 10 seconds before being laid protein side up on a piece of clear plastic (SARAN wrap is sufficient, or a cut three-ringed binder sheet protector). To each membrane is added 200 µl of West Dura solution (a mixture of 100 µl solution A and 100 µl solution B) and lightly tilted in several directions, by hand, to ensure that this 200 µl of solution covers the entire membrane. Another clear plastic sheet is laid over the protein side up membrane, creating a sandwich of two plastic pieces around the membrane, which is allow to sit for 30 seconds prior to ironing out excess liquid from inside the sandwich with a paper towel.

15. Plastic/membrane sandwiches are then taped to the inside of an imaging cassette, and exposed with x-ray developing film in a dark room for 15 seconds, 30 seconds, 1 minute and 5 minutes to produce varied exposures of the protein bands. Film is then developed using an automatic film developer.
16. This process from step 9 to step 15 is repeated with a 1:1000 dilution of mouse anti-Lamin A/C for one membrane, and a 1:1000 dilution of mouse anti-betaActin for the other membrane. Both antibody dilutions are made in 5 ml of 3 % (w/v) non-fat dry milk in TBS-t.

**Immunofluorescence Detection of Smad2 and Smad4**

1. Using immunofluorescent image analysis, we find that the ratio of cytoplasmic Smad4 to nuclear Smad4 (C:N) is 1.22 ± 0.15 in the basal state, 0.57 ± 0.08 when treated with TGF-beta, 0.53 ± 0.16 when treated with LMB, and 0.46 ± 0.08 when treated with both TGF-beta and LMB. Thus, either LMB or TGF-beta treatment is sufficient to drive the Smad4 from mostly cytoplasmic (C:N>1) to mostly nuclear (C:N<1) (Figure 6.1A and C). This was not observed to be case for Smad2, where the ratio of cytoplasmic Smad2 to nuclear Smad2 (C:N) is 0.78 ± 0.06 in the basal state, 0.54 ± 0.12 when treated with TGF-beta, 0.73 ± 0.07 when treated with LMB, and 0.32 ± 0.08 when treated with both TGF-beta and LMB (Figure 6.1A and B). We hypothesize that to cause for the C:N < 1 in the absence of both TGF-beta and LMB is due to high nuclear background of the anti-Smad2 antibody used for immunofluorescent staining.

2. 4 X round coverslips are placed one in each well of 4 wells in a 12 well plate tissue culture plate. To each well is added 1 ml of room temperature 1 mg/ml Poly-D-lysine hydrobromide solution, and incubated 1 hr in the dark. Poly-D-lysine Solution is removed and reused up to 10 times or
before 2 months if stored at -80 °C. Each well is washed with 1 ml of distilled water, and allowed to air dry in a sterile cell culture hood, with the UV light on. After 1 hr, 200,000 cells are added to each well in 1 ml of DMEM supplemented with 10 % FBS, 1 X Penecillin G/Streptomycin Sulfate Solution, 1 X L-Glutamine Solution (See Materials). Cells are allowed to adhere to the coverslips and grow for 24 hours at 37 °C in a 5 % CO₂ atmosphere.

3. One well is untreated, and media is exchange with fresh pre-warmed media. In two other wells, LMB is added at a final concentration of 20 ng/ml for 30 minutes prior to addition of TGF-beta1 at a final concentration of 100 pM to one of these wells and the remaining fourth well. TGF-beta1 treatment lasts for one hour, at which time cells are harvested.

4. All media is removed from each well by aspiration, and washed one time with 1 ml of PBS. 1 ml of room temperature 3.7 % paraformaldehyde (freshly made from 16 % stock, diluted with water), and incubated at room temperature for 30 minutes. Paraformaldehyde solution is removed and fixed cells are washed one time with 1 ml PBS.

5. Fixed cells are blocked with 1 ml of TBS-t supplemented with 5 % (v/v) normal goat serum (NGS), and allowed to incubate while rocking at room temperature for 1 hour. All liquid is removed prior to proceeding to the next step.

6. 1 ml of solution containing 1:400 dilution of Rabbit anti-Smad2 antibody in TBS-t supplemented with 5 % NGS is added to each well, and
incubated while rocking over-night (approximately 10 hours) at 4 °C.
Primary anti-smad2 solution is removed and cells are washed one time for
10 minutes at room temperature with 1 ml of PBS before proceeding to the
next step.

7. 1 ml of solution containing a 1:400 dilution of Mouse ant-Smad4 antibody
in TBS-t supplemented with 5 % NGS is added to each well, and
incubated for 4 hours while rocking at room temperature. Cells are washed
one time with 1 ml PBS for 10 minutes at room temperature before
proceeding to the next step.

8. 1 ml of solution containing a 1:400 dilution of Alexa488 conjugated Goat
anti-Mouse and 1:400 dilution of Alexa555 conjugated Goat anti-Rabbit
antibodies in TBS-t supplemented with 5 % NGS, and incubated at 4 °C
while rocking for 4 hours, wrapped in aluminum foil to completely
prevent exposure to light. This secondary staining solution is removed,
and cells are rinsed quickly with 1 ml of PBS, and then washed for 20
minutes with 1 ml PBS containing 10 µg/ml Hoescht 33258, while rocking
at room temperature without exposure to light.

9. Cells are rinsed twice with 1 ml PBS, and mounted on slides with 8 µl of
30 % glycerol/TBS-t solution and coverslips are secured and sealed to the
slides with a perimeter application of clear nail polish.

10. Cells are imaged using a Nikon microscope with a camera and metamorph
software. Images were integrated over 5 seconds.

Image Analysis for Immunofluorescence and Live Cell Imaging
1. For each image a ratio of the average cytoplasmic fluorescence intensity to the average nuclear fluorescence intensity was calculated using measurements made using the ImageJ program (See Note 11).

2. Four 20 X 20 Pixel squares were used to measure four values of integrated density for each of five cells for each condition. Two squares were used to obtain the integrated density of the cytoplasm, while two squares were used to do the same for the nucleus of the same cell. Integrated density was measured using the Analyze/Measure function in ImageJ. This process was repeated for five total representative cells for each condition tested in both immunofluorescence experiments and live cell imaging experiments.

3. Averages for each of the two measurements were used to calculate the average integrated density for both the cytoplasm and nucleus of each cell. Reported values for each Cytoplasmic Integrated Density: Nuclear Integrated Density (C:N) consisted of average C:N values from 5 cells. Error for C:N is reported as a single standard deviation of the 5 C:N values for the 5 cells.

Live cell imaging of GFP-Smad4 and YFP-Smad2

1. Using live cell imaging analysis, we find that the ratio of cytoplasmic Smad4 to nuclear Smad4 (C:N) is 1.28 ± 0.47 in the basal state, 0.60 ± 0.15 when treated with TGF-beta, 0.43 ± 0.04 when treated with LMB, and 0.41 ± 0.11 when treated with both TGF-beta and LMB. Thus, both
LMB and TGF-beta treatment is sufficient to drive the Smad4 from mostly cytoplasmic (C:N>1) to mostly nuclear (C:N<1) (Figure 6.2 A and C). This was not observed to be case for Smad2, where the ratio of cytoplasmic Smad2 to nuclear Smad2 (C:N) is 1.29 ± 0.15 in the basal state, 0.66 ± 0.10 when treated with TGF-beta, 1.68 ± 0.28 when treated with LMB, and 0.65 ± 0.16 when treated with both TGF-beta and LMB (Figure 6.2 A and B). Thus, our live cell imaging data supports our findings for endogenous Smad4 and Smad2 using immunofluorescence image analysis.

2. For both GFP-Smad4 and YFP-Smad2 HaCaT cells, 200,000 cells in 1 ml are added to 3.5 mm glass bottom Mat-tek Petri dishes and allowed to settle for 24 hours.

3. For each of the two cell lines 4 X 3.5 mm plates were prepared, where one plate was used as a control and media was exchange for 1 ml of prewarmed DMEM media lacking phenol red and supplemented with 10 % FBS, 1 % penicillin/streptomycin, and 1 % l-glutamine solution (referred to as media from here on). To another plate, media was exchanged with 1 ml prewarmed media supplemented with 100 pM TGF-B1. Two more plates were prepared the same way but both using media supplemented with leptomycin B at a final concentration of 2 ng/ml. Thus, we are testing two variables independently. Cells are treated for 1 hour prior to imaging.

4. GFP-Smad4 cells were imaged using the same methods as for AlexaFluor488, stated above. YFP-Smad2 cells were imaged using a
Figure 6.2 Live Cell Quantification of Smad Nucleo-Cytoplasmic Shuttling. A) Stable HaCaT cell lines expressing either GFP-Smad4 or YFP-Smad2 were used to visualize nuclear accumulation upon either ligand stimulation or leptomycin B treatment. Quantification of individual cells is displayed in B and C.
492/18 band pass excitation filter and a 535/30 band pass emission filter.

For both, images were integrations of 3 seconds.

5. Images were processed using ImageJ software, as stated above for immunofluorescence data.

NOTES

1. Cells prior to treatment should be fully spread out and 80% confluent. If too many cells are seeded at too high a density, clumps of cells will form in subsequent steps and lead to uneven lysing.

2. The term ‘rinse’ is used here to refer to brief addition, mixing, and removal of solutions. In contrast, the term ‘wash’ refers to the addition, timed incubation while mixing on a rocker, and removal of solutions.

3. The rate of cell swelling is not constant over different cell types, and the time of this incubation will have to be adjusted accordingly.

4. If cells are clumped together after this point, the final fraction of nuclear lysate will be partially contaminated with cytoplasm, due to incomplete lysis of plasma membrane prior to separation of nuclei by centrifugation.

5. If there are significant amounts of clumps of unbroken cells at this point, one may raise the amount in the hypotonic lysis buffer by adding an additional 30 µl, but this will dilute cytoplasmic fraction protein concentration. Alternatively, one may trypsinize cells at harvest, separate
cells from trypsin by centrifugation, wash cells 1 time with 1 ml D-PBS, separate cells again by centrifugation, and resuspend cells in hypotonic lysis buffer. This trypsinization step is in place of Steps 1 and 2.

6. When cells are scraped and transferred to an Eppendorf tube on ice, they will settle to the bottom of the tube within 5 minutes. However, when plasma membranes are disrupted and nuclei are released, nuclei will not settle in less than an hour, and no pellet will form by gravity.

7. If one desires to remove the plasma membrane component of this fraction, one may spin 13,200 rcf for 10 min, and separate the cytoplasmic supernatant from the plasma membrane pellet, but this is not done in our procedure.

8. If the nuclear pellet is washed with a greater volume or more times than once with hypotonic buffer, then nuclei with adsorbe to the inner walls of the eppendorf tube, and no pellet will form during separation of nuclei by centrifugation. However, this will not influence the rest of the procedure, as all liquid can be removed and RIPA buffer added to elute nuclei from the walls at the same time as causing complete lysis.

9. From here on the ‘cytoplasm’ fraction will refer to the fraction containing cytoplasmic proteins as well as plasma membrane proteins.

10. We have determined that under these conditions in HaCaT cells the total protein yield in the nuclear fraction is two-thirds that of the cytoplasmic fraction, allowing us accurately load equal ratios of cytoplasm and nuclear fractions. Thus, a different cell line may have a different ratio of total protein in the cytoplasm/plasma membrane relative to the nuclei, and that
ratio will have to be determined experimentally based on the total yield of protein in these fractions over several experiments.

Understanding How TGF-Beta Causes Cellular Spreading

In Chapter III, I showed that TGF-Beta stimulation results in cellular spreading and that cellular spreading results in MEK1 activation. However, it is not yet clear what biochemical mechanism drives these processes. I aim to identify the molecular events that both drive cellular spreading and MEK1 activation by partially depleting cells of the cellular junction protein alpha catenin, which has been shown by others to lead to cellular spreading [63, 107]. Thus, I will artificially induce weakening in cellular junctions in order to mimic the effect of TGF-Beta on HaCaT cells. Once junctions have been weakened in this manner, I will measure MEK1 activity by immunofluorescence. This approach should solidify our claim that cellular spreading governs MEK1 activity in HaCaT cells. Additionally, I plan on investigating whether alpha catenin is modified either in its subcellular localization or its protein expression level, upon stimulation of cells with TGF-Beta. In order to determine how weakening of cellular junctions leads to MEK1 activation, I will monitor the subcellular localization of both H and K-Ras in the aforementioned experiments. My preliminary inspection of H-Ras subcellular localization revealed that dense cells have H-Ras at cellular
junctions, while spread out cells have H-Ras at a perinuclear region (data not shown). Thus, it is entirely possible that membrane junctions sequester and inactivate Ras activity at high cell density, but not low cell density. This could be the key biochemical mechanism behind inhibition of MEK1 activity at high cell density, as Ras is a known activator of MEK1 kinase activity (reviewed in Chapter 4).

**Understanding How MEK1 Activity Suppresses Cellular Turning**

In Chapter III, I also showed that MEK1 activity suppresses cellular turning. As this role of MEK1 has never been detailed in the literature, the biochemical mechanism behind this process is of great value to the cell migration field. As a result, I will investigate the effect of altering MEK1 activity on cellular polarity, as I expect that migration persistence is heavily tied to cellular polarity. Particularly, I will look at endogenous CDC42 and Rac by immunofluorescence to determine if inhibition of MEK1 kinase activity abolishes cellular polarity. In addition, I will observe the polarity of CDC42 and Rho GTPase activity in live cells, as I am in the process of constructing stable cell lines that expresses either a GFP tagged CDC42 or a FRET based Rho sensor. I hypothesize that MEK1 activity stabilizes cellular polarity either by causing the recruitment of higher levels of CDC42 to the front end of migrating cells or by suppressing the development of alternative front end projections through the elevation of Rho GTPase activity.
Understanding How Migration Persistence in Individual Cells Drives Collective Cell Migration in Groups of Cells

In all of my experiments on collective migration, I observe that when collective migration occurs, it is always accompanied by the elevated migration persistence in individual cells. This begs the question of which cellular behavior comes first. I believe that migration persistence precedes collective migration, as HaCaT cell colonies migrate persistently in response to MEK1 activation (Figure 3.16). Therefore, I have devised a way to further support my claim that a group of persistently migrating cells will spontaneously migrate collectively, given only one low resistant path. I will used mathematical modeling to simulate what I believe are the three simple rules that govern cells during collective migration: 1) cells are attached 2) cells travel towards the path of least resistance, and 3) cells migrate persistently. I am currently working with a more qualified programmer than myself to achieve this task. If we are able to use these rules to recapitulate in a mathematical model the cellular behavior that we see in my experiments, then we can provide strong evidence that these three rules are at least sufficient to give rise to spontaneous collective migration behavior. Thus, I aim to complete an extremely simplified model for collective cell migration, where individual cell migration in certain circumstances leads to the appearance of collective migration. In other words, individual cells migrate according to the same principles regardless of whether they are alone, or are part of a group.


152. Millet, C., et al., *A negative feedback control of transforming growth factor-beta signaling by glycogen synthase kinase 3-mediated Smad3


