CROSSTALK BETWEEN SOLUBLE FACTORS AND CELL-CELL INTERACTIONS: IMPLICATIONS FOR CELL CYCLE CONTROL AND TUMOR DEVELOPMENT

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ABSTRACT

Crosstalk between Soluble Factors and Cell-Cell Interactions:

Implications for Cell Cycle Control and Tumor Development

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Precise and dynamic control of cell behaviors, including proliferation, adhesion, and migration, is required for proper tissue organization and homeostasis. A key element to understanding how cellular functions are controlled lies in uncovering the topology of the molecular signaling networks that couple environmental signals to cellular responses. In this study, we have parsed the signaling networks involved in cell cycle regulation and tumor development and uncovered novel mechanisms of crosstalk between soluble factors and cell-cell interactions.

Our findings demonstrate that extracellular cues, including the epidermal growth factor (EGF), stimulate proliferative signaling through β-catenin, an intracellular protein that participates in both cell adhesion and transcription of cell cycle genes. In fact, EGF-mediated β-catenin transcriptional activity is an essential signal for proliferation of normal epithelial cells. Additionally, in a cancer cell system, we discover that EGF cooperates with Wnt 3a, a classical agonist of β-catenin transcriptional activity, to induce greater signaling than either ligand alone. Notably, EGF and Wnt 3a activate

transcription using different sub-cellular pools of β-catenin. Because hyperactive β-catenin signaling drives proliferation in cancer, this suggests that attenuation of β-catenin signaling may require different therapeutic strategies for EGF- and Wnt-driven tumors.

Since β-catenin signaling can be antagonized by sequestration with the cell-cell contact protein E-cadherin at the plasma membrane, proliferative signals mediated by β-catenin may regulate growth suppression at high cell density, a property of normal cells that is often lost during tumorigenesis. Indeed, in non-tumorigenic epithelial cells, we demonstrate that E-cadherin is upregulated in contexts where β-catenin signaling and DNA synthesis are suppressed. Additionally, exogenous E-cadherin suppresses proliferation with a strict requirement for β-catenin binding. Future studies to test the hypothesis that E-cadherin regulates the growth of normal cells will benefit from a quantitative assay developed to measure E-cadherin:β-catenin complexes. Such quantitative measurements are likely to be important because contact-mediated growth suppression by E-cadherin is coupled with a density-dependent, ligand-depletion mechanism that concomitantly regulates proliferation.

Finally, we demonstrate that EGF and other soluble factors synergistically control cell-cell interactions governing organization of normal epithelial cells into multicellular structures. Notably, this behavior resembles the program initiated during metastatic cancer, thus illustrating the flexibility of the epithelial phenotype even in non-cancerous cells. Together, these studies illustrate how the topology of molecular signaling networks can couple environmental cues including soluble extracellular factors and cell-cell interactions to regulate fundamental cellular functions.

TABLE OF CONTENTS

Acknowledgments..... iii
Abstract..... v
Table of Contents..... vii
List of Tables..... xiii
List of Figures..... xiv
Abbreviations..... xvii

Chapter
I. Introduction..... I-1
1. Introduction..... I-1
2. Mechanisms of cell-cell adhesion..... I-2
3. The canonical Wnt pathway: soluble ligands promote signaling through the cell contact protein β-catenin..... I-3
4. E-cadherin and β-catenin in normal and pathological contexts..... I-4
4.1. De-regulation of β-catenin signaling drives proliferation..... I-5
4.2. Cadherins suppress tumorigenesis..... I-6
5. Current unresolved questions involving crosstalk between soluble factors and cell-cell interactions..... I-7
6. Current results..... I-10
7. References..... I-12

II. EGF-mediated Tcf/Lef transcriptional activity is essential but not sufficient for cell cycle progression in non-transformed mammary epithelial cells..... II-1
1. Introduction..... II-2
2. Materials and Methods..... II-6
2.1. Antibodies..... II-6
2.2. Cell Culture..... II-6
2.3. Plasmid Constructs..... II-7

2.4. Retroviral Infection..... II-7
2.5. GSK3β Serine 9 Phosphorylation Assay..... II-7
2.6. ERK Signaling Assay..... II-8
2.7. Cell Lysis..... II-8
2.8. Reporter Assays..... II-8
2.9. Integrated Reporter Response..... II-9
2.10. Western Blotting..... II-9
2.11. DNA Synthesis..... II-9
2.12. Immunofluorescence..... II-10
3. Results..... II-11
3.1. Re-entry into the Cell Cycle Correlates with Tcf/Lef Reporter Activity..... II-11
3.2. EGF Independently Induces Tcf/Lef Transcriptional Activity and DNA Synthesis..... II-13
3.3. Tcf/Lef Transcriptional Activity Is Required for EGF-mediated DNA Synthesis..... II-17
3.4. EGF-mediated Activation of Tcf/Lef Transcriptional Activity Is Upstream of Cyclin D1 Promoter Activity..... II-21
4. Discussion..... II-22
5. Acknowledgments..... II-27
6. References..... II-28

III. EGF and Wnt 3a differentially regulate Tcf/Lef transcription with implications for tumor development..... III-1
1. Introduction..... III-2
2. Results..... III-4
2.1. EGF activates Tcf/Lef transcriptional activity in 293T-EGFR cells..... III-4
2.2. EGF and Wnt 3a additively activate Tcf/Lef transcription..... III-6
2.3. Wnt 3a and EGF activate Tcf/Lef transcription via different mechanisms..... III-7
2.4. ERK is required for EGF- and Wnt 3a-mediated Tcf/Lef signaling..... III-10

2.5. PKC, but not PKA, is required for EGF- and Wnt 3a-mediated Tcf/Lef transcription..... III-13
2.6. Src family kinase activity is required for EGF-, but not Wnt 3a-, mediated Tcf/Lef transcription..... III-15
3. Discussion..... III-17
3.1. Physiological implications of EGF and Wnt co-regulation of Tcf/Lef transcription..... III-19
3.2. Mechanisms underlying EGF/Wnt crosstalk in regulating Tcf/Lef transcriptional activity..... III-21
4. Experimental Procedures..... III-26
4.1. Antibodies and Reagents..... III-26
4.2. Cell Culture..... III-26
4.3. Plasmid Constructs..... III-27
4.4. Cell Lysis..... III-27
4.5. ConA Fractionation..... III-28
4.6. Reporter Assays..... III-28
4.7. Immunoblotting..... III-29
4.8. siRNA knockdown of β-catenin..... III-29
5. Acknowledgments..... III-29
6. Supplemental Data..... III-30
7. References..... III-32

IV. A microtiter assay for quantifying protein-protein interactions associated with cell-cell adhesion..... IV-1
1. Introduction..... IV-2
2. Materials and Methods..... IV-4
2.1. Cell Culture..... IV-4
2.2. Cell Lysis..... IV-4
2.3. Protein complex and standard ELISAs..... IV-5
2.4. Validation of protein capture by Western blotting..... IV-6
2.5. Constructs..... IV-6

2.6. Retroviral Infection..... IV-7
2.7. Data Analysis and Statistical Calculations..... IV-7
3. Results and Discussion..... IV-8
3.1. Development and validation of a quantitative microtiter ELISA for E-cadherin:β-catenin protein complexes..... IV-8
3.2. Compatibility of the protein complex ELISA with the standard sandwich ELISAs..... IV-14
3.3. Quantitative comparision of E-cadherin:β-catenin interactions in transformed versus non-transformed cells..... IV-16
3.4. Quantitative analysis of the effect of constitutively-active Src on E-cadherin:β-catenin interactions..... IV-19
4. Conclusions..... IV-23
5. Acknowledgments..... IV-24
6. Supplemental Data..... IV-25
7. References..... IV-26

V. Mechanisms underlying growth saturation of epithelial cells..... V-1
1. Introduction..... V-2
2. Results..... V-6
2.1. Non-tumorigenic epithelial cells growth arrest at high cell density..... V-6
2.2. Receptor-mediated signaling is qualitatively similar in low- and high-density cells..... V-9
2.3. Increased expression of endogenous E-cadherin at high cell density inversely correlates with low levels of DNA synthesis and β-catenin:Tcf/Lef signaling..... V-11
2.4. Exogenous E-cadherin inhibits DNA synthesis..... V-13
2.5. Exogenous E-cadherin constructs interfere with EGFR signaling..... V-16
2.6. Growth factor availability contributes to growth arrest..... V-17
2.7. Diminished Akt, but not ERK, activity correlates with growth factor depletion-induced arrest..... V-21
3. Discussion..... V-23

3.1. Cadherins as anti-proliferative signals: Modulation of Tcf/Lef transcription via cell-cell contact..... V-25
3.2. Cadherins as anti-proliferative signals: Cell-cell contact mechanisms independent of Tcf/Lef transcription..... V-28
3.3. Density-dependent models of growth suppression: Phosphatase-mediated inhibition of mitogenic signaling pathways..... V-30
3.4. Mitogenic ligand depletion as a mediator of growth suppression..... V-31
4. Future Work..... V-32
4.1. The role of ERK and Akt in growth suppression..... V-32
4.2. Mechanisms underlying upregulation of endogenous E-cadherin..... V-34
4.3. Functional significance of E-cadherin upregulation for growth suppression..... V-35
5. Conclusions and Acknowledgments..... V-37
6. Experimental Procedures..... V-37
6.1. Antibodies..... V-37
6.2. Cell Culture..... V-37
6.3. Plasmid Constructs..... V-37
6.4. Retroviral Infection..... V-38
6.5. Cell Lysis..... V-38
6.6. Immunoblotting..... V-39
6.7. Reporter Assays..... V-40
6.8. DNA Synthesis Measurements..... V-40
6.9. Immunofluorescence..... V-41
6.10. Immunoprecipitation..... V-41
7. References..... V-42

VI. Epithelial cell patterning by soluble ligands..... VI-1
1. Introduction..... VI-2
2. Materials and Methods..... VI-4
2.1. Cell Culture..... VI-4
2.2. Phase Contrast Microscopy..... VI-4

2.3. Pharmacological Inhibition..... VI-4
3. Results and Discussion..... VI-5
3.1. Growth Medium controls the reversible formation of cell colonies..... VI-5
3.2. EGF, but not serum, can prevent cell aggregation..... VI-6
3.3. EGF and ChT induce mild dissociation of cell colonies..... VI-7
3.4. EGF and ChT cooperate to induce synergistic cell scattering..... VI-8
3.5. MAPK and PI3K control various aspects of cell scattering..... VI-10
3.6. Relevance of cell scattering to EMT..... VI-11
4. Future Work..... VI-13
4.1. Further elucidation of the mediators of EGF- and ChT-induced cell scatter..... VI-13
4.2. The role of cadherins and other adhesion proteins in cell scattering..... VI-14
4.3. Quantitative metrics of cell scatter..... VI-16
5. Conclusions..... VI-18
6. References..... VI-19

LIST OF TABLES

Number *Page*

IV-1. Quantitative performance of protein complex and sandwich ELISAs..... IV-12

VI-1. Potential Scatter Metric: Average and percent standard deviation of neighbor distance..... VI-18

LIST OF FIGURES

Number *Page*

I-1 Generalized structure of Adherens Junctions..... I-2

I-2 The canonical Wnt signaling pathway..... I-4

II-1. TOPFLASH and FOPFLASH reporter activity in SW480 and MCF-10A cells..... II-12

II-2. Dominant-negative Tcf4 effect on TOPFLASH reporter..... II-13

II-3. Growth medium constituents vary in the ability to induce Tcf/Lef transcriptional activity, GSK3β phosphorylation, and DNA synthesis..... II-16

II-4. Dominant-negative Tcf4 blocks DNA synthesis..... II-18

II-5. Dominant-negative Tcf4 does not affect EGF-mediated EGF receptor and ERK phosphorylation..... II-21

II-6. Tcf/Lef involvement in cyclin D1 promoter activity..... II-22

II-7. Proposed model for the strict requirement of Tcf/Lef signaling for EGF-mediated cell cycle progression..... II-26

III-1. EGF induces Tcf/Lef transcriptional activity in 293T-EGFR cells..... III-5

III-2. Wnt 3a and EGF cooperate to activate Tcf/Lef transcriptional activity..... III-7

III-3. Wnt 3a and EGF activate Tcf/Lef transcription via different mechanisms..... III-9

III-4. ERK is required for both EGF- and Wnt-3a-mediated Tcf/Lef signaling..... III-12

III-5. PKC, but not PKA, is required for EGF- and Wnt-3a-mediated Tcf/Lef transcriptional activity..... III-14

III-6. ERK, but not Wnt 3a, requires Src family kinase activity to activate Tcf/Lef transcriptional activity..... III-16

III-7. EGF and Wnt 3a activate Tcf/Lef signaling via a distinct but partially overlapping network..... III-18

III-S1. Wnt 5a does not activate Tcf/Lef signaling..... III-30

III-S2. Neither Wnt 3a nor EGF induces phosphorylation of GSK3β on serine 9..... III-30

III-S3. ConA pre-clearing of whole cell lysate depletes E-cadherin..... III-30
III-S4. ERK signaling is required for EGF-mediated Tcf/Lef signaling..... III-31
III-S5. The PKC inhibitor Calphostin C inhibits EGF-mediated Tcf/Lef signaling only at high concentrations..... III-31
III-S6. PKC does not lie upstream of EGF-mediated ERK activation, but Src possibly does..... III-32
III-S7. PKC and Src are not required for Wnt 3a-mediated stabilization of β-catenin..... III-32
IV-1. Antigen capture and protein:protein co-capture. IV-10
IV-2. Detection of E-cadherin:β-catenin protein complexes by protein complex ELISA..... IV-11
IV-3. Specificity test for E-cadherin:β-catenin ELISA..... IV-14
IV-4. Detection of E-cadherin and β-catenin total protein levels by sandwich ELISA..... IV-15
IV-5. Quantitative comparison of the levels of E-cadherin:β-catenin complexes, E-cadherin and β-catenin expression in normal and tumorigenic cell lines..... IV-18
IV-6. Quantifying the effect of constitutively-active Src on cellular levels of E-cadherin:β-catenin complexes and the expression of E-cadherin and β-catenin..... IV-21
IV-S1. Validation of standard sandwich and protein complex ELISAs in normal and tumorigenic cell lines..... IV-25
IV-S2. Validation of standard sandwich and protein complex ELISAs in cells expressing constitutively-active Src IV-26
V-1. Quantification of intercellular contact by measuring cell density in MCF-10A..... V-7
V-2. DNA synthesis is inversely correlated with cell density V-8
V-3. EGFR, ERK, and Akt signaling at various cell densities..... V-10
V-4. β-catenin-mediated transcription is inversely correlated with cell density..... V-12

V-5. Expression of endogenous E-cadherin, but not β-catenin, is cell density-dependent..... V-13
V-6. Full-length E-cadherin, but not the cytodomain-truncated mutant, reduces DNA synthesis..... V-15
V-7. Exogenous E-cadherin constructs do not affect ERK despite affecting EGFR and Akt phosphorylation..... V-17
V-8. Growth factor concentration-dependent saturation of cell growth..... V-18
V-9. Cell colony formation does not affect EGF-mediated proliferation..... V-19
V-10. Growth factor availability determines the cell density at growth saturation..... V-21
V-11. Akt signaling, but not ERK signaling, may control cell density at saturation..... V-22
V-12. Growth inhibition at high cell density in MCF-10A..... V-24
VI-1. Reversible formation of epithelial cell colonies by growth medium starvation or stimulation..... VI-5
VI-2. EGF, but not serum, prevents cell island aggregation..... VI-6
VI-3. EGF and ChT are the only components of growth medium that induce cell colony dissociation..... VI-7
VI-4. EGF and ChT can synergize to induce cell island dissociation..... VI-8
VI-5. PI3K and MAPK are required for different aspects of GM-induced cell scattering..... VI-11
VI-6. Average neighbor centroid distance as a metric for quantification of cell scattering..... VI-17

ABBREVIATIONS

Ab	Antibody
APC	*Adenomatous polyposis coli* gene product
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
ChT	Cholera toxin
CREB	cAMP-responsive element-binding protein
DAPI	4',6-diamidino-2'-phenylindole-dihydrochloride
E-	Epithelial
EGF	Epidermal growth factor
EGFR	EGF receptor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
Frz	Frizzled
GM	Growth medium
GSK3β	Glycogen synthase kinase 3β
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
IgG	Immunoglobulin G
IP	Immunoprecipitation
Lef	Lymphoid enhancer factor
LRP	Low-density lipoprotein receptor
MOI	Multiplicity of infection
PAGE	Poly-acrylamide gel electrophoresis
pAkt	Phospho-serine 473 Akt
PBS	Phosphate-buffered saline
ppERK	Dually-phosphorylated (Thr202/Tyr204) ERK
PI3K	Phosphatidylinostitol 3-kinase

PCR	Polymerase chain reaction
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
pRb	Retinoblastoma protein
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SDS	Sodium dodecyl sulfate
S.E.	Standard error
TBS	Tris-buffered saline
TBST	TBS plus 0.5% (v/v) Tween-20
Tcf	T-cell factor
TGFβ	Transforming growth factor β
VE	Vascular endothelial
VEGF	VE growth factor
VEGFR	VEGF receptor
v/v	Volume / volume

Chapter I. Introduction

1. Introduction

Precise and dynamic control of fundamental cell processes, including proliferation, adhesion and migration, is required for proper organization and homeostasis of mammalian organisms. De-regulation of the mechanisms regulating these behaviors underlies many pathologies, including cancer. For example, proliferation of non-cancerous mammalian cells requires properly-timed mitogenic signals, as well as avoidance of anti-proliferative stimuli, from the cellular microenvironment. However, cancer cells circumvent these requirements, becoming both self-sufficient in mitogenic signals and insensitive to anti-proliferative signals, permitting unchecked cell growth (Hanahan and Weinberg, 2000). With a fundamental understanding of the mechanisms underlying cell behaviors, it may be possible to manipulate these same processes, either for therapeutic benefit or technological applications. A key component of this strategy involves elucidating the topology of the molecular networks that regulate cellular functions.

In this study, we sought to identify mechanisms of crosstalk between soluble factors and cell-cell interactions that regulate the ability of cells to proliferate, migrate, and mediate intercellular adhesion. Probing these mechanisms, we elucidate the architecture of sophisticated molecular circuits that enable biological systems to control cell behaviors and guide multicellular organization. Notably, these studies highlight mechanisms by which pathologies de-regulate biochemical signaling networks in order to

achieve aberrant cellular behaviors. To motivate these studies, it is useful to have some background information on the key molecular players.

2. Mechanisms of cell-cell adhesion: Adherens junctions

One of the hallmarks of epithelial tissues is tight intercellular adhesion. Cell-cell contact not only permits epithelial tissues to serve as a physical barrier, but also encodes biochemical signals that regulate cell behaviors such as proliferation. Although there are several adhesive structures present in epithelial cells, including tight junctions, desmosomes, and gap junctions, the structure that is primarily responsible for intercellular adhesion is the adherens junction.

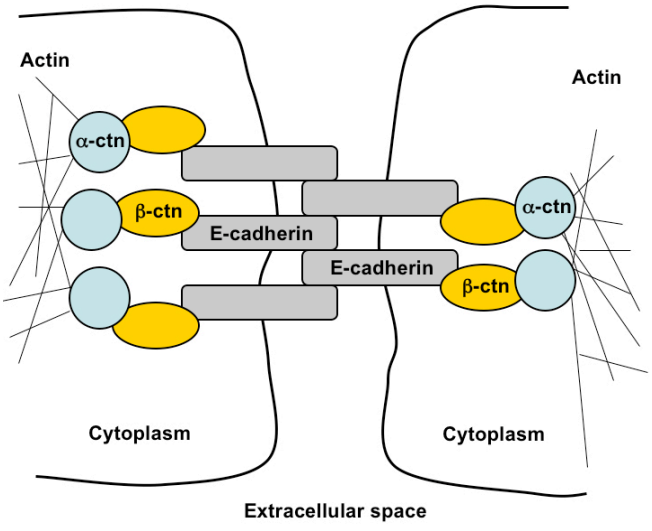

Figure I-1. **Generalized structure of adherens junctions**
The molecular constituents of adherens junctions are the cadherin and catenin proteins. Cadherins span the plasma membrane and bind to cadherins on neighboring cells. The cadherin intracellular domain binds β-catenin (β-ctn), which links to the actin cytoskeleton through α-catenin (α-ctn).

Adherens junctions are composed of cadherin and catenin proteins (Figure I-1). Cadherins are single-pass transmembrane glycoproteins that bind homotypically to cadherins on neighboring cells in a calcium-dependent manner (Angst et al., 2001). The

intracellular tail of cadherins binds β-catenin, which then recruits α-catenin and links to the actin cytoskeleton. As such, cadherin-mediated contacts link the cytoskeletons of neighboring cells and impart a structural rigidity to cell-cell contacts. E-(epithelial)cadherin is the predominant cadherin family member expressed in epithelial cells.

3. The canonical Wnt pathway: soluble ligands promote signaling through the cell contact protein β-catenin.

In addition to its adhesive role at the plasma membrane, β-catenin can function as a transcriptional activator when localized to the nucleus (Figure I-2). A key constraint on β-catenin-mediated transcription is the stability of β-catenin in the cytoplasm. In the absence of soluble Wnt factors, cytosolic β-catenin is phosphorylated on N-terminal serine and threonine residues by a multiprotein complex consisting of axin, APC, and glycogen synthase kinase 3β (GSK3β). Phosphorylated β-catenin is then ubiquitinated and degraded by the proteasome. Notably, this active degradation mechanism keeps cytosolic concentrations of β-catenin very low.

Signaling events that inhibit this degradation machinery, such as those initiated by a subset of Wnt family ligands, stabilize β-catenin. This allows β-catenin to accumulate and translocate to the nucleus, where it binds to the Tcf/Lef family of transcription factors. Together, this bipartite transcription factor induces expression of genes including *cyclin D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999) and *c-myc* (He et al.,

1998). The activation of gene transcription by β-catenin:Tcf/Lef complexes is generally referred to as β-catenin signaling; when Wnt ligands are the agonist of β-catenin signaling, this process is known as Wnt signaling.

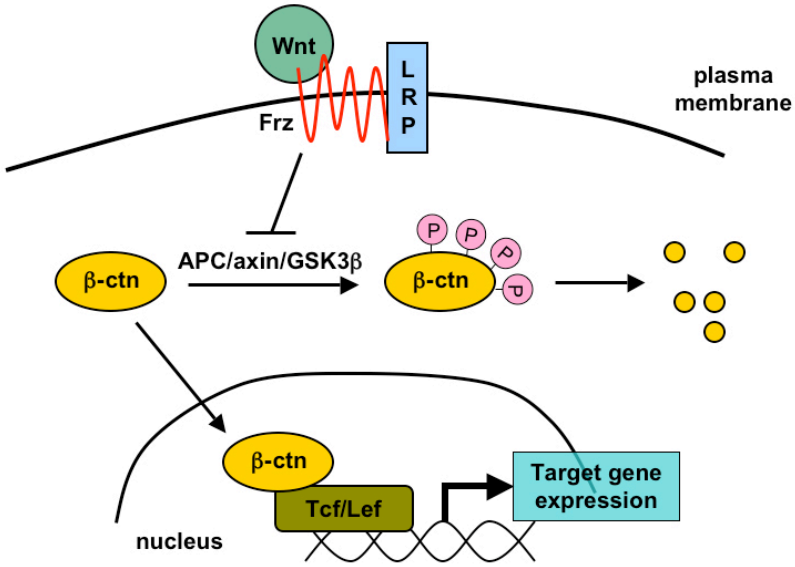

Figure I-2. **The canonical Wnt signaling pathway**
In the absence of Wnt ligands, cytosolic β-catenin (β-ctn) is phosphorylated by a multiprotein complex consisting of APC, axin, and GSK3β. Phosphorylated β-catenin is then degraded by the proteasome, keeping cytosolic concentrations of β-catenin low. When Wnt ligands bind to the co-receptor complex of Frizzled (Frz) and LRP 5/6, the cytosolic degradation machinery is inhibited, allowing β-catenin to accumulate in the cytoplasm and translocate to the nucleus. In the nucleus, β-catenin binds to the Tcf/Lef family of transcription factors and mediates expression of target genes including *cyclin D1* and *c-myc*.

4. E-cadherin and β-catenin in normal and pathological contexts

E-cadherin and β-catenin play prominent roles in both embryonic development and carcinogenesis (Clevers, 2006; Halbleib and Nelson, 2006; Wijnhoven et al., 2000). Morphogenetic and oncogenic signals are transmitted by both the adhesive function of cadherins and the nuclear signaling activity of β-catenin. Because of the functional effects that E-cadherin and β-catenin exert on cellular processes, extracellular cues - or

mutations that mimic these cues - regulate cell behaviors by regulating cadherin-mediated cell-cell interactions and β-catenin signaling.

4.1. De-regulation of β-catenin signaling drives proliferation.

Mutations that abnormally stabilize β-catenin and hard-wire Tcf/Lef transcription into a constitutively activate state occur in a diverse range of cancer types, implying a functional link between β-catenin signaling and tumor development. One such mechanism commonly found in breast cancers is autocrine secretion of β-catenin signaling agonists, including Wnt ligands (Bafico et al., 2004). Confirming that overexpression of β-catenin agonists can induce transformation, mammary-tissue-specific overexpression of Wnt-1 induces adenocarcinomas in mouse models (Tsukamoto et al., 1988). Consistent with these findings, studies using stabilized mutants of β-catenin or Tcf/Lef-VP16 fusion constructs have affirmed the capacity of β-catenin signaling to transform established cell lines and primary cells (Aoki et al., 1999; Kolligs et al., 1999; Orford et al., 1999).

In fact, antagonizing β-catenin signaling appears to be an effective method to curb the growth of cancer cell lines afflicted by elevated levels of nuclear β-catenin. Inhibitors of soluble Wnt factors decrease cell growth of human breast cancers that exhibit autocrine Wnt signaling (Bafico et al., 2004). Furthermore, overexpression of proteins – such as full-length E-cadherin or a truncated mutant that retains β-catenin binding – sequester stabilized β-catenin at the plasma membrane, precluding its association with Tcf/Lef transcription factors and effectively inhibiting proliferation of

colorectal cancer cell lines (Gottardi et al., 2001; Orsulic et al., 1999; Sadot et al., 1998).

Although the role of β-catenin in hyperproliferation of cancer cells is well established, the role of β-catenin and Tcf/Lef transcription factors in cell cycle progression of *normal* mammalian cells is only recently becoming apparent. Immunohistochemical data have shown that epithelial precursor cells in the intervillus regions of the small intestine may require β-catenin signaling for self-renewal (van de Wetering et al., 2002). In addition, Tcf4 knock-out mice lack proliferating stem cells and possess only differentiated villus cells, suggesting a causal role for Tcf/Lef in governing stem cell lineage commitment (Korinek et al., 1998). In addition to intestinal epithelia, Tcf/Lef signaling is involved in lineage commitment of human epidermal stem cells (Chenn, 2002; Chenn and Walsh, 2002; Hari et al., 2002; Zhu and Watt, 1996; Zhu and Watt, 1999), hematopoietic stem cells (Reya et al., 2003) and embryonic stem cells (Kielman et al., 2002). In all these cases, the upstream ligands that regulate β-catenin signaling are either Wnt or unidentified.

4.2. Cadherins suppress tumorigenesis.

In general, the attenuation of cell-cell adhesion plays a critical role in both early and late stages of oncogenesis (Wijnhoven et al., 2000). At early steps, reduced intercellular adhesion may attenuate contact-inhibition of proliferation, permitting unregulated cell division and tumor formation; at later stages, reduced cell-cell adhesion is often associated with invasion, metastasis, and poor patient prognosis (Christofori and Semb, 1999). In particular, expression of E-cadherin is frequently lost in cancers by

transcriptional inactivation (Giroldi et al., 1997; Hennig et al., 1996; Ji et al., 1997), but it is not clear whether loss of E-cadherin is a prerequisite for cancer progression or merely a consequence of the dedifferentiation that occurs during cancer progression (Wijnhoven et al., 2000). Since re-expression of E-cadherin inhibits invasion (Vleminckx et al., 1991) and tumorigenicity (Navarro et al., 1991) of some cancers, loss of E-cadherin may have a dual effect, permitting motility and invasion, as well as relaxing the constraints on proliferation (Sasaki et al., 2000).

The growth-suppressive effects of cadherins has been attributed to both sequestration of β-catenin outside of the nucleus (Sasaki et al., 2000; Stockinger et al., 2001) and the attenuation of receptor tyrosine kinase (RTK) (Grazia Lampugnani et al., 2003; Lampugnani et al., 2006; Qian et al., 2004; Takahashi and Suzuki, 1996). By inhibiting proliferation, cadherins may also play a role in contact inhibition of proliferation, whereby cells growth arrest even in the presence of mitogenic ligands (Motti et al., 2005; St Croix et al., 1998; Stockinger et al., 2001). Thus, cadherin-mediated cell-cell contacts may antagonize intracellular signaling pathways and subsequent cell responses that are initiated by soluble factors.

5. Current unresolved questions involving crosstalk between soluble factors and cell-cell interactions

The classical agonists of β-catenin transcriptional activity are the Wnt ligands (Figure I-2). However, it is becoming apparent that some ligands which activate RTKs,

including the epidermal growth factor (EGF), also provoke β-catenin signaling (Lu et al., 2003; Muller et al., 2002). If indeed non-Wnt ligands such as EGF can induce β-catenin transcriptional activity, it is unclear whether they utilize the canonical Wnt mechanism that stabilizes cytoplasmic β-catenin. In the case of EGF, this question is particularly interesting because EGF is known to inactivate GSK3β (Eldar-Finkelman et al., 1995), the kinase which primes cytosolic β-catenin for degradation (Aberle et al., 1997). Investigating this mechanism may shed insight on whether β-catenin is a primed or non-primed substrate of GSK3β (Ding et al., 2000; Liu et al., 2002). If EGF-mediated transactivation of β-catenin does not involve Wnt-like mechanisms such as the stabilization of β-catenin, what mechanisms are important? One possibility is that EGF transactivates β-catenin by modulating the adhesive and transcriptional properties of β-catenin through tyrosine phosphorylation (Harris and Peifer, 2005).

Whatever the mechanism of EGF-mediated β-catenin transactivation, it would also be interesting to test whether Wnt ligands and RTK ligands can co-regulate β-catenin:Tcf/Lef transcription. Some reports have suggested that specific signals downstream of RTKs, including constitutively-active Ras, can cooperate with constitutive inhibition of GSK3β to induce synergistic β-catenin signaling (Chen et al., 2000; Desbois-Mouthon et al., 2001). However, constitutive activation or inhibition of signaling pathways is clearly different from intracellular signals mediated by soluble ligands, precluding assessment of whether RTK and Wnt ligands co-regulate Tcf/Lef transcriptional activity.

In cancer, hyperactive β-catenin signaling drives unchecked proliferation (Clevers, 2006). Because the targets of β-catenin transcription include proteins that are ubiquitously required for cell cycle progression (e.g., cyclin D1, c-myc), the untested hypothesis remains that β-catenin signaling is important for proliferation of *normal* cells. Correlations between serum-mediated proliferation and Tcf/Lef transcriptional activity have been demonstrated in an engineered mammary cell system (Stockinger et al., 2001); however, the expression of a c-Fos:estradiol receptor fusion protein in these cells precludes an assessment of whether β-catenin nuclear activity is involved in proliferation, since c-Fos itself is itself critically involved in cell cycle control (Cook et al., 1999). Thus, it remains to be tested whether β-catenin signaling is involved in proliferation of normal cells, and if so, whether non-Wnt ligands utilize β-catenin:Tcf/Lef transcription to regulate passage through the cell cycle.

Because β-catenin signaling can be attenuated by binding to E-cadherin at the plasma membrane, the interplay between proliferative signals mediated by β-catenin and contact-induced, anti-proliferative signals may regulate growth. One cellular process that may be regulated by this mechanism is contact inhibition of proliferation, a property of normal cells that is often lost during tumorigenesis. As such, in the context of confluent, growth-arrested, epithelial cell monolayers, do cadherins antagonize β-catenin signaling and thereby inhibit proliferation? To test the hypothesis that E-cadherin regulates the growth of normal cells, it may be necessary to develop quantitative assays to measure the association of E-cadherin and β-catenin. Such quantitative measurements may help

distinguish between contact-mediated growth suppression and alternative mechanisms that concomitantly block proliferation of normal cells at high density.

Finally, although cell-cell interactions may regulate signaling initiated by soluble factors, the converse is also true. In particular, EGF signaling in carcinoma cells promotes the dissociation of cell-cell junctions, as seen in epithelial-mesenchymal transition (EMT) (Boyer et al., 1997; Edme et al., 2002; Lu et al., 2003), a process whereby tumor cells lose their epithelial characteristics and acquire invasive mesenchymal phenotypes. Although hyperactive EGF signaling induces cell scatter in epithelial cells (Khoury et al., 2001), it is not clear whether the soluble factor EGF initiates similar phenomena in normal epithelia, or whether EGF cooperates with other signaling pathways to induce synergistic responses.

6. Current results

In this report, we have investigated the molecular networks that control fundamental cellular processes including proliferation, adhesion, and multicellular organization. Chapter II elucidates how the soluble factor EGF promotes proliferation through the cell-cell contact protein β-catenin. In fact, transactivation of β-catenin:Tcf/Lef target genes is an essential signal for EGF-mediated proliferation of normal cells. Because Wnt ligands are the classical activators of β-catenin:Tcf/Lef transcription, Chapter III compares and contrasts the mechanisms by which Wnt 3a and EGF activate β-catenin signaling in cancer cells overexpressing EGFR. These chapters

illustrate the sophisticated molecular circuitry that regulates activation of β-catenin:Tcf/Lef transcription and highlight the importance of this process for proliferation of normal cells.

One of the key mechanisms regulating β-catenin signaling may be tuning the ability of E-cadherin to bind β-catenin. Thus, in Chapter IV, a quantitative method for measuring the association of endogenous E-cadherin and β-catenin is developed. In two case studies closely related to cancer cell biology, we use this quantitative method to observe the regulation of adherens junctions *in vivo*. Because E-cadherin can attenuate β-catenin signaling, this suggests that E-cadherin:β-catenin interactions may mediate growth suppression of normal cells at high density, a property of normal cells that is often lost during tumorigenesis. In Chapter V, evidence for both contact-dependent and density-dependent mechanisms of growth inhibition in normal cells is presented.

Finally, in Chapter VI, we probe the role of soluble ligands in promoting aggregation of individual epithelial cells into multicellular structures with extensive intercellular adhesions. We demonstrate that EGF and other soluble factors synergistically govern the cell-cell interactions that guide multicellular organization. Notably, this behavior resembles the program initiated during metastatic cancer, thus illustrating the flexibility of the epithelial phenotype even in non-cancerous cells.

Together, these studies illustrate how the topology of molecular signaling networks can couple environmental cues to regulate fundamental cellular functions.

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Chapter II. EGF-mediated Tcf/Lef transcriptional activity is essential but not sufficient for cell cycle progression in non-transformed mammary epithelial cells

Abstract

Because β-catenin target genes such as *cyclin D1* are involved in cell cycle progression, we examined whether β-catenin has a more pervasive role in normal cell proliferation, even upon stimulation by non-Wnt ligands. Here, we demonstrate that epidermal growth factor (EGF) stimulates T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcriptional activity in nontransformed mammary epithelial cells (MCF-10A), and that its transcriptional activity is essential for EGF-mediated progression through G_1/S phase. Thus, expression of dominant-negative Tcf4 blocks EGF-mediated Tcf/Lef transcriptional activity and bromodeoxyuridine uptake. In fact, the importance of EGF-mediated Tcf/Lef transcriptional activity for cell cycle progression may lie further upstream at the G_1/S phase transition. We demonstrate that dominant-negative Tcf4 inhibits a reporter of cyclin D1 promoter activity in a dose-dependent manner. Importantly, dominant-negative Tcf4 suppresses EGF-mediated cell cycle activity specifically by thwarting EGF-mediated Tcf/Lef transcriptional activity, not by broader effects on EGF signaling. Thus, although expression of dominant-negative Tcf4 blocks EGF-mediated TOPFLASH activation, it has no effect on either EGF receptor or ERK phosphorylation, further underscoring the fact that Tcf/Lef-mediated transcription is essential for cell cycle progression, even when other pro-mitogenic signals are at normal levels. Yet, despite its essential role, Tcf/Lef transcriptional activity alone is not

sufficient for cell cycle progression. Serum also stimulates Tcf/Lef transcriptional activation in MCF-10A cells but is unable to promote DNA synthesis. Taken together, our data support a model wherein EGF promotes Tcf/Lef transcriptional activity, and this signal is essential but not sufficient for cell cycle activity.

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1. Introduction

β-catenin is a 90-kDa intracellular protein whose functions range from stabilization of cell-cell adhesion to control over gene expression. These functions are tightly regulated through its association with various proteins such as the transmembrane protein E-cadherin and Tcf/Lef transcription factors (1,2). E-cadherin is a major constituent of adherens junctions where it promotes epithelial cell-cell contact through homotypic interactions mediated by its extracellular domain (3). Meanwhile, its cytoplasmic domain binds to β-catenin, whose association with α-catenin and other structural proteins bridges E-cadherin-mediated contacts to the actin cytoskeleton (4). In addition to regulation by sequestration to the plasma membrane, β-catenin is tightly regulated by cytosolic degradation via a multiprotein complex consisting of Axin, APC, and glycogen synthase kinase 3β (GSK3β) (5). Signaling events that inhibit this cytosolic degradation machinery, such as those initiated by a subset of Wnt family ligands, help to stabilize β-catenin, which then translocates to the nucleus where it serves as a transactivator for the

Tcf/Lef family of transcription factors.

Mutations that abnormally stabilize β-catenin occur in a diverse range of cancer types. In colorectal carcinomas and melanomas, these mutations include the loss and/or truncation of *APC* and mutations among critical N-terminal serine residues of β-catenin whose phosphorylation flags it for ubiquitin-mediated degradation (5-7). Evidence of β-catenin stabilization has also been shown in hepatomas and prostate cancers wherein loss of *axin* and *PTEN*, respectively, leads to accumulation of nuclear β-catenin and increased Tcf/Lef-mediated transcription (8,9). In the mammary gland, transgenic expression of Wnt family ligands induces mammary adenocarcinomas in mice (10). Consistent with this finding, mammary-tissue-specific overexpression of a constitutively stable β-catenin mutant induces hyperplasia and adenocarcinoma in the mammary gland (11). Finally, studies using stabilized mutants of β-catenin or Tcf/Lef-VP16 fusion constructs have affirmed the capacity of this signaling pathway to transform established cell lines and primary cells (12-14).

In fact, antagonizing β-catenin signaling appears to be an effective method to curb the growth of cancer cell lines afflicted by elevated levels of nuclear β-catenin. Exogenous expression of APC, axin, or PTEN reinstates β-catenin turnover and suppresses growth of hepatocellular and prostate carcinoma cells (8,9). Inhibition of integrin-linked kinase, a serine/threonine kinase that inhibits GSK3β and thereby stabilizes β-catenin, reduces growth in prostate cancer lines (15). Finally, overexpression of proteins, such as full-length E-cadherin or a truncated mutant possessing just the C-terminal β/γ-catenin-

binding domain, sequesters stabilized β-catenin, precludes its association with Tcf/Lef transcription factors, and effectively inhibits proliferation of colorectal cancer cell lines (16-18). Although the transformation potential of β-catenin has been closely examined, the role of β-catenin and Tcf/Lef transcription factors in cell cycle progression among *normal* mammalian cells is just now beginning to emerge. Immunohistochemical data have shown that self-propagating precursor cells in the intervillus regions of the small intestine epithelium – but not the well-differentiated cells at the villi tip – exhibit nuclear β-catenin and express several Tcf/Lef target genes, including c-*myc* and *CD44* (19). In addition, Tcf4 knock-out mice lack proliferating stem cells and possess only differentiated villus cells, suggesting a causal role for Tcf/Lef in governing stem cell lineage commitment (20). In addition to intestinal epithelia, Tcf/Lef signaling is involved in lineage commitment of human epidermal stem cells (21-25), hematopoietic stem cells (26), and embryonic stem cells (27). However, the ligand(s) implicated in stimulating Tcf/Lef signaling and dictating stem cell fate are largely unknown, although Wnt is clearly involved in some instances (26,28).

It is unclear whether non-Wnt ligands also utilize the Tcf/Lef pathway to regulate proliferation. Recently, a correlation between serum-mediated proliferation and Tcf/Lef transcriptional activity has been suggested in a study using an engineered mammary cell system (29). These cells express a c-Fos-estradiol receptor fusion protein that permits switching from epithelial to fibroblastoid phenotype upon estradiol-mediated activation of c-Fos (30). In both phenotypes, conditions that inhibited proliferation, such as serum starvation, also downregulated β-catenin transcriptional activity. However, a causal role

for β-catenin in serum-induced cell cycle progression was not clearly established in the epithelial cell phenotype. Taken together, inducible activation of c-Fos, which is a component of the AP-1 transcriptional machinery and itself critically involved in cell cycle control (31), and the inability of β-catenin suppression to consistently inhibit proliferation preclude an assessment of whether β-catenin nuclear activity is mechanistically involved in proliferation.

Interestingly, several reports have indicated that specific growth factors such as insulin and insulin-like growth factor I induce β-catenin transcriptional activity (32). Although these studies were conducted with cancer cell lines lacking normal β-catenin degradation machinery, HGF and certain members of the Wnt family of ligands induce β-catenin transcriptional activity in normal cells (33,34). Although the importance of HGF-mediated β-catenin signaling for normal cell cycle progression has not been examined, certain members of the Wnt family of ligands regulate proliferation in a β-catenin-dependent manner (35). Nevertheless, because β-catenin target genes include *c-myc* and *cyclin D1*, whose protein products are ubiquitously crucial for cell cycle progression (36-38), the untested hypothesis remains that β-catenin has a more pervasive role in normal epithelial cell proliferation, even in response to growth-stimulating cues from non-Wnt ligands.

We examined this hypothesis pertaining to the role of β-catenin in cell cycle progression in the normal mammary epithelial cell line MCF-10A. We demonstrate that EGF stimulates Tcf/Lef transcriptional activity and that this transcriptional activity is

necessary but not sufficient for cell cycle progression of normal epithelial cells. Thus, inhibition of Tcf/Lef transcriptional activity using dominant-negative Tcf4 prevents EGF-mediated cell cycle progression. Since dominant-negative Tcf4 inhibits cyclin D1 promoter activity and BrdU uptake without affecting other EGF-mediated signals such as ERK that also regulate proliferation, we conclude that Tcf/Lef-mediated transcription is required for cell cycle progression.

2. Experimental Procedures

2.1. Antibodies

The following antibodies were used in this study: anti-actin (Santa Cruz), anti-BrdU (Roche Applied Science), anti-ERK2 (Santa Cruz), anti-GSK3β (BD Transduction Laboratories), anti-phospho-Ser9-GSK3β (BIOSOURCE), monoclonal and polyclonal anti-FLAG (Sigma), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology), anti-phosphotyrosine (Santa Cruz), and anti-Tcf4 (Upstate Biotechnology, Inc.).

2.2. Cell Culture

SW480 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine (Invitrogen), 10% (v/v) fetal bovine serum (Invitrogen), and 1% (v/v) penicillin/streptomycin (Invitrogen). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 0.5 μg/ml hydrocortisone (Sigma), 0.1 μg/ml cholera toxin (Sigma), 10

μg/ml insulin (Sigma), and 1% (v/v) penicillin/streptomycin. For serum starvation, the cells were washed twice in PBS and then cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 1% (v/v) penicillin/streptomycin and 0.1% bovine serum albumin (Sigma) for 24 h.

2.3. Plasmid Constructs

pcDNA-myc-ΔN-Tcf4 was generously provided by K. W. Kinzler (Johns Hopkins University) (7). pPGS and pPGS-ΔNTcf4 were kindly donated by E. Fearon (University of Michigan, Ann Arbor) (14). VSV-G and gag-pol vectors were gifts from D. Schaffer (University of California, Berkeley). Luciferase-based reporters pTOPFLASH and pFOPFLASH were purchased from Upstate Biotechnology, Inc., whereas 1745CD1 was a gift from R. Pestell (Georgetown University, Washington, D.C.) (39).

2.4. Retroviral Infection

Retrovirus was produced by either by single transfection of the packaging cell line 293GPG with 15 μg of retroviral plasmid (40) or by triple transfection of 293T cells with 5 μg each of VSV-G, gag-pol and a retroviral vector using LipofectAMINE (Invitrogen). For infection, MCF-10A cells were incubated with retrovirus-containing medium and 8 μg/ml polybrene for 24 h.

2.5. GSK3β Serine 9 Phosphorylation Assay

MCF-10A cells were plated at a subconfluent density (10^5 cells/35-mm dish) and allowed to adhere for 48 h, followed by serum starvation for 24 h. The cells were stimulated with either full growth medium or serum-free medium supplemented with either 10 μg/ml insulin or 20 ng/ml EGF and then lysed in modified RIPA buffer at desired times.

2.6. ERK Signaling Assay

MCF-10A cells were plated at a subconfluent density (10^5 cells/35-mm dish), allowed to adhere for 24 h, and then infected with retrovirus encoding pPGS or pPGS-FLAG-ΔN-Tcf4 at multiplicity of infection equal to 1. Twenty-four hours after infection, the cells were starved in serum-free medium for 24 h, stimulated with 20 ng/ml EGF in serum-free medium, and then lysed in modified RIPA buffer at desired times.

2.7. Cell Lysis

The stimulated cells were washed twice in ice-cold PBS and scraped into cold lysis buffer. After incubating on ice for 15 min, the cell lysates were clarified by centrifugation, and the supernatant was collected as whole cell lysate. The protein concentrations were determined using BCA reagents (Sigma). The samples prepared to assay EGF-mediated activation of the ERK pathway were lysed in modified RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM β-glycerophosphate (pH 7.3), 10 mM NaPP, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Finally, cell lysis for all reporter measurements was performed in 1X passive lysis buffer provided by the manufacturer (Promega).

2.8. Reporter Assays

SW480 or MCF-10A cells were plated at a subconfluent density (10^5 cells/35-mm dish) and co-transfected with 1 μg of the appropriate reporter and 0.1 μg of pRL-TK using FuGENE 6 (Roche Applied Science). SW480 cells were always maintained in growth medium and lysed 48 h after transfection. Meanwhile, MCF-10A cells were

serum-starved for 24 h, stimulated with appropriate medium, and lysed at desired times. In both cases, reporter activity was measured using the dual luciferase assay according to the manufacturer instructions (Promega). To normalize for potential variations in transfection or lysis efficiency, luciferase signals were normalized to control *Renilla* luciferase signal.

2.9. Integrated Reporter Response

The reporter signal response above its initial value was integrated numerically over time as follows,

$$\int_0^{12} R(t) - R(t_0)dt = \left(\left(\frac{1}{2}\sum_{k=1}^{n} R(t_k) + R(t_{k-1})\right) - (n-1)R(t_0)\right)\Delta t \qquad \text{(Eq. II-1)}$$

where $R(t)$ is the reporter signal, $R(t_0)$ is its basal, initial value, t_k is the time ranging from 0 to 12 h in discrete intervals of Δt (3 h), n is the number of time points (n = 5), and k is the index of summation.

2.10. Western Blotting

Whole cell lysates were resolved by SDS-PAGE on 7.5-10% gels and blotted onto polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked overnight and then incubated sequentially with primary and corresponding horseradish peroxidase-conjugated secondary antibody. The blots were treated with SuperSignal West Femto Substrate (Pierce) and imaged on VersaDoc 3000 (Bio-Rad) using Quantity One software (Bio-Rad).

2.11. DNA Synthesis

DNA synthesis was assayed by either [^{3}H]thymidine or BrdU incorporation. In both cases, MCF-10A cells were seeded at the indicated cell densities. After 24 h, the cells were either infected with retrovirus or left in growth medium. On the following

day, the cells were serum-starved. Notably, the 48 h of duration between cell seeding and serum starvation was chosen to match the time required for plating and transfecting cells in reporter assays, allowing direct comparison between DNA synthesis and reporter experiments. Following 24 h of serum starvation, the cells were stimulated with appropriate medium. Sixteen hours after stimulation, the medium was replaced with identical medium supplemented with either 10 μCi/ml [^{3}H]thymidine (ICN Biomedicals) or 10 μmol/liter BrdU (Roche Applied Science) and further incubated for 6 h. In the case of [^{3}H]thymidine incorporation, the cells were washed twice in ice-cold PBS, incubated in 5% trichloroacetic acid for 20 min at 4 °C, washed twice with cold 70% ethanol, and incubated with 0.1 M NaOH, 2% Na_2CO_3, and 1% SDS for 30 min at 37 °C. The solution was collected and mixed with CytoScint (ICN Biomedicals) for scintillation counting. For BrdU detection, the cells were fixed and co-stained with DAPI, anti-BrdU antibody, and polyclonal anti-FLAG antibody. The number of nuclei stained positive for BrdU and FLAG were quantified in 3-10 different fields on 2-5 independent trials using the Zeiss Axiovert 200M microscope.

2.12. Immunofluorescence

For Tcf/FLAG co-staining, the cells grown on glass coverslips were washed three times in ice-cold PBS, fixed in 4% formalin in PBS, and permeabilized in 0.2% Triton X-100. After blocking overnight in BB (10% goat serum, 0.1% bovine serum albumin in PBS), the coverslips were sequentially incubated with primary and corresponding Alexa dye-labeled secondary antibodies (Molecular Probes). Following antibody incubations, the coverslips were stained with DAPI (Sigma) and mounted using Prolong Anti-Fade (Molecular Probes). For BrdU/FLAG co-staining, the cells were fixed and permeabilized

in 4% formalin and 0.2% Triton X-100, respectively, and then incubated with polyclonal FLAG antibody and Alexa 594-conjugated secondary antibody. The antibodies were then fixed in 4% formalin, followed by a second fixation in 15 mM glycine in 70% ethanol (pH 2). The coverslips were incubated with monoclonal BrdU antibody and then Alexa 488-conjugated secondary antibody, stained with DAPI, and mounted as described above.

3. Results

3.1. Re-entry into the Cell Cycle Correlates with Tcf/Lef Reporter Activity

Target genes for Tcf/Lef include *cyclin D1* and c-*myc*, suggesting a role for this family of transcription factors in cell cycle progression not only among cancer cells with stabilized nuclear β-catenin, but also among normal epithelial cells. Therefore, we determined whether a correlation exists between Tcf/Lef transcriptional activity and cell cycle progression in nontransformed mammary epithelial (MCF-10A) cells. Tcf/Lef transcriptional activity was monitored with TOPFLASH reporter (7), a plasmid containing consensus Tcf-binding sites upstream of the *luciferase* gene. In contrast, the negative control FOPFLASH reporter carries mutations at these Tcf/Lef-binding sites. Performance of TOPFLASH and FOPFLASH reporters was confirmed in SW480 colon carcinoma cells in which TOPFLASH, but not FOPFLASH, is constitutively active because of a truncation of the *APC* gene and consequent stabilization of β-catenin (7) (Fig. II-1*A*). Subconfluent MCF-10A cells transfected with TOPFLASH or FOPFLASH reporters were growth-arrested by serum starvation and then stimulated to re-enter the

cell cycle by treatment with growth medium. As shown in Fig. II-1*B*, growth medium stimulation activated TOPFLASH reporter, which gradually increased to a near-maximum level within the first 9 h. Meanwhile, FOPFLASH negative control reporter did not respond to growth medium stimulation. Taken together, this establishes a correlation between re-entry into the cell cycle and Tcf/Lef-mediated transcription.

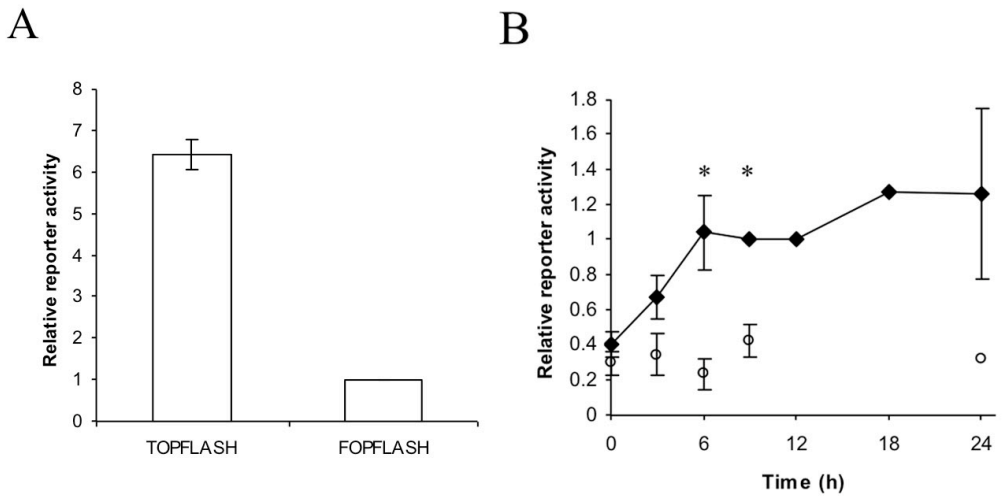

FIG. II-1. **TOPFLASH and FOPFLASH reporter activity in SW480 and MCF-10A cells**
A, TOPFLASH, but not FOPFLASH, reporter is triggered in SW480 colon carcinoma cells. SW480 cells were co-transfected with 0.1 μg of pRL-TK and 1 μg of either TOPFLASH or FOPFLASH. Forty-eight hours after transfection, the cells were lysed, and the ratio of luciferase to *Renilla* luciferase signal was quantified. *B*, TOPFLASH, but not FOPFLASH, reporter is activated upon growth medium stimulation of normal mammary epithelial cells. MCF-10A cells were co-transfected with 0.1 μg of pRL-TK and 1 μg of TOPFLASH (◆) or FOPFLASH (○). After serum starvation, the cells were stimulated with growth medium and luciferase:*Renilla* luciferase signal ratio was quantified at desired time points. Reporter activity relative to the TOPFLASH response at 6 h is shown. The *error bars* represent ± S.E. from two to five independent experiments. The *asterisk* denotes $p < 0.05$ (Student's *t*-test) in comparing TOPFLASH signal to the zero time response.

To confirm further that the observed TOPFLASH signal was specifically monitoring Tcf/Lef transcription factor activity, a dominant-negative Tcf4 construct (myc-ΔN-Tcf4) was employed. This construct possesses the DNA-binding domain of Tcf4, but lacks the N-terminal 31 amino acids that mediate its association with its transactivating catenin partner (7). As expected, dominant-negative Tcf4 inhibited TOPFLASH activity in

SW480 cells (Fig. II-2*A*). Co-transfection of ΔN-Tcf4 into MCF-10A cells decreased growth medium-induced TOPFLASH response in a dose-dependent fashion (Fig. II-2*B*), indicating that the TOPFLASH signal was mediated specifically by Tcf/Lef transcription factors.

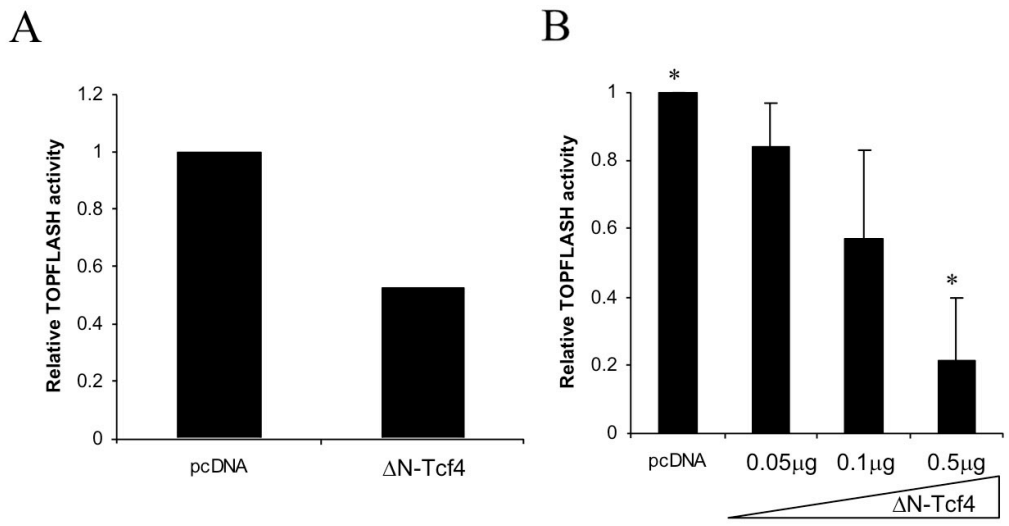

FIG. II-2. **Dominant-negative Tcf4 effect on TOPFLASH reporter**
A, dominant-negative Tcf4 inhibits TOPFLASH signal in SW480 colon carcinoma cells. SW480 cells were co-transfected with 1 μg of TOPFLASH, 0.1 μg of pRL-TK, and either 0.5 μg of empty vector (pcDNA) or dominant-negative Tcf4 (ΔN-Tcf4). Forty-eight hours after transfection, the luciferase:*Renilla* luciferase signal ratio was quantified. *B*, dominant-negative Tcf4 inhibits growth medium-mediated TOPFLASH signal in MCF-10A cells. MCF-10A cells were co-transfected with 1 μg of TOPFLASH, 0.1 μg of pRL-TK, and different amounts (0, 0.05, 0.1, and 0.5 μg) of dominant-negative Tcf4 (ΔN-Tcf4), always with a balancing amount (0.5, 0.45, 0.4, and 0 μg, respectively) of empty vector (pcDNA). Serum-starved cells were stimulated with growth medium for 9 h, after which the luciferase:*Renilla* luciferase signal ratio was quantified. Co-transfection with increasing amount of ΔN-Tcf4 correspondingly attenuated TOPFLASH induction by growth medium. The *error bars* indicate ± S.E. ($n = 3$). The *asterisk* indicates $p < 0.01$ (Student's *t*-test).

3.2. EGF Independently Induces Tcf/Lef Transcriptional Activity and DNA Synthesis

Because MCF-10A growth medium contains a complex mixture of stimuli, including serum factors, insulin, and EGF, it is unclear whether a single constituent is capable of inducing Tcf/Lef transcriptional activity and, moreover, whether the same constituent also functions as a mitogen. To address this issue, the cells were stimulated with each

constituent of growth medium separately, and Tcf/Lef transcriptional activity and DNA synthesis were assessed by measuring TOPFLASH reporter signal and [^{3}H]thymidine uptake, respectively. EGF independently induced TOPFLASH signal to a level distinctly above the corresponding FOPFLASH control (Fig. II-3*A*). At early times, EGF-mediated TOPFLASH signal mirrors growth-medium-induced TOPFLASH activity. However, whereas full growth medium sustains TOPFLASH signal to 24 h (Fig. II-1*A*), EGF promotes a transient signal that reaches its peak intensity of nearly 3-fold above basal level at 3 h. Meanwhile, in contrast to EGF, insulin-mediated TOPFLASH activation more closely matches the FOPFLASH negative control, except at 3 h, where a transient signal that is 50% of the EGF-mediated TOPFLASH signal is observed. Taken together, growth medium constituents quantitatively vary in their ability to promote Tcf/Lef transcriptional activity, with EGF, more so than insulin, resembling the response to full growth medium.

The current paradigm for Wnt-mediated Tcf/Lef transcriptional activity involves inhibition of GSK3β-mediated phosphorylation of β-catenin, which in turn stabilizes β-catenin and ultimately enables its translocation into the nucleus (41-43). Interestingly, both EGF and insulin have been reported to inhibit GSK3β kinase activity toward primed substrates by inducing phosphorylation of GSK3β at serine 9 (44-48). Because there is conflicting evidence as to whether β-catenin qualifies as a primed or nonprimed substrate of GSK3β (49-52), we investigated whether EGF, insulin, and growth medium affected GSK3β phosphorylation at serine 9 in a manner that is quantitatively consistent with their differential effects on Tcf/Lef transcriptional activity. Within 10 min of stimulation,

GSK3β was phosphorylated at serine 9 among serum-starved MCF-10A cells in response to either insulin or EGF (Fig. II-3*B*). However, in insulin-stimulated cells, dephosphorylation of GSK3β was evident by 60 min, whereas in EGF-stimulated cells, serine 9 phosphorylation of GSK3β was more sustained. Stimulation with growth medium, which contains insulin, EGF, and serum factors, induced more sustained phosphorylation of GSK3β than either EGF or insulin alone. Taken together, the stimuli (growth medium and EGF) that yield a qualitatively durable phosphoserine 9 GSK3β signal also induce stronger Tcf/Lef transcriptional activity. This correlation suggests, but does not unequivocally demonstrate, a role for serine 9 phosphorylation of GSK3β in EGF-mediated Tcf/Lef transcriptional activity, further raising the issue of whether β-catenin is a primed or nonprimed substrate of GSK3β.

Although ongoing work is focused on parsing the role of serine 9 phosphorylation of GSK3β and other mechanisms by which EGF stimulates TOPFLASH activity, we focused in this work on understanding the downstream significance of EGF-mediated Tcf/Lef transcriptional activity. Since the kinetics of TOPFLASH reporter response varied among stimuli, we calculated the time integral of each signal as a single quantitative metric capable of capturing effects on both signal magnitude and dynamics (53). Using this metric, we determined whether the ability to induce Tcf/Lef transcriptional activity quantitatively relates to the mitogenic potency of the stimulus as measured by DNA synthesis using a [^{3}H]thymidine uptake assay. EGF independently induces DNA synthesis and accounts for 70% of the mitogenic activity of complete growth medium (Fig. II-3*C*); insulin, however, fails to promote DNA synthesis. Thus,

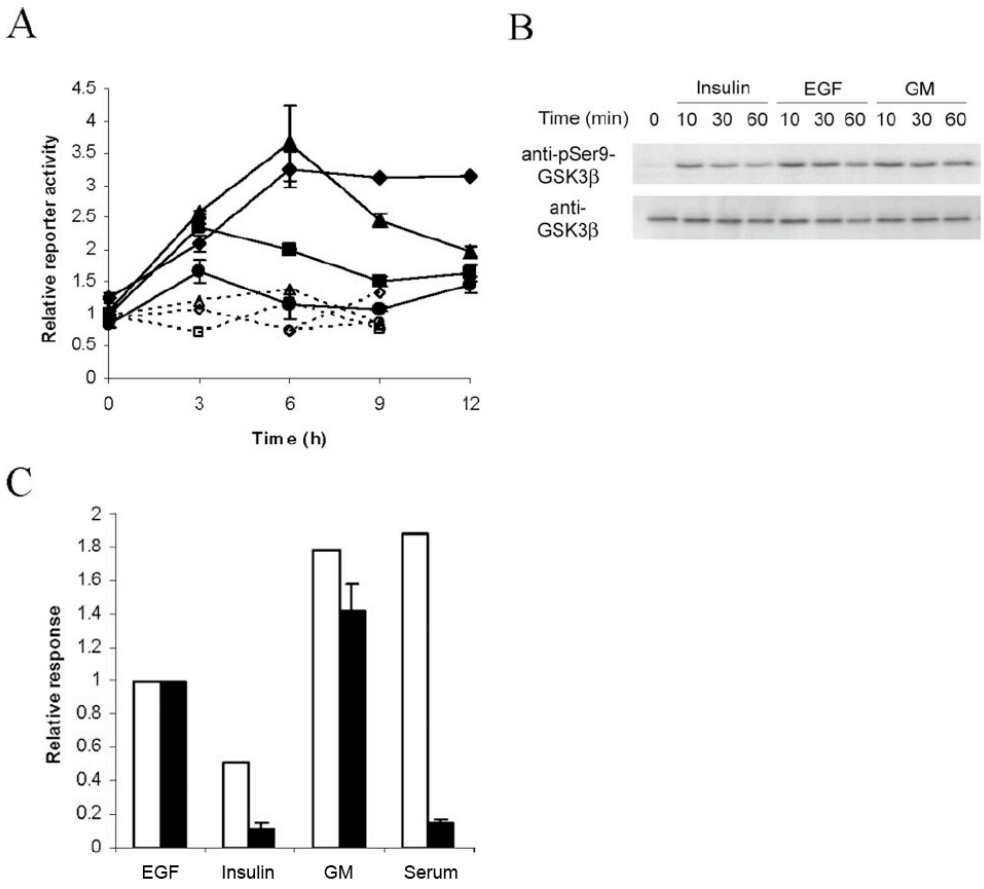

FIG. II-3. **Growth medium constituents vary in the ability to induce Tcf/Lef transcriptional activity, GSK3β phosphorylation, and DNA synthesis.**
A, EGF transiently stimulates Tcf/Lef transcriptional activity. MCF-10A cells were co-transfected with 0.1 μg of pRL-TK and 1 μg of TOPFLASH (*filled symbols*, *solid line*) or FOPFLASH (*open symbols*, *dotted line*). After serum starvation, the cells were stimulated with full growth medium (◆ and ◇) or serum-free medium supplemented with 20 ng/ml EGF (■ and □), 5% horse serum (▲ and △), or 10 μg/ml insulin (● and ○). The concentration of each supplement was chosen to match the concentration of the supplement in growth medium. Luciferase:*Renilla* luciferase signal ratio was quantified at the desired time points and is reported relative to its initial, basal level. The *error bars* represent ± S.E. from two to five independent experiments. *B*, insulin, EGF, and growth medium induce serine 9 phosphorylation of GSK3β with qualitatively distinct time courses. Serum-starved MCF-10A cells were stimulated with either full growth medium (*GM*) or serum-free medium supplemented with 10 μg/ml insulin or 20 ng/ml EGF and then lysed at indicated times. Western blotting of cell lysates with a phospho-specific antibody shows that serine 9 of GSK3β is phosphorylated within 10 min of stimulation with insulin, EGF, or growth medium. After 10 min, the levels of phosphorylated GSK3β decrease in insulin-stimulated cells, whereas cells stimulated with EGF show relatively more sustained phosphorylation (compare the 30-min bands). Finally, the cells stimulated with growth medium possess the most sustained phosphorylation response (compare the 60-min bands). Taken together, EGF and growth medium promote a longer lifetime of serine 9 phosphorylation of GSK3β than insulin. Blotting with a total GSK3β antibody confirmed equal protein loading. The data shown are representative of two independent trials. *C*, stimuli that promote DNA synthesis also activate TOPFLASH reporter, but not all stimuli that induce TOPFLASH promote DNA synthesis. TOPFLASH data (*white bars*) are expressed as the integrated response over a 12-h stimulation period. Both TOPFLASH reporter response and [^{3}H]thymidine incorporation (*black bars*) are expressed relative to the EGF response. Although EGF, growth medium, and horse serum induced significant TOPFLASH responses, only EGF and growth medium stimulate [^{3}H]thymidine incorporation.

the ability of EGF, insulin, and full growth medium to induce Tcf/Lef transcriptional activity strictly correlates with their ability to induce DNA synthesis, because stimuli that induce high levels of Tcf/Lef transcription also promote DNA synthesis (e.g., EGF and growth medium), whereas those stimuli that do not induce Tcf/Lef-mediated transcription (e.g., insulin) do not promote DNA synthesis. The exception to this apparent correlation between TOPFLASH response and DNA synthesis involves serum stimulation, which induced strong integrated TOPFLASH signal but failed to promote DNA synthesis. Taken together, stimuli that induce DNA synthesis also promote Tcf/Lef transcriptional activity (e.g., EGF), but the converse is not necessarily true (e.g., serum). Therefore, Tcf/Lef transcriptional activity is by itself insufficient to promote proliferation.

3.3. Tcf/Lef Transcriptional Activity Is Required for EGF-mediated DNA Synthesis

Although Tcf/Lef transcriptional activity is not sufficient for proliferation, we examined whether its activity is required for cell cycle progression using dominant-negative Tcf4. MCF-10A cells were infected with retrovirus encoding FLAG-tagged, dominant-negative Tcf4 (pPGS-FLAG-ΔN-Tcf4), and expression was confirmed by Western blot (data not shown) and immunofluorescence (Fig. II-4*A*). Exogenous dominant-negative Tcf4 was clearly identifiable among ΔN-Tcf4-infected cells because of distinct anti-FLAG staining, which was only observed at background levels in control (pPGS)-infected cells. Staining with anti-Tcf4 antibody revealed that both endogenous Tcf4 and FLAG-ΔN-Tcf4 were localized to the nucleus, and expression of the dominant-negative construct greatly enhanced the intensity of anti-Tcf4 stain, consistent with the expected overexpression of this exogenous protein. To determine the role of Tcf/Lef in

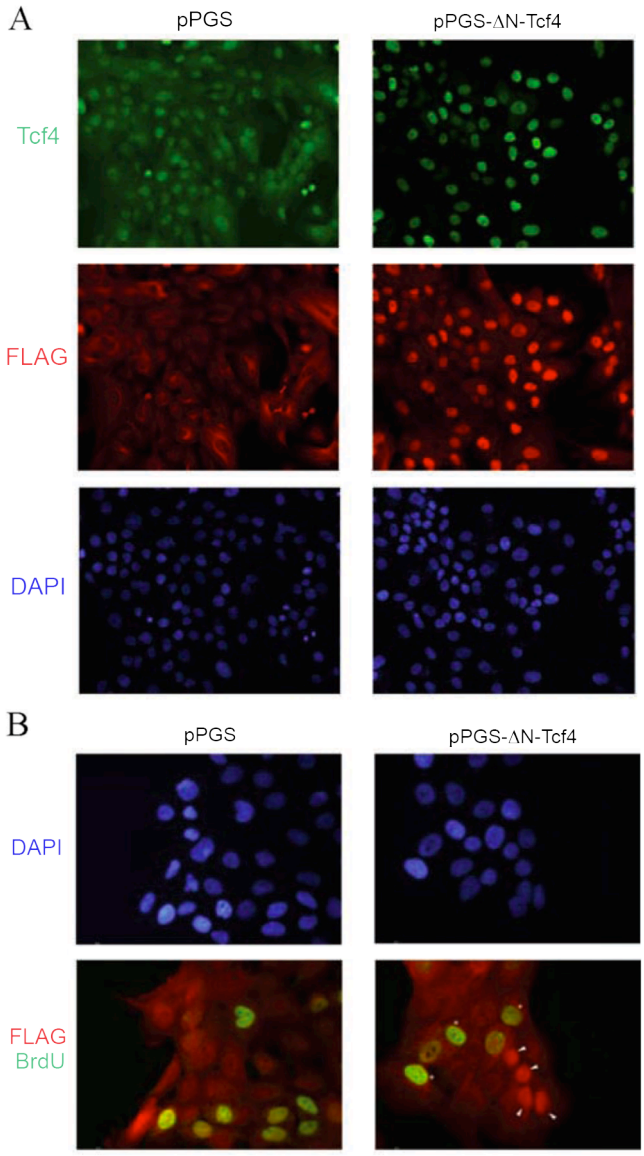

FIG. II-4. **Dominant-negative Tcf4 blocks DNA synthesis.**
A, both endogenous Tcf4 and exogenous ΔN-Tcf4 localize to cell nuclei. MCF-10A cells were retrovirally infected with either the empty vector (pPGS) or FLAG-tagged dominant-negative Tcf4 (pPGS-ΔN-Tcf4) and costained for Tcf4 and FLAG. Among pPGS-infected cells, endogenous Tcf4 (*green*) was visible in the nucleus (DAPI, *blue*), whereas an anti-FLAG antibody (*red*) revealed only background staining within the cell body. Among cells infected with pPGS-ΔN-Tcf4, anti-FLAG staining produced intense signal from cell nuclei, demonstrating that dominant-negative Tcf4, like its endogenous counterpart, localizes to the nucleus. Consistently, Tcf4 staining in ΔN-Tcf4-expressing cells was significantly more intense than endogenous Tcf4 levels in pPGS-infected cells. *B*, ΔN-Tcf4 completely blocks BrdU incorporation at a single-cell level. MCF-10A cells were infected with either pPGS or pPGS-ΔN-Tcf4 at a multiplicity of infection less than 1. After serum starvation, the cells were stimulated to re-enter the cell cycle with either full growth medium, serum-free medium, or serum-free medium supplemented with 20 ng/ml EGF. After a 6-h pulse with BrdU, the cells were co-stained with anti-FLAG antibody (*red*), anti-BrdU (*green*) antibody, and DAPI (*blue*). Images depict immunofluorescence results of EGF-stimulated cells. The BrdU and FLAG images have been superimposed to demonstrate that cells incorporating BrdU never express FLAG-tagged ΔNTcf4. The *arrowheads* denote FLAG-positive cells, and the *asterisks* denote BrdU-positive cells.

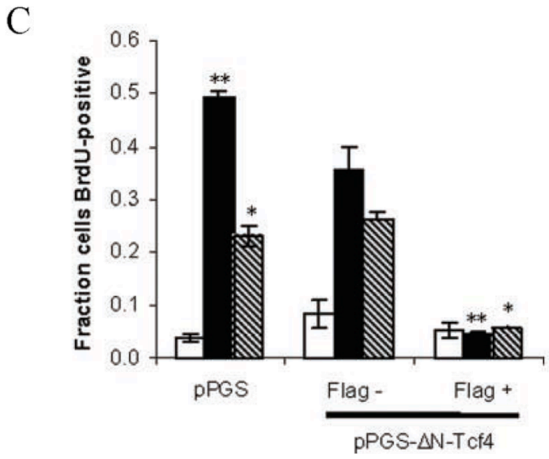

FIG. II-4. **Dominant-negative Tcf4 blocks DNA synthesis.**
C, quantification of immunostaining reveals that ΔN-Tcf4 completely inhibits BrdU incorporation in MCF-10A cells. The fraction of nuclei positive for BrdU was quantified from immunostained samples of cells treated with serum-free medium (*open bars*), growth medium (*black bars*), and EGF (*hatched bars*) medium as described above for *B*. Among pPGS-infected cells, both growth medium and EGF substantially increase the fraction of cells incorporating BrdU. Within the population targeted for infection by pPGS-ΔN-Tcf4, those cells lacking expression of FLAG-tagged, ΔN-Tcf4 responded to growth medium and EGF similar to pPGS-infected cells. Notably, FLAG-positive cells failed to incorporate BrdU upon stimulation by either EGF or growth medium. The *error bars* are ± S.E. (n = 6). The *asterisk* denotes $p <$ 0.05 (Student's *t*-test) in comparing delineated data pairs.

cell cycle progression, EGF- and growth-medium-induced DNA synthesis were measured by assessing BrdU incorporation in control and dominant-negative Tcf4-infected MCF10A cells. Because the multiplicity of infection was less than 1, BrdU uptake was observed among both cell populations upon stimulation (Fig. II-4*B*). Importantly, co-staining for FLAG revealed that cells clearly expressing FLAG-tagged, dominant-negative Tcf4 never incorporated BrdU, showing a strict Tcf/Lef requirement for DNA synthesis.

More quantitatively, both growth medium and EGF stimulation induced BrdU uptake in pPGS-infected cells (Fig. II-4*C*). Consistent with thymidine incorporation in uninfected cells (Fig. II-3*C*), growth medium was more mitogenic than EGF alone. In the case of pPGS-ΔN-Tcf4-infected cells, the fraction of nuclei positive for BrdU was

determined among FLAG-positive and FLAG-negative subpopulations. Although the FLAG-negative cells incorporated BrdU at levels similar to control pPGS-infected cells, growth medium- and EGF-mediated BrdU incorporation in FLAG-positive cells was inhibited completely. This result quantitatively demonstrates that inhibition of Tcf/Lef-mediated transcription thwarts EGF-mediated S phase progression.

In addition to Tcf/Lef transcriptional activity, EGF induces other signaling pathways that regulate cell proliferation, including the ERK pathway. To verify that dominant-negative Tcf4 inhibited EGF-mediated DNA synthesis by specifically blocking Tcf/Lef signaling rather than by more globally affecting EGF receptor signaling, we examined EGF-mediated tyrosine phosphorylation and ERK signaling among control pPGS-infected cells and pPGS-ΔN-Tcf4-infected cells. Anti-phosphotyrosine Western blotting revealed that EGF receptor (180 kDa) becomes heavily tyrosine-phosphorylated within 15 min of EGF stimulation in pPGS-infected cells and that infection with pPGS-ΔN-Tcf4 alters neither the magnitude nor the dynamics of EGF receptor phosphorylation (Fig. II-5). Similarly, ERK1/2 undergoes rapid and sustained dual phosphorylation after EGF stimulation in both pPGS- and pPGS-ΔN-Tcf4-infected cells. Expression of FLAG-tagged, dominant-negative Tcf4 was confirmed by Western blot (Fig. II-5) and immunofluorescence (data not shown). Taken together, this confirms that the inhibitory effect of dominant-negative Tcf4 on proliferation is specifically due to inhibition of EGF-mediated Tcf/Lef transcriptional activity.

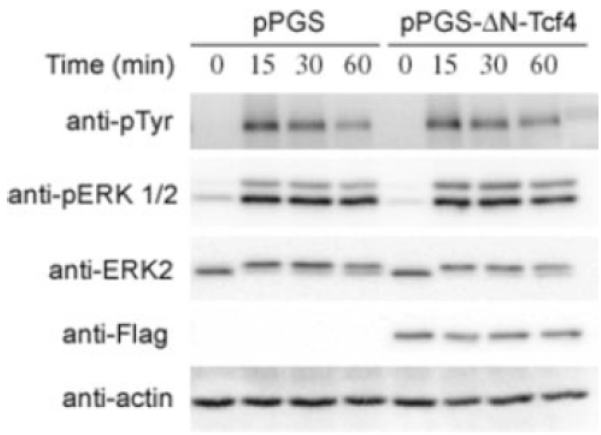

FIG. II-5. **Dominant-negative Tcf4 does not affect EGF-mediated EGF receptor and ERK phosphorylation.**
MCF-10A cells were plated at a subconfluent density (1 x 10^5 cells/35-mm dish), allowed to adhere for 24 h, and then retrovirally infected at a multiplicity of infection of 1 with empty vector (pPGS) or dominant-negative Tcf4 (pPGS-ΔN-Tcf4). Forty-eight hours after plating, the cells were serum-starved for 24 h, stimulated with 20 ng/ml EGF, and then lysed at indicated times afterward. The lysates were analyzed by Western blot using anti-phosphotyrosine and anti-phospho-ERK 1/2 antibodies. Expression of dominant-negative Tcf4, as confirmed by an anti-FLAG blot, did not affect either the magnitude or the dynamics of EGF receptor tyrosine phosphorylation (180-kDa band shown) and ERK phosphorylation. Furthermore, probing for total ERK2 revealed that expression level of this signaling protein was unaffected by dominant-negative Tcf4 expression. An anti-actin blot demonstrated equal loading of cell lysate.

3.4. EGF-mediated Activation of Tcf/Lef Transcriptional Activity Is Upstream of Cyclin D1 Promoter Activity

Upstream of S phase entry, cyclin D1 regulates passage through mid-G_1 phase of the cell cycle and is also a Tcf/Lef target gene (36,37). Therefore, we examined whether Tcf/Lef regulates cyclin D1 induction using the 1745CD1 reporter that monitors cyclin D1 promoter activity (39). In serum-starved MCF-10A cells, stimulation with growth medium initiated cyclin D1 reporter activity at 12 h (Fig. II-6*A*). The observation that the time course of TOPFLASH activation (Fig. II-1*B*) precedes timing of cyclin D1 promoter activity is consistent with, but does not prove the fact, that Tcf/Lef lies upstream of cyclin D1 upregulation.

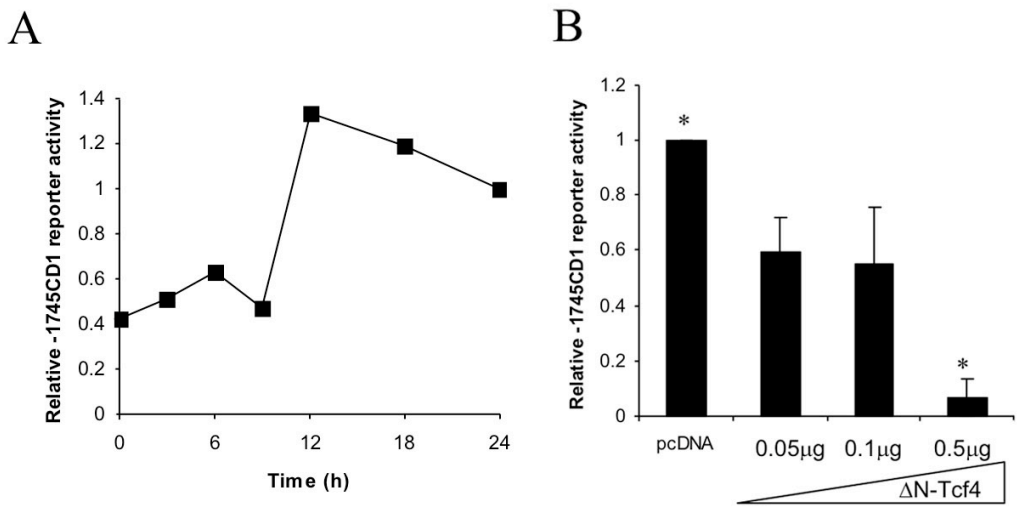

FIG. II-6. **Tcf/Lef involvement in cyclin D1 promoter activity**
A, growth medium treatment of MCF-10A cells stimulates cyclin D1 promoter activity. MCF-10A cells were co-transfected with 1 μg of 1745CD1 reporter and 0.1 μg of pRL-TK. After serum starvation, the cells were stimulated with growth medium, and luciferase:*Renilla* luciferase signal was monitored at different times. Maximum promoter activity occurred at 12 h after stimulation. *B*, dominant-negative Tcf4 blocks induction of cyclin D1 promoter activity. MCF-10A cells were transfected with 1 μg of 1745CD1 reporter, 0.1 μg of pRL-TK, and different amounts (0, 0.05, 0.1, and 0.5 μg) of dominant-negative Tcf4 (ΔN-Tcf4) in balance with empty pcDNA vector (0.5, 0.45, 0.4, and 0 μg, respectively). Following serum starvation, the cells were stimulated with growth medium for 12 h, and the luciferase:*Renilla* luciferase signal was measured. Increasing the dose of ΔN-Tcf4 reduced the induction of cyclin D1 promoter activity. The *error bars* are ± S.E. ($n = 2$), and the *asterisk* denotes $p < 0.05$ (Student's *t*-test).

To determine whether Tcf/Lef activity is in fact required for cyclin D1 promoter activity, different amounts of dominant-negative Tcf4 (pcDNA-myc-ΔN-Tcf4) balanced with empty vector (pcDNA) were co-transfected, and cyclin D1 promoter activity was measured 12 h after growth medium stimulation. The reporter response was abolished in MCF-10A cells co-transfected with myc-ΔN-Tcf4 in a dose-dependent fashion (Fig. II-6*B*), establishing a causal link between Tcf/Lef activity and cyclin D1 promoter activity.

4. Discussion

In this work, we demonstrate that EGF stimulates Tcf/Lef transcriptional activity in normal mammary epithelial cells and that its transcriptional activity is essential for EGF-

mediated cyclin D1 induction and DNA synthesis. Thus, expression of dominant-negative Tcf4 inhibits EGF-mediated Tcf/Lef transcriptional activity and also blocks EGF-mediated BrdU uptake. To our knowledge, this report offers the first demonstration that a specific growth factor, other than Wnt ligands, stimulates cell cycle progression in a Tcf/Lef-dependent manner in an untransformed epithelial cell line.

Although complex medium containing fetal calf serum has been shown to stimulate TOPFLASH reporter (29), the only specific, non-Wnt growth factors implicated in Tcf/Lef signaling among normal epithelial cells are HGF and Gas6 (34,54). Although Gas6 was shown to induce both Tcf/Lef-mediated transcription and proliferation, these results were correlative and did not establish a mechanistic role for β-catenin signaling in cell proliferation. Nevertheless, a role for Tcf/Lef in HGF-mediated proliferation may be inferred cautiously from the finding that overexpression of oncogenic MET and RON (receptors for HGF and macrophage-stimulating protein/HGF-like protein, respectively) induces transformation in a Tcf/Lef-dependent manner (55). Because these studies involved overexpression of oncogenic receptors, it is difficult to conclude whether HGF-mediated Tcf/Lef signaling will have similar functional significance in normal cells. Indeed, our findings suggest that such extrapolation may prove quantitatively inaccurate, especially in the case of insulin-induced Tcf/Lef signaling. Insulin and insulin-like growth factor I stabilize β-catenin and induce TOPFLASH reporter activity in cancer cell lines with constitutive defects in β-catenin degradation machinery (32). However, our observations reveal that, in the context of normal β-catenin regulation, insulin stimulates Tcf/Lef transcriptional activity only to a relatively minor extent and to a level that is

insufficient to promote cell cycle activity.

Interestingly, our results suggest a correlation between the level of EGF- and insulin-mediated Tcf/Lef transcriptional activity and their differential ability to phosphorylate GSK3β at serine 9. Serine 9 phosphorylation inhibits GSK3β kinase activity toward primed substrates (49). However, published reports support both the possibility that β-catenin is a primed substrate (51,52) and the possibility that it may be a nonprimed substrate (49,50) for GSK3β. *In vitro* kinase assays have demonstrated that mutations in GSK3β that abolish kinase activity toward primed substrates do not affect its activity toward β-catenin (49). Consistent with this finding, Wnt-mediated stabilization of β-catenin signaling does not coincide with phosphorylation of GSK3β at serine 9, whereas insulin-mediated phosphorylation of GSK3β at serine 9 correlates with its ability to activate a primed substrate, glycogen synthase (50). Taken together, these reports support a model where β-catenin is a nonprimed substrate whose phosphorylation is regulated by GSK3β, but not in a serine 9 phosphorylation-dependent manner. In contrast, other reports have identified members of the casein kinase I family as priming kinases that are required for GSK3β-mediated phosphorylation of N-terminal β-catenin serine residues (51,52). Thus, assuming β-catenin stabilization via inhibition of GSK3β activity plays a crucial role in EGF-mediated Tcf/Lef transcriptional activation as it does in Wnt signaling, the mechanistic significance of our observed correlation between the level of phosphorylation of GSK3β at serine 9 and the intensity of Tcf/Lef transcriptional activity will depend on whether EGF employs a primed or nonprimed mechanism to affect β-catenin stabilization. Further quantitative experiments to test this and other

hypotheses regarding the mechanisms by which EGF stimulates Tcf/Lef transcriptional activity are underway.

Although the mechanisms connecting EGF stimulation to Tcf/Lef transcriptional activity remain to be deciphered, this work focused on the essential role that EGF-mediated Tcf/Lef activation plays in stimulating DNA synthesis. Further upstream within the cell cycle, we show that EGF-mediated induction of cyclin D1 promoter activity was blocked by expression of dominant-negative Tcf4, consistent with the fact that *cyclin D1* is a putative target gene for Tcf/Lef transcription factors (36,37).

Importantly, although Tcf/Lef transcriptional activity is essential for G_1/S phase progression, it alone is insufficient for DNA synthesis. Thus, serum induces TOPFLASH reporter signal but does not elicit DNA synthesis, clearly underscoring that other signaling pathways, such as PI3K and ERK, are likely to be important for the ultimate mitogenic response. Some clues to how this combination of signals impinges on cell cycle regulation are beginning to emerge. An important point of convergence may involve the upregulation of cyclin D1 during late G_1 phase of the cell cycle. The cyclin D1 promoter contains both Tcf/Lef-binding sites that are essential for β-catenin responsiveness and Ets and CREB sites that are essential for Ras-mediated activation (37). Studies using cyclin D1 promoter reporters carrying mutations in Tcf/Lef, Ets, and/or CREB sites revealed that exogenous expression of a stabilized β-catenin mutant stimulates cyclin D1 promoter activity independent of Ets/CREB sites, whereas RasV12-mediated reporter stimulation is independent of the Tcf/Lef sites. Yet, maximal response

was observed only when all sites were intact. Hence, these findings argue for an additive effect of Ras and β-catenin signaling on cyclin D1 promoter activity. More recently, PI3K has been implicated in regulating Tcf/Lef-dependent cyclin D1 induction, as inhibition of PI3K or its downstream target IKKα thwarts serum-mediated induction of cyclin D1 (56). Furthermore, the sensitivity of cyclin D1 induction to PI3K/IKKα signaling was traced to a single Tcf-binding site on the cyclin D1 promoter.

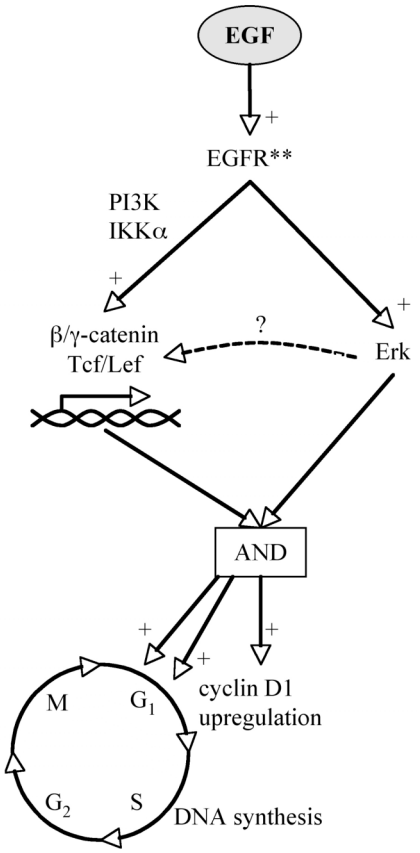

FIG. II-7. **Proposed model for the strict requirement of Tcf/Lef signaling for EGF-mediated cell cycle progression** EGF-induced progression from G_1 into S phase is controlled by cyclin D1 upregulation and is known to require signals such as ERK. Here, we report that EGF also induces Tcf/Lef-mediated transcription and that this transcriptional activity is essential for cyclin D1 upregulation and DNA synthesis. Other studies have suggested that Tcf/Lef transcription may require PI3K/IKKα and/or PKC (50,56). Although ERK signaling is not affected by inhibition of Tcf/Lef transcription, ERK may either lie upstream of β/γ-catenin:Tcf/Lef transcription or act as a parallel signal. Taken together, both Tcf/Lef signaling and parallel signals such as ERK may cooperate to upregulate cyclin D1, which leads to cell cycle progression and, ultimately, cell proliferation.

Whether working additively with Ras-mediated signals, possibly including ERK, or synergistically via PI3K, Tcf/Lef transcriptional activity seems to be essential for EGF-mediated DNA synthesis, since blocking Tcf/Lef transcriptional activity using a dominant-negative Tcf4 inhibits EGF-mediated DNA synthesis (Fig. II-7). In addition to

the β/γ-catenin:Tcf/Lef signal, other canonical growth factor signals, such as ERK, are crucial mediators of cell cycle activity (57). Here, we demonstrate that inhibition of Tcf/Lef transcription by dominant-negative Tcf4 does not affect EGF-mediated activation of the ERK pathway (Fig. II-5). Thus, ERK signaling is not downstream of Tcf/Lef-mediated transcription; however, the ERK pathway may work synergistically with Tcf/Lef signaling to provide multiple, essential signals that initiate cell cycle progression. Alternatively, ERK signaling may lie upstream of Tcf/Lef signaling. Although current work is focused on understanding the relationship among ERK, Tcf/Lef signaling, and cell cycle progression, our data demonstrate that Tcf/Lef-mediated transcription is one of several intracellular signals that are essential for cell cycle progression.

Taken together, our findings in MCF-10A normal mammary epithelial cells, along with recent reports in a range of stem cells (19,21,25-27), underscore the important role β/γ-catenin:Tcf/Lef signaling plays in normal cell proliferation. On the other extreme, mutations that constitutively aggrandize β-catenin nuclear activity lead to transformation (5). Thus, finding strategies that attenuate hyperactive β-catenin signaling in cancer cells (8,9,15-18), while minimizing deleterious effects in normal cells, will clearly be important to the success of this family of therapeutic strategies.

5. Acknowledgments

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Chapter III. EGF and Wnt 3a differentially regulate Tcf/Lef transcription with implications for tumor development

Abstract

Tcf/Lef-mediated transcription plays a prominent role in development and oncogenesis. While Wnt ligands are the classical agonists of Tcf/Lef signaling, epidermal growth factor (EGF) also stimulates Tcf/Lef transcriptional activity with potential implications for breast cancer development. However, the mechanisms by which EGF stimulates Tcf/Lef signaling and the potential crosstalk between EGF and Wnt are poorly understood. Here, we demonstrate that EGF and Wnt 3a tap distinct sub-pools of β-catenin to stimulate Tcf/Lef transcriptional activity, even though both factors rely on some common intermediate signals (Erk and PKC). While Wnt 3a operates by the canonical β-catenin stabilization pathway, EGF utilizes a siRNA-resistant sub-pool of β-catenin in a Src-dependent manner. We further show that these distinct sub-pools of β-catenin provide independent and additive contributions to Tcf/Lef signaling when cells are co-stimulated with EGF and Wnt 3a. These results suggest therapeutic strategies to selectively target EGF and Wnt contributions to Tcf/Lef signaling.

Manuscript prepared for submission by N. A. Graham, J. H. Kim, M. D. Pope, and A. R. Asthagiri

1. Introduction

The Wnt family of ligands regulates both embryonic and adult development in several tissues, including the gastrointestinal tract, the neural crest and the mammary gland (Hatsell et al., 2003; Ille and Sommer, 2005). Furthermore, hyperactivation of Wnt-mediated signaling plays a key role in the development of cancers of the colon, breast, and prostate (Polakis, 2000). The broad role of Wnt in development and cancer stems from its ability to coax an otherwise structural protein, β-catenin, into inducing the expression of target genes within the nucleus.

β-catenin is a 90 kDa proto-oncogene that mediates intercellular adhesion by bridging transmembrane cadherin proteins to the actin cytoskeleton. Canonical Wnt ligands, including Wnt 3a, induce developmental cues through transcription of gene targets by nuclear β-catenin, which forms a bipartite transcription factor with the Tcf/Lef family of proteins. A key aspect of transcriptional activation by Wnts is the tight regulation of β-catenin stability. In the absence of Wnt ligands, β-catenin is phosphorylated on N-terminal serine and threonine residues by a multiprotein complex consisting of axin, APC, and glycogen synthase kinase 3β (GSK3β). Phosphorylated β-catenin is then ubiquitinated and subsequently degraded by the proteasome. Upon binding to the co-receptor complex of Frizzled (Frz) and LRP 5/6, canonical Wnt ligands inhibit the β-catenin degradation complex, allowing accumulation of cytoplasmic β-catenin. Stabilized β-catenin then translocates to the nucleus and, in partnership with Tcf/Lef transcription factors and a multitude of co-activators, catalyzes transcription of oncogenes such as *cyclin D1* and *c-myc* (He et al., 1998; Tetsu and McCormick, 1999).

Mutations that impair the β-catenin degration complex stabilize β-catenin and permit constitutive transcription of Tcf/Lef gene targets in several cancer types (Polakis, 2000). Interestingly, similar mutations are not found in human breast cancers even though hyperactive β-catenin signaling is observed in approximately 60% of cases (Lin et al., 2000). Thus, the mechanistic basis of hyperactive β-catenin signaling in human breast cancer remains unclear. Recent evidence suggests that epidermal growth factor (EGF) signaling may be involved. We and others have shown that EGF activates Tcf/Lef-mediated transcription in a variety of cell systems (Graham and Asthagiri, 2004; Lu et al., 2003; Muller et al., 2002). Furthermore, Wnt 1 and Wnt 3 can cooperate with EGFR ligands such as TGFα to initiate neoplastic progression in murine mammary glands, although the mechanism is not known (Schroeder et al., 2000). In addition, Wnt-mediated tumorigenesis in both murine models and humans may require association of β-catenin with EGFR (Schroeder et al., 2002).

These reports suggest the intriguing possibility that Wnt and EGF may co-regulate Tcf/Lef signaling in physiological contexts such as human breast cancer and have raised several important questions. What are the mechanisms by which EGF stimulates Tcf/Lef transcriptional activity? Do EGF and Wnt operate through the same canonical pathway, or do these factors utilize complementary and distinct mechanisms? Are the pathways configured such that EGF and Wnt work together to stimulate Tcf/Lef signaling to a level beyond what each factor promotes by itself? To address these questions, we sought to delineate the intracellular signaling pathways by which EGF

stimulates Tcf/Lef signaling and to quantify the coordinate effects of EGF and Wnt on Tcf/Lef signaling.

2. Results

2.1. EGF activates Tcf/Lef transcriptional activity in 293T-EGFR cells.

To investigate the possibility of EGF/Wnt crosstalk in Tcf/Lef signaling, we used the HEK-293T cell line, in which both EGF (Lu et al., 2003) and canonical Wnts (Chen et al., 2000) induce Tcf/Lef transcriptional activity. Because HEK-293T cells express low levels of endogenous EGFR (Johns et al., 2004), cells were transfected with a plasmid encoding human EGFR or an empty control vector. As expected, EGF treatment of empty vector transfectants (293T-LPCX) yielded minimal tyrosine phosphorylation (Figure III-1A). Meanwhile, EGF treatment of 293T cells expressing exogenous EGFR (293T-EGFR) stimulated robust tyrosine phosphorylation near the molecular weight of EGFR (~180 kDa).

Using the Tcf/Lef-dependent reporter TOPFLASH, which contains consensus Tcf/Lef binding sites upstream of the *luciferase* gene (Korinek et al., 1997), we monitored Tcf/Lef transcription in response to EGF stimulation. In 293T-EGFR cells, EGF stimulated TOPFLASH reporter activity within 6 h of stimulation; by 12 h, the reporter response had increased ~ 3-fold above its initial basal level (Figure III-1B). In contrast, EGF did not activate the TOPFLASH reporter in 293T-LPCX cells, consistent with the inability to generate a measurable phospho-tyrosine response. As a second

negative control, we employed the FOPFLASH reporter, which carries mutated Tcf/Lef binding sites. EGF treatment yielded a significantly weaker signal from the FOPFLASH negative control than the TOPFLASH reporter (Figure III-1C). Furthermore, EGF-mediated Tcf/Lef signaling was dose-dependent, reaching maximal response near a dosage of 40 ng/ml (Figure III-1D).

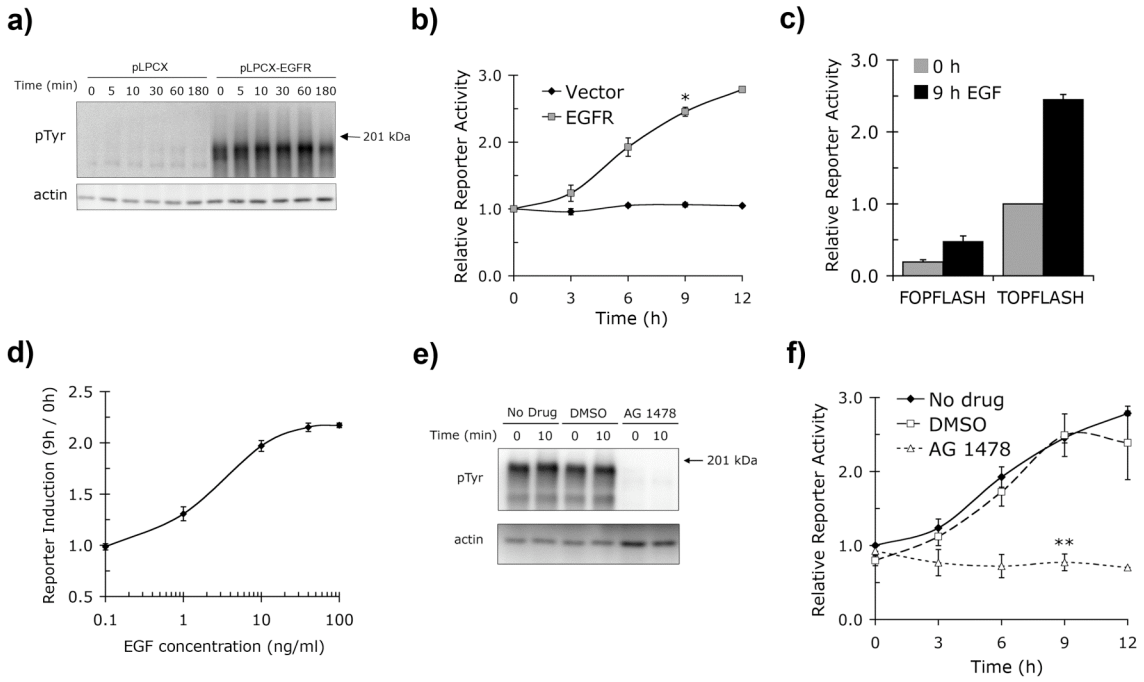

Figure III-1. EGF induces Tcf/Lef transcriptional activity in 293T-EGFR cells.
a) 293T cells were transfected with pLPCX-EGFR or the empty vector pLPCX, serum-starved and then stimulated with EGF (40 ng/ml) before lysis at the indicated times. Whole cell lysates were immunoblotted for phospho-tyrosine and actin.
b) TOPFLASH reporter activity was quantified at the indicated times after stimulation of serum-starved 293T-LPCX or 293T-EGFR cells with EGF. The *asterisk* denotes $P < 0.01$ comparing reporter activity at 0 and 9 h in 293T-EGFR cells.
c) 293T-EGFR were transfected with either TOPFLASH or the negative control FOPFLASH, serum-starved, stimulated with EGF, and the reporter activity was quantified.
d) 293T-EGFR were stimulated with varying concentrations of EGF, lysed at 0 h and 9 h following stimulation, and the extent of TOPFLASH reporter induction (9h / 0h) is reported.
e) After pre-treatment with AG 1478 (5 μM) or the solvent DMSO, serum-starved 293T-EGFR were stimulated with EGF (40 ng/ml) and lysed at the indicated times. Whole cell lysates were immunoblotted for phospho-tyrosine and actin.
f) 293T-EGFR pre-treated with AG 1478 or the solvent DMSO were stimulated with EGF before lysis at the indicated times and quantification of TOPFLASH reporter activity. The *double asterisk* denotes $P < 0.001$ comparing reporter activity at 9 h in the absence and presence of AG 1478.

To further demonstrate the specific requirement for EGFR in EGF-induced Tcf/Lef transcriptional activity, we treated cells with AG 1478, a pharmacological inhibitor of EGFR kinase activity, effectively blocking EGFR tyrosine phosphorylation (Figure III-1E). In the presence of AG 1478, the EGF-mediated TOPFLASH response was ablated in 293T-EGFR cells (Figure III-1F). Meanwhile, the solvent control had no effect, demonstrating a strict requirement for EGFR kinase activity in EGF-mediated Tcf/Lef transcription.

2.2. EGF and Wnt 3a additively activate Tcf/Lef transcription.

Having quantified the magnitude and kinetics of EGF-mediated Tcf/Lef transcriptional activity, we sought to compare and contrast signaling by this non-Wnt ligand to the canonical Wnt pathway. Stimulating 293T-EGFR with recombinant mouse Wnt 3a provoked a ~ 2.5-fold increase in TOPFLASH reporter activity within 9 h (Figure III-2A). Notably, the magnitude and kinetics of Wnt 3a-mediated TOPFLASH activity were similar to that of EGF. As negative controls, we verified that Wnt 3a did not activate the FOPFLASH reporter (Figure III-2B) and that recombinant mouse Wnt 5a, a non-canonical Wnt ligand (Weeraratna, 2005), did not induce TOPFLASH reporter activity (Figure III-S1).

Because EGF and Wnt 3a both activate the TOPFLASH reporter with similar magnitude and dynamics, we asked whether these two ligands could cooperate to induce Tcf/Lef reporter activity in an additive or synergistic fashion. Indeed, co-stimulation of 293T-EGFR cells with EGF and Wnt 3a provoked a stronger reporter response than

either ligand alone (Figure III-2C). In fact, adding the reporter response from each individual ligand closely matches the data from co-stimulated cells, demonstrating that EGF and Wnt cooperate in an additive manner. Synergistic activation of the TOPFLASH reporter was never observed, even at sub-saturating doses of EGF and Wnt 3a (data not shown).

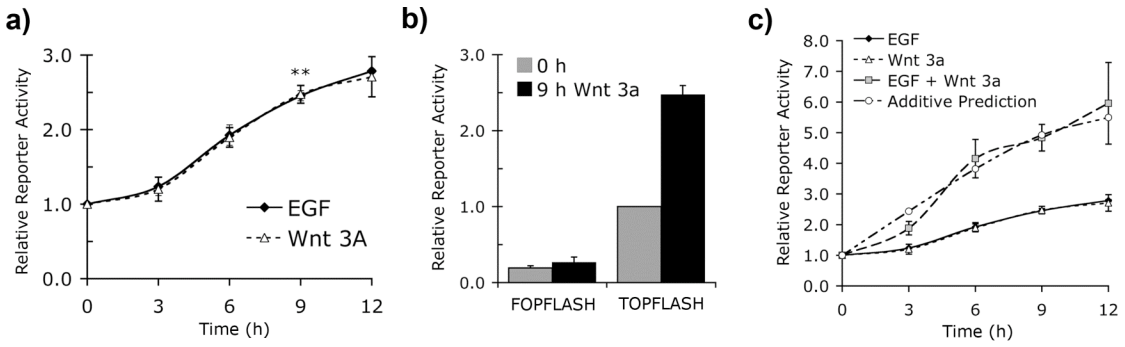

Figure III-2. Wnt 3a and EGF cooperate to activate Tcf/Lef transcriptional activity.
a) 293T-EGFR cells were serum-starved, stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml) and then lysed at the desired times before quantification of TOPFLASH reporter activity. The *double asterisk* denotes $P < 0.001$ in comparing reporter activity between 0 and 9 h for the Wnt 3a-mediated response.
b) 293T-EGFR transfected with either TOPFLASH or the negative control FOPFLASH were serum-starved, stimulated with Wnt 3a, and lysed at the desired times before quantification of the reporter activity.
c) Serum-starved 293T-EGFR were stimulated with either EGF, Wnt 3a, or EGF plus Wnt 3a, and the TOPFLASH reporter activity was quantified at the indicated times. *Additive prediction* denotes the sum of the reporter activities induced by EGF and Wnt 3a alone.

2.3. Wnt 3a and EGF activate Tcf/Lef transcription via different mechanisms.

Because EGF and Wnt 3a additively activate Tcf/Lef transcriptional activity, it raises the possibility that EGF and Wnt 3a function through divergent mechanisms. To test this hypothesis, we determined whether only Wnt 3a, or both Wnt 3a and EGF, induce Tcf/Lef transcriptional activity via the canonical Wnt mechanism. Since serine 9 phosphorylation of GSK3β can inhibit kinase activity towards primed substrates like β-catenin (Shaw and Cohen, 1999), we examined the effect of EGF and Wnt 3a treatment on GSK3β serine phosphorylation. Western blotting demonstrated that neither EGF nor

Wnt 3a increased levels of phospho-serine 9 (Figure III-S2), suggesting EGF and Wnt 3a do not regulate GSK3β activity through inhibitory phosphorylation in 293T-EGFR. However, inferring effects on β-catenin stability based on GSK3β phosphorylation is somewhat tenuous, since Wnt may function independently of GSK3β phosphorylation (Ding et al., 2000; McManus et al., 2005).

To more directly assess β-catenin stability, we measured the effect of Wnt 3a or EGF on the pool of β-catenin that is not associated with the transmembrane glycoprotein E-cadherin, and therefore, available for nuclear translocation. We assayed this "free" pool of β-catenin using an established technique based on pre-clearing of E-cadherin and other glycoproteins from cell lysates using beads coated with concanavalin A (ConA), a lectin that binds glycoproteins with high affinity (Fagotto et al., 1999). Following Wnt 3a stimulation, the level of free β-catenin was substantially increased (Figure III-3A), confirming that Wnt 3a does inhibit β-catenin degradation. As a control for the efficacy of the ConA pre-clearing procedure, E- cadherin was undetectable in pre-cleared lysates (Figure III-S3). In contrast to Wnt 3a, EGF stimulation induced only a small but reproducible increase in the pool of free β-catenin. Thus, although Wnt 3a stabilizes β-catenin, elevating the level of free β-catenin available for nuclear translocation and transactivation, EGF does not, suggesting that EGF activates Tcf/Lef transcription through a non-canonical mechanism.

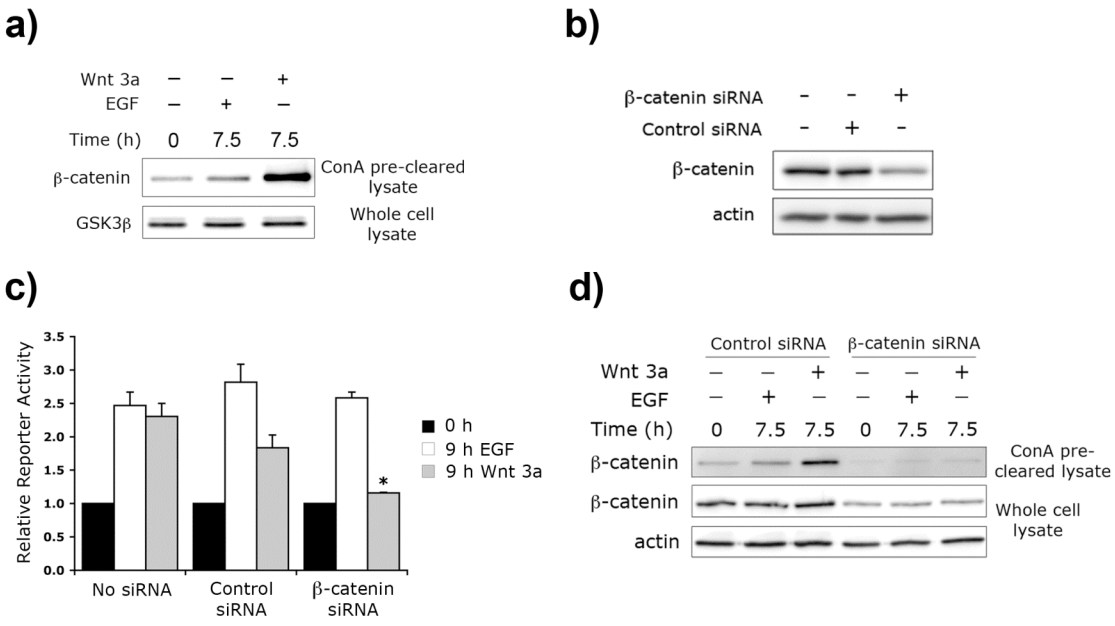

Figure III-3. Wnt 3a and EGF activate Tcf/Lef transcription via different mechanisms.
a) 293T-EGFR were serum-starved, stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml), and lysed at the indicated times. Whole cell lysates were pre-cleared with ConA beads before immunoblotting for β-catenin. Immunoblotting of whole cell lysate for GSK3β confirmed equal protein loading.
b) 293T-EGFR were transfected with β-catenin or control siRNA (10 nM). Whole cell lysates were immunoblotted for β-catenin and actin.
c) 293T-EGFR were transfected with β-catenin or control siRNA (10 nM) or not transfected with siRNA. After serum starvation, cells were stimulated with either EGF or Wnt 3a and the TOPFLASH reporter activity was quantified at the indicated times. The *asterisk* denotes $P < 0.05$ comparing reporter activity at 9 h between cells transfected with control and β-catenin siRNA. The observed difference in Wnt-mediated reporter activity between control siRNA and no siRNA cells was not statistically significant ($P = 0.12$).
d) 293T-EGFR were transfected with β-catenin or control siRNA (15 nM) or not transfected with siRNA. After serum starvation, cells were stimulated with either EGF or Wnt 3a, and ConA pre-clearing and immunoblotting was performed as in (A).

To explore this possibility, we sought to determine whether β-catenin was required for EGF-mediated activation of the TOPFLASH reporter. Transfection of β-catenin-specific siRNA caused substantial, but not complete, knockdown of total cellular β-catenin, whereas transfection of control siRNA did not affect β-catenin expression (Figure III-3B). In cells transfected with control siRNA, TOPFLASH reporter induction by EGF and Wnt 3a was not significantly different than in cells untreated with siRNA (Figure III-3C). Transfection of β-catenin siRNA, however, resulted in complete

inhibition of Wnt 3a-mediated TOPFLASH activation. In contrast, β-catenin siRNA did not affect the induction of Tcf/Lef transcription by EGF. These results raise two possibilities: either β-catenin is not required for EGF-mediated TOPFLASH reporter activation, or the pool of β-catenin that is resistant to siRNA treatment contributes selectively to EGF-mediated Tcf/Lef transcriptional activity.

To examine the latter possibility, we reasoned that subcellular pools of β-catenin whose turnover is significantly less than that of free β-catenin might be resistant to siRNA treatment. One such highly-stable pool is the fraction of β-catenin that is associated with E-cadherin (Papkoff, 1997). In cells transfected with control siRNA, EGF and Wnt 3a induced accumulation of free β-catenin similar to cells untreated with siRNA (Figure III-3D). However, in cells treated with β-catenin siRNA, the pool of free β-catenin was completely ablated in both EGF- and Wnt 3a-treated cells, even though total cellular β-catenin was only partially reduced. Thus, free β-catenin is highly sensitive to siRNA treatment. In contrast, the glycoprotein-associated fraction of β-catenin, including β-catenin bound to E-cadherin, is at least partially resistant to siRNA, raising the possibility that this siRNA-resistant pool of β-catenin is involved in EGF-mediated Tcf/Lef transcriptional activity.

2.4. ERK is required for EGF- and Wnt 3a-mediated Tcf/Lef signaling.

Since EGF-mediated Tcf/Lef transcriptional activity does not occur via the canonical Wnt pathway, we sought to determine which EGF-mediated signaling pathways might be involved. We first examined the role of the MAP kinase ERK, a

prominent EGF-related signal. Upon treatment with EGF, 293T-EGFR exhibit robust, sustained activation of ERK 1/2 (Figure III-4A). ERK phosphorylation in response to EGF was significantly diminished by treatment with the pharmacological agent PD 98059, which inhibits MEK, the upstream activator of ERK (Figure III-4A). EGF-mediated Tcf/Lef transcription was also completely blocked by inhibition of ERK signaling with PD 98059 (Figures III-4B and III-S4A). Corroborating this observation, a second, structurally distinct MEK inhibitor (U1026) blocked TOPFLASH reporter activity in a dose-dependent manner (Figures III-4B and III-S4B). Additionally, because pharmacological inhibitors can have non-specific effects, we tested TOPFLASH reporter activity in cells transfected with MKK1-K97M, an inactive mutant of MEK (Mansour et al., 1994). Compared to 293T-EGFR cells transfected with an empty vector, EGF-mediated Tcf/Lef transcriptional activity was blocked by dominant-negative MEK in a dose-dependent fashion (Figure III-4C), clearly demonstrating that ERK signaling is required for EGF-mediated Tcf/Lef transcriptional activity.

Because Wnt 3a has also been reported to stimulate ERK activation (Almeida et al., 2005; Yun et al., 2005), we examined whether Wnt 3a can induce ERK activity in 293T-EGFR. Indeed, Wnt 3a treatment transiently activated ERK in a manner sensitive to MEK inhibitors (Figure III-4D), although the magnitude and duration of ERK phosphorylation were less than that induced by EGF. Despite reports that Wnts can stimulate MAPK through transactivation of EGFR (Civenni et al., 2003), Wnt 3a-mediated ERK activity was not affected by pharmacological inhibition of EGFR (data not shown).

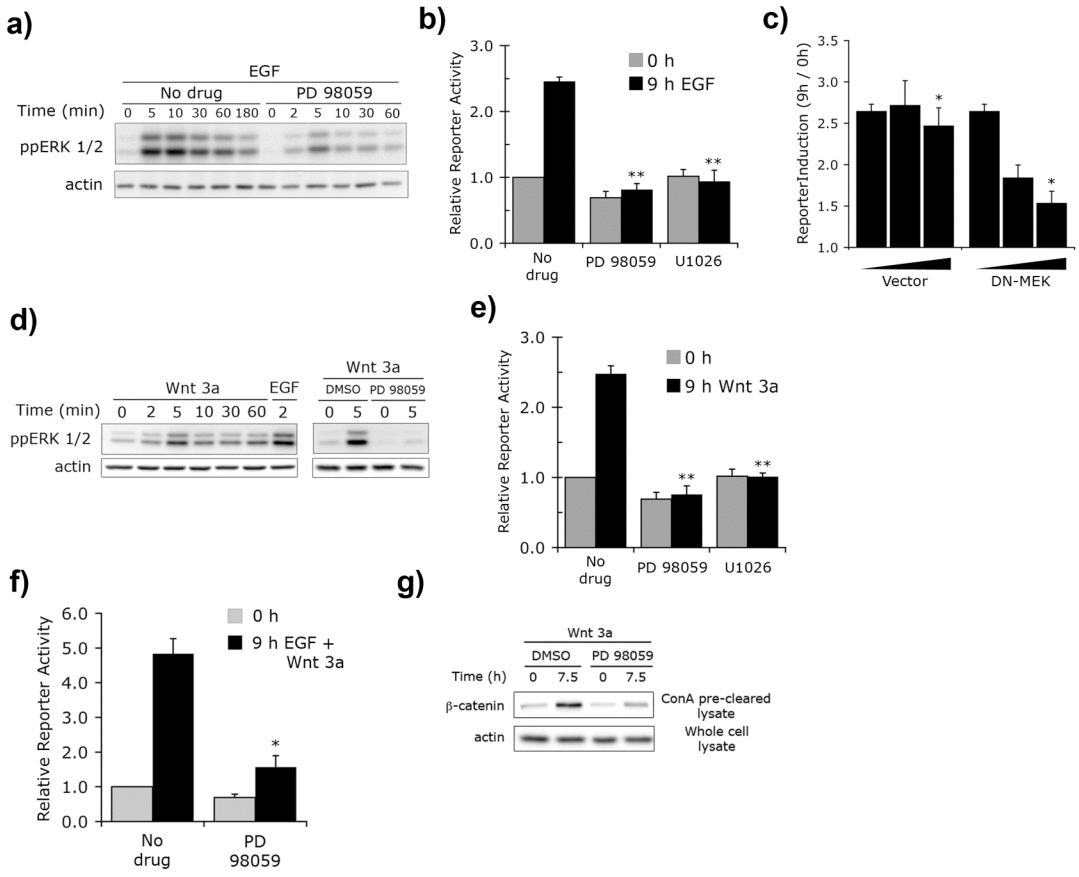

Figure III-4. ERK is required for both EGF- and Wnt 3a-mediated Tcf/Lef signaling.
a) 293T-EGFR were pre-treated with PD 98059 (50 μM), stimulated with EGF (40 ng/ml), and then lysed at the desired times. Whole cell lysates were immunoblotted for dually-phosphorylated ERK 1/2 and actin.
b) After pre-treatment with PD 98059, U1026 (10 μM), or no drug, serum-starved 293T-EGFR were stimulated with EGF, and the TOPFLASH reporter activity was quantified at the indicated times. The *double asterisk* denotes $P < 0.01$ comparing reporter activity at 9 h in the absence and presence of the pharmacological agents.
c) 293T-EGFR were transfected with increasing amounts (0, 5, or 10 μg) of the dominant-negative MEK mutant or the corresponding empty vector. After serum starvation, cells were treated with EGF and the extent of TOPFLASH reporter induction (9h / 0h) is reported. The *asterisk* denotes $P < 0.05$ comparing TOPFLASH reporter activity between the MEK mutant and the empty vector.
d) 293T-EGFR were either stimulated with Wnt 3a (50 ng/ml) or EGF (left panel) or pre-treated with PD 98059 or the solvent DMSO before stimulation with Wnt 3a (right panel). Whole cell lysates were immunoblotted for dually-phosphorylated ERK 1/2 and actin.
e) 293T-EGFR were pre-treated with PD 98059, U1026, or no drug, and then stimulated with Wnt 3a before quantification of the TOPFLASH reporter activity. The *double asterisk* denotes $P < 0.01$ in comparing reporter activity at 9 h in the absence and presence of the indicated pharmacological agents.
f) 293T-EGFR were pre-treated with PD 98059 or no drug, stimulated with Wnt 3a, and the TOPFLASH reporter activity was quantified. The *asterisk* denotes $P < 0.05$ comparing reporter activity at 9 h in the absence and presence of PD 98059.
g) After pre-treatment with PD 98059 or the solvent DMSO, 293T-EGFR were stimulated with Wnt 3a and lysed at the indicated times. Whole cell lysates were pre-cleared with ConA beads before immunoblotting for β-catenin. Whole cell lysates were also immunoblotted for actin.

To determine whether ERK was involved in Wnt 3a-mediated Tcf/Lef signaling, we again employed the MEK inhibitors PD98059 and U1026. Similar to EGF-mediated signaling, Wnt 3a-induced Tcf/Lef reporter activity was completely ablated by both MEK inhibitors (Figure III-4E). Additionally, PD 98059 substantially reduced TOPFLASH induction by co-stimulation with EGF and Wnt 3a (Figure III-4F), demonstrating that EGF- and Wnt-3a-mediated Tcf/Lef transcription, which proceed via distinct mechanisms, have a common requirement for ERK signaling.

We next examined whether ERK signaling is involved in Wnt-mediated stabilization of β-catenin. Although the vehicle control did not affect stabilization of free β-catenin by Wnt 3a, PD 98059 treatment of 293T-EGFR diminished β-catenin stabilization (Figure III-4G), corroborating observations that ERK signaling is partially required for Wnt-mediated stabilization of cytoplasmic β-catenin (Almeida et al., 2005).

2.5. PKC, but not PKA, is required for EGF- and Wnt 3a-mediated Tcf/Lef transcription.

To further parse the divergent mechanisms by which EGF and Wnt 3a activate Tcf/Lef transcription, we examined the role of protein kinase C (PKC), which can mediate Tcf/Lef transcription in several contexts (Chen et al., 2000; Yang et al., 2006). We first probed EGF-mediated TOPFLASH activity with calphostin C, a pharmacological inhibitor of all PKC isoforms. In 293T-EGFR cells, low doses of calphostin C (0.1 and 0.5 μM) did not block EGF-mediated TOPFLASH activity (Figure III-S5). At a dose of 1 μM, however, calphostin C reduced EGF-mediated Tcf/Lef transcriptional activity by ~60 % (Figure III-5A).

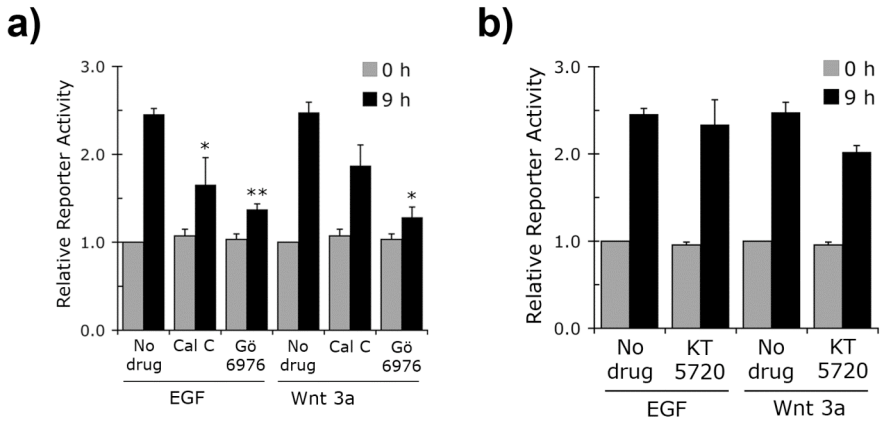

Figure III-5. PKC, but not PKA, is required for EGF- and Wnt 3a-mediated Tcf/Lef transcriptional activity.
a) After pre-treatment with either calphostin C (1 μM) or Gö 6976 (0.5 μM), 293T-EGFR were stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml), and the TOPFLASH reporter activity was quantified. The *asterisks* and the *double asterisk* denote $P < 0.05$ and $P < 0.01$, respectively, comparing the reporter activity at 9 h in the absence and presence of the indicated pharmacological inhibitor.
b) 293T-EGFR were pre-treated with KT 5720 (1 μM), stimulated with either EGF or Wnt 3a, and the TOPFLASH reporter activity was quantified. The observed differences in reporter activity were not statistically significant ($P = 0.63$ and 0.12 for EGF and Wnt 3a, respectively).

Since calphostin C only partially blocked EGF-mediated TOPFLASH reporter activity, we sought to confirm the effect of PKCs in the induction of Tcf/Lef transcription by EGF. Using a second pharmacological inhibitor, Gö 6976, which selectively inhibits the α/β1 isoforms of PKC, confirmed that PKC is functionally required for EGF-mediated Tcf/Lef signaling (Figure III-5A). Some reports have suggested that PKC lies upstream of ERK activation (Schonwasser et al., 1998); however, treatment with Gö 6976 did not reduce ERK phosphorylation in response to EGF (Figure III-S6). Thus, PKC and ERK are distinct, parallel requirements for EGF-mediated Tcf/Lef transcription.

Similar to EGF, Wnt-3a-mediated induction of Tcf/Lef transcriptional activity was also blocked by the PKC inhibitors calphostin C and Gö 6976 (Figure III-5A), confirming that activation of the TOPFLASH reporter by canonical Wnts is PKC-

dependent (Chen et al., 2000). Thus, similar to ERK, PKC α/β1 is not a unique requirement of EGF-mediated Tcf/Lef signaling. However, in contrast to ERK, the PKC α/β1 inhibitor Gö 6976 did not affect Wnt 3a-mediated stabilization of β-catenin (Figure III-S7), indicating that PKC is essential for Wnt signaling at a step downstream of β-catenin stabilization.

Because several agonists of protein kinase A (PKA) reportedly induce Tcf/Lef transcriptional activity (Hino et al., 2005; Taurin et al., 2006), we next tested the role of PKA in EGF- and Wnt 3a-mediated Tcf/Lef transcription. Inhibiting PKA with the drug KT 5720, which does not affect PKC, did not affect TOPFLASH reporter activity in response to stimulation with EGF or Wnt 3a (Figure III-5B).

2.6. Src family kinase activity is required for EGF-, but not Wnt 3a-, mediated Tcf/Lef transcription.

The Src family of kinases have also been implicated in Tcf/Lef transcription (Coluccia et al., 2006; Haraguchi et al., 2004; Ress and Moelling, 2006). To parse the role of Src in EGF- and Wnt-3a-mediated β-catenin signaling, we used the drug PP2, which inhibits all Src family members. Treating 293T-EGFR with PP2 blocked EGF-mediated induction of TOPFLASH by ~ 70 % (Figure III-6A), suggesting that Src kinase activity is required for induction of EGF-mediated Tcf/Lef transcriptional activity. Because Src may lie upstream of ERK activation (Daulhac et al., 1999), we tested whether the PP2 drug affected EGF-mediated phosphorylation of ERK. At 10 min after EGF stimulation, levels of dually-phosphorylated ERK were not changed in PP2-treated

cells; however, at 30 min, ERK phosphorylation was slightly reduced (Figure III-S6). Thus, Src family kinases may partially mediate ERK signaling, as opposed to being fully distinct, parallel requirements.

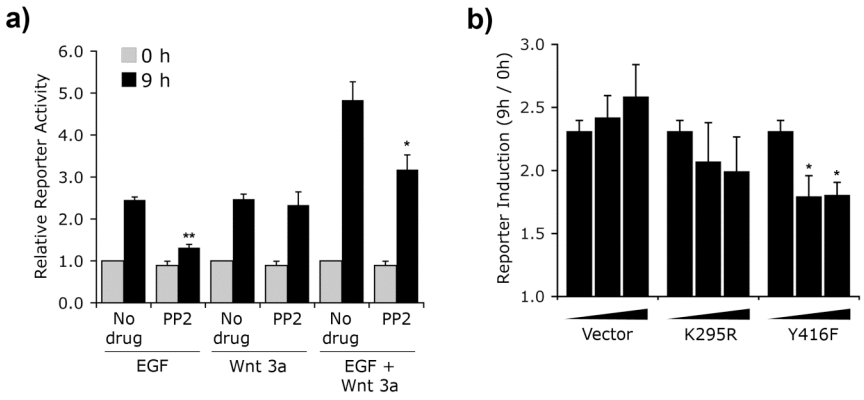

Figure III-6. EGF, but not Wnt 3a, requires Src family kinase activity to activate Tcf/Lef transcriptional activity.
a) Serum-starved 293T-EGFR were pre-treated with PP2 (10 μM), stimulated with EGF (40 ng/ml), Wnt 3a (50 ng/ml), or EGF plus Wnt 3a, and the TOPFLASH reporter activity was quantified. The *asterisk* and *double asterisk* denote $P < 0.05$ and $P < 0.001$, respectively, comparing reporter activity in the absence and presence of PP2.
b) 293T-EGFR were transfected with increasing amounts (0, 5, 10 μg) of the indicated dominant-negative Src mutants or the corresponding empty vector. After serum starvation, cells were treated with EGF, and the extent of TOPFLASH reporter induction (9h / 0h) is reported. The *asterisks* denote $P < 0.05$ comparing reporter induction of empty vector-transfected cells to Src-Y416F-transfected cells.

In contrast to EGF, Wnt-3a-mediated TOPFLASH reporter activity was unaffected by Src inhibition (Figure III-6A). Consistent with its inability to block Wnt-3a-mediated Tcf/Lef signaling, the Src inhibitor PP2 did not block stabilization of free β-catenin by Wnt 3a (Figure III-S7). As such, it appears that the kinase activity of Src family members is required for activation of Tcf/Lef transcription by EGF, but not Wnt 3a. In support of this conclusion, 293T-EGFR co-stimulated with EGF and Wnt 3a in the presence of PP2 activate the TOPFLASH reporter to the same magnitude as Wnt 3a alone (Figure III-6A), demonstrating that Src inhibitors can selectively ablate the EGF-mediated contribution to Tcf/Lef transcription in co-stimulated cells.

To verify the requirement of the Src family kinases in EGF-mediated Tcf/Lef signaling, we tested two dominant-negative mutants of Src, one deficient in ATP binding (K297R) (Kamps and Sefton, 1986) and one lacking a key phosphorylation residue (Y416F) (Piwnica-Worms et al., 1987). Compared to the empty vector, both inactive Src mutants reduced EGF-mediated TOPFLASH reporter activity (Figure III-6B), corroborating results from pharmacological inhibition of Src.

3. Discussion

A central question in biology is how cells make appropriate response decisions in the presence of numerous environmental cues. Part of the answer lies in uncovering the topology of the molecular signaling networks that couple different environmental signals. In this study, we investigated crosstalk between two extracellular factors – EGF and Wnt – that play prominent roles in both normal and pathological physiology. We demonstrate that EGF and Wnt 3a utilize distinct but partially overlapping pathways that converge on Tcf/Lef transcriptional activity (Figure III-7). Because Tcf/Lef signaling regulates the expression of several prominent gene switches, including the oncogenes *cyclin d1* and *c-myc*, the intricate connectivity between EGF and Wnt signaling pathways raises the intriguing possibility that crosstalk between these two factors may synergistically regulate development and tumorigenesis.

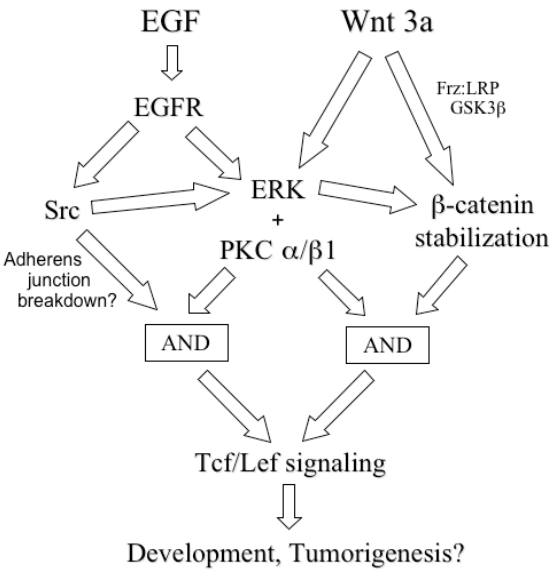

Figure III-7. EGF and Wnt 3a activate Tcf/Lef signaling via a distinct but partially overlapping network.
Wnt 3a signals via the canonical Wnt mechanism involving stabilization of cytoplasmic β-catenin, presumably through the Frz:LRP receptor complex and inhibition of GSK3β. In contrast, EGF induces Tcf/Lef signaling without affecting β-catenin stability. Both ligands require activation of ERK and PKC, although only ERK is involved in β-catenin stabilization. In addition to ERK and PKC, EGF also requires Src kinase activity, presumably because Src promotes adherens junction dissociation and β-catenin nuclear activity through tyrosine phosphorylation. This strongly-coupled signaling network indicates that crosstalk between EGF and Wnt may regulate Tcf/Lef-dependent phenomena such as development and oncogenesis.

Our data show that EGF and Wnt 3a stimulate Tcf/Lef transcriptional activity by regulating β-catenin in distinct ways. While Wnt 3a functions through the canonical mechanism involving β-catenin stabilization, EGF targets an siRNA-resistant, more stable sub-pool of β-catenin. The net quantitative effect is that EGF and Wnt 3a co-stimulate Tcf/Lef-mediated transcription in an additive manner. This additive convergence on Tcf/Lef transcriptional activity masks an intricate signaling network in which EGF and Wnt 3a utilize both distinct (Src) and common (Erk and PKC) signaling molecules to regulate Tcf/Lef-mediated transcription (Figure III-7).

To our knowledge, this is the first report to demonstrate the coordinate effect of EGF and Wnt 3a on Tcf/Lef-mediated transcription and to elucidate the signaling network underlying this effect. Previous reports have suggested that specific signaling pathways downstream of EGF and Wnt might crosstalk in some cellular contexts. In 293 cells, for example, the GSK3β inhibitor LiCl cooperates with EGF to induce greater Tcf/Lef transcriptional activity than either stimulus alone (Chen et al., 2000). Additionally, co-expression of dominant-negative GSK3β and constitutively-active Ras in hepatocytes synergistically activates the TOPFLASH reporter (Desbois-Mouthon et al., 2001). However, the inhibition of GSK3β and activation of Ras are only partial surrogates for the full signaling capacity of Wnt 3a and EGF ligands, respectively. Moreover, constitutive activation or inhibition of signaling pathways is clearly different from the induction of these intracellular signals by soluble ligands. Thus, it was unclear whether EGF and Wnt 3a could co-regulate Tcf/Lef transcriptional activity, and if so, through what signaling network. Our work addresses this question and reveals a model (Figure III-7) where several EGF- and Wnt 3a-mediated signaling pathways coordinate to stimulate Tcf/Lef transcriptional activity.

3.1. Physiological implications of EGF and Wnt co-regulation of Tcf/Lef transcription

Because co-stimulation with EGF and Wnt 3a provokes quantitatively greater Tcf/Lef signaling than either ligand alone (Figure III-2C), embryonic and adult developmental processes that are concomitantly regulated by these two factors may be shaped by their crosstalk. In the adult mammary gland, multiple developmental stages require signals from both EGF and Wnt ligands, including ductal elongation during

adolescence and alveolar morphogenesis during pregnancy (Brennan and Brown, 2004; Troyer and Lee, 2001). Even in simpler organisms such as *C. elegans*, EGF and Wnt receptors (LET-23 and LIN-17/LIN-18, respectively) operate concomitantly to guide vulval development in the adult hermaphrodite (Inoue et al., 2004; Sternberg and Horvitz, 1989). Our findings raise the intriguing hypothesis that developmental fates may be shaped by cooperative regulation of Tcf/Lef by EGF and Wnt in these contexts.

Crosstalk between EGF- and Wnt-mediated Tcf/Lef signaling may also play a role in the development of human breast cancers. Mutations that deactivate the cytoplasmic degradation machinery of β-catenin, causing abnormal stabilization and nuclear localization of β-catenin, are pervasive in many human cancers, especially those of the colon (Polakis, 2000). As in other cancer types, upregulation of β-catenin signaling is prominent in human breast cancers. One study found that 60% of patient samples tested positive for cytoplasmic and nuclear β-catenin staining (Lin et al., 2000). Furthermore, suggestive of its biological significance, positive staining for β-catenin correlated with cyclin D1 upregulation and poor prognosis.

While there is strong evidence of a role for hyperactivated β-catenin signaling in human breast cancer, the underlying molecular causes are less clear. Mutations that stabilize β-catenin have been found in numerous cancer types, but not in human breast cancer (Hatsell et al., 2003). Autocrine production of Wnt ligands may play a role in breast cancer, since mammary-tissue-specific overexpression of Wnt-1 induces adenocarcinomas in mouse models (Tsukamoto et al., 1988) and some human breast

cancers exhibit autocrine Wnt signaling (Bafico et al., 2004). Our results raise the possibility that hyperactive EGF signaling may independently, or in collaboration with autocrine Wnt production, stimulate Tcf/Lef signaling during breast cancer development. In support of a role for EGF/Wnt synergism in breast cancer development, the EGFR ligand TGFα has been reported to cooperate with Wnt 1 and Wnt 3 to initiate neoplastic progression in murine mammary glands, although the mechanism is not known (Schroeder et al., 2000). Furthermore, there is evidence for interactions between ErbB2/Neu and β-catenin in metastatic human breast cancer (Schroeder et al., 2002).

Uncovering the precise role of EGF in regulating β-catenin signaling during human breast cancer development will be an important future direction. In the meantime, our results suggest that specific therapeutic strategies may provide selective control over EGF- versus Wnt-mediated Tcf/Lef transcription. RNAi-based therapeutic strategies may prove ineffectual in antagonizing Tcf/Lef signaling arising from hyperactive EGF signaling, since EGF utilizes a highly stable subpool of β-catenin. Instead, siRNA targeting the Src family of kinases should selectively inhibit EGF-mediated Tcf/Lef transcriptional activity.

3.2. Mechanisms underlying EGF/Wnt crosstalk in regulating Tcf/Lef transcriptional activity

Although Wnt ligands are the classical agonists of β-catenin:Tcf/Lef transcriptional activity, it is becoming clear that ligands which activate receptor tyrosine kinases (RTKs) also induce Tcf/Lef transcriptional activity. RTK-activating soluble

factors that have been linked to activation of Tcf/Lef transcriptional activity include EGF (Graham and Asthagiri, 2004; Lu et al., 2003; Muller et al., 2002), hepatocyte growth factor (Monga et al., 2002), insulin-like growth factors (Desbois-Mouthon et al., 2001), fibroblast growth factor 2 (Holnthoner et al., 2002), platelet-derived growth factor (Yang et al., 2006), and the Gas6 growth factor (Goruppi et al., 2001). Most of these non-Wnt ligands have been suggested to activate Tcf/Lef transcription via Wnt-like mechanisms that stabilize cytoplasmic β-catenin, often through the inhibition of GSK3β.

In contrast, our results demonstrate that EGF-mediated Tcf/Lef transcriptional activity proceeds via a non-canonical mechanism and does not involve an increase in the level of free β-catenin (Figure III-3A). In fact, we observed that a β-catenin-targeting siRNA completely downregulates free β-catenin, yet has no effect on EGF-mediated Tcf/Lef transcription. Meanwhile, siRNA treatment only partially downregulated total cellular β-catenin, suggesting that EGF activates Tcf/Lef transcription using a highly-stable, siRNA-resistant sub-cellular pool of β-catenin, such as the fraction bound to E-cadherin (Papkoff, 1997). Indeed, in 293T-EGFR cells, depletion of glycoproteins from whole cell lysates demonstrated that the residual pool of β-catenin in siRNA-treated cells includes E-cadherin-bound β-catenin (Figure III-3D). As such, EGF might induce Tcf/Lef transcriptional activity by shifting this adhesive fraction of β-catenin into a transcriptionally-competent state, without substantially increasing the cytoplasmic pool of β-catenin. Such a mechanism would be consistent with the current paradigm that β-catenin may be primed to selectively function in either adhesion or transcription (Gottardi and Gumbiner, 2004).

In fact, in cancer cells that overexpress EGFR and undergo epithelial-to-mesenchymal transition upon EGF treatment, a stabilization-independent mechanism of β-catenin transcriptional activity has been proposed (Lu et al., 2003). In these cell types, EGF treatment induces caveolin-mediated endocytosis of E-cadherin, causing breakdown of adherens junctions and releasing β-catenin for transcriptional activation. This mechanism would also be consistent with our observation that Src is required for EGF-mediated Tcf/Lef transcriptional activity, since Src-mediated tyrosine phosphorylation has been shown to disrupt adherens junctions both *in vitro* (Piedra et al., 2003; Roura et al., 1999) and *in vivo* (Behrens et al., 1993; Owens et al., 2000). Notably, if Src does disrupt adherens junctions in 293T-EGFR cells, it does not concomitantly induce significant accumulation of cytoplasmic β-catenin (Figure III-3A).

In addition to regulating the adhesive properties of β-catenin, tyrosine phosphorylation by Src kinases may also promote β-catenin transcriptional activity by modifying the affinity of β-catenin for nuclear binding partners such as BCL9-2 (Brembeck et al., 2004). Src-mediated phosphorylation of intracellular proteins other than β-catenin may also facilitate Tcf/Lef transcriptional activity, since phosphorylation of the BCR kinase by Src induces dissociation of BCR from Tcf1, thereby promoting Tcf/Lef transcription (Ress and Moelling, 2006). As such, Src may play multiple roles in priming β-catenin for Tcf/Lef transcription in EGF-treated cells, including dissociation from adherens junctions, nuclear localization, and association with Tcf/Lef.

In addition to Src, our results suggest EGF-mediated Tcf/Lef transcriptional activity is completely ERK-dependent (Figure III-4). Recently, EGFR has been shown to relieve Groucho-mediated repression of the Notch signaling pathway via ERK (Hasson et al., 2005). Since the Groucho family of repressors also regulate β-catenin-mediated transcription (Brantjes et al., 2001), it raises the intriguing possibility that de-repression of Groucho via EGFR signaling is involved in EGF-mediated Tcf/Lef signaling. Precisely how ERK- and Src-mediated control of Groucho and tyrosine phosphorylation, respectively, contribute to EGF-mediated Tcf/Lef transcriptional activity is currently under investigation.

Notably, in some cell systems, EGF treatment is not sufficient to induce Tcf/Lef transcriptional activity (Mizushima et al., 2002). For example, A431 cancer cells, which overexpress EGFR, do not exhibit TOPFLASH reporter induction upon stimulation with EGF (Yan et al., 2006). However, in this same system, Tcf/Lef transcriptional activity does become responsive to EGF when levels of the phosphatase PCP-2 are downregulated by siRNA. Thus, the induction of Tcf/Lef transcription by EGF, which has been reported in a variety of cell types (Graham and Asthagiri, 2004; Lu et al., 2003; Muller et al., 2002), may critically depend on the balance between phosphatase and kinase activity.

Although the mechanisms controlling Tcf/Lef transcription by EGF are still under investigation, canonical Wnt signaling has been studied in great detail. While the key event in Wnt signaling is stabilization of cytoplasmic β-catenin, our results also

demonstrate a requirement for other intracellular pathways including ERK and PKC for full activation of Tcf/Lef transcription by Wnt 3a. This observation is underscored by the fact that PKC inhibitors block Wnt-mediated Tcf/Lef transcription even without affecting stabilization of β-catenin (Figure III-S7). Precisely how PKC is involved Wnt signaling is unknown at this time.

In contrast to PKC, ERK is required for both Wnt-mediated β-catenin stabilization and Tcf/Lef transcriptional activity. Previous reports have implicated ERK in the phosphorylation and inactivation of GSK3β (Almeida et al., 2005; Ding et al., 2005); however, since we did not observe GSK3β phosphorylation in response to Wnt 3a (Figure III-S2), this mechanism does not appear to be relevant in 293T-EGFR. Interestingly, siRNA targeting β-catenin preferentially ablated the free pool of β-catenin utilized in Wnt 3a-mediated Tcf/Lef transcription (Figure III-3D). This result suggests that the transcriptionally-active pool of β-catenin in Wnt-3a-treated cells may consist primarily of newly-synthesized β-catenin. In fact, since ERK has been reported to control β-catenin levels by regulating the activity of eukaryotic translation iniation factor 4E (Karni et al., 2005), Wnt 3a may cause accumulation of β-catenin via ERK-dependent regulation of β-catenin synthesis.

In summary, our results reveal an intricate coupling between EGF and Wnt 3a in activation of Tcf/Lef transcriptional activity. Parsing the topology of the signaling networks that couple these prominent extracellular factors reveals that EGF and Wnt stimulate distinct but partially overlapping pathways which converge on Tcf/Lef

transcription. Because the transcriptional targets of Tcf/Lef include several prominent gene switches, including the oncogenes *cyclin d1* and *c-myc*, the connectivity between EGF and Wnt signaling pathways may synergistically regulate development and tumorigenesis. Probing the specific mechanistic roles of Src, ERK, and PKC in this signaling network will provide valuable insight into the role of EGF/Wnt crosstalk in development and cancer, as well as how cells mediate appropriate responses in the presence of numerous environmental cues.

4. Experimental Procedures

4.1. Antibodies and Reagents

Recombinant mouse Wnt 3a and Wnt 5a were purchased from R&D Systems. Recombinant human EGF was obtained from Peprotech. The pharmacological inhibitors AG 1478, calphostin C, Gö 6976, KT 5720, PD 98059, PP2, and U1026 were purchased from Calbiochem and reconstituted in DMSO. The following antibodies were used in this study: anti-actin (Santa Cruz Biotechnology), monoclonal anti-β-catenin (BD Transduction Laboratories), anti-GSK3β (BD Transduction Laboratories), anti-phospho-Ser9-GSK3β (Biosource), monoclonal anti-E-cadherin (BD Transduction Laboratories), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology), anti-phosphotyrosine (Santa Cruz Biotechnology).

4.2. Cell Culture

293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine (Invitrogen), 10% (v/v) fetal bovine serum (Invitrogen), and 1%

(v/v) penicillin/streptomycin (Invitrogen). For serum starvation, the cells were washed once in PBS and then cultured in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) penicillin/streptomycin and 0.1% bovine serum albumin (Sigma) for 24 h. For studies involving pharmacological inhibitors, cells were starved for 23 h and then pre-treated with the drug in serum-free medium for 1 h prior to stimulation in the continued presence of the drug.

4.3. Plasmid Constructs

pLPCX, pLPCX-EGFR, pLNCX, pLNCX-Src-K295R, and pLNCX-Src-Y416F were generously provided by J. Brugge (Harvard Medical School). The luciferase-based reporters pTOPFLASH and pFOPFLASH were purchased from Upstate Biotechnology, Inc. pRL-TK was purchased from Promega. pMCL -MKK1-K97M and the parental vector pCEP4L were kind donations of N. Ahn (University of Colorado at Boulder)

4.4. Cell Lysis

Cells were washed once in ice-cold PBS and scraped into cold lysis buffer. After incubation on ice for 15 min, the cell lysates were clarified by centrifugation, and the supernatant was collected as whole cell lysate. Protein concentrations were determined using BCA reagents (Sigma). For immunoblotting, samples were lysed in modified RIPA buffer, as described elsewhere (Graham and Asthagiri, 2004), except for the ConA pre-clearing assays, where cells were lysed in Triton-only lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, β-glycerophosphate (pH 7.3) 10 mM NaPP, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). For luciferase reporter measurements, cell lysis was

performed in 1X passive lysis buffer provided by the manufacturer (Promega).

4.5. ConA fractionation

Separation of the glycoprotein-bound and un-bound fractions of β-catenin was performed as described elsewhere (Fagotto et al., 1999). Briefly, cells were plated at a subconfluent density (10^5 cells/35-mm dish) and then transfected with 1 μg pLPCX or pLPCX-EGFR before serum starvation, stimulation with EGF or Wnt 3a in serum-free medium, and lysis. 50 μg of whole cell lysate were then incubated with 100 μl of ConA-sepharose 4B beads (Sigma) in 500 μl Triton-only lysis buffer for 90 min at 4 ˚C. The beads were then spun down and the supernatant (pre-cleared lysate) was collected for immunoblotting.

4.6. Reporter Assays

293T cells were plated at a subconfluent density (10^5 cells/35-mm dish) and co-transfected with 1 μg of the reporter plasmid (pTOPFLASH or pFOPFLASH), 0.1 μg of pRL-TK, and 1 μg of pLPCX-EGFR or the empty vector pLPCX using FuGENE 6 (Roche Applied Science). 24 h after transfection, cells were serum-starved, stimulated and then lysed at desired times. Reporter activity was measured using the dual luciferase assay (Promega), and luciferase activity was normalized to *Renilla* luciferase activity. To pool data from multiple trials, the luciferase:*Renilla* luciferase ratio from all experiments was normalized to serum-starved cells in the absence of pharmacological agents. Error bars represent the sample standard error of at least three independent experiments, and two-tailed Student's *t*-tests were performed to P values. For studies involving siRNA-mediated knockdown of β-catenin, siRNA were transfected approximately 12 h after transfection of the reporter plasmids and 12 h before serum-starvation.

4.7. Immunoblotting

Whole cell lysates or pre-cleared lysates were resolved by SDS-PAGE on 10% gels and blotted onto nitrocellulose membrane (Bio-Rad). The membranes were blocked overnight and then incubated sequentially with primary and corresponding horseradish peroxidase-conjugated secondary antibody. The blots were treated with SuperSignal West Femto Substrate (Pierce) and imaged on the VersaDoc 3000 (Bio-Rad) using Quantity One software (Bio-Rad). All immunoblots presented are representative of at least two independent experiments.

4.8. siRNA knockdown of β-catenin

siRNA directed against β-catenin, as well as non-specific, negative control siRNA, were purchased from Ambion. siRNAs were transfected in antibiotic-free medium using Lipofectamine RNAiMAX (Invitrogen).

5. Acknowledgements

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6. Supplemental Data

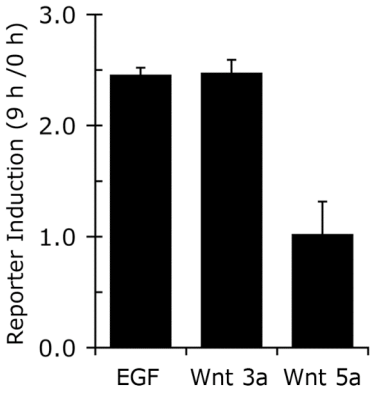

Figure III-S1. Wnt 5a does not activate Tcf/Lef signaling.
Serum-starved 293T-EGFR cells were stimulated with EGF (40 ng/ml), Wnt 3a (50 ng/ml), or Wnt 5a (50 ng/ml). Induction of the Tcf/Lef-dependent reporter TOPFLASH (9h / 0h) was quantified.

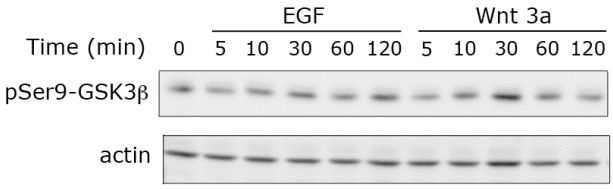

Figure III-S2. Neither Wnt 3a nor EGF induces phosphorylation of GSK3β on serine 9.
Serum-starved 293T-LPCX and 293T-EGFR cells were stimulated with either EGF (40ng/ml) or Wnt 3a (50 ng/ml) and lysed at the indicated times. Whole cell lysates were immunoblotted for phospho-serine 9 GSK3β and actin.

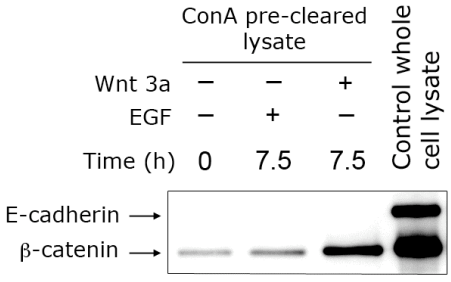

Figure III-S3. ConA pre-clearing of whole cell lysate depletes E-cadherin.
293T-EGFR were serum-starved and then stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml) for the indicated times. Whole cell lysates were pre-cleared with ConA beads, and the pre-cleared lysates were immunoblotted for E-cadherin and β-catenin. Non-pre-cleared whole cell lysate was included as a positive control for E-cadherin immunoblotting.

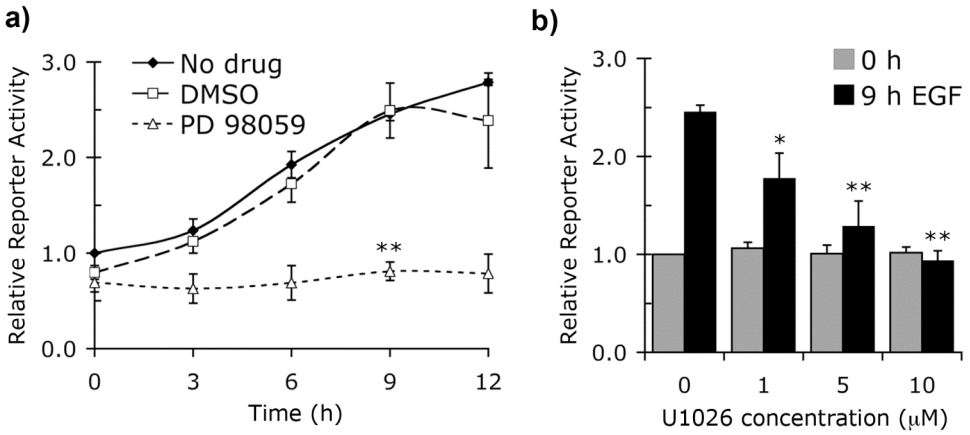

Figure III-S4. ERK signaling is required for EGF-mediated Tcf/Lef signaling
a) Serum-starved 293T-EGFR cells were pre-treated with PD 98059 (50 μM) or DMSO, stimulated with EGF (40 ng/ml), and the TOPFLASH reporter activity was quantified at the indicated times. The *double asterisk* denotes $P < 0.01$ (Student's *t*-test) in comparing EGF-induced TOPFLASH reporter activity in the absence and presence of PD 98059 at 9 h.
b) Serum-starved 293T-EGFR cells were pre-treated with the indicated range of concentrations of U1026, stimulated EGF, and the TOPFLASH reporter activity was quantified at the indicated times. The *asterisk* denotes $P < 0.05$ (Student's *t*-test) and the *double asterisk* denotes $P < 0.01$ (Student's *t*-test) in comparing EGF-induced TOPFLASH reporter activity in the absence and presence of U1026 at 9 h.

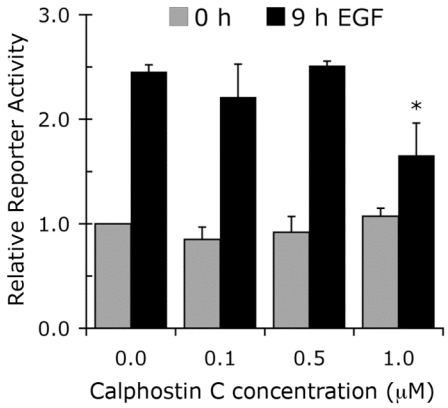

Figure III-S5. The PKC inhibitor calphostin C inhibits EGF-mediated Tcf/Lef signaling only at high concentrations.
Serum-starved 293T-EGFR cells were pre-treated with calphostin C at the indicated concentrations, stimulated with EGF (40 ng/ml), and the TOPFLASH reporter activity was measured at the indicated times. The *asterisk* denotes $P < 0.01$ (Student's *t*-test) in comparing TOPFLASH reporter activity at 9 h in the absence and presence of calphostin C.

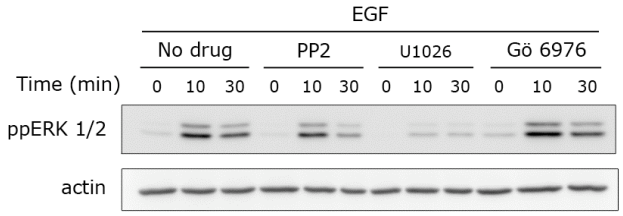

Figure III-S6. PKC does not lie upstream of EGF-mediated ERK activation, but Src possibly does. Serum-starved 293T-EGFR cells were pre-treated with either the solvent DMSO, the Src family kinase inhibitor PP2 (10 μM), the MEK inhibitor U1026 (10 μM), or the PKC α/β1 inhibitor Gö 6976 (0.5 μM), then stimulated with EGF (40 ng/ml) and lysed at the indicated times. Whole cell lysates were immunoblotted for dually-phosphorylated ERK and the equal loading control actin.

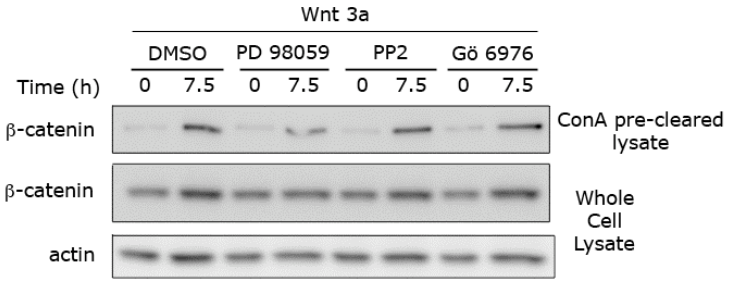

Figure III-S7. PKC and Src are not required for Wnt 3a-mediated stabilization of β-catenin. Serum-starved 293T-EGFR were pre-treated with either the solvent DMSO, the MEK inhibitor PD 98059 (50 μM), the Src family kinase inhibitor PP2 (10 μM), or the PKC α/β1 inhibitor Gö 6976 (0.5 μM), stimulated with Wnt 3a (50 ng/ml) and lysed at the indicated times. Whole cell lysates were pre-cleared with ConA beads, and the pre-cleared lysates were immunoblotted for β-catenin. Whole cell lysates were immunoblotted for total cellular β-catenin and the equal loading control actin.

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Chapter IV. A microtiter assay for quantifying protein-protein interactions associated with cell-cell adhesion

Abstract

Cell-cell adhesions are a hallmark of epithelial tissues, and the disruption of these contacts plays a critical role in both the early and late stages of oncogenesis. The interaction between the transmembrane protein E-cadherin and the intracellular protein β-catenin plays a crucial role in the formation and maintenance of epithelial cell-cell contacts, and is known to be down-regulated in many cancers. We have developed a protein complex enzyme-linked immunosorbent assay (ELISA) that can quantify the amount of β-catenin bound to E-cadherin in unpurified whole cell lysates with a Z' factor of 0.74. The quantitative nature of the E-cadherin:β-catenin ELISA represents a dramatic improvement over the low-throughput assays currently used to characterize endogenous E-cadherin:β-catenin complexes. In addition, the protein-complex ELISA format is compatible with standard sandwich ELISAs for parallel measurements of total levels of endogenous E-cadherin and β-catenin. In two case studies closely related to cancer cell biology, we utilize the protein complex ELISA and traditional sandwich ELISAs to provide a detailed, quantitative picture of the molecular changes occurring within adherens junctions *in vivo*. Because the E-cadherin:β-catenin protein complex plays a crucial role in oncogenesis, this protein complex ELISA may prove to be a valuable quantitative prognostic marker of tumor progression.

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1. Introduction

Cell-cell adhesions are an integral aspect of epithelial tissues. These adhesions provide a physical barrier between two compartments, permitting the epithelial cell layer to serve as a selective transporter. Moreover, the attenuation of cell-cell adhesion plays a critical role in both early and late stages of oncogenesis.[1] At early steps, reduced intercellular adhesion may attenuate contact-inhibition of proliferation, permitting unchecked cell division and tumor formation; at later stages, reduced cell-cell adhesion is often associated with invasion, metastasis, and poor patient prognosis.[2]

In epithelial tissues, adherens junctions play a central role in the establishment and maintenance of cell-cell adhesions.[3] These junctions are composed of the cadherin and catenin families of proteins, which link sites of cell-cell contact to the actin cytoskeleton. Cadherins are transmembrane proteins that bind homotypically to cadherins on neighboring cells; meanwhile, the intracellular tail of cadherins binds β-catenin, which then recruits α-catenin and links to the actin cytoskeleton. In epithelial tissues, E-(epithelial)cadherin is the predominant member of the cadherin family expressed, and the loss of E-cadherin via genetic and epigenetic mechanisms is common in tumor progression.[4]

Given their integral role in forming and maintaining cell-cell adhesion, the interaction of E-cadherin and β-catenin has been widely studied. The most common method for assaying the expression and subcellular localization of endogenous E-cadherin and β-catenin in pathological contexts is histochemistry.[5,6] While informative, this method is

low-throughput and offers only qualitative information. Other more quantitative approaches have been developed to examine the molecular mechanisms governing E-cadherin:β-catenin association, including bead-based co-immunoprecipitation techniques (co-IP),[7-9] semi-quantitative immunofluorescence,[10] recombinant protein pull-down assays,[11,12] and chip-based biosensors.[13] Co-IP assays, in particular, have been widely used to measure the association of endogenous E-cadherin and β-catenin; however, this technique involves cumbersome and repetitive centrifugation and wash steps, as well as low-throughput handling of multiple test tubes. Furthermore, the proteins isolated by co-IP are typically analyzed by Western blotting, which offers a limited linear range of detection. In contrast, other techniques using recombinant proteins provide quantitative measures of E-cadherin:β-catenin interactions over a wide linear range;[12,13] however, these *in vitro* binding assays may not reflect *in vivo* biology.

We have developed a protein complex enzyme-linked immunosorbent assay (ELISA) that addresses many of the limitations in current methods for quantifying the amount of endogenous E-cadherin:β-catenin complexes. The microtiter format is also highly compatible with the standard sandwich ELISA for parallel measurements of total protein levels. In two case studies closely related to cancer cell biology, we demonstrate that the protein complex ELISA, in conjunction with parallel measurements of total protein, offers a detailed, quantitative picture of the molecular changes occurring within adherens junctions *in vivo*.

2. Materials and Methods

2.1. Cell Culture

MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), 0.5 μg/ml hydrocortisone (Sigma, Saint Louis, MO), 0.1 μg/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and 1% (v/v) penicillin/streptomycin (Invitrogen). SW480 and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine (Invitrogen), 10% (v/v) fetal bovine serum (Invitrogen), and 1% (v/v) penicillin/streptomycin (Invitrogen).

2.2. Cell Lysis

Cells were washed twice in ice-cold PBS and scraped into cold modified RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 0.25% (v/v) sodium deoxycholate, 50 mM β-glycerophosphate (pH 7.3), 10 mM NaPP, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 15 min, the cell lysates were clarified by centrifugation, and the supernatant was collected as whole cell lysate. The protein concentrations were determined using BCA reagents (Sigma, Saint Louis, MO).

2.3. Protein complex and standard ELISAs

Mouse monoclonal capture antibodies against either E-cadherin or β-catenin (BD Transduction Laboratories, San Jose, CA) were diluted to 2.5 μg/ml in Tris-buffered saline (10 mM Tris (pH 8.0), 150 mM NaCl) containing 0.02% (v/v) sodium azide. 100 μl of the diluted capture antibody was then added to a flat-bottom, high-protein-binding 96-well microtiter plate (Corning, Corning, NY) and incubated overnight at room temperature. The next day, the wells were blocked with 150 μl of blocking buffer (10% (v/v) horse serum (Invitrogen, Carlsbad, CA) in TBST (TBS plus 0.05% (v/v) Tween-20)) for 2 h at room temperature. After washing the wells three times with TBST, whole cell lysate was diluted in modified RIPA buffer to a final volume of 100 μl and added to each well for 2 h at 37 ºC. The wells were then washed three times with TBST, and 100 μl of either anti-E-cadherin or anti-β-catenin rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1 μg/ml in blocking buffer was added to each well and incubated for 1 h at 37 ºC. The wells were washed three times with TBST, and the alkaline phosphatase-conjugated, anti-rabbit IgG secondary antibody (Biosource, Camarillo, CA) was diluted to 1 μg/ml in blocking buffer, and 100 μl was added to each well for 1 h at 37 ºC. For detection, the wells were washed three times with TBST and once with TBS, and then 100 μl of 1 mg/ml *p*-nitrophenyl phosphate (Sigma, Saint Louis, MO) dissolved in reaction buffer (1 M diethanolamine, 0.05 mM $MgCl_2$, pH 9.5) was added to each well. The absorbance at 405 nm was monitored by kinetic read at 37 ºC at 17 second intervals over a period of 22 minutes using a microplate reader (Molecular Devices, Sunnyvale, CA). The rate of change in A405 over time was taken as the assay signal.

For the standard sandwich ELISA, both the capture and the detection antibody targeted the same protein (e.g., monoclonal and polyclonal anti-E-cadherin); for the protein complex ELISAs, the capture and detection antibodies targeted different halves of the E-cadherin:β-catenin protein complex (e.g., monoclonal anti-E-cadherin followed by polyclonal anti-β-catenin).

2.4. Validation of protein capture by Western blotting

Varying amounts of whole cell lysate were incubated in the wells of a microtiter plate that had been coated with either monoclonal anti-E-cadherin or anti-β-catenin antibody and blocked with blocking buffer. After incubation of the whole cell lysate, the plate was washed three times with TBST, and then 100 μl of 1X SDS sample buffer (2% SDS, 0.1 M dithiothreitol, 60 mM Tris (pH 6.8), and 5% (v/v) glycerol diluted in modified RIPA buffer) was added to the wells. The microtiter plate was then incubated for 5 min at 100 ^{o}C, and the contents of the well were collected, resolved by SDS-PAGE on 10% gels, and blotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked overnight and then incubated sequentially with primary monoclonal and corresponding horseradish peroxidase-conjugated secondary antibody. The blots were treated with SuperSignal West Femto Substrate (Pierce, Rockford, IL) and imaged on VersaDoc 3000 (Bio-Rad) using Quantity One software (Bio-Rad).

2.5. Plasmid Constructs

pLNCX-Src-Y527F was generously provided by J. Brugge (Harvard Medical School). VSV-G and gag-pol vectors were gifts from D. Schaffer (University of

California, Berkeley).

2.6. Retroviral Infection

Retrovirus was produced by triple transfection of 293T cells with 5 μg each of VSV-G, gag-pol and the retroviral vectors pLNCX or pLNCX-Src-Y527F using LipofectAMINE (Invitrogen, Carlsbad, CA) as previously described.[14] For infection, MCF-10A cells were incubated with retrovirus-containing growth medium and 8 μg/ml polybrene (Sigma, Saint Louis, MO) for 24 h; after infection, cells were selected with 200 μg/ml Geneticin (Invitrogen) for 14 days.

2.7. Data Analysis and Statistical Calculations

The Signal/Noise ratio (S/N) was calculated as:

$$\text{S/N} = \frac{(\mu_s - \mu_b)}{\sigma_s} \qquad \text{(Eq. IV-1)}$$

where μ_s and μ_b represent the means of the signal and the background, respectively, and σ_s denotes the standard deviation of the assay signal. Z' factors were calculated according to the equation:

$$Z' = 1 - \frac{3 \times (\sigma_s + \sigma_b)}{|\mu_s - \mu_b|} \qquad \text{(Eq. IV-II)}$$

where μ_s, μ_b, and σ_s are as defined above and σ_b is the standard deviation of the blank.[15] The Signal/Noise ratio and the Z' factor were calculated at 200 μg and 130 μg of whole cell lysate for the protein complex ELISAs and the sandwich ELISAs, respectively.

For the studies comparing the expression of E-cadherin, β-catenin, and E-cadherin:β-catenin complexes between cell types (Fig. IV-5) or between cells infected with a dominant-active oncogene or an empty vector control (Fig. IV-6), linear regressions were performed with total cell protein as the dependent variable and the assay signal (rate of change in optical density at 405 nm over time) as the independent variable, as shown in Fig. IV-S1 and IV-S2. The statistical significance of the observed difference in slopes was calculated by analysis of covariance (ANCOVA).

3. Results and Discussion

3.1. Development and validation of a quantitative microtiter ELISA for E-cadherin: β-catenin protein complexes

Changes in E-cadherin:β-catenin interactions modulate cell-cell interactions and contribute to cell invasiveness and epithelial-mesenchymal transition.[1] To better quantify the level of E-cadherin:β-catenin association, we sought to develop a microtiter immunoassay built on the format of the traditional sandwich ELISA. In contrast to the sandwich ELISA, which measures the levels of a single protein, the protein complex ELISA quantifies the level of an endogenous protein complex, as previously described for several other protein complexes.[16-18] The assay utilizes an antibody to capture an antigen from unpurified whole cell lysate, similar to a sandwich ELISA; however, rather than employ a detection antibody against the captured antigen, as in a sandwich ELISA, the protein complex ELISA utilizes a detection antibody targeting a purported binding partner of the captured antigen. For example, to measure the E-cadherin:β-catenin

protein complex, one might use an anti-E-cadherin antibody for the initial capture step, followed by an anti-β-catenin antibody for detection of β-catenin associated with the captured E-cadherin. Thus, although both free and β-catenin-bound E-cadherin would be captured in the initial step, the protein complex ELISA is designed to selectively detect β-catenin in complex with E-cadherin. The amount of detection antibody can then be measured using an alkaline phosphatase-conjugated secondary antibody and the colorimetric substrate *p*-nitrophenyl phosphate (PNPP), where the rate of change in optical density at 405 nm provides a quantitative measure of detection antibody present.

The protein complex ELISA hinges on the possibility that capturing an antigen from crude cell lysates will co-precipitate associated proteins. This co-precipitation is exploited in IP-based detection of protein-protein complexes, a technique that involves the low-throughput, cumbersome use of test tubes in repeated centrifugation and wash steps. To verify that co-precipitation would occur in a microtiter format, we used a monoclonal anti-E-cadherin antibody to capture E-cadherin from whole cell lysate and subsequently treated the wells with SDS sample buffer to extract all proteins. For this initial test, we used lysates from an immortalized mammary epithelial cell line (MCF-10A) that forms adherens junctions rich in E-cadherin:β-catenin complexes.

Analyzing protein extracts from microtiter wells by SDS-PAGE and Western blot demonstrated that the anti-E-cadherin monoclonal antibody efficiently captured E-cadherin from whole cell lysates (Fig. IV-1A). Importantly, β-catenin co-precipitated with E-cadherin; in fact, the amount of β-catenin increased with the amount of whole cell

lysate used in the assay. Furthermore, the "reverse" co-capture also worked in the microtiter format (Fig. IV-1B). Using a monoclonal anti-β-catenin antibody as the capture antibody allowed efficient capture of β-catenin and associated E-cadherin. Again, the amount of captured β-catenin and co-captured E-cadherin increased with the amount of whole cell lysate used in the assay, suggesting that co-capture may have a broad dynamic range.

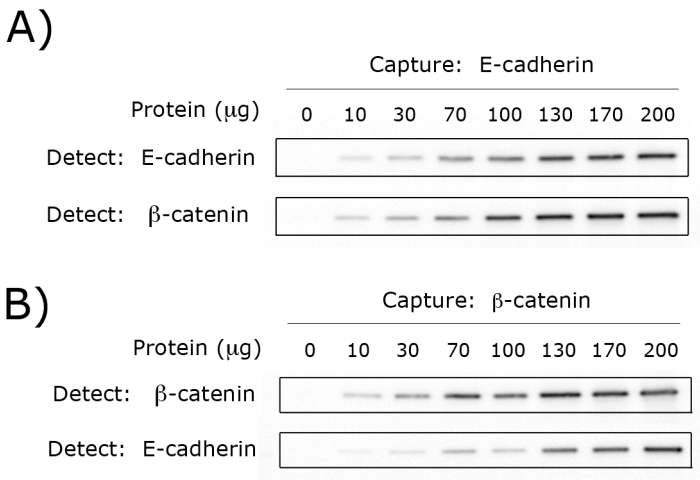

Fig. IV-1. Antigen capture and protein:protein co-capture
Mouse monoclonal antibodies against either (A) E-cadherin or (B) β-catenin were adsorbed to a microtiter plate, and varying amounts of whole cell lysates from MCF-10A cells were incubated in the wells. Protein fractions isolated by the capture antibodies were collected and analyzed by Western blot for the presence of E-cadherin or β-catenin.

While the co-captured protein is detectable by Western blot after stripping the wells, it is not clear whether the amount of co-captured protein is sufficient to detect using the microtiter immunoassay protocol. To address this question, we applied the protein complex ELISA to detect the amount of β-catenin that is bound to E-cadherin. We used a monoclonal anti-E-cadherin antibody to capture E-cadherin from cell lysate and then detected co-captured β-catenin using a polyclonal anti-β-catenin antibody. The protein

complex ELISA exhibits a linear response over the entire range of whole cell lysate used in this study (0 - 200 μg) (Fig. IV-2A). Furthermore, the amount of β-catenin co-captured with E-cadherin was detectable in as little as 10 μg of whole cell lysate, and the signal/noise ratio was approximately 8 (Table IV-1). Indicative of the protein complex ELISA's robustness, the statistical parameter used for evaluation of high-throughput screens (Z') for this E-cadherin:β-catenin ELISA was 0.74.

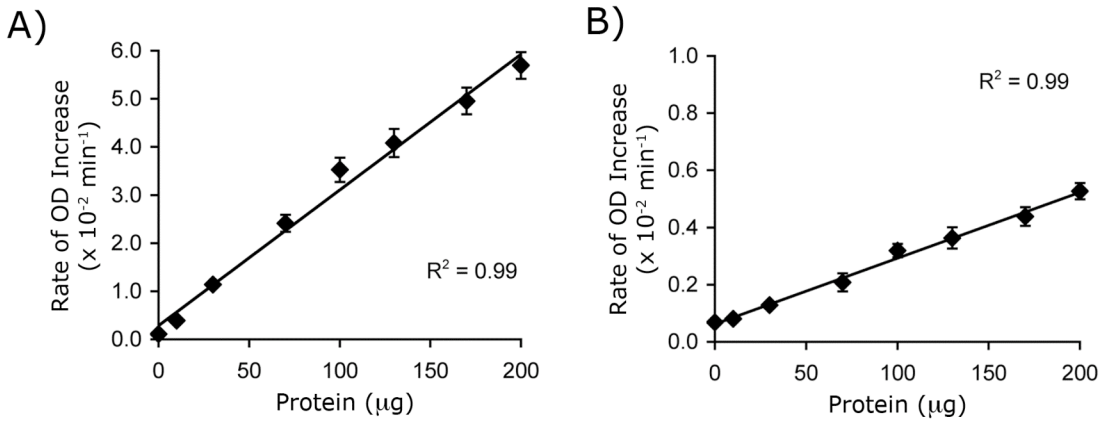

Fig. IV-2. Detection of E-cadherin:β-catenin protein complexes by protein complex ELISA
Whole cell lysate from MCF-10A cells was analyzed by protein complex ELISA for E-cadherin:β-catenin complexes by either (A) E-cadherin capture or (B) β-catenin capture. The rate of change in the optical density over time was plotted versus total cell lysate, and a linear regression was performed. Error bars represent the sample standard error (n = 3).

We also tested the reverse protein complex ELISA by capturing β-catenin with the mouse monoclonal antibody and then detecting E-cadherin with a polyclonal anti-E-cadherin antibody. The β-catenin:E-cadherin ELISA also demonstrated a broad linear dynamic range over 0 - 200 μg of whole cell lysate (Fig. IV-2B). However, the β-catenin:E-cadherin format was slightly less sensitive than the E-cadherin:β-catenin

ELISA, as the signal/noise ratio of this assay was only 6.2 (Table IV-1). However, this assay still demonstrates good suitability to screening assays, with a Z' factor of 0.59.

The relatively poorer performance of the β-catenin:E-cadherin ELISA is consistent with the full profile of *in vivo* protein:protein interactions in which β-catenin and E-cadherin participate. Whereas the β-catenin-binding domain of E-cadherin also recruits plakoglobin, a close homolog of β-catenin,[3] the Armadillo repeat domains of β-catenin bind numerous proteins including α-catenin, Tcf/Lef and components of the Axin-APC degradation machinery.[19] Thus, in principle, for a fixed E-cadherin:β-catenin binding affinity in a particular cell lysate, captured E-cadherin should generate more co-captured β-catenin signal than the co-capture of E-cadherin by β-catenin. However, while this physiological explanation could explain the better performance of the E-cadherin:β-catenin ELISA, we cannot rule out assay-related issues, such as relatively poorer efficacy of the monoclonal capture antibody for β-catenin versus E-cadherin.

Table IV-1. Quantitative performance of protein complex and sandwich ELISAs

Assay type	Captured protein	Detected protein	Signal/Noise ratio [a]	Z′ factor [a]
Protein complex ELISAs	E-cadherin	β-catenin	7.7	0.74
	β-catenin	E-cadherin	6.2	0.59
Sandwich ELISAs	E-cadherin	E-cadherin	48	0.80
	β-catenin	β-catenin	3.6	0.19

[a]Calculated as described in Materials and Methods.

The results reported for the protein complex ELISAs were collected using optimal assay conditions; all other formats and conditions tested were found to be sub-optimal in terms of signal strength and signal/noise ratio. For example, we attempted to increase the

amount of properly-oriented capture antibody coated on the well by first adsorbing neutravidin, followed by incubation with a biotinylated secondary antibody that would bind the Fc domain of the capture antibody. Although this approach has been reported to increase antigen capture elsewhere,[20] the neutravidin/biotin approach reduced the signal/background ratio of our protein complex ELISA by approximately 5-fold compared to direct adsorption of the capture antibody (data not shown). The reduced sensitivity resulted from a non-specific interaction between neutravidin and the alkaline phosphatase-conjugated secondary antibody used in the detection phase of the assay (data not shown). In other stages of assay development, we observed that the monoclonal antibody outperformed the polyclonal antibody in the capture step. For example, in the case of the E-cadherin:β-catenin ELISA, using the mouse monoclonal antibody for antigen capture step yielded a ~ 6-fold higher signal/background ratio than when the rabbit polyclonal antibody was used for capture (data not shown). Additionally, we explored the effect of the incubation temperature on assay sensitivity, but protein complex ELISAs carried out at 4 ºC and 37 ºC showed no difference in assay sensitivity (data not shown). All results reported here are from assays conducted at 37 ºC.

To verify that the protein complex ELISA specifically measures the interaction of E-cadherin and β-catenin, we performed the E-cadherin:β-catenin ELISA but omitted portions of the capture antibody:protein complex:detection antibody bridge that presumably forms in the microtiter well. Omission of either the cell lysate, the anti-E-cadherin capture antibody, or the anti-β-catenin detection antibody completely ablated the assay signal (Fig. IV-3), demonstrating that a measurable assay signal is produced

only when the complete antibody:protein:antibody sandwich is established. Given that these antibodies specifically recognize only one protein in Western blots (data not shown), combined with the fact that these antibodies can mediate capture of protein complexes in a microtiter well (Fig. IV-1), this demonstrates that the protein complex ELISA is specifically measuring the interaction of E-cadherin and β-catenin.

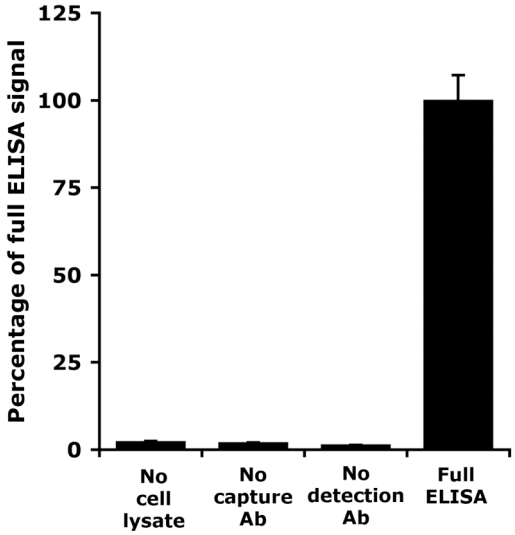

Fig. IV-3. Specificity test for E-cadherin:β-catenin ELISA
The E-cadherin:β-catenin ELISA was performed on 100 μg of MCF-10A whole cell lysate. At the appropriate step, either the whole cell lysate, the anti-E-cadherin capture antibody (Ab), or the anti-β-catenin detection antibody was omitted from the assay. Values are presented as the percentage of the rate of change in optical density over time relative to the control ELISA, where no reagents were omitted (Full ELISA). Error bars represent the sample standard error (n = 3).

3.2. Compatibility of the protein complex ELISA with standard sandwich ELISAs

Our results demonstrate that the protein complex ELISA offers a quantitative method for measuring the amount of endogenous E-cadherin:β-catenin complexes in crude cell lysates. A key consideration, however, is that any measured change in the level of protein:protein complexes may be to due to a change in either protein:protein affinity or protein expression level. To discriminate between these possibilities, it is essential to

measure the total amount of each protein in whole cell lysates. To address this issue, we exploited the flexibility of the protein complex ELISA to accommodate the traditional sandwich ELISA.

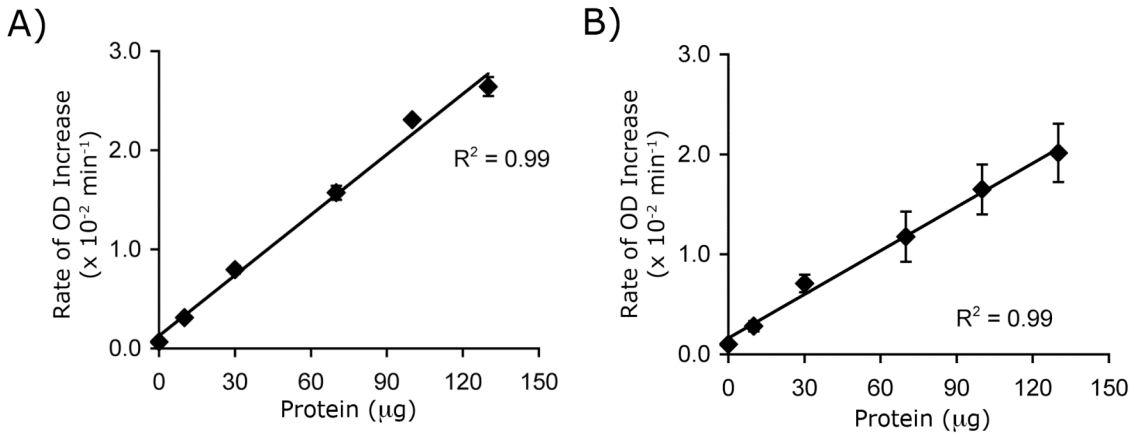

Fig. IV-4. Detection of E-cadherin and β-catenin total protein levels by sandwich ELISA
Whole cell lysate from MCF-10A cells was analyzed by sandwich ELISA for (A) total E-cadherin expression or (B) total β-catenin expression. The rate of change in optical density over time was plotted versus total cell lysate, and a linear regression was performed. Error bars represent the sample standard error (n = 3).

Having demonstrated that both E-cadherin and β-catenin are captured effectively in microtiter wells (Fig. IV-1), we determined whether the amount of captured antigen may be detected using polyclonal anti-E-cadherin and anti-β-catenin antibodies in a sandwich ELISA format. Under the optimal detection conditions, E-cadherin was detected over a linear range spanning a wide range of whole cell lysate (0 - 130 μg) (Fig. IV-4A). Notably, the linear range of the E-cadherin sandwich ELISA extended only to 130 μg, as opposed to the protein complex ELISAs, which demonstrated linearity to at least 200 μg of total cell protein. The linear dynamic range of the standard sandwich ELISA may saturate at a lower amount of total cell protein compared to the protein complex ELISA because not all E-cadherin molecules are bound to β-catenin and vice-versa.

Nonetheless, the E-cadherin sandwich ELISA demonstrated an outstanding signal/noise ratio of 48, with a Z' factor of 0.80 (Table IV-1). Using the same conditions as in the E-cadherin sandwich ELISA, we tested the detection of β-catenin using monoclonal and polyclonal anti-β-catenin antibodies for capture and detection, respectively. As for E-cadherin, β-catenin detection was linear up to 130 μg of total cell protein (Fig. IV-4B), with a signal/noise ratio of 3.6 (Table IV-1). However, in contrast to the E-cadherin sandwich ELISA. the β-catenin ELISA, appears only marginally suitable for screening assays, with a Z' factor of only 0.19. However, these results show that the protein complex ELISA is readily compatible with the sandwich ELISA for detecting total protein levels. This compatibility suggests that these assays may be operated on the same microtiter platform in parallel, reducing interplate variabilities and enhancing measurement throughput.

3.3. Quantitative comparison of E-cadherin:β-catenin interactions in transformed versus non-transformed cells

To test the applicability of the protein complex and the total protein ELISAs, we quantified the amount of E-cadherin:β-catenin complexes and the expression of E-cadherin and β-catenin in a transformed and a non-transformed cell system. The non-transformed mammary epithelial cell line, MCF-10A, exhibits a normal epithelial phenotype, including E-cadherin-mediated intercellular adhesion;[21] meanwhile, SW480 is a colon carcinoma cell line with dysfunctional β-catenin degradation machinery that permits β-catenin to build up to high levels in the cytoplasm.[22]

To confirm that SW480 cells express greater amounts of β-catenin than MCF-10A cells, we used the β-catenin sandwich ELISA. The β-catenin sandwich ELISA yielded a linear response with respect to the amount of whole cell lysate loaded in the assay for both cell types (Fig. IV-S1). We quantified the slope of the ELISA signal versus the amount of whole cell lysate as a metric of β-catenin expression. As shown in Figure IV-5A, the amount of β-catenin per μg of cell lysate was nearly 10-fold higher in SW480 cells than in MCF-10A cells, consistent with the known dysfunction of β-catenin degradation in SW480.

We next examined how excessive stabilization of β-catenin might affect the level of E-cadherin:β-catenin complexes in SW480 cells relative to those found in the MCF-10A cell line. As shown in Figure IV-5B, the E-cadherin:β-catenin ELISA revealed that MCF-10A cells exhibit approximately 2-fold higher levels of E-cadherin:β-catenin complexes than SW480 cells. Thus, despite a 10-fold relative abundance in β-catenin expression, the amount of E-cadherin:β-catenin complexes is fewer in SW480 cells than in MCF-10A cells.

To gain more insight into what may be limiting E-cadherin:β-catenin complex formation in SW480 cells, we quantified the E-cadherin expression level in the two cell lines using the sandwich ELISA, revealing that SW480 cells express approximately 2-fold lower levels of E-cadherin per μg of total cell lysate than MCF-10A cells (Fig. IV-5C). This 2-fold reduction in E-cadherin expression mirrors the 2-fold decrease in E-cadherin:β-catenin complexes in SW480 cells, suggesting that the limiting factor in

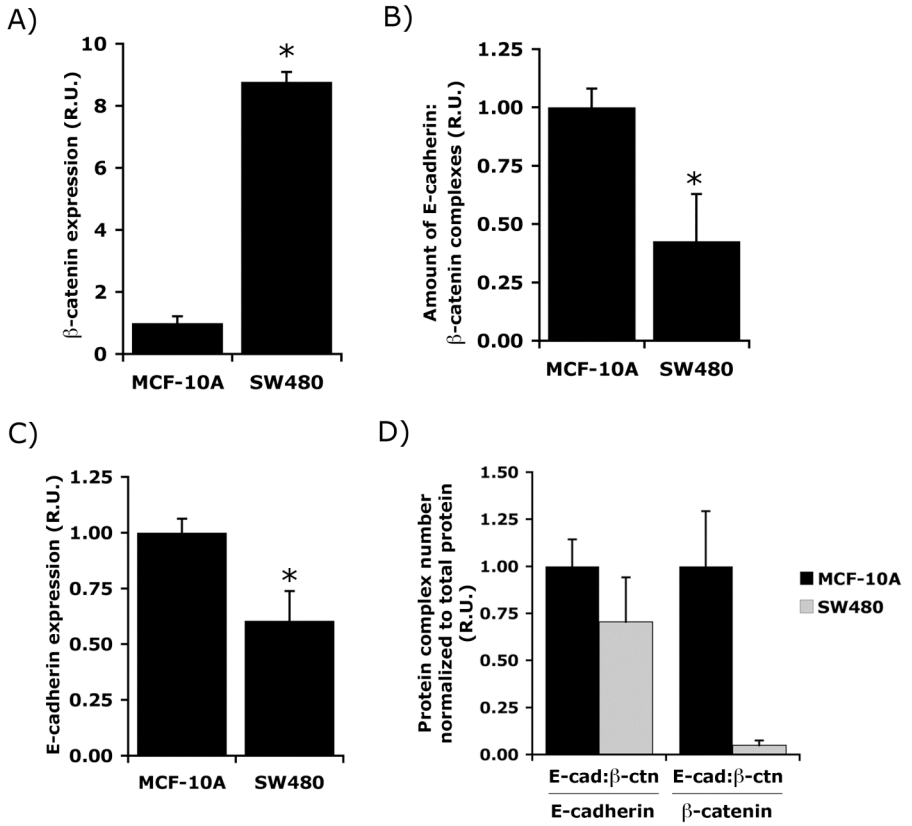

Fig. IV-5. Quantitative comparison of the levels of E-cadherin:β-catenin complexes, E-cadherin and β-catenin in normal and tumorigenic cell lines
Whole cell lysates were prepared from MCF-10A and SW480 cells, and were analyzed either by (A) sandwich ELISA for total β-catenin levels, (B) protein complex ELISA for E-cadherin:β-catenin complexes, or (C) sandwich ELISA for total E-cadherin levels. For all ELISAs, the rate of change in optical density over time was plotted versus total cell lysate, as shown in Figure IV-S1. The slopes of these curves were normalized to the value of the MCF-10A sample and then reported in relative units (R.U.). Error bars represent 95% confidence intervals on the slope; the asterisk denotes $P < 0.001$, as calculated by ANCOVA. (D) The level of E-cadherin:β-catenin complexes from the protein complex ELISA in MCF-10A and SW480 was normalized to total cellular levels of E-cadherin or β-catenin, as measured by the sandwich ELISA.

E-cadherin:β-catenin complex formation in SW480 cells is the reduced expression of E-cadherin. In fact, when the levels of E-cadherin:β-catenin are normalized to the total amount of E-cadherin, MCF-10A and SW480 cells show nearly identical values, with SW480 showing only a 30 ± 24% decrease compared to MCF-10A cells (Fig. IV-5D). This suggests that, per E-cadherin molecule, there is no significant difference in binding affinity for β-catenin between the two cell types, although direct measurements are

needed to confirm this hypothesis. Thus, the limiting factor in the formation of adherens junctions in SW480 appears to be expression of E-cadherin, especially since SW480 cells express roughly 10-fold higher levels of β-catenin than MCF-10A cells.

To gauge the relative differences in the fraction of cellular β-catenin engaged with E-cadherin between the two cell lines, we normalized the amount of E-cadherin:β-catenin complexes to total levels of β-catenin, as measured by the β-catenin sandwich ELISA. The fraction of cellular β-catenin that is engaged with E-cadherin is approximately 20-fold higher in MCF-10A cells relative to SW480 cells (Fig. IV-5D). This result indicates that SW480 possess a much larger pool of β-catenin that is not bound to E-cadherin. Evidently, this arises because SW480 cells express very high levels of total β-catenin with a concomitant decrease E-cadherin expression, leaving a large fraction of β-catenin unbound to E-cadherin. This pool of unbound β-catenin molecules may contribute to the constitutive activation of transcriptional targets by β-catenin in SW480.[23]

3.4. Quantitative analysis of the effect of constitutively-active Src on E-cadherin:β-catenin interactions

As another application of our quantitative protein complex ELISA, we quantified the effect of the tyrosine kinase Src on the disruption of adherens junctions in MCF-10A, since several lines of evidence have implicated Src in regulating intercellular adhesion.[7,8,10,24] To study the quantitative effects of Src activity on E-cadherin:β-catenin interactions *in vivo* using our protein complex ELISA, MCF-10A cells were infected with retrovirus encoding a constitutively-active mutant of Src (Y527F);[25,26] as a negative

control, cells were infected with retrovirus generated using an empty vector control. Cell lysates were prepared and analyzed for the amount of E-cadherin:β-catenin complexes and for the levels of E-cadherin and β-catenin expression. All assays demonstrated a broad linear range with respect to total cell protein (Fig. IV-S2).

Using the protein complex ELISA revealed that activated Src reduced levels of the E-cadherin:β-catenin complex by ~ 40% in MCF-10A cells (Fig. IV-6A), corroborating reports that Src can disrupt E-cadherin mediated adhesions *in vivo*.[7,8,10,24] These results are consistent with our qualitative observation that sub-confluent MCF-10A cells expressing activated Src formed fewer cell-cell contacts in culture as compared to their empty-vector counterparts (data not shown). To determine whether the reduction in the amount of E-cadherin:β-catenin complexes correlated with a decrease in E-cadherin:β-catenin interactions or with a decreased expression of these proteins, we quantified E-cadherin expression using the sandwich ELISA. MCF-10A cells expressing activated Src exhibited a reduction in E-cadherin of 40% when compared to cells infected with empty vector control virus (Fig. IV-6B). Thus, there is a striking quantitative similarity between the decrease in the levels of E-cadherin:β-catenin complexes and E-cadherin expression. In fact, when the levels of the E-cadherin:β-catenin complex are normalized to total levels of E-cadherin, the ratio is nearly equivalent in cells expressing activated Src and the negative control counterparts (Fig. IV-6C).

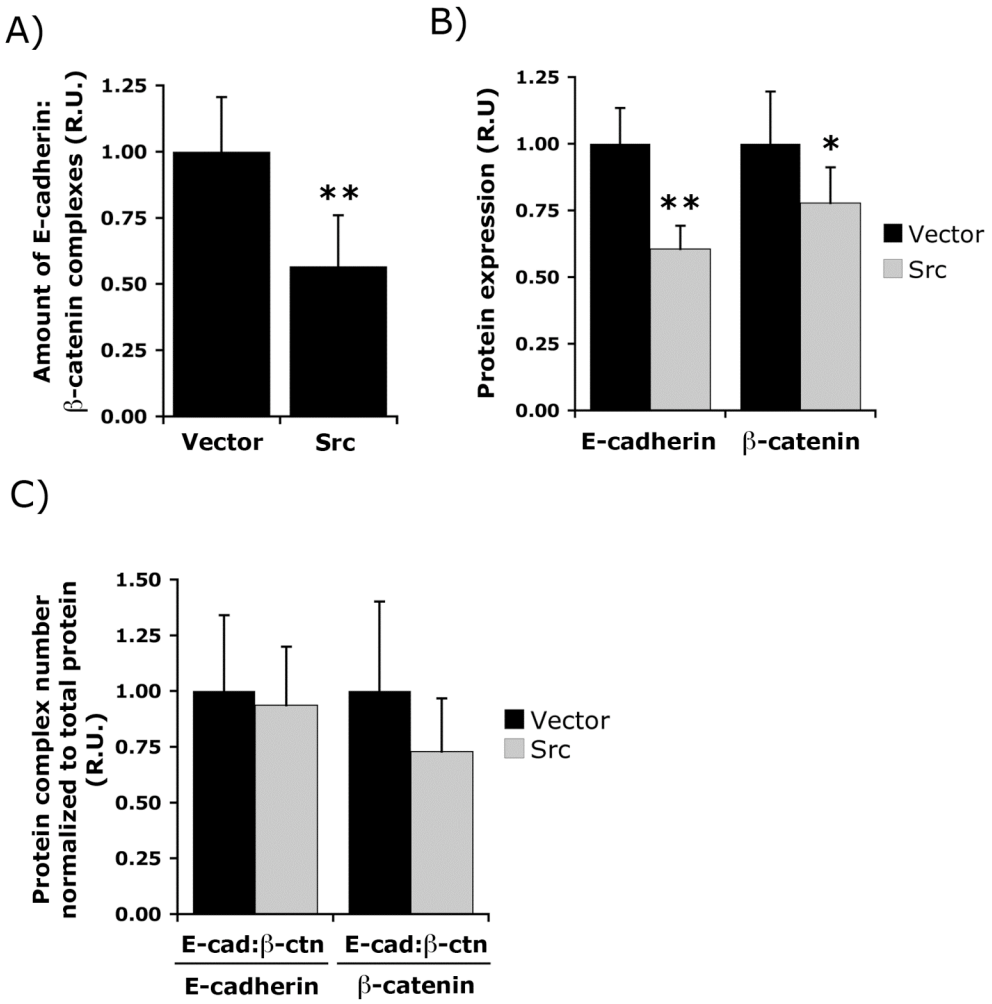

Fig. IV-6. Quantifying the effect of constitutively-active Src on cellular levels of E-cadherin: β-catenin complexes and the expression of E-cadherin and β-catenin

MCF-10A cells were infected with either activated Src (Y527F) or the empty vector control, lysed, and then analyzed either by (A) the E-cadherin:β-catenin ELISA or (B) sandwich ELISAs. For all assays, the rate of change in optical density over time was plotted against total cell lysate, as shown in Figure IV-S2. The slopes of these curves were normalized to the value of the empty vector control and then reported in relative units (R.U.). Error bars represent 95% confidence intervals on the slope; the asterisk and double asterisk denote $P < 0.05$ and 0.001, respectively, as calculated by ANCOVA. (D) The level of E-cadherin:β-catenin complexes as measured by the protein complex ELISA was normalized to total cellular levels of either E-cadherin or β-catenin as measured by the sandwich ELISA for both Src-infected and empty vector-infected MCF-10A cells.

Because Src-mediated disruption of adherens junctions *in vivo* correlates with tyrosine phosphorylation of both E-cadherin and β-catenin,[7,8,10,24] it has been speculated that Src disrupts adherens junctions by phosphorylating β-catenin and thus reducing its

binding affinity for E-cadherin. In support of this model, *in vitro* studies have demonstrated that Src-mediated phosphorylation of β-catenin Tyr654 causes a 6-fold decrease in the affinity of β-catenin for E-cadherin.[12] Additional *in vitro* studies have also shown that phosphorylation of E-cadherin by Src decreases the affinity constant of E-cadherin for β-catenin from approximately 260 nM to 4 μM *in vitro*.[13]

While our results are not wholly incompatible with this model, the striking correlation between Src-induced decreases in E-cadherin:β-catenin complexes and E-cadherin itself strongly suggests that active Src may reduce the levels of E-cadherin:β-catenin complexes by a simpler mechanism, namely the downregulation of E-cadherin expression. By decreasing the availability of E-cadherin in MCF-10A cells, Src could reduce levels of the endogenous E-cadherin:β-catenin complex without modulating the protein binding affinity through phosphorylation. In another non-transformed epithelial cell line (MDCK), activation of Src induced ubiqutination and endocytosis of E-cadherin through the Hakai ubiquitin ligase, leading to decreased E-cadherin expression and the disruption of cell-cell contacts.[8] Thus, altering the expression level of E-cadherin may be a mechanism by which Src regulates adherens junctions in non-transformed epithelial cells. In fact, Src-mediated downregulation of E-cadherin may contribute to oncogenic phenomena, such as transforming growth factor β-induced epithelial-mesenchymal transition.[27]

The observed 40% decrease in E-cadherin expression raises the question of the fate of its binding partner β-catenin. It is reasonable to hypothesize that a reduction in

E-cadherin expression may cause a parallel decrease in β-catenin expression, since when β-catenin is bound to E-cadherin, it is protected from proteasomal degradation.[28] However, once released from E-cadherin, β-catenin is subject to degradation by the APC/axin/GSK3β/casein kinase I degradation machinery. Indeed, the β-catenin ELISA revealed that the expression level of β-catenin also decreased in cells expressing activated Src, but only by a relatively modest 20% (Fig. IV-6B). Compared to the 40% reduction in E-cadherin levels, the modest reduction in β-catenin levels suggests that some of the E-cadherin-free β-catenin lingers in the cell, avoiding degradation. Indeed, when the levels of E-cadherin:β-catenin complexes are normalized to total levels of β-catenin, the ratio decreases by ~ 25% in cells expressing activated Src (Fig. IV-6C). This result suggests that among total cellular β-catenin, 25% fewer are associated with E-cadherin in response to constitutive activation of Src, which may contribute to Src-mediated activation of β-catenin:Tcf/Lef transcription as reported by others.[29,30]

4. Conclusions

In summary, we have developed a protein complex ELISA to quantify the level of endogenous E-cadherin:β-catenin complexes in unpurified whole cell lysates. Furthermore, we have exploited the compatibility of our protein complex ELISA format to quantify total cellular levels of E-cadherin and β-catenin using traditional sandwich ELISAs. Using these techniques, we have characterized the quantitative differences in E-cadherin:β-catenin complexes between normal and tumorigenic cells as well as the effects of a dominant-active oncogene on E-cadherin:β-catenin complexes *in vivo*. These

measurements provide insight into not only the expression levels of adherens junctions constituents, but also their ability to form multiprotein complexes, which is an integral feature of their biological functionality. Since the association of E-cadherin and β-catenin is an established predictor of tumor cell invasiveness and patient prognosis,[1] the E-cadherin:β-catenin protein complex ELISA may prove to be a powerful tool for diagnostic characterization of tumors. In fact, other protein complex ELISAs have demonstrated that protein complexes can serve as better biomarkers of disease than individual proteins.[16,18] In addition to the powerful prognostic value of protein:protein association data, the quantitative nature of the protein complex ELISA offers advantages over the qualitative, low-throughput techniques currently used to characterize E-cadherin:β-catenin association. As such, the E-cadherin:β-catenin ELISA is a powerful tool for quantitative characterization of cell-cell adhesion.

5. Acknowledgments

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6. Supplemental Data

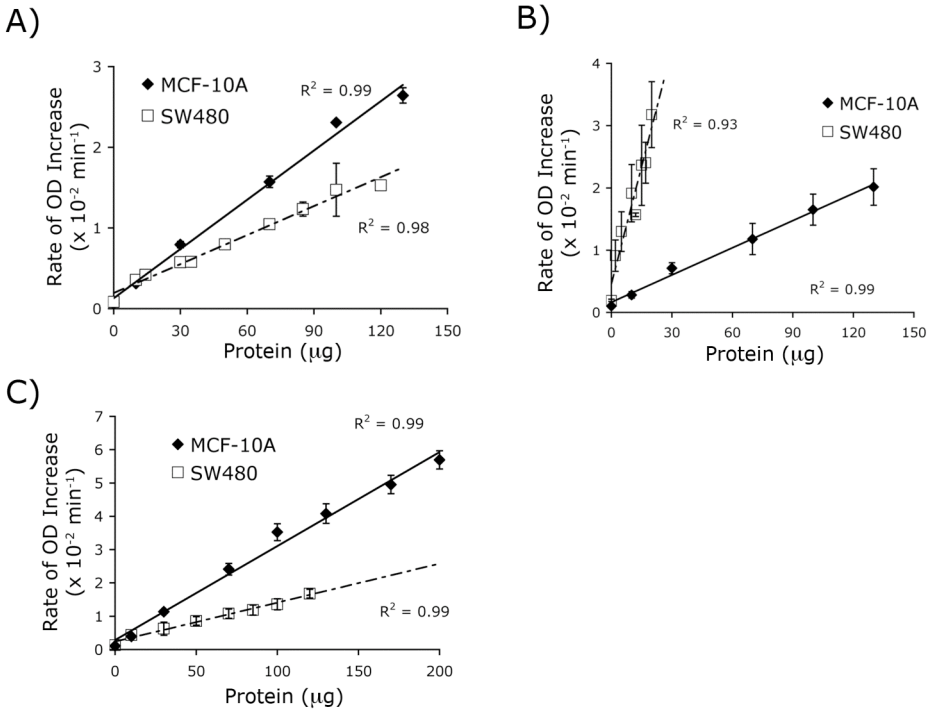

Fig. IV-S1. Validation of standard sandwich and protein complex ELISAs in normal and tumorigenic cell lines

Confluent cultures of MCF-10A and SW480 cells were lysed and analyzed by sandwich ELISA for either (A) E-cadherin or (B) β-catenin or by protein complex ELISA for (C) E-cadherin:β-catenin complexes. For all assays, the rate of change in optical density over time was plotted versus total cell lysate, and linear regressions were performed to calculate the slopes of these curves. Error bars represent sample standard error (n=3).

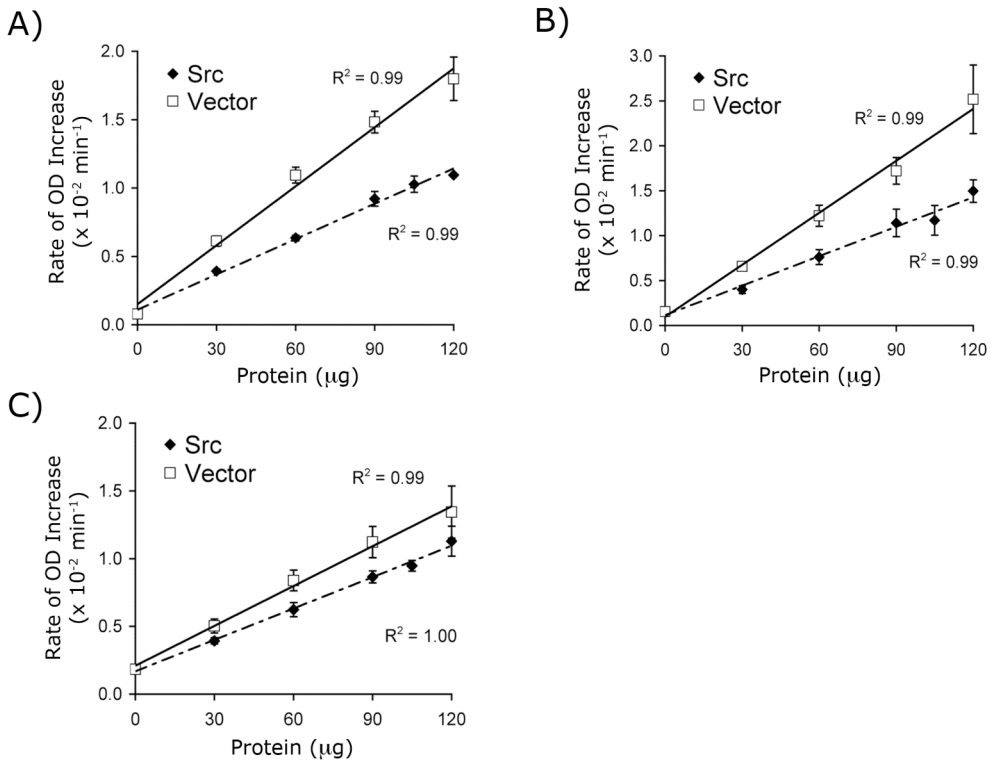

Fig. IV-S2. Validation of standard sandwich and protein complex ELISAs in cells expressing constitutively-active Src
Subconfluent cultures of MCF-10A cells infected with either Src Y527F or the empty vector were lysed and analyzed by sandwich ELISA for (A) E-cadherin or (B) β-catenin expression or by protein complex ELISA for (C) E-cadherin:β-catenin complexes. For all assays, the rate of change in optical density over time was plotted against total cell lysate, and linear regressions were performed to calculate the slopes of these curves. Error bars represent sample standard errors (n = 3).

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Chapter V. Mechanisms underlying growth saturation of epithelial cells

Abstract

Deregulation of mammalian cell proliferation underlies many diseases, including cancer. Here, we investigate the mechanistic basis of growth inhibition in non-transformed epithelial cells at high cell density, a property that is often lost during tumorigenesis. We demonstrate that growth saturation at high cell density is controlled by multiple anti-proliferative signals, including cell contact and cell density. First, we present correlative data suggesting that the cell adhesion molecular E-cadherin mediates contact-induced growth suppression. As cells grow to confluency, E-cadherin expression is upregulated, thereby inhibiting expression of crucial cell cycle genes by β-catenin:Tcf/Lef transcription complexes. Expression of exogenous mutants confirms that E-cadherin can inhibit growth in a manner consistent with inhibition of Tcf/Lef transcription. Second, we demonstrate that growth saturation is also controlled by a density-dependent mechanism, whereby high cell density depletes mitogens from the cellular microenvironment, depriving cells of proliferative signals, possibly including Akt. Notably, since this mechanism is independent of cell-cell contact, cell growth can saturate even at subconfluent densities. Taken together, this report describes both contact- and density-dependent mechanisms regulating growth inhibition of normal epithelial cells in culture. Furthermore, we suggest a global model whereby cells integrate both contact- and density-dependent mechanisms to mediate growth saturation at high cell density.

1. Introduction

Mammalian cell proliferation is highly-regulated. Precise and dynamic control of cell division is essential for proper organization and remodeling of multicellular organisms. In tissue culture, one hallmark of non-tumorigenic cells is the saturation of cell division at high cell density. This property is often lost during tumorigenesis, leading to de-regulation of cell growth (Wijnhoven et al., 2000). Although tissue culture does not fully recapitulate the complexity of the *in vivo* microenvironment, the physiological significance of this phenomenon is underscored by the observation that normal cells will divide in culture until reaching confluency and then cease proliferating. In contrast, cells derived from aggressive, invasive tumors do not arrest at confluency and will proliferate in multi-layer patterns. Despite research spanning over 40 years (Eagle and Levine, 1967), the precise mechanisms controlling growth inhibition of non-cancerous cells at high density are not fully understood. The confusion surrounding this phenomenon is highlighted by the two distinct names often used in the literature: contact-dependent (Grazia Lampugnani et al., 2003; Motti et al., 2005; Uegaki et al., 2006; Vizirianakis et al., 2002) and density-dependent inhibition of proliferation (Hornberg et al., 2006; Pino et al., 2006; Zhang et al., 2000). The former suggests that a local sensing mechanism drives cell cycle arrest: cell contact generates intracellular growth-inhibitory signals. Meanwhile, density-dependent inhibition suggests a population-scale mechanism independent of cell contact that resembles quorum sensing in bacterial communities (Camilli and Bassler, 2006). Precisely which of these two extremes determines growth saturation of normal epithelial cells remains unclear.

In non-transformed somatic cells, progression through the cell cycle is dependent on both proper timing of mitogenic signals (Evan and Vousden, 2001) and avoidance of anti-proliferative signals (Hanahan and Weinberg, 2000). Examples of anti-proliferative signals range from soluble, growth-inhibitory molecules to immobilized inhibitors embedded either in the extracellular matrix or on the surfaces of neighboring cells. Many, if not all, of these anti-proliferative signals are funneled through the retinoblastoma protein (pRb), which blocks proliferation when hypophosphorylated. Although a multitude of anti-proliferative signals have been identified, how normal cells integrate multiple growth-inhibitory signals to cease proliferating at high cell density is still unclear.

The fundamental difference between contact- and density-dependent mechanisms of growth saturation lies in the identity of the growth-inhibitory signal. Contact-dependent mechanisms assert that non-tumorigenic cells sense the presence of neighboring cells, typically through cell adhesion molecules such as cadherins, and cease dividing when this contact signal passes a critical threshold. In contrast, density-dependent mechanisms ascribe growth arrest to a contact-independent ability to sense cell density and halt cell division. One such density-dependent mechanism asserts that cells secrete growth-inhibitory peptides, which accumulate in dense cultures and inhibit proliferation above a critical concentration (Koga et al., 1986; Steck et al., 1982).

For contact-mediated mechanisms of growth inhibition at high cell density, multiple adhesion proteins reportedly serve as anti-proliferative signals. Examples

include various members of the connexin gap junction family (Fujimoto et al., 2004; Ruch et al., 1995), the tight junction protein occludin (Li and Mrsny, 2000), and the immunoglobulin superfamily cell adhesion protein CEACAM1 (Scheffrahn et al., 2005). However, the adhesion proteins most often invoked in contact inhibition of proliferation are the cadherins, a family of transmembrane glycoproteins which localize to adherens junctions (Angst et al., 2001).

Cadherins mediate intercellular contact through their extracellular domain, which binds homotypically to the extracellular domain of cadherins on opposing cells. Intracellularly, cadherins bind β-catenin, which links to the actin cytoskeleton-binding protein α-catenin. Interestingly, β-catenin is a proto-oncogene that controls the expression of cell cycle genes including *cyclin d1* and *c-myc* by binding to and activating the Tcf/Lef family of transcription factors (He et al., 1998; Tetsu and McCormick, 1999). As such, cadherins have been suggested to mediate contact inhibition of proliferation through suppression of β-catenin-mediated transcription (St Croix et al., 1998; Stockinger et al., 2001). Cadherins may also control contact inhibition of growth by regulating mitogenic signaling, since cadherin-mediated adhesion can inhibit the activation of receptor tyrosine kinases (RTKs) including the epidermal growth factor (EGF) receptor (Qian et al., 2004; Takahashi and Suzuki, 1996) and the vascular endothelial growth factor (VEGF) receptor (Grazia Lampugnani et al., 2003; Lampugnani et al., 2006). Other mechanisms, including upregulation of the atm phosphoprotein (Vutskits et al., 2006) and baso-lateral sequestration of RTKs in ligand-inaccessible compartments (Guo

et al., 2005), may also be involved in cadherin-mediated contact inhibition of proliferation.

Similar to growth suppression by cadherins, density-dependent mechanisms often invoke reduced mitogenic signaling to explain growth suppression at high cell density. In particular, density-dependent increases in phosphatase activity can attenuate mitogenic signals such as ERK and thus block cell division (Ishibe et al., 2006; Machide et al., 2006; Sorby and Ostman, 1996; Suzuki et al., 2000; Vinals and Pouyssegur, 1999; Wayne et al., 2006). Density-dependent decreases in the availability of RTK (Rizzino et al., 1990), have also been implicated in control of growth suppression at high cell density. Notably, the mechanisms underlying density-dependent upregulation of these anti-proliferative signals are generally unknown.

Another mechanism by which high cell density can inhibit proliferation is by the depletion of mitogenic ligands from the culture medium. This mechanism was originally observed by Holley and colleagues, who found that dense cultures of NIH-3T3 fibroblasts which were ostensibly growth arrested would re-enter the cell cycle by stimulation with a fresh bolus of mitogens (Holley, 1975). More recently, it has been demonstrated that proliferation can be controlled by receptor-ligand trafficking (Starbuck et al., 1990), and that engineering ligands with enhanced lifetimes in culture can enhance mitogenic potency (Reddy et al., 1996). Additionally, density-dependent inhibition of proliferation is undoubtedly linked to other mechanisms which are yet to be understood, including the *ING4* gene (Kim et al., 2004), nucleo-cytoplasmic shuttling of the Ca^{2+}-

binding proteins S100B and S100C (Sakaguchi et al., 2000; Scotto et al., 1998), and sustained activation of the p38α MAPK at confluence (Faust et al., 2005).

With so many reported mechanisms controlling growth suppression of non-tumorigenic cells at high cell density, it is unclear which anti-proliferative signals might be important for normal epithelial cells. For example, which mitogenic signals are reduced at high density? What role do cell-cell contact and cell density play? Can contact- and density-dependent mechanisms cooperate to mediate growth saturation at high density, and if so, how do cells integrate multiple anti-proliferative signals to turn off the cell cycle? To address these questions, we sought to quantitatively interrogate how intercellular contact and cell density contribute to growth inhibition of non-tumorigenic epithelial cells.

2. Results

2.1. Non-tumorigenic epithelial cells growth arrest at high cell density.

To investigate growth saturation at high cell density, we chose the immortalized but non-tumorigenic mammary epithelial cell line MCF-10A, which retains many characteristics of normal epithelial cells (Soule et al., 1990; Tait et al., 1990). In fact, MCF-10A have been previously reported to undergo cell cycle arrest in high density cultures (LeVea et al., 2004; Liu et al., 2006). Before testing whether we could observe growth arrest of MCF-10A at high densities, we characterized how cell phenotype varies as a function of cell density. After seeding a range of initial cell numbers and allowing

culture for 72 h, various levels of cell density, and thus varying degrees of cell-cell contact, were achieved (Figure V-1A). At low cell densities (9.1 x 10^3 cm^{-2}), most cells were isolated from neighboring cells and thus form virtually no cell-cell contacts. As cell density increased, cells began to form contacts with neighbors (3.9 x 10^4 cells cm^{-2}) and eventually reached monolayer status (1.6 x 10^5 cells cm^{-2}), where every cell is entirely surrounded by neighbors.

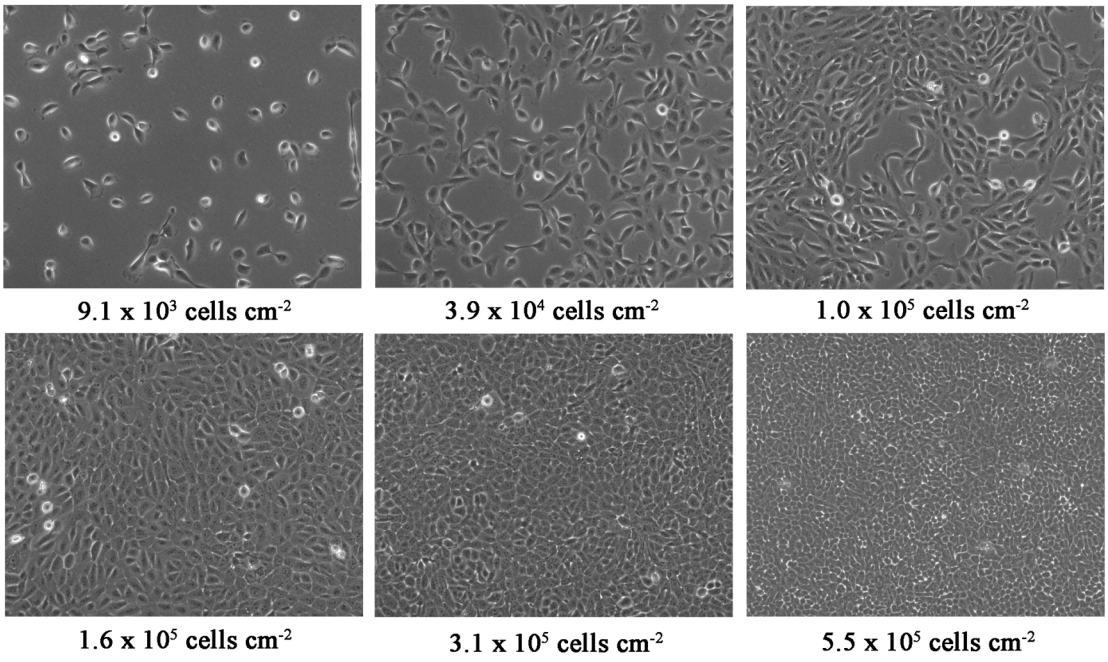

Figure V-1. Quantification of intercellular contact by measuring cell density in MCF-10A
Varying numbers of MCF-10A were plated and cultured for 72 h in GM before trypsinization and quantification of cell density.

Although MCF-10A grew densities higher than 1.6 x 10^5 cells cm^{-2}, if one gauges intercellular contact by the absence or presence of neighbors alone, cell-cell contact does not increase beyond 1.6 x 10^5 cells cm^{-2}. However, since the cell diameter clearly decreases as cell density is increased from 1.6 x 10^5 cells cm^{-2} to 5.5 x 10^5 cells cm^{-2}, we assumed that cells maintained a constant total cell surface area (i.e., plasma membrane is

neither created nor destroyed). Under this assumption, increasing cell density also increases the cell-cell contact area. To more conclusively quantify intercellular contact, one could estimate the cell-cell contact area by multiplying the cell height (measured by confocal microscopy) by the projected cell area.

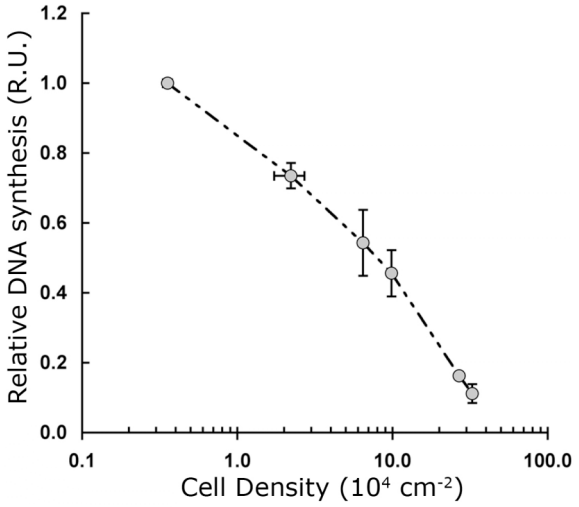

Figure V-2. DNA synthesis is inversely correlated with cell density.
Serum-starved MCF-10A were stimulated with GM, and the amount of DNA synthesis was measured by ^{3}H-thymidine incorporation as described in Experimental Procedures. DNA synthesis data was normalized to the response of cells at 3.6 x 10^{3} cells cm^{-2} and is expressed in relative units (R.U.).

After characterizing cell phenotype as a function of cell density, we tested whether MCF-10A would growth arrest at high cell density. We measured DNA synthesis in cells stimulated with growth medium (GM), which contains EGF, insulin, hydrocortisone, cholera toxin, and serum factors, as a function of cell density. Normalizing ^{3}H-thymidine incorporation data to total cell number, DNA synthesis was highest in subconfluent cells and decreased monotonically with increasing cell density (Figure V-2). Compared to subconfluent cells (4 x 10^{3} cells cm^{-2}), DNA synthesis was reduced ~ 90 % in confluent cells (2.8 x 10^{5} cells cm^{-2}); thus, MCF-10A do growth arrest

at high cell density, even in the presence of mitogenic cytokines, as previously reported (LeVea et al., 2004; Liu et al., 2006).

2.2. Receptor-mediated signaling is qualitatively similar in low- and high-density cells.

In a variety of cell lines, cell cycle arrest at high cell density has been linked to the inability of growth factors to activate mitogenic signaling pathways (e.g., ERK) (Conacci-Sorrell et al., 2003; Grazia Lampugnani et al., 2003; LeVea et al., 2004; Mansbridge et al., 1992; Qian et al., 2004; Sorby and Ostman, 1996; Takahashi and Suzuki, 1996). Thus, we speculated that proliferation of MCF-10A at high densities might be caused by reduced EGFR activation or attenuated downstream signaling. To examine this possibility, we examined levels of active (dually-phosphorylated) ERK over a range of densities, and found that ERK phosphorylation was strongly reduced at high cell densities (Figure V-3A). We reasoned that reduced ERK signaling at high cell density could result from at least two factors: 1) a molecular mechanism restricting RTK signaling (e.g., phosphatase-mediated deactivation of EGFR), or 2) depletion of mitogenic growth factors from the cellular microenvironment.

To test the latter hypothesis, serum-starved cells at low and high density were stimulated with a fresh bolus of EGF, and immunoblotting demonstrated that phosphorylation of both EGFR and ERK was not attenuated at high cell density (Figure V-3B). Thus, growth arrest in dense cultures is not caused by an inability to activate EGFR or signal to ERK. In addition to ERK, the PI3K/Akt signaling pathway is crucially required for proliferation (Lawlor and Alessi, 2001). To investigate Akt

activation, we probed for Akt phosphorylated on serine 473 Akt (Figure V-3B). Although Akt was strongly activated above vassal levels in low and high density cells, qualitative analysis revealed a slight reduction in phospho-Akt at high cell density. However, without semi-quantitative immunoblotting data, we are unable to definitively conclude that Akt signaling is attenuated at high density in MCF-10A.

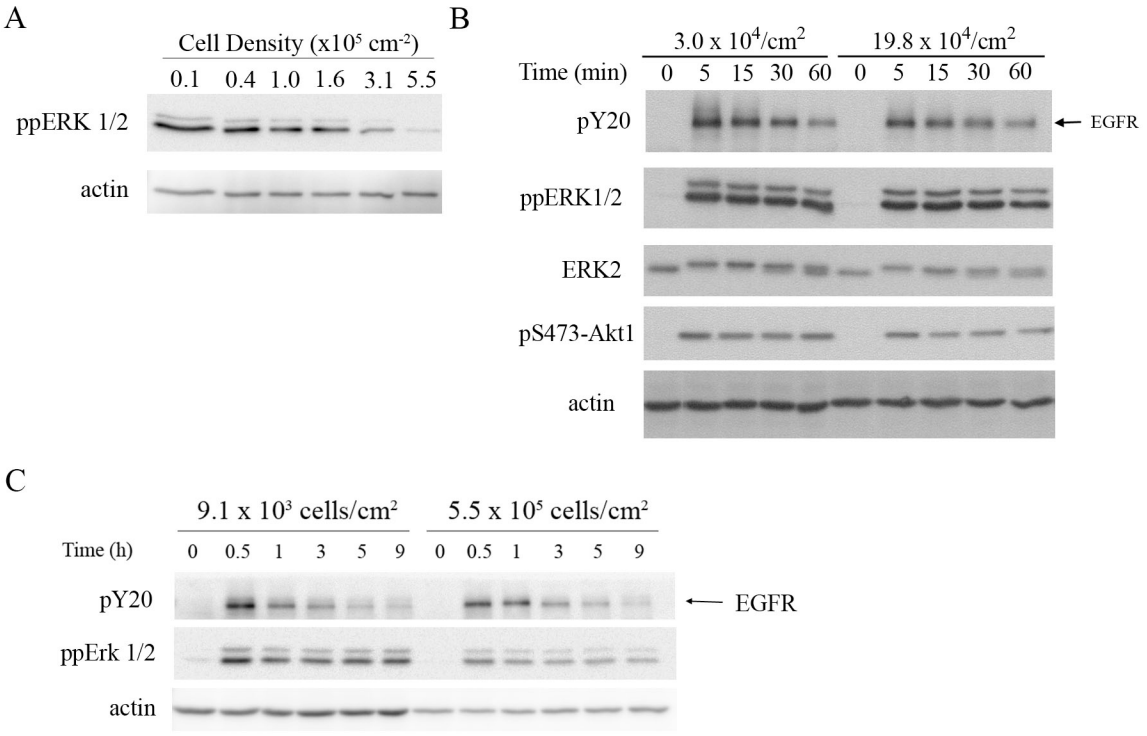

Figure V-3. EGFR, ERK, Akt signaling at various cell densities
Whole cell lysates from MCF-10A at the indicated cell densities were immunoblotted for dually-phosphorylated ERK 1/2 (ppERK 1/2), phospho-tyrosine, total ERK2, phospho-serine 473 Akt, or the equal loading control actin. (A) Cells were cultured in GM as in Figure V-1A. (B) Serum-starved cells cultured at low and high density in 35 mm dishes were stimulated with EGF (20 ng/ml) for the indicated times. (C) Serum-starved cells cultured at low and high density in 35 and 60 mm dishes, respectively, were stimulated with EGF and lysed at the indicated times.

Thus, MCF-10A growth arrest at high density in the presence of mitogens despite qualitatively similar signaling at early times (< 60 minutes). Because proliferation of fibroblasts requires both early (~ 1 h) and late (~ 8 h) phases of RTK signaling (Jones and

Kazlauskas, 2001), we speculated that growth arrest at high cell density might be due to attenuated mitogenic signaling at late times. To test this hypothesis, we attempted to measure ERK and Akt signaling in low- and high-density cells over an extended duration; however, we repeatedly encountered a problem with the equal loading control actin (Figure V-3C). Without confirmed equal protein loading, we cannot comment on the status of these signaling pathways at late times. This technical issue has yet to be resolved, but we speculate that it may be an artifact due to culturing cells in different size dishes. Therefore, it is possible that high density cells may growth arrest due to significant attenuation of EGFR, ERK, and/or Akt signaling.

2.3. Increased expression of endogenous E-cadherin at high cell density correlates with low levels of DNA synthesis and β-catenin:Tcf/Lef signaling.

In addition to ERK and Akt signaling, we have previously demonstrated that transcription of cell cycle genes by β-catenin:Tcf/Lef complexes is required for EGF-mediated proliferation of MCF-10A (Graham and Asthagiri, 2004). Therefore, we measured the ability of GM to induce β-catenin:Tcf/Lef transcriptional activity as a function of cell density. To monitor β-catenin-mediated transcription, we used the TOPFLASH reporter, a plasmid containing four consensus Tcf-binding sites upstream of the *luciferase* gene; as a negative control, the FOPFLASH reporter carries mutations at these Tcf/Lef binding sites (Korinek et al., 1997). Using these reporter constructs, we observed that TOPFLASH activity was initially high in subconfluent cells (Figure V-4). Moderate increases in cell density (1.4×10^4 to 4.0×10^4 cells cm^{-2}) did not affect reporter activity, but cell densities in excess of 10^5 cells cm^{-2} did decrease TOPFLASH

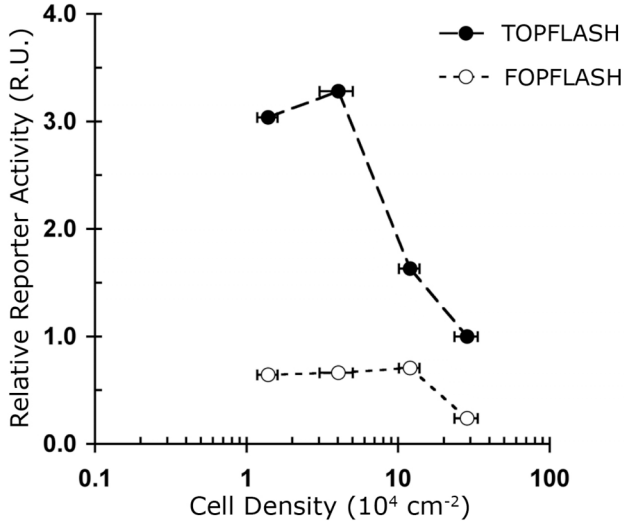

Figure V-4. β-catenin-mediated transcription is inversely correlated with cell density. MCF-10A that had been transfected with the Tcf/Lef reporter TOPFLASH or the negative control FOPFLASH were plated at various densities, serum-starved, and stimulated with GM for 9 h. All reporter activities were normalized to the TOPFLASH signal of cells at 2.8 x 10^5 cells cm^{-2}.

reporter activity between two- and three-fold. Notably, 10^5 cells cm^{-2} is the cell density at which cells first form a complete monolayer (Figure V-1A). In contrast, over the same range of cell densities, activity of the negative control FOPFLASH reporter was not strongly diminished. Thus, high-density MCF-10A exhibit a density-dependent reduction in β-catenin:Tcf/Lef-transcriptional activity, a crucial mitogenic signal.

Because the cell-cell contact protein E-(epithelial-)cadherin can bind β-catenin and sequester it outside of the nucleus (Orsulic et al., 1999; Sadot et al., 1998), we wondered if E-cadherin might play a role in attenuation of β-catenin:Tcf/Lef signaling at high cell density. Semi-quantitative immunoblotting demonstrated that E-cadherin expression increased ~ 4-fold as cells grew from low to high cell density (Figure V-5A). Meanwhile, the expression of β-catenin was independent of cell density. Thus, as illustrated by the ratio of E-cadherin to β-catenin (Figure V-5B), intercellular adhesion selectively enriches expression of E-cadherin. Because the E-cadherin:β-catenin ratio is

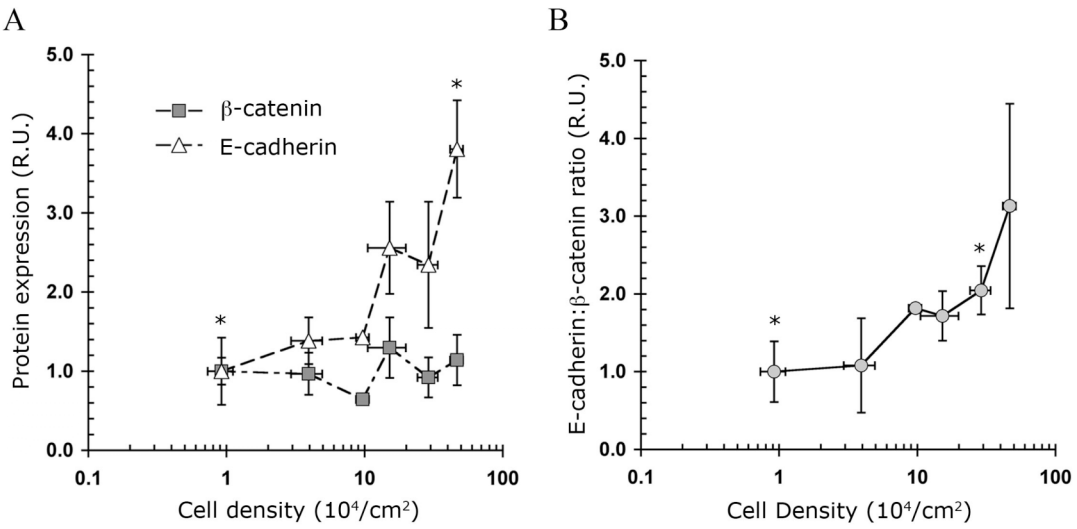

Figure V-5. Expression of endogenous E-cadherin, but not β-catenin, is cell density-dependent. MCF-10A were cultured in GM as described in Figure V-1A and levels of E-cadherin, β-catenin, and actin were measured by semi-quantitative immunoblotting as detailed in Experimental Procedures. Protein expression was normalized to that of cells at 9.2 x 10^3 cells cm^{-2} and is depicted in relative units (R.U.). Error bars represent sample standard errors (n = 3), and the *asterisks* denote $P < 0.05$ by Student's *t*-test. (A) Expression of E-cadherin and β-catenin relative to the equal loading control actin. (B) The ratio of E-cadherin to β-catenin.

highest at cell densities where β-catenin:Tcf/Lef transcriptional activity is suppressed, high cell density may induce the sequestration of β-catenin at the plasma membrane with E-cadherin. Spatial sequestration would prevent β-catenin from binding to Tcf/Lef transcription factors, thwarting β-catenin:Tcf/Lef transcription and depriving high-density cells of an essential mitogenic signal.

2.4. Exogenous E-cadherin inhibits DNA synthesis.

An E-cadherin-dependent mechanism of growth saturation assumes that E-cadherin can suppress proliferation by modulating β-catenin signaling. To explicitly test this assumption, we retrovirally overexpressed epitope-tagged, full-length E-cadherin (E-cadHA). As a negative control, we also expressed a mutant that lacks the cytoplasmic (and thus β-catenin-binding) domain of E-cadherin (E-cadΔcytoHA). Characterization of

these exogenous proteins by immunostaining revealed that E-cadHA localized to the plasma membrane and overlaid with β-catenin (Figure V-6A). E-cadΔcytoHA, in contrast, localized to intracellular vesicles, as reported elsewhere (Chen et al., 1999), and did not overlay with β-catenin. Immunoprecipitation of whole cell lysates demonstrated two principal HA-tagged proteins, one migrating at ~ 120 kDa, the expected size of E-cadherin, and the other migrating at ~ 100 kDa, the expected size of the cytodomain-truncated mutant (Figure V-6B). The secondary, higher-molecular-weight bands HA-tagged proteins are likely E-cadherin precursors that are not expressed on the cell surface (Shore and Nelson, 1991). Furthermore, the HA immunoprecipitates revealed that β-catenin co-immunoprecipitated with E-cadHA, but not with E-cadΔcytoHA, consistent with the fact that E-cadΔcytoHA lacks the β-catenin binding domain.

To test the effects of E-cadHA and E-cadΔcytoHA on cell cycle progression, we infected MCF-10A with the empty vector (pLPCX) or either of the E-cadherin constructs at a multiplicity of infection (MOI) less than one. In cells infected with the empty vector pLPCX, EGF and GM induced DNA synthesis in ~ 20 % and ~ 40 % of cells, respectively (Figure V-6C). Because E-cadHA or E-cadΔcytoHA cells were infected at a MOI of less than one, cells were classified as HA-positive or -negative. In E-cadΔcytoHA cells, both HA-positive and HA-negative cells synthesized DNA at rates comparable to empty vector cells, indicating that this construct had no effect on cell cycle progession. In cells infected with E-cadHA, the HA-negative cells (i.e., those not expressing E-cadHA) also synthesized DNA at rates comparable to the empty vector. However, HA-positive cells (i.e., cells expressing E-cadHA) showed a roughly 2-fold

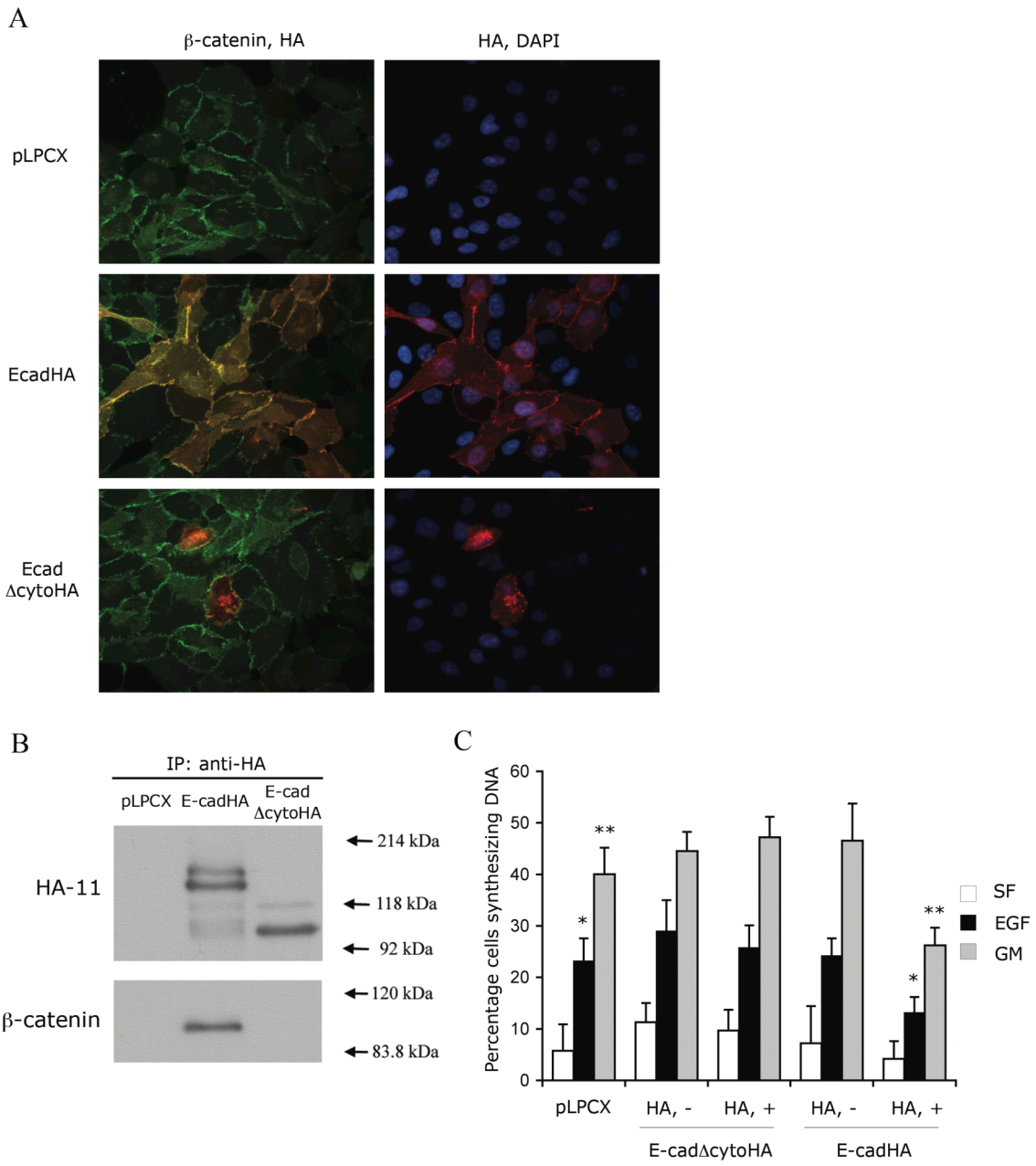

Figure V-6. Full-length E-cadherin, but not the cytoplasmic-domain truncated mutant, reduces DNA synthesis.
MCF-10A cells were retrovirally transduced with full-length E-cadherin (E-cadHA), the cytodomain-truncated E-cadherin mutant (E-cadΔcytoHA), or retrovirus generated with the empty vector pLPCX. (A) Immunofluorescent staining (β-catenin, green; HA, red; DAPI, blue). (B) Whole cell lysates were immunoprecipitated for the epitope tag HA and then immunoblotted for HA and β-catenin. (C) After serum-starvation, MCF-10A were stimulated with serum-free medium, EGF (20 ng/ml), or GM, and DNA synthesis was assessed by BrdU incorporation. Because cells had been infected at an MOI less than one, cells were classified as either HA-negative or HA-positive.

decrease in DNA synthesis in response to either EGF or full GM. Thus, overexpression of E-cadherin reduces cell cycle progression with a strict requirement for the β-catenin-binding domain.

2.5. Exogenous E-cadherin constructs interfere with EGFR signaling.

Because cadherins have been linked to decreased RTK signaling (Fedor-Chaiken et al., 2003; Grazia Lampugnani et al., 2003; Qian et al., 2004; Takahashi and Suzuki, 1996), we sought to verify that E-cadHA suppressed proliferation of MCF-10A without perturbing other RTK-mediated signals that are required for proliferation. In cells expressing E-cadHA or E-cadΔcytoHA, EGF-mediated phosphorylation of ERK was unaffected (Figure V-7). Akt activity, however, appeared slightly reduced in both E-cadHA and E-cadΔcytoHA cells. Surprisingly, E-cadΔcytoHA cells exhibited greatly reduced levels of EGFR tyrosine phosphorylation, even though ERK activity was unaffected. Assuming that phosphorylation is a relevant metric for signaling downstream of EGFR, this demonstrates that ERK activation is ultrasensitive with respect to EGFR phosphorylation, as demonstrated for other MAPK cascades (Ferrell and Machleder, 1998). Additionally, the fact that E-cadΔcytoHA does not affect proliferation yet diminishes EGFR and Akt activity illustrates the difficulty often encountered in parsing the effects of exogenous constructs on proliferation. Another complicating factor revealed by this experiment was the appearance of a heavily-phosphorylated species near 120 kDa, the approximate size of E-cadHA, in cells expressing E-cadHA. As such, additional approaches will be required to verify that E-cadherin-mediated growth

saturation of normal epithelial cells is specifically due to attenuation of β-catenin:Tcf/Lef transcription.

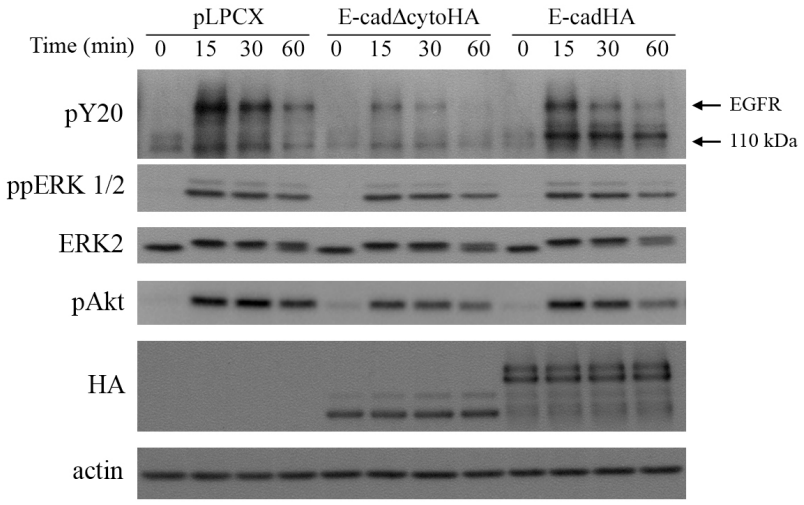

Figure V-7. Exogenous E-cadherin constructs do not affect ERK despite affecting EGFR and Akt phosphorylation.
MCF-10A cells retrovirally transduced with the empty vector pLPCX, exogenous full-length E-cadherin (E-cadHA), or the cytodomain-truncated mutant of E-cadherin (E-cadΔcytoHA). Cells were serum-starved, stimulated with EGF (20 ng/ml), and then lysed at the indicated times. Whole cell lysates were immunoblotted for phospho-tyrosine, dually-phosphorylated ERK (ppERK) 1/2, total ERK2, phospho-serine 473 Akt (pAkt), the epitope tag HA and the equal loading control actin.

2.6. Growth factor availability contributes to growth arrest.

Because there is some evidence that MCF-10A can deplete mitogenic growth factors from the culture medium (Figure V-3), we asked whether receptor-ligand trafficking and intracellular ligand degradation might also contribute to growth saturation. To test if the availability of mitogens affects proliferation, subconfluent MCF-10A were stimulated with low, intermediate, or high EGF concentrations (0.1, 1.0, or 10.0 ng/ml, respectively). To counteract mitogen depletion, EGF was refreshed every 24 h. Measurable proliferation was induced by all three growth factor concentrations (Figure V-8). Within 96 h of stimulation, however, cell growth reached a plateau that

was maintained for at least 10 days at all three EGF concentrations. Notably, the saturation density correlated with the growth factor concentration, suggesting that proliferation was controlled by the availability of EGF. Additionally, in cells treated with EGF at low concentration (0.1 ng/ml), growth saturation occurred at a subconfluent density (~ 2 x 10^4 cm^{-2}, see Figure V-1A for reference), implying that growth saturation may be independent of cell-cell contact.

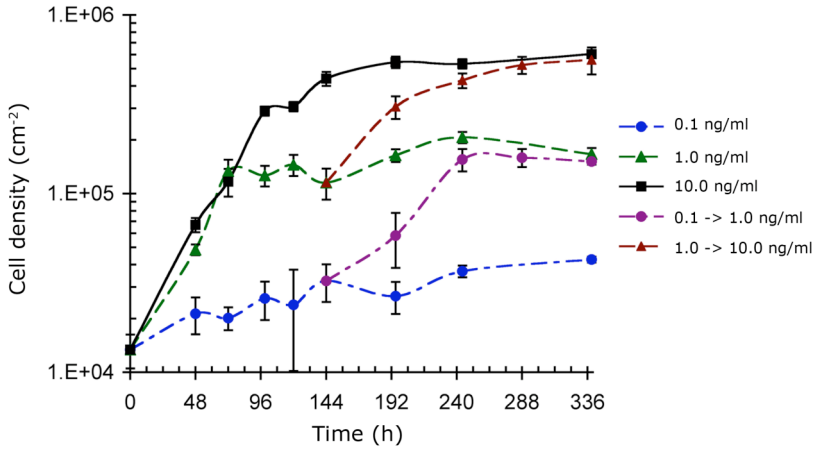

Figure V-8. Growth factor concentration-dependent saturation of cell growth
Serum-starved MCF-10A cells were stimulated with EGF (0.1, 1.0, or 10.0 ng/ml) and cell density was monitored by enzymatic lifting and counting cells approximately every 24 h. To prevent growth factor depletion, EGF was refreshed every 24 h. For some cells initially stimulated with 0.1 and 1.0 ng/ml EGF, the EGF concentration was increased to 1.0 and 10.0 ng/ml EGF, respectively, at 144 h.

Because the time when growth saturation occurred was independent of EGF concentration, we speculated that growth arrest may be controlled by the time in culture. To test whether the observed growth arrest was reversible, cells which had been growth saturated by repeated stimulation with low EGF concentration were stimulated with the intermediate EGF concentration (i.e., the concentration of EGF was increased from 0.1 ng/ml to 1.0 ng/ml). These cells immediately re-commenced proliferating, demonstrating that growth saturation is reversible and not simply a function of time. Consistent with the

hypothesis that growth factor concentration determines the saturation density, these cells growth saturated a second time, and the new saturation density exactly matched that of cells which had been originally growth arrested on the intermediate concentration of EGF (Figure V-8). Identical behavior was exhibited by cells switched from intermediate to high concentrations of EGF.

During the course of these growth experiments, we noted that subconfluent cells undergoing repeated exposure to low growth factor concentrations (0.1 ng/ml) formed cell colonies, similar to the phenotype exhibited by starved MCF-10A (Figure V-9A). Since cells on the interior of cell colonies can exhibit reduced tyrosine phosphorylation compared to perimeter cells (Lichtner and Schirrmacher, 1990), we reasoned that cell-cell contact might inhibit proliferation. However, measuring DNA synthesis as a function of

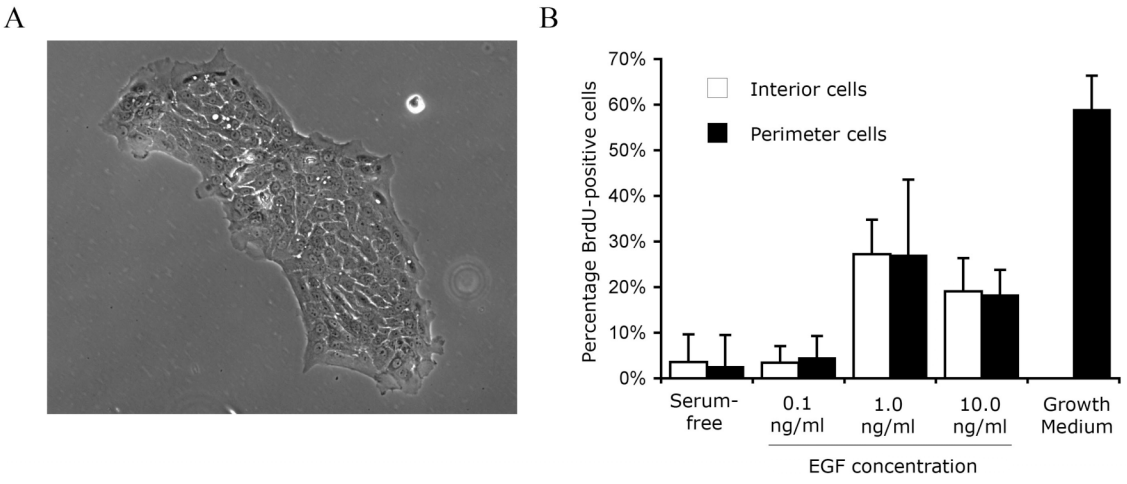

Figure V-9. Cell colony formation does not affect EGF-mediated proliferation.
(A) Phase contrast image of a cell colony formed by serum-starvation of MCF-10A. (B) Serum-starved MCF-10A, which had formed cell islands, were stimulated with serum-free medium, EGF at the indicated concentrations, or GM, and DNA synthesis was measured by BrdU incorporation. Cells that had incorporated BrdU were classified as interior or perimeter cells, according to their position within the cell islands. Because GM induces cell scatter, all GM-stimulated cells were considered perimeter cells.

location within cell islands revealed no difference in DNA synthesis between interior and exterior cells (Figure V-9B). Therefore, proliferation of MCF-10A organized in cell colonies appears independent of cell-cell contact status.

In Figure V-8, we found that EGF concentration determined the cell density at growth saturation. In that experiment, cells had been stimulated with varied concentrations of EGF in a constant volume of culture medium. Based on this data, we speculated that growth saturation occurred when cells had depleted EGF from the culture medium by receptor-ligand trafficking. According to this hypothesis, growth saturation depends on the total amount of EGF available to cells. To test this possibility, serum-starved cells were stimulated with different volumes of culture medium containing a constant, low concentration of EGF (0.1 ng/ml). As such, cells stimulated with larger volumes of culture medium will have the same initial concentration of growth factor, but will deplete the growth factor less quickly, perhaps leading to increased proliferation. Indeed, we found that cell density correlated linearly with the total quantity of EGF (Figure V-10A), supporting a model where the total availability of mitogenic ligands controls the density at growth saturation.

Moreover, because MCF-10A stimulated every 24 h with a high concentration of EGF (10 ng/ml) growth saturated at ~ 5 x 10^5 cells cm^{-2} (Figure V-8), we asked whether this was the true upper limit on cell density, or whether this was simply the point at which cells undergoing this stimulation regimen reached ligand-controlled growth arrest. To test if we could increase the saturation density of cells, we increased the total amount of

EGF 4-fold by increasing both the concentration of EGF (20 ng/ml, rather than 10 ng/ml) and the frequency of stimulation (every 12 h, rather than every 24 h). Indeed, this stimulation regimen induced cells to grow to a new saturation density of ~ 8 x 10^5 cells cm^{-2} (Figure V-10B). However, increasing the EGF dosage even further (50 ng/ml every 12 h) did not boost the saturation density above ~8 x 10^5 cells cm^{-2}, suggesting that there is a some physical limit beyond which MCF-10A cannot proliferate.

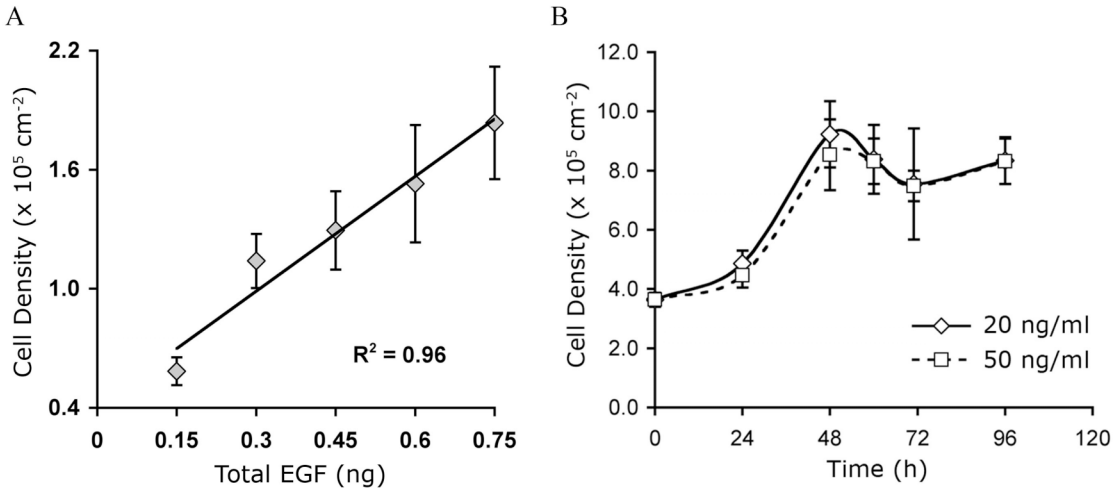

Figure V-10. Growth factor availability determines the cell density at growth saturation. (A) Serum-starved MCF-10A cells were stimulated with varied amounts of total EGF by supplying different volumes of culture medium while holding the growth factor concentration constant (0.1 ng/ml). To prevent growth factor depletion, EGF was refreshed every 24 h. After 7 days, cells were enzymatically lifted and counted in a hemocytometer. The total EGF is calculated as media volume times EGF concentration. (B) MCF-10A at high cell density were serum-starved and then stimulated every 12 h with EGF (20 or 50 ng/ml). At the indicated times, cells were enzymatically lifted and counted.

2.7. Diminished Akt, but not ERK, activity correlates with growth factor depletion-induced arrest

Although it is clear that the availability of mitogenic factors such as EGF can control growth arrest in MCF-10A, the relevant molecular mechanisms are unknown. We surmised that depletion of mitogenic factors would induce growth arrest when one or

more of the RTK-mediated signaling pathways crucially required for proliferation dips below some critical threshold. At this purported threshold, the amount of ligand present is unable to activate sufficient mitogenic signaling to sustain cell cycle progression. Since both ERK and Akt signaling are activated by EGF and required for proliferation of MCF-10A, we examined levels of these signaling molecules in cells stimulated every 24 h with low, intermediate, or high concentrations of EGF. Semi-quantitative immunoblotting revealed that neither ERK nor Akt activity decayed to basal levels at ~ 96 h, the time at which cells growth saturate (Figure V-8). However, at ~ 104 h, when all cells have growth saturated, cells stimulated with high EGF concentrations have noticeably more ERK activity than cells stimulated with low EGF concentrations (Figure V-11A), suggesting that ERK is not the limiting signal in density-dependent growth saturation.

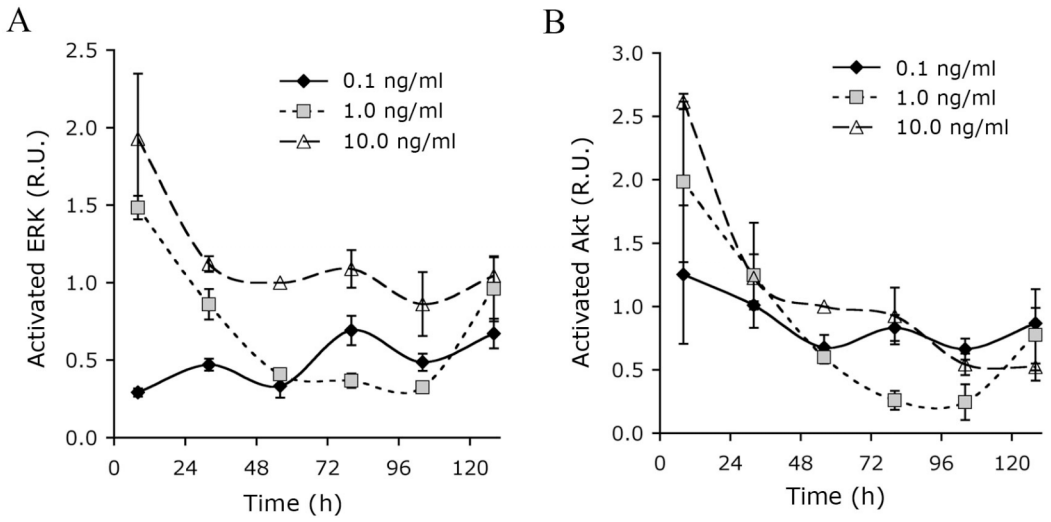

Figure V-11. Akt signaling, rather than ERK signaling, may control cell density at saturation. Serum-starved MCF-10A were stimulated with the indicated concentrations of EGF every 24 h for a total of 5 days. 8 h after every stimulation, cells were lysed and whole cell lysates were analyzed by semi-quantitative immunoblotting for (A) dually-phosphorylated ERK and (B) phospho-serine 473-Akt. Error bars represent sample standard errors (n=2).

In contrast, it appears that Akt activity in cells stimulated with low, intermediate, and high concentrations of EGF does converge to the same value at the approximate time when growth saturation occurs (~ 104 h) (Figure V-11B). However, because only two independent trials were performed, these conclusions are preliminary. Nevertheless, this data suggests that Akt, which is crucially required for proliferation, may be the signal which mediates growth factor depletion-induced growth saturation.

3. Discussion

Cell cycle arrest in dense cultures is a hallmark of non-cancerous cells that is thought to have relevance to oncogenesis. In this work, we have demonstrated that growth arrest of normal mammary epithelial cells (MCF-10A) is controlled by two anti-proliferative signals: contact-mediated suppression of β-catenin:Tcf/Lef signaling and density-dependent depletion of mitogens. Based on the data presented here, we propose that proliferation is controlled by a network that incorporates both of these anti-proliferative signals (Figure V-12).

According to this model, subconfluent cells, which have a low degree of cell-cell contact, proliferate when stimulated with mitogenic ligands, which induce multiple signals that mediate cell cycle progression (e.g., ppERK, pAkt, β-catenin:Tcf/Lef transcription, among others). As cells divide, cell density (and thus cell-cell contact) increases, stabilizing E-cadherin. Upregulation of E-cadherin sequesters β-catenin at the plasma membrane, attenuating β-catenin:Tcf/Lef signaling, a crucial mediator of cell

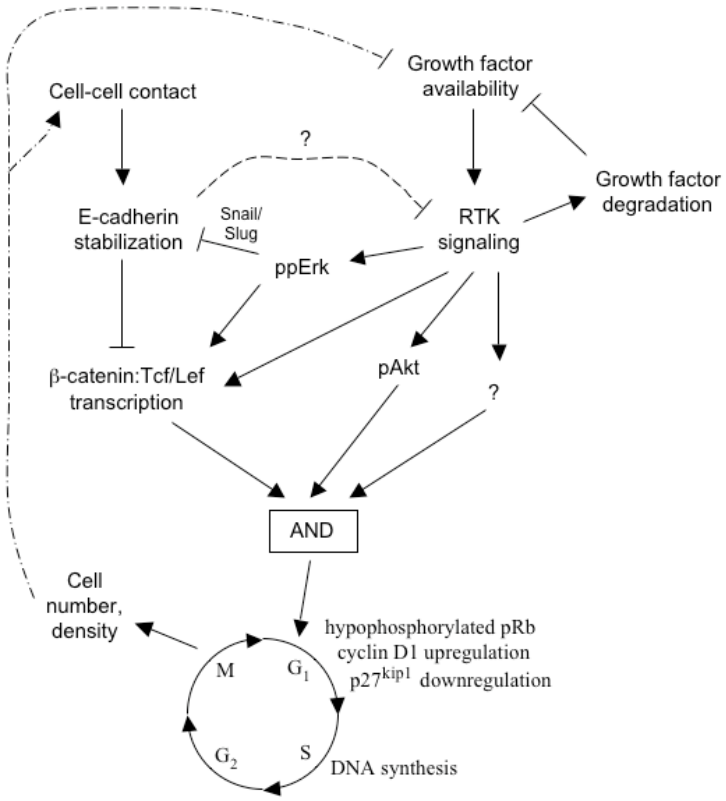

Figure V-12. Growth inhibition at high cell density in MCF-10A.
In non-tumorigenic cells, growth inhibition is mediated by two interconnected signals, one contact-dependent and one density-dependent. Contact inhibition of proliferation of MCF-10A is mediated by E-cadherin, which is stabilized by intercellular contact and inhibits β-catenin signaling through sequestration of β-catenin at the plasma membrane. Density-mediated inhibition of proliferation is controlled by receptor-ligand trafficking, which depletes mitogenic ligands from the cellular microenvironment. At high cell density, the available amount of growth factors is not sufficient to promote signals downstream of RTKs that are required for cell cycle progression, perhaps including Akt. Notably, the mechanisms of growth suppression by contact- and density-mediated signals are interconnected, since ppERK can cause transcriptional repression of E-cadherin through Snail/Slug, while E-cadherin-mediated adhesion may also inhibit RTK signaling.

cycle progression in MCF-10A (Graham and Asthagiri, 2004). Higher cell density also increases the rate at which receptor-ligand trafficking depletes mitogens from the cellular microenvironment. As a result, RTK signaling is decreased, attenuating downstream signaling pathways that are required for proliferation. As an additional consequence of decreased RTK signaling, E-cadherin expression may be increased through de-repression of Snail and Slug, transcriptional repressors of *E-cadherin* (Barrallo-Gimeno and Nieto, 2005). In turn, increased cell-cell contact may interfere with RTK signaling. Eventually,

all of these anti-proliferative signals funnel into the cell cycle, which is controlled by pRb, cyclins, and cyclin-dependent kinase inhibitors such as $p27^{kip1}$. Together, these factors prevent cell cycle progression of non-tumorigenic epithelial cells. Loss of one or all of these anti-proliferative mechanisms may underlie de-regulation of proliferation during oncogenesis.

It is noteworthy that growth saturation at high cell density is controlled by both contact- and density-mediated signals. Our data demonstrate that these signals can act semi-independently in certain contexts. In MCF-10A overexpressing cell-cell contact proteins, for example, cell cycle progression is suppressed in a manner strictly dependent on the cadherin-binding protein β-catenin (Figure V-6C). Alternatively, the density signal mediated by growth factor availability in MCF-10A stimulated with low mitogen concentrations appears independent of cell-cell contact (Figure V-9). However, the anti-proliferative signals from cell-cell contact and cell density are clearly not fully independent, a point underscored by the observation that overexpression of cell-cell contact proteins can perturb RTK signaling (Figure V-7). Additionally, cell cycle progression at low EGF concentrations may depend on cell-cell contact in certain situations (Jin-Hong Kim, personal communication).

3.1. Cadherins as anti-proliferative signals: Modulation of Tcf/Lef transcription via cell-cell contact

Cadherins have long been known to suppress both invasion (Vleminckx et al., 1991) and tumorigenicity (Navarro et al., 1991) of cancerous cells. The concept of

cadherins as anti-proliferative signals that modulate β-catenin:Tcf/Lef transcription has also received extensive support. Since the ability of cadherins to inhibit growth is dependent on β-catenin binding, rather than their adhesive function (Gottardi et al., 2001; Sasaki et al., 2000), upregulation of E-cadherin is likely the key step for contact inhibition of growth in MCF-10A. In similar fashion, another normal epithelial cell system exhibits increased expression of E-cadherin at confluency (Takahashi and Suzuki, 1996). Takahashi and Suzuki also demonstrated that increased E-cadherin expression was not accompanied by a concomitant increase in E-cadherin mRNA levels, suggesting that adhesion may indirectly inhibit β-catenin:Tcf/Lef signaling by stabilizing E-cadherin. Notably, density-mediated signals caused by growth factor depletion may also contribute to upregulation of E-cadherin, since the expression of Slug, a transcriptional repressor of E-cadherin, is regulated by ERK (Conacci-Sorrell et al., 2003).

Similar to our observations in MCF-10A, density-dependent Tcf/Lef transcription has been observed in bronchial epithelial cells (Steel et al., 2005), the engineered Fos:ER mammary epithelial cell line (Stockinger et al., 2001), and the colon carcinoma cell line SW480 (Conacci-Sorrell et al., 2003). Although SW480 do retain expression of E-cadherin, Conacci-Sorrell et al. made no link between Tcf/Lef signaling and proliferation, perhaps because these cancerous cells have lost the ability to contact inhibit. In fact, the key difference between this cancerous cell system and our normal cell system might be that SW480 express stabilized β-catenin due to inactive cytosolic degradation machinery (Korinek et al., 1997), whereas MCF-10A actively degrade β-catenin (data not shown). Thus, high cell density may reduce Tcf/Lef transcriptional activity in SW480, but perhaps

not sufficiently to block proliferation. By contrast, in non-transformed MCF-10A, which exhibit lower Tcf/Lef transcriptional activity than SW480 (Graham and Asthagiri, 2004), the density-dependent reduction of Tcf/Lef signaling is sufficient to inhibit proliferation. As such, this could be one reason that nontransformed cell lines contact inhibit, while many cancerous cell lines do not.

The concept of a threshold of Tcf/Lef signaling below which proliferation is inhibited may explain why we observe only partial (~ 50%) reduction in DNA synthesis upon retroviral expression of E-cadHA (Figure V-6C). By infecting cells with E-cadHA retrovirus at a MOI much less than one, we ensured that each cell carries at most one copy of *E-cadHA*, causing average overexpression of only ~ 2-fold (data not shown). As such, we hypothesize that only a fraction of cells expressing with E-cadHA had total E-cadherin levels sufficient to inhibit Tcf/Lef transcriptional activity and block DNA synthesis. Supporting this hypothesis, in cells transiently transfected with E-cadherin, Stockinger et al. observed only partial inhibition of DNA synthesis (~ 50-60%) at low expression levels, but nearly complete inhibition of DNA synthesis (> 90%) in cells expressing high levels of E-cadherin (Stockinger et al., 2001). Taken together, our data supports a mechanism whereby increasing the binding sites for β-catenin outside of the nucleus, whether by intercellular contact or overexpression of E-cadHA, induces cell cycle arrest by inhibiting β-catenin:Tcf/Lef transcriptional activity.

3.2. Cadherins as anti-proliferative signals: Cell-cell contact mechanisms independent of Tcf/Lef transcription

In addition to anti-proliferative signaling through β-catenin, cadherins also negatively affect proliferation via mechanisms independent of Tcf/Lef transcription. H-cadherin, for example, which lacks the catenin-binding domain, inhibits growth of several cancerous cell lines (Lee et al., 1998; Zhong et al., 2004), although the mechanism of growth suppression is unclear. The growth suppressive effects of cadherins have also been linked to attenuation of RTK signaling. According to these models, cadherin-mediated adhesion prevents activation of RTKs, inhibiting downstream signaling pathways that are required for cell cycle activity. The presence of vascular-endothelial-(VE-)cadherin, for example, can reduce VEGF signaling at high cell density, leading to decreased ERK phosphorylation and growth arrest (Grazia Lampugnani et al., 2003). VE-cadherin was found to bind the VEGF receptor (VEGFR) and exert its anti-proliferative effect by recruiting the junctional phosphatase DEP-1 to VEGFR:VE-cadherin complexes. This phosphatase then de-phosphorylates and inactivates VEGFR, ablating ERK signaling. Later studies in this same system found that VE-cadherin also negatively affects VEGFR signaling by preventing the internalization of VEGFR into signaling compartments (Lampugnani et al., 2006). In contrast to cadherin-mediated inhibition of β-catenin signaling, the ability of cadherins to inhibit RTK signaling requires the adhesive function of cadherins, as antibodies that inhibit E-cadherin function restore RTK signaling and DNA synthesis to growth-arrested cultures (Qian et al., 2004; Takahashi and Suzuki, 1996).

Notably, we have demonstrated that growth-arrested MCF-10A can still signal through EGFR and canonical pathways like ERK and Akt at levels similar to proliferating, subconfluent cells (Figure V-3). Thus, increased expression of endogenous E-cadherin at high cell density does not appear to perturb growth factor signaling, making MCF-10A a unique and interesting system even among normal epithelial cells. Interestingly, however, some domains of E-cadherin can affect RTK signaling in MCF-10A, since the exogenous construct E-cadΔcytoHA affected EGFR tyrosine phosphorylation, even without affecting proliferation (Fig V-7). Because E-cadΔcytoHA localizes to endocytotic vesicles (Figure V-6A), E-cadΔcytoHA may sequester EGFR in a subcellular locale that is inaccessible to EGF.

Nevertheless, our findings that contact inhibition of proliferation in normal epithelial cells is regulated by E-cadherin have implications for cancer biology. Since re-expression of E-cadherin can inhibit migration and invasion in some cancers (Chen and Obrink, 1991; Frixen et al., 1991; Vleminckx et al., 1991), it has been suggested that loss of E-cadherin may have a dual effect, permitting motility and invasion, as well as relaxing the constraints on proliferation. This work demonstrates the importance of E-cadherin in regulating proliferation of normal mammary epithelial cells, it seems plausible that the transcriptional inactivation of the *E-cadherin* gene may be a genetic lesion that confers multiple cancerous capabilities simultaneously (Hanahan and Weinberg, 2000; Stockinger et al., 2001; Wijnhoven et al., 2000).

3.3. Density-dependent models of growth suppression: Phosphatase-mediated inhibition of mitogenic signaling pathways

Independent of cadherins, reduced RTK signaling, particularly by upregulation of phosphatase activity, has been linked to growth suppression at high cell density (Machide et al., 2006; Mansbridge et al., 1992; Sorby and Ostman, 1996; Wayne et al., 2006). In primary cultured hepatocytes, for example, the HGF-receptor c-Met associates with the protein tyrosine phosphatase LAR at high cell density, downregulating c-Met activity and downstream signaling (Machide et al., 2006). Similarly, normal fibroblasts exhibit density-dependent decreases in ERK signaling due to upregulation of MAPK phosphatases (Wayne et al., 2006). According to these models, association of RTKs with phosphatases limits the duration of mitogenic signaling, preventing cell cycle progression. Confirming that phosphatases can mediate growth inhibition in these systems, functional inhibition of phosphatase activity, either by pharmacological agents or protein knockdown, releases cells from growth arrest. However, while it is clear that phosphatases inhibit growth in these systems, the density-dependent mechanisms that cause upregulation of phosphatase expression or activity are generally unknown.

Growth suppression of MCF-10A, by contrast, does not appear to be controlled by phosphatases. First, if phosphatases were mediating density-dependent growth suppression, one would expect significant downregulation of canonical signaling pathways such as ERK and Akt at high cell density. Second, treatment of EGF-stimulated cells with the phosphatase inhibitor sodium orthovanadate did not increase the density at growth saturation (data not shown).

3.4. Mitogenic ligand depletion as a mediator of growth suppression

In MCF-10A, density-mediated control of proliferation appears to be controlled by mitogenic ligand depletion. Upon binding of ligands such as EGF to RTK, the ligand:receptor complexes are internalized through clathrin-dependent and -independent pathways. In many cases, this process terminates RTK signaling via degradation of both the receptor and the ligand. Hence, proliferation can depend on the rate at which mitogenic ligands are depleted from the culture medium via receptor:ligand trafficking (Starbuck et al., 1990). In growth suppression of normal cells, ligand depletion was first observed by Holley and colleagues in NIH-3T3 fibroblasts (Holley, 1975). Supporting the hypothesis that ligand depletion can control growth in MCF-10A, increasing the quantity of EGF in the cellular microenvironment increased the density at growth saturation (Figure V-10).

For ligand depletion to induce cell cycle arrest, one or more of the proliferative signals mediated by RTKs must be attenuated at low EGF concentrations. Our data suggest that Akt may be this signal, which is intriguing because LeVea et al. attributed growth-inhibition of MCF-10A cells to highly transient Akt signaling (LeVea et al., 2004), although the mechanism underlying the transience of Akt signaling was unexplained. In our hands, however, Akt signaling did not decay to basal levels, even hours after stimulation with low concentrations of EGF (0.1 ng/ml). The basis for the discrepancies between LeVea et al. and this report is a topic for future investigation. Additionally, we cannot rule out the possibility that density-dependent inhibition of growth in MCF-10A is controlled by secretion of a growth-inhibitory molecule (Koga et

al., 1986; Steck et al., 1982), although this seems unlikely since the culture medium was replaced daily.

4. Future Work

In this report, we have demonstrated that growth inhibition of normal epithelial cells is controlled by both contact-mediated signals (E-cadherin expression) and density-mediated signals (growth factor depletion). However, there remain several questions that require further investigation in order to gain a more complete understanding of growth inhibition in MCF-10A.

4.1. The role of ERK and Akt in growth suppression

Our data suggest dense cultures of MCF-10A can activate mitogenic signaling pathways such as ERK and Akt equally as well as sparse cultures in some contexts (Figure V-3B). However, diminished Akt signaling may be functionally involved in growth suppression of MCF-10A, as suggested by two lines of evidence. First, although high-density MCF-10A are capable of activating Akt over short time scales, Akt signaling may be quantitatively reduced at late times. Second, semi-quantitative Western blotting of MCF-10A stimulated with low, intermediate, or high concentrations of EGF suggested that growth saturation occurs when Akt signaling converges to a common value (Figure V-11B), suggesting that Akt may the limiting factor in ligand depletion-mediated arrest. A functional role for Akt in growth suppression would particularly interesting since LeVea et al. reported that MCF-10A cells stimulated with intermediate

concentrations of EGF (5 ng/ml) demonstrated highly transient Akt signaling (LeVea et al., 2004).

Even if Akt signaling is reduced in magnitude or duration in high-density MCF-10A, other tests would be needed to confirm the hypothesis that a critical threshold of Akt signaling determines growth saturation. One tool that may be useful for parsing the role of Akt signaling in growth inhibition is LY 294002, a pharmacological inhibitor of PI3K, the kinase which activates Akt. At high doses, LY 294002 will completely block EGF-mediated proliferation of MCF-10A (LeVea et al., 2004); however, sub-saturating doses of Akt signaling inhibitors might partially, but not completely, reduce Akt activity. Then, if Akt signaling determines the point at which growth saturation occurs (Figure V-11), partially reducing Akt activity with LY 294002 would shift the density at growth saturation to a lower value. Alternatively, if Akt signaling is not controlling growth inhibition, partial reductions in its activity will not affect the cell density at saturation.

As a control, it might be possible to use sub-saturating doses of pharmacological inhibitors of the ERK pathway (e.g., PD98059, U1026) in the same manner. Since this signal does not appear to control the saturation density (Figure V-11), partial blockage may not affect saturation density. Finally, since complete inhibition of ERK will block cell cycle progression (Pages et al., 1993), it might be possible to use pharmacological agents to shift cells into a regime where growth is limited by ERK, rather than Akt.

4.2. Mechanism underlying upregulation of endogenous E-cadherin

Although overexpression of exogenous E-cadherin can partially suppress DNA synthesis (Figure V-6C), the mechanism by which E-cadherin expression increases ~ 4-fold in dense cultures (Figure V-5A), as well as the functional significance of this increased expression, remain to be explored. One possible mechanism underlying upregulation of E-cadherin is that formation of cadherin-cadherin bonds may stabilize E-cadherin protein, protecting it from degradation. As such, increased cell-cell contact could increase cellular levels of E-cadherin, even without increased transcription of the *E-cadherin* gene. As a first test of this mechanism, it is necessary to quantify *E-cadherin* mRNA levels by RT-PCR. Assuming that *E-cadherin* mRNA does not increase with cell density, the stability of E-cadherin protein in low and high-density cells could be measured by ^{35}S-methionine incorporation.

An alternative explanation for E-cadherin upregulation at high cell density could be downregulation of Slug, a transcriptional repressor of *E-cadherin* that is controlled by ERK signaling in human colon cancer cells (Conacci-Sorrell et al., 2003). Because ERK signaling can be repressed in high-density MCF-10A due to growth factor-depletion (Figure V-3), it would be interesting to construct a culture system that prohibited growth factor depletion (e.g., a perfusion culture system). Compared to parallel cultures without growth factor replenishment, is E-cadherin upregulated when ERK signaling is always active? Additionally, does expression of Slug decrease at high-cell density in non-growth factor-depleted cultures? Alternatively, one could test the role of ERK and Slug in upregulation of E-cadherin by introduction of a constitutively active mutant of ERK into

MCF-10A cells. Does constitutively active ERK induce Slug expression and thus repress E-cadherin upregulation? Together, these experiments could elucidate the role of increased protein stability and transcriptional repression in upregulation of E-cadherin at high density.

4.3. Functional significance of E-cadherin upregulation for growth suppression

In addition, whether E-cadherin upregulation at high cell density has functional significance for growth suppression remains unclear. In this report, we have demonstrated that E-cadherin expression is inversely correlated with β-catenin:Tcf/Lef transcription and DNA synthesis. To test whether endogenous E-cadherin is functionally related to these phenomena, one could knockdown expression of E-cadherin via lentiviral expression of E-cadherin shRNA. In MCF-10A cells expressing less E-cadherin, do cells still exhibit density-dependent inhibition of the TOPFLASH reporter and DNA synthesis? Additionally, does E-cadherin knockdown shift the DNA synthesis curve (Figure V-2) to the right? Conversely, using RNA duplexes that target the E-cadherin promoter (Li et al., 2006), one could increase expression of E-cadherin from the endogenous gene and then ask the same questions. For example, do cells expressing higher levels of E-cadherin growth inhibit at a lower saturation density?

5. Conclusions

Complex cell behaviors, such as proliferation, are generally not controlled by a single signal, pathway, or protein; rather, cells must integrate multiples signals to make

decisions. Using a non-tumorigenic mammary epithelial cell system, we have investigated the mechanisms controlling growth suppression at high cell density, a property of normal cells that is often lost during tumorigenesis. Notably, it seems that proliferation of normal cells is controlled by a complex interplay of contact- and density-dependent anti-proliferative signals including cadherins and growth factor depletion (Figure V-12). Further quantitative experiments are needed to fully understand how cells integrate multiple proliferative signals in order to achieve precise and dynamic control of cell growth. Along with other reports detailing the mechanisms of growth inhibition, these studies may pinpoint how cancer is able to hijack signaling pathways to drive hyperproliferation. Additionally, a quantitative understanding of growth reguation may suggest therapeutic targets that could curb aberrant proliferation of cancer cells without triggering deleterious effects on normal cells.

Acknowledgments

We would like to thank P. Wheelock and D. Schaffer for reagents, and M. Pope and J.-H. Kim for helpful discussions.

6. Experimental Procedures

6.1. Antibodies

The following antibodies were used in this study: anti-actin (Santa Cruz), anti-BrdU (Roche Applied Science), anti-ERK2 (Santa Cruz), anti-phospho-ERK 1/2 (Cell Signaling Technology), anti-phosphotyrosine (Santa Cruz), anti-phospho-serine 473-Akt (Cell Signaling Technology), anti-E-cadherin (BD Transduction Laboratories), anti-β-catenin (BD Transduction Laboratories), and anti-HA-11 (Covance).

6.2. Cell Culture

MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/mL EGF (Peprotech), 0.5 μg/ml hydrocortisone (Sigma), 0.1 μg/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and 1% penicillin/streptomycin. For serum starvation, cells were washed twice in PBS and then cultured with Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 1% penicillin/streptomycin and 0.1% bovine serum albumin (Sigma) for 24 h.

6.3. Plasmid Constructs

The human cDNA of E-cadherin (a kind donation of P. Wheelock, University of Nebraska Medical Center) was used for the generation of retroviral plasmids encoding full-length E-cadherin and the cytodomain-truncation mutant. The E-cadherin gene was amplified by PCR using the forward primer 5'-AAAAGATCTCACCATGGGCCCTTG-GAGCCGCAGC-3' and the reverse primer 5'-AAACTCGAGTCAGGCGTAGTCGGG-CACGTCGTAGGGGTAGGCGTAGCGGGCACGTCGTAGGGGTAGTCGTCCTCG-CCGCCTCC-3'. PCR products were then ligated into the retroviral backbone pLPCX

using BglII/XhoI sites, and the construct was confirmed by DNA sequencing. To generate the cytodomain-truncated mutant, the reverse primer 5'-AAACTCGAGTCAG-GCGTAGTCGGGCACGTCGTAGGGGTAGGCGTAGTCGGGCACGTCGTAGGGG-TATCTCCTCCGAAGAAACAGCAA-3' was used in PCR to delete residues 734-882 of E-cadherin. To facilitate detection of the exogenous proteins, two HA epitopes (YPYDVPDYA) were added to the C-terminus of each construct. The reporter plasmids pTOPFLASH and pFOPFLASH were purchased from Upstate Biotechnology, Inc., and pRL-TK was purchased from Promega. VSV-G and gag-pol vectors were gifts from D. Schaffer (University of California, Berkeley).

6.4. Retroviral Infection

Retrovirus was produced by triple transfection of 293T cells with 5 μg each of VSV-G, gag-pol, and the appropriate retroviral expression vector using LipofectAMINE (Invitrogen). For infection, MCF-10A were incubated with retrovirus-containing medium and 8 μg/mL polybrene for 24 h.

6.5. Cell Lysis

Cells were washed twice in ice-cold PBS and scraped in cold lysis buffer. After incubation on ice for 15 min, cell lysates were clarified by centrifugation, and the supernatant was collected as whole cell lysate. Protein concentrations were determined using BCA reagents (Sigma). For immunoblotting, cells were lysed in modified RIPA buffer, as described elsewhere (Graham and Asthagiri, 2004). Cell lysis for reporter assays was performed in 1X passive lysis buffer provided by the manufacturer (Promega).

6.6. Immunoblotting

Whole cell lysates were resolved by SDS-PAGE on 10% gels and blotted onto polyvinylidene difluoride membrane (Biorad). The membranes were blocked overnight and then incubated sequentially with primary and corresponding horseradish peroxidase-conjugated secondary antibody. The blots were treated with SuperSignal West Femto Substrate (Pierce) and imaged on VersaDoc 3000 (Biorad) using Quantity One software (Biorad).

For quantitative immunoblotting of E-cadherin, β-catenin, and actin in Figure V-5, whole cell lysates were loaded in increasing amounts in multiple lanes (typically 4 lanes with 3-6 μg of protein for each sample). The volume of each band was calculated as:

$$\text{Volume} = (\text{Band intensity} - \text{Background intensity}) \times (\text{No. pixels}) \qquad \text{(Eq.V-1)}$$

and the volume of each band was plotted versus μg of whole cell lysate. For samples that exhibited linear relationships between the band volume and total μg whole cell lysate ($R^2 > 0.95$), the ratios of the slopes for each lysate were then calculated, and this value was taken as the value of protein expression. This method not only ensures that each Western blots is in a linear dynamic range, but also automatically controls for variation in the protein concentrations of the lysates. For all samples, protein expression was internally normalized to that of cells at 9.7×10^4 cells cm^{-2} sample, and values from three independent experiments were averaged. For simplicity of interpretation, all values were then expressed relative to cells at the lowest measured density, 9.1×10^3 cells cm^{-2}.

For quantitative immunoblotting of dually-phosphorylated ERK and phospho-serine 473 Akt in Figure V-11, band volumes from whole cell lysates were calculated

using Equation V-1 and then normalized to standard curves. All samples were normalized expression of the equal loading control total ERK2 or total Akt, and then expressed as a value relative to a common standard.

6.7. Reporter Assays

MCF-10A were transfected with pTOPFLASH or pFOPFLASH and pRL-TK using Fugene-6 (Roche Applied Science), and then enzymatically lifted before replating of 0.5, 1.0, 3.0, and 10.0 x 10^5 cells per well of 6-well plate. Following serum-starvation, cells were stimulated with GM and reporter activity was quantified 9 h later (Promega). At the time of lysis, cell densities were determined by enzymatic lifting using trypsin, followed by cell counting in a hemocytometer.

6.8. DNA Synthesis Measurements

DNA synthesis was assayed by either ^{3}H-thymidine or BrdU incorporation. For DNA synthesis as a function of cell density, MCF-10A were plated at various initial seeding densities, serum-starved, and then stimulated with GM. To measure the effect of exogenous E-cadherin mutants on DNA synthesis, MCF-10A were plated at a subconfluent density of 10^5/35 mm dish, infected with either pLPCX, pLPCX-E-cadherinHA, or pLPCX-E-cadherinΔcytoHA retrovirus the following day, serum-starved, and then with serum-free medium, serum-free medium supplemented with 20 ng/mL EGF, or GM. 16 h after stimulation, the culture medium was replaced with identical medium supplemented with either 10 μCi/mL 3H-thymidine (ICN Biomedicals) or 10 μmol/liter BrdU (Roche Applied Science) and further incubated for 6 h. For thymidine incorporation assays, cells were then washed twice in ice-cold PBS, incubated in 5% trichloroacetic acid for 20 min at 4 °C, washed twice with cold 70% ethanol, and

incubated with 0.1 M NaOH, 2% Na_2CO_3, and 1% SDS for 30 min at 37 °C. The solution was collected and mixed with CytoScint (ICN Biomedicals) for scintillation counting. To normalize ^{3}H-thymidine incorporation, total cell numbers were calculated using a hemacytometer at the time of ^{3}H-thymidine addition. For BrdU detection, cells were fixed and co-stained with DAPI, anti-BrdU antibody, and anti-HA-11 antibody. The number of nuclei stained positive for BrdU and HA were quantified in 5-10 different fields from 5 independent trials using a Zeiss Axiovert 200M inverted microscope.

6.9. Immunofluorescence

For HA/BrdU co-staining, cells grown on glass coverslips were washed two times in ice-cold PBS, fixed in 4% formalin in PBS, and permeabilized in 0.2% Triton X-100. After blocking with 10% goat serum and 0.1% bovine serum albumin, the coverslips were sequentially incubated with primary and corresponding Alexa dye-labeled secondary antibodies (Molecular Probes). Following antibody incubations, the coverslips were stained with DAPI (Sigma) and mounted using Prolong Anti-Fade (Molecular Probes)

6.10. Immunoprecipitation

Approximately 200 μg of whole cell lysate was pre-cleared with Protein G beads (Pierce) in PBS plus 0.05 % Triton X-100. Pre-cleared lysates were then immunoprecipitated with 2 μg of anti-HA antibody coated on Protein G beads. After incubation for 90 min at 4 °C, beads were spun down by centrifugation, washed five times, and then boiled with sample loading buffer to elute the immunoprecipitated proteins from the beads.

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Chapter VI. Epithelial cell patterning by soluble ligands

Abstract

In metazoans, the epithelial-mesenchymal transition (EMT) is a fundamental process governing embryonic morphogenesis and metastatic cancer. In non-tumorigenic epithelial cells, we have investigated how soluble ligands regulate an EMT-like phenomenon, the aggregation and dissociation of cell-cell contacts. We demonstrate that deprivation of the soluble factors contained in growth medium induces cell aggregation, a phenomenon that is reversible upon restoration of growth medium. Among the soluble factors constituting growth medium, only epidermal growth factor and cholera toxin contribute to cell scattering; notably, these factors can synergize to induce greater scattering than either factor alone. Using pharmacological inhibitors, we begin to parse the mechanisms that control dissociation of intercellular junctions and cell migration. Taken together, these studies provide insight into the control of epithelial cell aggregation and dissociation by soluble factors, which may prove relevant to understanding EMT and metastasis.

1. Introduction

In general, epithelial cells are highly polarized, with distinct apical and basal domains, and characterized by strong intercellular adhesions. In contrast, mesenchymal cells are migratory, non-polarized cells embedded in an extracellular matrix. The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose many of their epithelial characteristics and acquire properties associated with mesenchymal cells (Thiery and Sleeman, 2006). During embryonic development, metazoans require the precise control of EMT to form many multicellular structures, including the parietal endoderm, the mesoderm, and the primitive streak during gastrulation (Vincent-Salomon and Thiery, 2003).

Notably, EMT is also prominent in the development of metastatic cancer. The majority of human cancers arise in epithelial tissues, which undergo an EMT to become migratory and invasive. EMT is characterized by several criteria, including the loss of epithelial polarity, separation into individual cells, and subsequent dispersion after the acquisition of cell motility (Vincent-Salomon and Thiery, 2003). Additionally, EMT is typically accompanied by loss of E-cadherin expression and a shift from expression of epithelial markers such as cytokeratins to mesenchymal markers like vimentin (Christofori, 2006).

Most studies in EMT involve *in vitro* assays where epithelial cell lines can be converted into fibroblast-like cells. Studies into the molecular basis of EMT have demonstrated that these phenomena can be controlled by soluble ligands in the cellular

microenvironment or by mutations that mimic ligand-mediating signaling. The ligands most prominently associated with EMT are hepatocyte growth factor (HGF) and transforming growth factor β (TGF-β). HGF was originally discovered as a protein secreted by stromal fibroblasts that could induce scattering of normal epithelial cells (Stoker and Perryman, 1985). Subsequent studies revealed that HGF binds to and activates the c-Met receptor tyrosine kinase, recruiting a number of adaptor molecules that activate downstream signaling pathways (Birchmeier et al., 2003). Similarly, TGF-β ligands mediate the assembly and activation of receptor complexes that activate the Smad family of transcription factors (Massague et al., 2000). For full induction of EMT, both HGF and TGF-β crucially require several common downstream signaling molecules, including small GTPases and signaling cascades such as ERK (Thiery and Sleeman, 2006). Together with cues from the extracellular matrix and cell-cell interactions, these extracellular signals then execute the complex genetic program involved in EMT.

Although several of the molecular pathways and cellular mechanisms controlling EMT have been identified, little is known about EMT-like phenomena in non-cancerous cells. In particular, what aspects of EMT can be recapitulated by non-tumorigenic epithelial cells *in vitro*? Using a non-tranformed, epithelial cell line (MCF-10A), we investigate how soluble factors such as the epidermal growth factor (EGF) and cholera toxin (ChT) influence epithelial cell plasticity. Notably, we demonstrate a synergistic interaction between EGF and ChT to induce dissociation of intercellular contacts, a phenomenon similar to the early stages of EMT. Quantitative studies of the mechanisms

controlling this EMT-like phenomenon should lead to a better understanding of EMT in development and metastasis.

2. Materials and Methods

2.1. Cell Culture

MCF-10A cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 containing HEPES and L-glutamine (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 0.5 μg/ml hydrocortisone (Sigma), 0.1 μg/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and 1% penicillin/streptomycin. For serum starvation, the cells were washed twice in PBS and then cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 1% (v/v) penicillin/streptomycin and 0.1% bovine serum albumin (Sigma) for 24 h.

2.2. Phase Contrast Microscopy

Phase contrast images were captured at 10X magnification using a Zeiss Axiovert 200M inverted microscope.

2.3. Pharmacological Inhibition

LY 294002 and PD 98059 were purchased from Calbiochem and reconstituted in DMSO. For experiments, cells were pre-treated with LY 294002 and PD 98059 for 15 or 60 min, respectively, before stimulation in the continued presence of these inhibitors.

3. Results and Discussion

3.1. Growth Medium controls the reversible formation of cell colonies.

To test whether normal epithelial cells can exhibit EMT-like behavior, we investigated whether MCF-10A cells, a non-tumorigenic epithelial cell line (Soule et al., 1990; Tait et al., 1990), exhibited EMT-like characteristics in tissue culture. When cultured at a subconfluent density in growth medium (GM), MCF-10A cells exhibit random scattering and minimal intercellular contacts (Figure VI-1A). However, when MCF-10A cells were starved of GM, we noted the formation of cell colonies, whereby cells formed "islands" with substantial cell-cell contacts with neighbors. In this starved state, cells were non-motile, although perimeter cells occasionally exhibited membrane ruffling or membrane protrusions (data not shown). Notably, the formation of cell colonies was reversible, as dissociation of cell colonies was induced by re-stimulation with growth medium (Figure VI-1B).

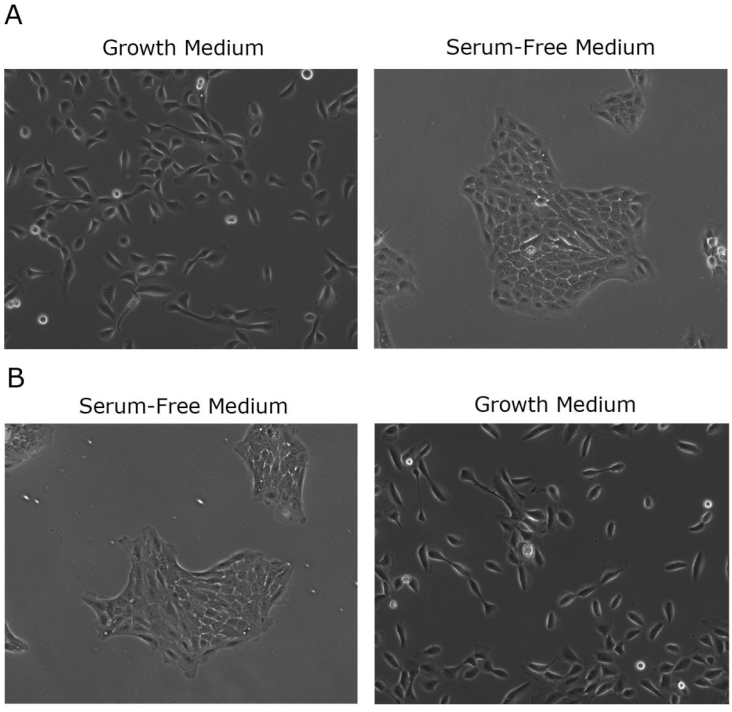

Figure VI-1. Reversible formation of epithelial cell colonies by growth medium starvation or stimulation (A) Subconfluent MCF-10A cells were either serum-starved or maintained in growth medium, and phase contrast images were captured 24 h later. (B) Serum-starved MCF-10A were stimulated with serum-free medium or growth medium, and phase contrast images were captured 24 h later.

3.2. EGF, but not serum, can prevent cell aggregation.

MCF-10A GM contains multiple soluble factors, including EGF, ChT, insulin, hydrocortisone, and serum factors, and the scattered phenotype of MCF-10A maintained in GM could be due to one or all of these factors. To parse the requirements for maintenance of the scattered phenotype, subconfluent cells that were initially scattered were deprived of various soluble factors. Surprisingly, in cells maintained in serum factors, formation of cell islands was robust and exhibited virtually no difference compared to fully-starved MCF-10A (Figure VI-2). In contrast, cells stimulated with EGF showed only partial aggregation into islands, with many EGF-stimulated cells remaining outside of cell islands and exhibiting migratory phenotypes. Additionally, cells treated with GM minus EGF (i.e., supplemented with ChT, hydrocortisone, insulin,

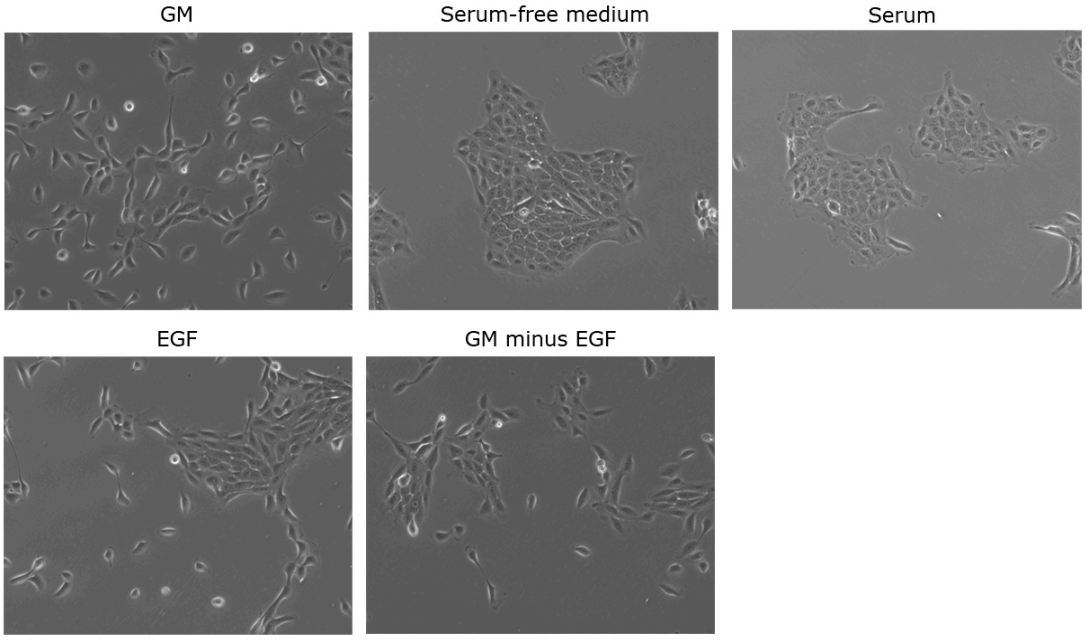

Figure VI-2. EGF, but not serum, prevents cell island aggregation.
Subconfluent MCF-10A cells maintained were either maintained in full GM or transferred to serum-free medium, serum-containing medium, EGF (20 ng/ml), or GM containing all factors except for EGF. Phase contrast images were captured 24 h later.

and serum factors) became partially aggregated, with many cells remaining outside of cell colonies.

3.3. EGF and ChT induce mild dissociation of cell colonies.

Having demonstrated that MCF-10A could partially maintain the scattered phenotype in the presence of either EGF or GM minus EGF, we asked whether these same factors could induce dissociation of cell islands. To test this question, we stimulated starved cell colonies with each factor individually (ChT, EGF, hydrocortisone, insulin, or serum factors) and assessed the degree of cell dissociation. Interestingly, EGF and ChT were the only components of growth medium to induce mild cell dissociation (Figure VI-3). However, even upon treatment with EGF or ChT, the majority of cells

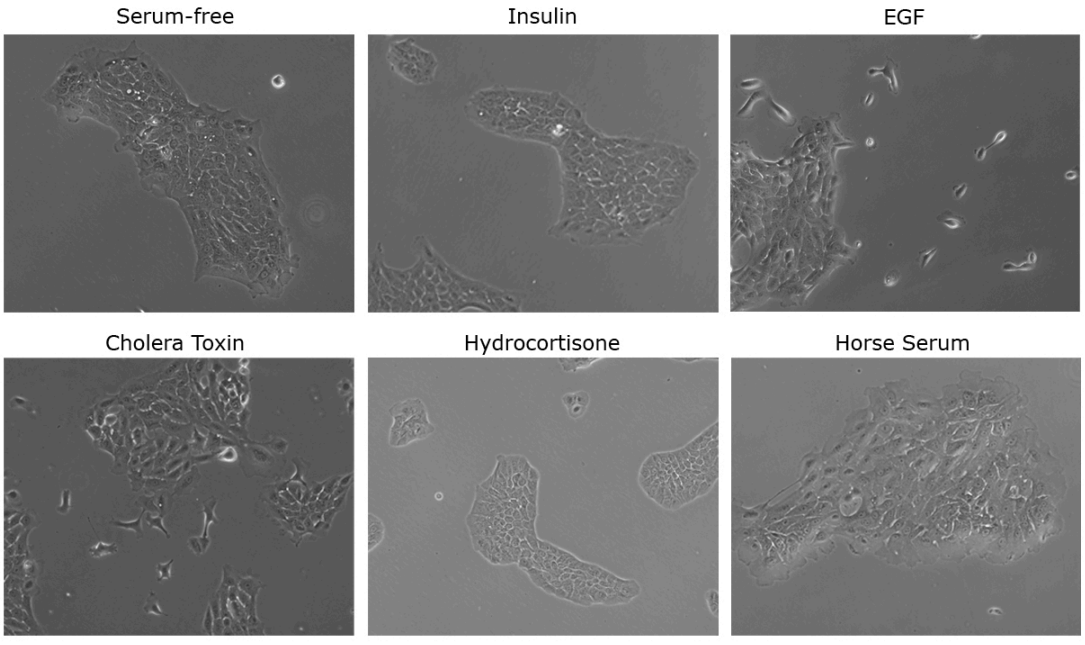

Figure VI-3. EGF and ChT are the only components of growth medium that induce cell colony dissociation.
Serum-starved MCF-10A cells were stimulated with serum-free medium, insulin, EGF, cholera toxin, hydrocortisone, or horse serum at concentrations identical to those of full GM. Phase contrast images were captured 24 h later.

remained in islands, with only a minority of cells "escaping." In contrast, hydrocortisone caused perimeter cells to form a smooth, rounded exterior, suggesting that hydrocortisone may inhibit basal membrane ruffling.

3.4. EGF and ChT cooperate to induce synergistic cell scattering.

Since neither EGF nor ChT alone reproduced the highly-scattered phenotype of GM-treated cells, we asked whether co-stimulation with these two factors could induce greater scattering than either factor alone. Indeed, co-stimulation of MCF-10A cell colonies with EGF and ChT demonstrated a synergistic response (Figure VI-4). Notably, co-stimulation with EGF and ChT still did not reproduce the fully-scattered phenotype of GM-treated cells, suggesting that there may exist further synergy between EGF, ChT, and another of the factors present in GM.

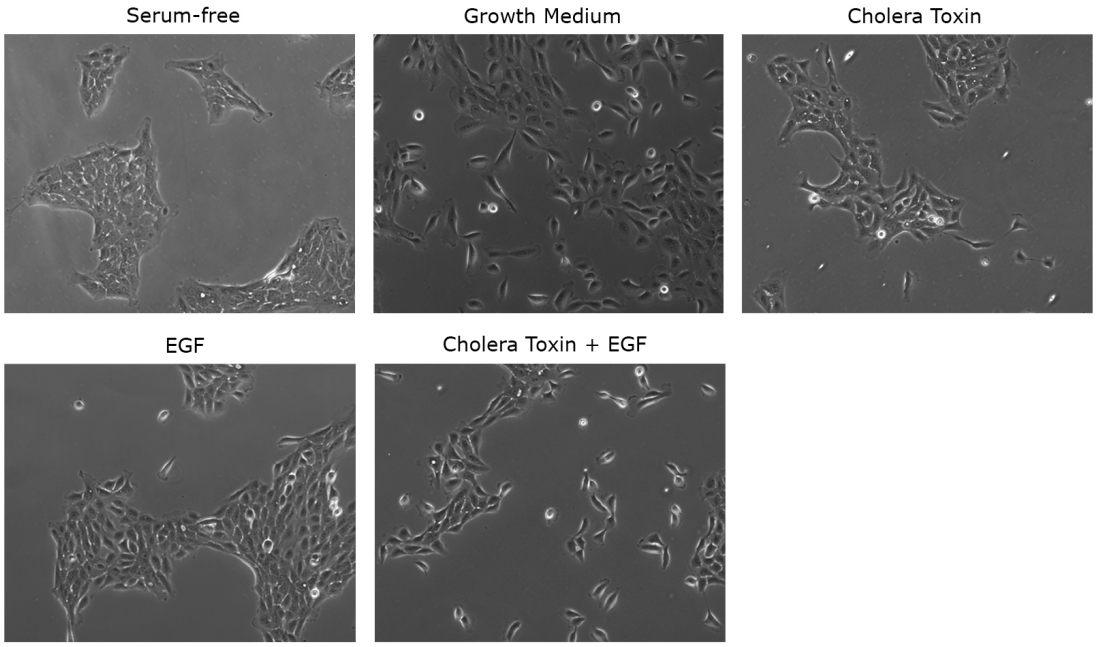

Figure VI-4. EGF and ChT can synergize to induce cell island dissociation.
Serum-starved MCF-10A cells were stimulated with either serum-free medium, full growth medium, cholera toxin (0.1 μg/ml), EGF (20 ng/ml), or cholera toxin plus EGF. Phase contrast images were captured 24 h later.

Among the components of GM, it is not surprising that EGF is a pro-scatter factor, since previous reports have demonstrated that EGF induces scatter in both non-tumorigenic epithelial cells (Matthay et al., 1993) and some carcinoma systems (Boyer et al., 1997; Edme et al., 2002; Lu et al., 2003). Additional support for the scatter capacity of EGF comes from the observation that overexpression of an active mutant of the EGFR family member ErbB-2 induced cell scattering in MDCK normal epithelial cells (Khoury et al., 2001).

In contrast to EGF and other RTK-binding ligands, ChT has not been associated with dissociation of intercellular junctions or induction of cell migration. *In vivo*, ChT exists as a hexamer, with one A subunit and five B subunits. The B subunits bind to cell surface gangliosides and mediate entry of the hexamer into the cell. Once inside the cell, the A subunit activates the intracellular G-protein $G_{s\alpha}$, inducing dissociation of $G_{s\alpha}$ from $G_{s\beta\gamma}$. This in turn activates adenylate cyclase, increasing in intracellular cAMP concentrations and activating protein kinase A (PKA) (Salmond et al., 2002). Because PKA has been shown to be active in leading-edge, protrusive structures and essential for chemotaxis in fibroblasts (Howe et al., 2005), PKA may be responsible for the pro-migratory effects of ChT MCF-10A.

In addition to its effects on intracellular cAMP, ChT stimulation has been reported to induce secretion of a variety of cytokines, including TNFα (Viana et al., 2002; Yan et al., 1999) and a variety of interleukin family members (Braun et al., 1999; Bromander et al., 1991; Cong et al., 2001; McGee et al., 1993). These cytokines,

however, are not known to stimulate dissociation of intercellular junctions or cell migration. In addition to TNFα and interleukins, ChT has also been reported to cause a 2-fold increase in TGF-β1 mRNA levels (Kim et al., 1998), which is intriguing because TGF-β signaling plays a significant role in EMT (Christofori, 2006; Thiery, 2003). In support of the connection between ChT and TGF-β, Lewis rats which were administered a conjugate of ChT subunit B and myelin basic protein were found to have increased levels of TGF-β in the spinal cord (Sun et al., 2000). Because TGF-β can induce scattering of pancreatic cancer cells (Ellenrieder et al., 2001), ChT-induced scattering of MCF-10A cells might be an indirect result of ChT-induced TGF-β secretion.

3.5. MAPK and PI3K control various aspects of cell scattering.

Because studies have demonstrated that phosphatidylinositol 3-kinase (PI3K) is required for EMT in several contexts (Larue and Bellacosa, 2005), we investigated the role of PI3K in MCF-10A cell scattering. In cells treated with LY 294002, a pharmacological inhibitor of PI3K kinase activity, GM-induced scattering was minimal (Figure VI-5A). However, the intercellular contacts did appear to loosen, with some cells dissociating from their neighbors but unable to migrate away from cell islands. This "loosening" of cell junctions could be a result of incomplete inhibition of PI3K activity by LY 294002. Alternatively, PI3K may not be involved in the breakdown of cell-cell junctions, a necessary step for dissociation of cell colonies. Similarly, inhibition of PI3K in MDCK transformed with active ErbB-2 did not affect breakdown of cell-cell junctions (Khoury et al., 2001).

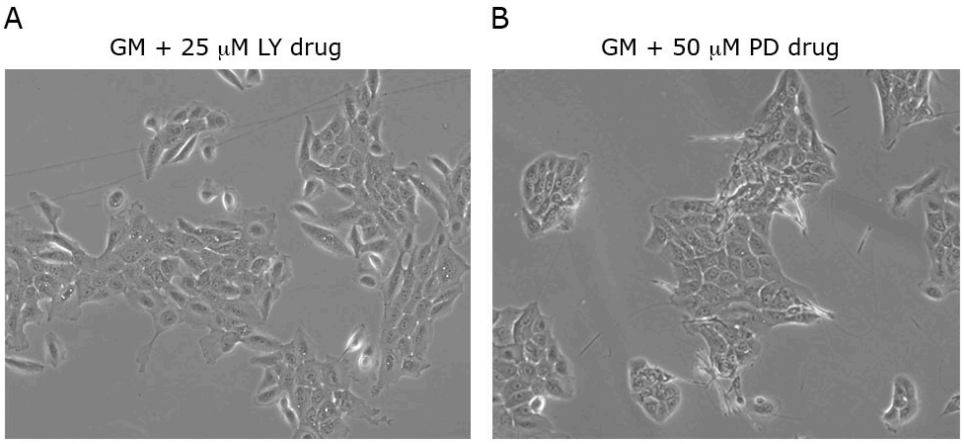

Figure VI-5. PI3K and MAPK are required for different aspects of GM-induced cell scattering. Serum-starved MCF-10A cells were pre-treated with the indicated concentrations of (A) the PI3K inhibitor LY 294002 or (B) the MAPK inhibitor PD 98059 and then stimulated with GM in the continued presence of the pharmacological inhibitors. Phase contrast images were captured 24 h later.

In other epithelial cell models, the ERK signaling pathway is required for HGF-mediated breakdown of cell junctions (Potempa and Ridley, 1998). To test the role of ERK in scatter of MCF-10A, we inhibited MEK, the kinase upstream of ERK, with the pharmacological agent PD 98059. In contrast to PI3K, blocking activation of ERK completely ablated both dissociation of cell junctions and cell scattering in MCF-10A stimulated with GM (Figure VI-5B). This confirms other reports that have demonstrated an absolute requirement for ERK activity in EGF-induced cell scattering (Boyer et al., 1997; Edme et al., 2002; Khoury et al., 2001). Also, given the possible link to TGF-β signaling through ChT, it is noteworthy that ERK is required for TGF-β mediated disruption of adherens junctions, cell scattering, and EMT (Ellenrieder et al., 2001; Zavadil et al., 2001).

3.6. Relevance of cell scattering to EMT

Two of the criteria for an EMT are separation of epithelial cells into individual cells and subsequent dispersion after acquisition of cell motility (Vincent-Salomon and

Thiery, 2003). As such, the aggregation and scatter phenomena exhibited by MCF-10A cells recapitulate a small portion of the EMT. Other criteria for a full EMT include the loss of epithelial polarity, expression of mesenchymal proteins such as vimentin, and the loss of E-cadherin expression. While we have not explicitly tested for these characteristics, it is doubtful that MCF-10A cells undergo a full EMT. For example, the scattering phenomena observed in MCF-10A are fully reversible on a short time scale (~ 24 h) (Figure VI-1). In a similar but transformed mammary epithelial cell system (EpRas), FGF and HGF induced an EMT-like, spindle migratory phenotype, but neither growth factor was able to induce a full mesenchymal phenotype (Janda et al., 2002). Given the similarity between our system and growth factor-stimulated EpRas cells, MCF-10A probably do not exhibit additional EMT characteristics such as lasting loss of E-cadherin expression or mesenchymal gene expression patterns. In addition, when MCF-10A reach confluency, cells become non-motile and express high levels of E-cadherin, even in the presence of GM (data not shown). Thus, the EMT-like phenomena in MCF-10A likely do not represent a full EMT.

The inability of *in vitro* culture assays to recapitulate the full extent of EMT is well-known (Vincent-Salomon and Thiery, 2003); however, phenomena such as cell scattering still can provide some insight into the mechanisms regulating EMT *in vivo*. For example, native MCF-10A cells are considered non-tumorigenic, as evidenced by the fact that MCF-10A do not form tumors in nude mice (Soule et al., 1990). Thus, the ability of MCF-10A to scatter in response to soluble factors such as EGF and ChT demonstrates the degree of plasticity exhibited by normal epithelial cells. By comparing

the EMT-like capabilities of normal epithelial cell systems to other systems that exhibit metastatic phenotypes *in vivo*, it may be possible to delineate the acquired capabilities of cancer cells that permit EMT, invasion, and metastasis (Hanahan and Weinberg, 2000).

4. Future Work

The results discussed here describe a system for the study of cell aggregation and scattering in non-tumorigenic epithelial cells. These preliminary studies suggest several additional lines of experimentation needed to gain a more thorough understanding of the molecular mechanisms underlying these EMT-like phenomena.

4.1. Further elucidation of the mediators of EGF- and ChT-induced cell scatter

Although preliminary examination of GM-mediated cell scatter has pinpointed specific roles for PI3K and ERK, the contribution of other signaling molecules remains unexplored. Since EGF and ChT can synergize to induce cell scattering (Figure VI-4), a first test should be to probe the role of several other prominent signaling molecules using pharmacological inhibitors. In particular, because ChT upregulates intracellular cAMP concentrations, it would be interesting to test whether inhibition of PKA affects cell scattering. Additionally, since the Src kinase has been implicated in EGF-induced scattering of NBT-II epithelial cells (Boyer et al., 1997), it would be interesting to test whether the Src inhibitor PP2 affects cell scattering. To strengthen conclusions made with pharmacological agents, retroviral expression of dominant-negative proteins or siRNA-mediated protein knockdown could be used to test the role of MAPK, PI3K, Src,

and/or PKA in cell scattering. Finally, because ChT may induce autocrine production of migratory ligands, particularly TGF-β, it would be interesting to probe the existence of autocrine signaling loops using either neutralizing antibodies or soluble decoy receptors.

4.2. The role of cadherins and other adhesion proteins in cell scattering

In epithelial cells, E-cadherin regulates intercellular adhesion and has been implicated in both EMT and cancer progression (Thiery, 2002). E-cadherin is a single-pass transmembrane protein whose extracellular domain homotypically binds E-cadherin molecules on neighboring cells. The intracellular domain of E-cadherin binds β-catenin, which in turn binds α-catenin and bridges E-cadherin to the actin cytoskeleton. Notably, it has been suggested that the reorganization of cells into colonies, such as that observed upon growth factor deprivation of MCF-10A cells (Figure VI-1A), is mediated by E-cadherin and the subsequent re-organization of the actin cytoskeleton (Adams et al., 1998).

The loss of E-cadherin function has also been implicated in EMT, since blocking E-cadherin interactions with inhibitory antibodies can induce mesenchymal phenotypes (Imhof et al., 1983). Some scatter-inducing growth factors have also been linked to downregulation of E-cadherin. For example, HGF has been shown to induce cell scattering through MAPK/Egr-1-mediated upregulation of Snail, a transcriptional repressor of E-cadherin (Grotegut et al., 2006). As such, it would be interesting to test whether E-cadherin is functionally involved in both the cell aggregation (Figure VI-1A) and cell scattering (Figure VI-1B) phenomena in MCF-10A. One method for testing the

role of E-cadherin in these processes would be to selectively deplete E-cadherin by siRNA. Alternatively, one could inhibit E-cadherin function via anti-E-cadherin antibodies. Using these tools, one could ask, do MCF-10A deprived of soluble factors aggregate in absence of cadherin-mediated adhesion? Alternatively, if E-cadherin-mediated cell contacts are required for cell colony formation, does E-cadherin overexpression reduce cell scattering? In addition to classical overexpression techniques, it might be possible to increase E-cadherin expression using RNA duplexes that target the endogenous E-cadherin promoter (Janowski et al., 2007).

Although the adhesive function of E-cadherin may play a role in EMT-like phenomena, the E-cadherin-binding partner β-catenin may also promote cell scatter, since EGF-mediated β-catenin:Tcf/Lef transcription can contribute to processes resembling EMT (Lu et al., 2003; Muller et al., 2002). Other growth factors such as IGF-II also induce β-catenin signaling during EMT (Morali et al., 2001). Although these reports did not demonstrate a functional requirement for β-catenin in EMT, siRNA knockdown of β-catenin levels ablated HGF-induced cell scattering (Rasola et al., 2007). Additionally, Rasola et al. demonstrated that cell scattering was induced by expression of a constitutively active, non-degradable β-catenin mutant, even in the absence of HGF. Thus, β-catenin may promote EMT though gene transcription, particularly because Tcf/Lef target genes include matrix metalloproteinases (Brabletz et al., 1999; Takahashi et al., 2002), which can degrade the basement matrix during metastasis, and Slug (Vallin et al., 2001), a transcriptional repressor of E-cadherin.

In MCF-10A, we have previously demonstrated that EGF induces β-catenin:Tcf/Lef signaling (Graham and Asthagiri, 2004). As such, does β-catenin signaling play a role in EGF- and ChT-induced cell scattering? As a first test of this question, does siRNA-mediated depletion of β-catenin inhibit cell scattering? Since β-catenin regulates expression of Tcf/Lef gene targets in MCF-10A, would expression of dominant-negative Tcf4, which ablates Tcf/Lef transcription, affect cell scattering? If Tcf/Lef gene targets are functionally involved in EGF-mediated cell scatter, would inhibition of individual Tcf/Lef target genes block cell scatter? Methods to test this last question include pharmacological inhibition of matrix metalloproteinases or siRNA directed against individual Tcf/Lef target gene products. Answers to these questions would provide meaningful insight into the cellular mechanisms regulating cell scattering by soluble factors.

4.3. Quantitative metrics of cell scatter

The observations of cell aggregation and scatter in this report are highly qualitative. While qualitative analysis is informative, quantitative metrics might permit more rigorous dissection of the mechanisms underlying cell scattering. One semi-quantitative metric for characterization of cell scattering is the percentage of cells expressing membrane-localized desmoplakin (Boyer et al., 1997; Edme et al., 2002), since desmoplakin is indicative of desmosomes, which mediate intercellular contact. However, quantification of desmoplakin membrane localization requires fixation and immunostaining of cells, as well as subjective judgments about what constitutes membrane localization.

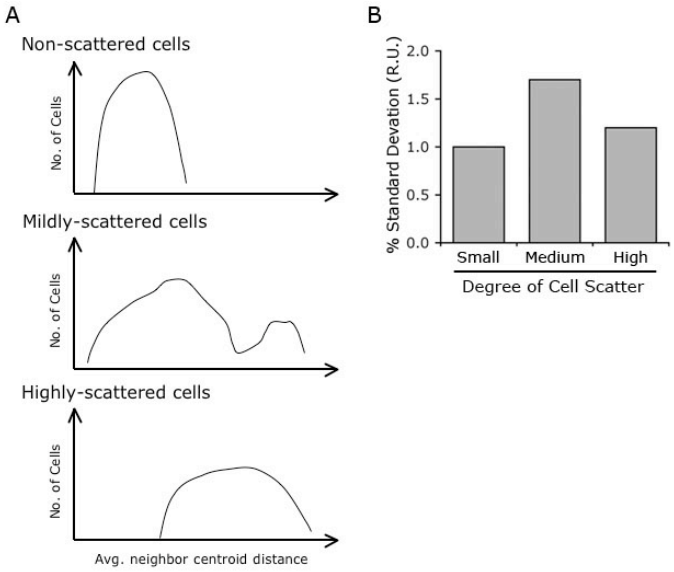

Figure VI-6. Average neighbor centroid distance as a metric for quantification of cell scattering
A representative image of the proposed metrics for quantification of cell scattering: (A) the average distance between neighboring cells and (B) the percent standard deviation of the average neighbor distance, expressed in Relative Units (R.U.)

Ideally, a quantitative metric of cell scattering would be objective and derived from live cell imaging, so that one could track the temporal evolution of scatter. One technique that has been used for quantification of cell movement in collagen gels is the annular grayscale method (Vernon and Gooden, 2002), which measures how far cells migrate away from an initially circular colony. Although MCF-10A cell colonies are not strictly circular (Figure VI-1), adaptation of this method for quantification of irregularly-shaped colonies is theoretically possible. Other simple metrics for quantification of cell colony dispersal include the percent of colonies that are dissociating, as well as the percentage of single cells migrating away from colonies (Jourquin et al., 2006).

Another possible metric for cell scatter is the average distance between a cell's centroid and the centroids of its nearest-neighbors (i.e., the ten closest cells). Highly

scattered cells (e.g., GM-treated cells) would exhibit average neighbor distances much greater than one cell diameter (Figure VI-6A), whereas cells organized in colonies (e.g., fully-starved cells) would have average neighbor distances of approximately one cell diameter (Table VI-1). One drawback of this metric is that distinguishing mildly-scattered cells (e.g., EGF-treated cells) from non-scattered cells would be difficult because the small percentage of mildly-scattered cells would only slightly increase the average neighbor distance above one cell diameter. To distinguish these relatively similar cell patterns, a useful metric might be the percent standard deviation of the average neighbor distances (Figure VI-6B). Because mildly-scattered cells will have a few cells that are highly detached from neighbors, the percent standard deviation of the average neighbor centroid distances should be much higher for mildly-scattered cells than for non-scattered cells.

Table VI-1. Potential Scatter Metric: Average and percent standard deviation of neighbor distance

	Low Scatter	Medium Scatter	High Scatter
Average distance of 10 nearest neighbors	Low (~1 cell diameter)	Fairly low (<2 cell diameters)	High (>2 cell diameters)
% Standard Deviation of Average Nearest Neighbor Distance	Low	High	Low (?)

5. Conclusions

In this report, we demonstrate a system that recapitulates one of the earliest hallmarks of EMT, the detachment of epithelial cells from their neighbors and the acquisition of a migratory phenotype. Using the non-tumorigenic epithelial cell line MCF-10A, we show that subconfluent cells aggregate into islands when deprived of GM;

this aggregation phenomenon is reversible, as restoration of GM re-establishes cell scattering. By individually assessing the contributions of each of the soluble factors in GM, we find that cell scattering is controlled by both EGF, a ligand known to regulate EMT, and ChT, a ligand not known to be involved in epithelial cell plasticity. Interestingly, these ligands can function synergistically to induce greater cell scatter than either ligand alone. Finally, pharmacological inhibition of the PI3K and MAPK signaling pathways demonstrates that PI3K may be involved in cell-cell junction disassembly, but not migration, whereas MAPK may be essential for both processes. Notably, this report leaves several questions unanswered. In particular, the mechanisms connecting EGF and ChT to dissolution of cell-cell junctions remain to be elucidated. Some intriguing possibilities, including the involvement of the cell-cell adhesion protein E-cadherin, intracellular kinases such as Src and PKA, and autocrine production of TGF-β, also remain to be explored. As such, the MCF-10A system may prove ideal for investigating the mechanisms underlying the plasticity of non-tumorigenic epithelial phenotypes, EMT, and metastasis.

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