

Chapter III. AUTOMATED QUANTITATIVE ANALYSIS OF EPITHELIAL CELL SCATTER

1. Abstract

Epithelial cell scatter is a well-known *in vitro* model for the study of epithelial-mesenchymal transition (EMT). Scatter recapitulates many of the events that occur during EMT, including the dissociation of multicellular structures and increased cell motility. Because it has been implicated in tumor invasion and metastasis, much effort has been made to identify the molecular signals that regulate EMT. To better understand the quantitative contributions of these signals, we have developed metrics that quantitatively describe multiple aspects of cell scatter. One metric (cluster size) quantifies the disruption of intercellular adhesions while a second metric (nearest-neighbor distance) quantifies cell dispersion. We demonstrate that these metrics delineate the effects of individual cues and detect synergies between them. Specifically, we find epidermal growth factor (EGF), cholera toxin (CT) and insulin to synergistically reduce cluster sizes and increase nearest-neighbor distances. To facilitate the rapid measurement of our metrics from live-cell images, we have also developed automated techniques to identify cell nuclei and cell clusters in fluorescence images. Taken together, these studies provide broadly applicable quantitative image analysis techniques and insight into the control of epithelial cell scatter, both of which will contribute to the understanding of EMT and metastasis.

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2. Introduction

Epithelial cells have an intrinsic ability to self-assemble into multicellular structures. For example, submandibular gland (SMG) epithelial cells isolated from embryonic mice retain the ability to self-organize into branched tissue aggregates in a manner analogous to that which occurs *in vivo*.¹ These epithelial tissues have a distinct, well-ordered structure. Namely, constituent cells tightly adjoin to their neighbors and form highly polarized multicellular sheets that provide physical barriers between external and internal environments. In addition, epithelial cells are motile, in that they can move away from their neighbors, but generally remain within the epithelial layer.²

Many epithelial tissues are dynamic structures that undergo constant regeneration. Disruptions in the self-assembly and maintenance of epithelial cell structures can have drastic pathological consequences such as cancer development.³ These physical disruptions are driven by molecular perturbations that alter cell behavior. For example, oncogenes such as *c-met* break up cellular aggregates and promote cell dispersion.⁴ At a single cell level, these molecular perturbations induce an epithelial-mesenchymal transition (EMT). During EMT, epithelial cells lose apical-basolateral polarity and transform into a more migratory phenotype, resembling fibroblasts. This allows cells to dissociate from the epithelial layer and disperse.⁵

Because of the clinical and physiological significance of EMT, much effort has been made to identify the molecular signals that control this process. An important tool for the *in vitro* study of EMT is the cell scatter assay, in which two-dimensional epithelial

aggregates dissociate in response to extracellular stimuli. While advances are being made in cataloguing the signaling pathways that control scatter, an emerging challenge is to understand the quantitative contributions of these signals and any coupling between them. For example, which stimuli are the most potent effectors? Which signals work synergistically? Which signals work antagonistically?

A challenge to answering these questions is that current studies of cell scatter are largely qualitative. Prevalent in the limited quantitative literature are studies that score cellular aggregates as “scattered” based on an observed morphological change – for example the appearance of space between cells, the disappearance of membrane-localized desmoplakin or a reduction in the number of cell-cell adhesions.⁶⁻⁹ Such analyses permit comparison between experimental conditions but provide little insight into the cellular-level response.

In this work, we introduce quantitative metrics to systematically characterize multiple aspects of epithelial cell scatter. One metric is the aggregate size, which quantifies the degree to which cells have disrupted intercellular adhesions. Another metric is the distance between a cell and its nearest neighbor, which evaluates the extent to which cells disperse. Our data shows that these metrics delineate the effects of individual molecular signals and detect synergies between them. Specifically, we find epidermal growth factor (EGF) to be essential for scattering non-transformed human mammary epithelial cells (MCF-10A) and to synergize with both cholera toxin (CT) and insulin to reduce aggregate size and increase internuclear distances. To facilitate the

rapid measurement of our metrics from live-cell images, we have also developed automated techniques to identify cell nuclei and multicellular aggregates in fluorescence images. In summary, this work provides an experimental methodology and high throughput techniques that will prove useful for gleaning quantitative insights into EMT.

3. Results

3.1. EGF regulates MCF-10A scatter

When deprived of the soluble factors contained in growth medium (GM), MCF-10A cells aggregate into well-defined clusters (Figure III-1A). Upon re-addition of GM, cells scatter (Figure III-1B). GM has several components, among them EGF, which has been shown to induce scatter in multiple cell lines.^{7, 8, 10, 11} When EGF is omitted from GM, cell scatter is noticeably reduced (Figure III-1C). However, EGF alone is unable to induce scatter (Figure III-1D). Therefore, EGF appears to be required for scatter but not sufficient to induce a response. We next performed a qualitative screen to identify additional components of GM that contribute to scatter. Among them were CT and insulin. CT appears to synergize with EGF to induce scatter (Figure III-1E), while the further addition of insulin makes little noticeable enhancement (Figure III-1F) even after comparing across 16 different fields (Supplemental Data). Thus, based on this qualitative analysis, we conclude that (1) although EGF is essential for cell scatter, it alone does not induce cell scatter, and (2) CT, but not insulin, significantly synergizes with EGF to induce cell scatter. To test these assessments more rigorously, we sought to quantify the extent of cell scatter induced by these different treatments.

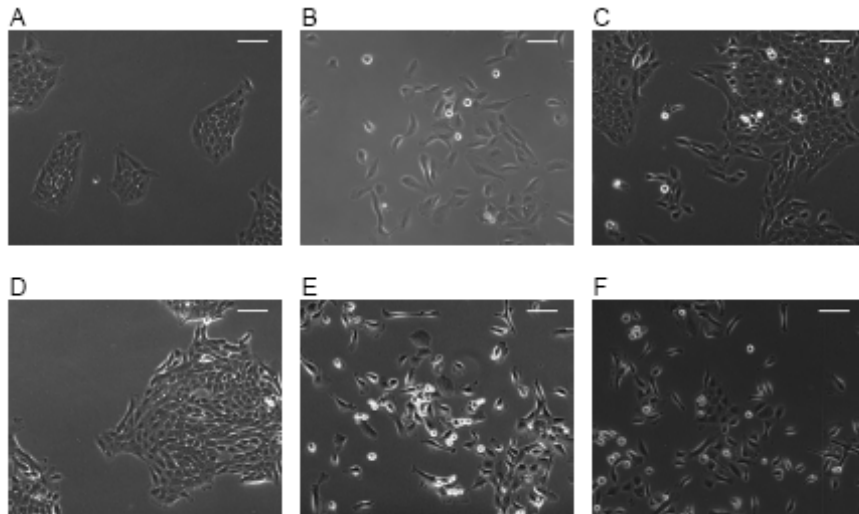


Figure III-1. EGF is a key regulator of epithelial cell scatter.

(A) MCF-10A cells were maintained in SFM for 24 hours to induce the formation of multicellular aggregates. To induce scatter, aggregates were then treated for 24 hours with multiple soluble factors. GM (B), EGF + CT (E) and EGF + CT + insulin (F) induce cell scatter, while EGF (D) and GM containing all factors except for EGF (C) do not. EGF, CT and insulin were used at concentrations identical to those of GM: 20 ng/ml, 100 ng/ml, and 10 μ g/ml respectively. Scale bars are 100 μ m.

3.2. Quantitative metrics of cell scatter

We propose two metrics to quantify the extent of cell scatter. The first metric is the number of cells in a cluster, i.e. the cluster size. A cell cluster is defined as a group of cells in which every member is in physical contact with at least one other member. This metric quantifies the degree to which cells have disrupted intercellular adhesions. Therefore, the mean cluster size is expected to decrease as cells scatter. This metric would not, however, effectively gauge the degree to which cells have dispersed. Thus, a loosely disaggregated cluster would score equivalently to a completely scattered population. To address this issue, we propose a second metric, the nearest-neighbor distance, to evaluate cell dispersion. The nearest-neighbor distance measures the distance

between a cell and its nearest neighbor. We expect the mean nearest-neighbor distance to increase as cells scatter. This metric of cell scatter would have a lower limit (the diameter of a single cell) and an upper limit that depends on the surface density of cells.

3.3. The distribution of cluster sizes is differentially altered by EGF-containing media

After serum-starvation, the distribution of cluster sizes is bimodal, with one peak centered at small sizes (2-5 cells per cluster) and a second peak centered at moderate sizes (20-30 cells per cluster) (Figure III-2A). Upon treatment with EGF for 24 hours, the fraction of isolated cells (i.e. 1 cell per cluster) remains unchanged. This confirms that EGF is not sufficient to disrupt multicellular aggregates (Figure III-1D). However, unexpectedly, treatment with EGF dramatically reduces the fraction of cells in small- and moderate-sized clusters (2-70 cells per cluster), leading to the emergence of a new population of large aggregates (100+ cells per cluster) (Figure III-2B).

This observation from our quantitative measurements led us to probe several mechanisms that may underlie these changes. One possibility is that proliferation within small- and moderate-sized aggregates causes an increase in cluster size. Because MCF-10A proliferation occurs on a timescale of 18 hours post-treatment with EGF, we would expect proliferation effects in our experiments to be minimal. Indeed, we find that these large aggregates form even in the presence of aphidicolin, an inhibitor of proliferation (data not shown). A second possibility is that small- and moderate-sized clusters aggregate to form large ones. To test this possibility, we collected time-lapse videos of

serum-starved cells treated with EGF. These videos clearly show the amalgamation of small/moderate-sized aggregates into large aggregates (Figure III-2C). It therefore appears that although EGF is required for scatter, treatment with this factor alone promotes the formation of new cell-cell adhesions and the amalgamation of pre-existing aggregates.

Treatment with CT in conjunction with EGF increases the fraction of cells that are isolated or in small clusters after 24 hours compared to treatment with EGF alone. This result is consistent with the apparent cell scatter induced by co-treatment with EGF and CT (Figure III-1E). Furthermore, inclusion of CT reduces the formation of new large clusters. CT therefore synergizes with EGF in both disrupting cell-cell adhesions and reducing the formation of new adhesions.

The further addition of insulin results in nearly complete dissociation of moderate-sized aggregates (15-100 cells per cluster), and predominantly small clusters and isolated cells remain after 24 hours. Thus, insulin makes a striking contribution to the disruption of cell clusters that was not evident from our qualitative analysis (Figure III-1F). Our quantitative measurements reveal that insulin-mediated signals are essential for maximum scatter.

Notably, GM, which in addition to EGF, CT and insulin contains serum and hydrocortisone, is a less potent promoter of scatter than EGF + CT + insulin. This

suggests that additional components contained within GM may counteract insulin-mediated effects and reduce scatter.

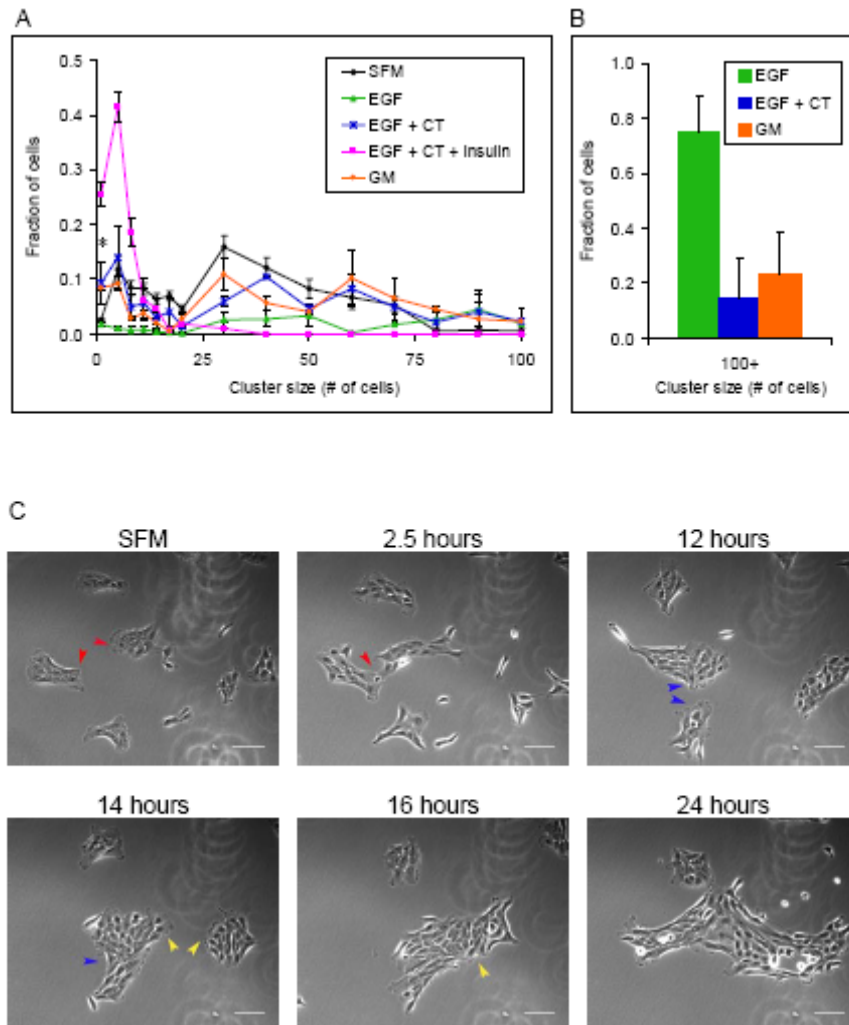


Figure III-2. Distribution of cluster sizes for scattering cells.

MCF-10A cells were maintained in SFM for 24 hours to induce the formation of multicellular aggregates. (A, B) Aggregates were treated with the indicated factor(s) for 24 hours to induce scatter. 15 – 30 epifluorescence images were acquired per condition per experiment and cluster size was determined for all clusters completely contained within an image (150 – 250 clusters per condition per experiment). EGF, EGF + CT and GM induce the formation of large aggregates (100+ cells per cluster) while SFM and EGF + CT + insulin do not. Note that since the fraction of cells in aggregates of 100+ cells is equal to zero for the SFM and EGF + CT + insulin conditions, they are not included in Figure III-2B. Data are means \pm standard error; $n=3$. Asterisk denotes $p <$

0.05 (Student's t test) in comparing the fraction of cells that are isolated after treatment with EGF + CT to the fraction of cells that are isolated after culture in SFM and comparing the fraction of cells that are isolated after treatment with GM to the fraction of cells that are isolated after culture in SFM. (C) Aggregates were treated with EGF and imaged via time-lapse microscopy for 24 hours. Arrowheads indicate locations where new adhesions are formed. Scale bars are 100 μm .

3.4. The distribution of nearest-neighbor distances is differentially altered by EGF-containing media

After serum-starvation, nearly every cell is a member of an aggregate and therefore in contact with its nearest neighbor. Upon stimulation with EGF-containing media, the fraction of cells in contact with their nearest neighbor (i.e. a nearest-neighbor distance of ≤ 1 cell diameter) decreases (Figure III-3). EGF alone produces little change in the distribution of nearest-neighbor distances. However, the additions of CT and insulin produce stepwise increases in the fraction of cells that have distanced themselves from their neighbors by multiple cell diameters. GM induces the greatest response, scattering a small population of cells by greater than 2 cell diameters.

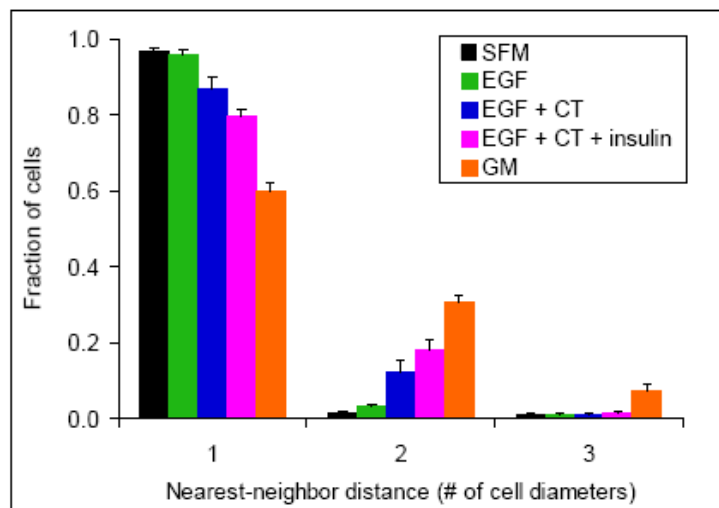


Figure III-3. Distribution of nearest-neighbor distances for scattering cells.

MCF-10A cells were maintained in SFM for 24 hours to induce the formation of multicellular aggregates. Aggregates were then treated with the indicated factor(s) and imaged via time-lapse microscopy for 24 hours. Nearest-neighbor distances were determined for all cells within the first (SFM) and last frames. A cell diameter was taken to be the greatest nearest-neighbor distance measured for contacting serum-starved cells (42 μm), and all nearest-neighbor distances were expressed as multiples of this distance. Data are means \pm standard error; n=2.

To determine if the measured nearest-neighbor distances approach those expected at maximal scatter, we calculated a theoretical maximum internuclear distance that corresponds to the case where all cells are equally spaced from one another. This distance was calculated as follows: the surface area contained within an image was divided by the number of cells within the image to determine an area per cell. Assuming each cell to be a circle of the resulting area with a nucleus at its center, the maximum internuclear distance was calculated to be $76.9 \pm 4.8 \mu\text{m}$ or ~ 1.8 cell diameters. Therefore, the distances presented in Figure III-3 approach the values expected at the time of maximal scatter.

3.5. Automated image processing

From the data presented above, it is clear that our metrics provide useful quantitative insight into the regulation of epithelial cell scatter and exhibit promise for use in future studies. The manual measurement of these metrics, however, is time-consuming. We have therefore developed a simple, high throughput method for identifying cell nuclei and multicellular aggregates in fluorescence images.

MCF-10A cells expressing nuclear-localized GFP and membrane-localized mCherry were imaged using epifluorescence and a digital CCD camera (Figure III-4A,A'). GFP-channel intensity images were segmented using the MATLAB function `edge`. `edge` creates a binary image with 1's where the function finds edges and 0's elsewhere. Next, the function `imfill` was used to fill holes (areas of black pixels surrounded by white pixels) in the segmented images (Figure III-4B). This algorithm correctly reconstructs isolated nuclei, but fails to resolve contacting nuclei into distinct structures. In fact, contact between nuclei is occasionally created by under-segmentation. To address this issue, the watershed algorithm was applied to the image and successfully divides overlapping nuclei into distinct objects.¹²

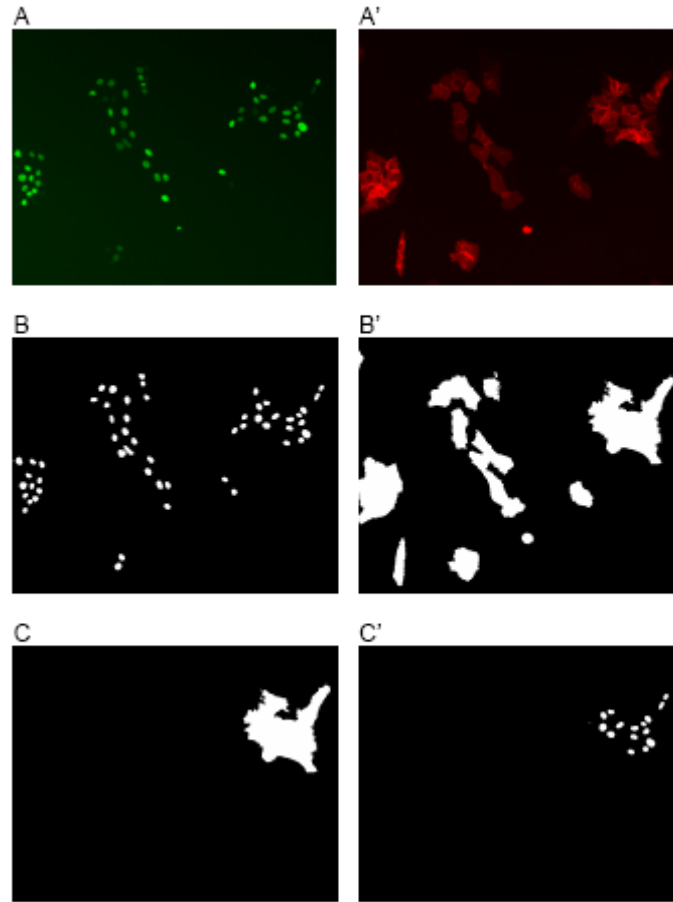


Figure III-4. Automated image processing using MATLAB.

(A, A') Nuclear- and membrane-localized fluorescent proteins, H2B-GFP and mCherry respectively, were co-expressed in MCF-10A cells. (B, B') Epifluorescence images of scattering cells were segmented using thresholding and edge detection algorithms in MATLAB. (C) Masks were created such that each mask contained a single cluster. (C') Masks were then applied to the corresponding H2B-GFP image and nuclei that co-localized with the mask were identified. These nuclei were counted to determine the cluster size.

To identify cell clusters, a global threshold was applied to red-channel intensity images using the MATLAB function `graythresh`. `Graythresh` computes a global threshold for each image using Otsu's algorithm.¹³ This method correctly identifies all cell clusters, but also introduces additional small objects into the image. To eliminate these non-cellular components, a size threshold was applied to the image using the function

bwareaopen. Objects smaller than the area of a single cell ($\sim 400 \mu\text{m}^2$) were eliminated from the image, resulting in a binary image containing distinct cell clusters (Figure III-4B').

Cluster size and nearest-neighbor distance metrics were easily and quickly extracted from the processed images. To determine cluster sizes, clusters in membrane-mCherry frames were first indexed using the function `bwlabel`. `Bwlabel` creates a matrix in which pixels labeled 0 make up the background, pixels labeled 1 make up one object, pixels labeled 2 make up a second object, and so on. From each indexed image, a series of masks was created such that each mask contained a single cluster (Figure III-4C). Masks were then applied to the corresponding H2B-GFP image and the nuclei overlapping with each were indexed and counted using `bwlabel` (Figure III-4C'). To determine nearest-neighbor distances, nuclear centroids were determined from the segmented H2B-GFP frames using the function `regionprops`. Internuclear distances were calculated and the minimum distance measured for each cell was recorded.

We next performed a trial experiment to determine the precision with which these automated techniques evaluate our metrics. Scattering MCF-10A cells co-expressing H2B-GFP and membrane-mCherry were imaged via phase contrast and epifluorescence. Cluster sizes and nearest-neighbor distances were determined (1) manually from phase contrast images and (2) from fluorescence images of the same fields using the described automated method. For both the SFM and GM conditions, results obtained via automated image processing were similar to those obtained manually (Figure III-5).

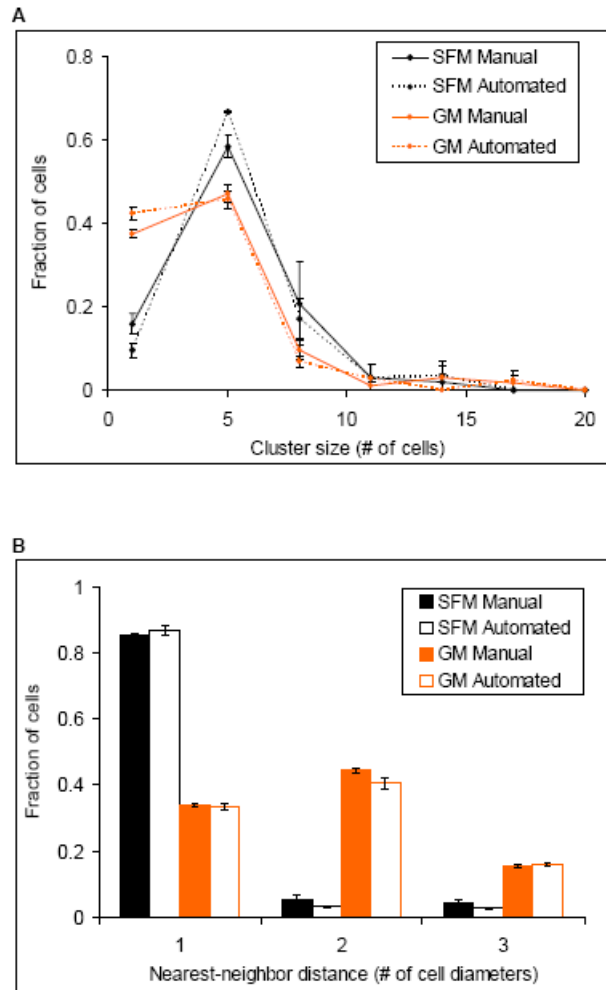


Figure III-5. Comparison of manual and automated techniques.

MCF-10A cells co-expressing H2B-GFP and membrane-mCherry were maintained in SFM for 24 hours to induce the formation of multicellular aggregates. Aggregates were then treated with GM for 24 hours to induce scatter. For each field, both phase contrast and epifluorescence images were collected. Cluster sizes (A) and nearest-neighbor distances (B) were determined manually from phase contrast images and from epifluorescence images using the described automated techniques. Both cluster size and nearest-neighbor distance distributions are similar for the manual and automated techniques.

We note that for each application (i.e. cell line and stimulation conditions), one must also test whether the H2B-GFP and membrane-mCherry constructs perturb cell behavior. This is best accomplished by comparing cluster sizes and nearest-neighbor distances determined manually for uninfected cells to those determined manually for cells expressing the fluorescent constructs.

4. Discussion

Epithelial organization is regulated by a complex signaling network. Many scatter-promoting factors have been identified, among them EGF, hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and transforming growth factor β (TGF- β). These extracellular cues trigger various intracellular signals – Src, Rac, PI3K and Erk, for example – that drive scatter.^{3, 14, 15} A key limiting factor in our understanding of this phenomenon is that the phenotypic changes associated with scatter are characterized largely in qualitative terms. Such assessment is inadequate for several reasons. First, qualitative characterizations do not provide insight into how important a particular factor might be. Is the degree of scatter induced by EGF the same as that induced by HGF? Is there quantitative synergy when both signals are received? Second, cell scatter is a complex phenomenon involving several events. These events include de-compaction of the aggregate, cell-cell dissociation and cell migration. However, because cell scatter is currently evaluated in a lumped fashion, the precise facet(s) of cell scatter a given signal affects remain unclear.

This work introduces quantitative metrics to describe multiple aspects of epithelial cell scatter. We demonstrate that these metrics gauge the potencies with which specific signals induce scatter and the synergies among them. Our measurements show that although EGF alone is unable to induce cell scatter, it synergizes with CT to reduce aggregate sizes and increase nearest-neighbor distances. Furthermore, our quantitative metrics extracted information regarding the role of insulin that would be missed from a qualitative analysis. Inspecting images of cells treated with EGF, CT and insulin suggested that insulin provided no major enhancement to the scatter induced by EGF and CT. However, quantitative measurements of cluster sizes and nearest-neighbor distances revealed that insulin provides a striking improvement in cell scatter, essentially ablating all clusters. This type of quantitative analysis will prove useful for categorizing scatter-promoting factors according to their ability to alter epithelial structures and for grouping synergistic cues. Moreover, identifying potent scatter-inducing cues may provide more pivotal targets for anti-cancer therapeutics.

This quantitative approach also provides new insights into the role of EGF in multicellular epithelial organization. Our measurements revealed that treatment with EGF alone induced the formation of large aggregates through the amalgamation of small/moderate-sized aggregates. Thus, although EGF is essential for inducing cell scatter in the presence of co-factors, our data suggest that EGF alone promotes cell-cell adhesion and the emergence of large clusters.

The metrics proposed in this study quantify distinct aspects of scatter, and therefore, may not always be correlated to each other. For example, GM treatment does not score as the most efficient at breaking clusters apart (Figure III-2), but still mediates the largest increase to nearest-neighbor distance (Figure III-3). This counterintuitive observation is due to a phenomenon called de-compaction, where cells in a cluster relax cell-cell adhesions and enhance cell spreading against the substratum. In this manner, GM-treated cells distance themselves from their neighbors without breaking cell-cell contacts. Thus, the proposed metrics gauge distinct aspects of multicellular organization and analyzing how both metrics respond to molecular perturbations can provide mechanistic insights.

Because the metrics capture distinct facets of cell scatter, they will prove useful in exploring synergisms between signals. Some cues, for instance, will have a profound effect on breaking cell-cell contacts but little effect on the nearest-neighbor distance. Other cues will have exactly the opposite effect. Combined exposure to such complementary cues may have a synergistic effect on cell scatter that is significantly greater than their individual contributions. Identifying such synergistic cues may reveal “multi-hit” pathways that contribute to cancer development and thereby guide therapeutic strategies.

In this work, we also present a simple, versatile and high-throughput method for measuring morphological changes to epithelial structures from live-cell images. These techniques may be a valuable tool not only for in-depth study of cell scatter, but also for

clinical applications. First, quantitative metrics combined with automated image analysis may facilitate *in vitro* high-throughput screening of anti-cancer therapeutics. Furthermore, since inspection of tumor morphology is widely used to categorize disease and decide on a treatment option, the techniques described here may facilitate advancements in cancer diagnostics.^{3, 5} Overall, we believe our methods can provide necessary quantitative insight into the regulation of epithelial structures, which will lead to advancements in our understanding of scatter, EMT and metastasis.

5. Materials and Methods

5.1. 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 $\mu\text{g/ml}$ hydrocortisone (Sigma), 0.1 $\mu\text{g/ml}$ cholera toxin (Sigma), 10 $\mu\text{g/ml}$ insulin (Sigma), and 1% penicillin/streptomycin (Invitrogen). For serum starvation, cells were washed twice with 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.

5.2. Plasmid Constructs

H2B-GFP and membrane-mCherry were gifts from S. Fraser (California Institute of Technology). The membrane-mCherry construct consists of monomeric mCherry fused to the first 20 amino acids of zebrafish Gap43.¹⁶ Palmitoylation at cysteine residues within the Gap43 sequence directs the mCherry protein to the membrane.^{17, 18} VSV-G and gag-pol vectors were gifts from D. Schaffer (University of California, Berkeley).

5.3. Retroviral Infection

H2B-GFP and membrane-mCherry genes were subcloned into retroviral vectors (pLHCX and pLPCX respectively) and expressed in epithelial cell lines via retroviral

infection. Retrovirus was produced by triple transfection of 293T cells with 5 μg each of VSV-G, gag-pol and the retroviral vector using LipofectAMINE (Invitrogen). For infection, cells were incubated with retrovirus-containing medium and 8 $\mu\text{g/ml}$ polybrene for 24 h. Puromycin (2 $\mu\text{g/ml}$) and hygromycin (100 $\mu\text{g/ml}$) were used for selection.

5.4. Cell scatter assay

MCF-10A cells were seeded in GM at a density of 8×10^4 per 35 mm culture dish and, 18-24 hours later, serum starved for 24 hours to induce aggregate formation. Cell aggregates were then stimulated with the indicated factor(s) and imaged. MCF-10A cells co-expressing membrane-mCherry and H2B-GFP were seeded at 2×10^4 per 35 mm dish.

5.5. Live cell microscopy

Images were captured at 10x magnification using a Zeiss Axiovert 200M microscope equipped with a digital CCD camera. Time-lapse microscopy experiments were performed using an environmental chamber that maintains temperature, humidity and CO_2 levels.

6. Supplemental Data

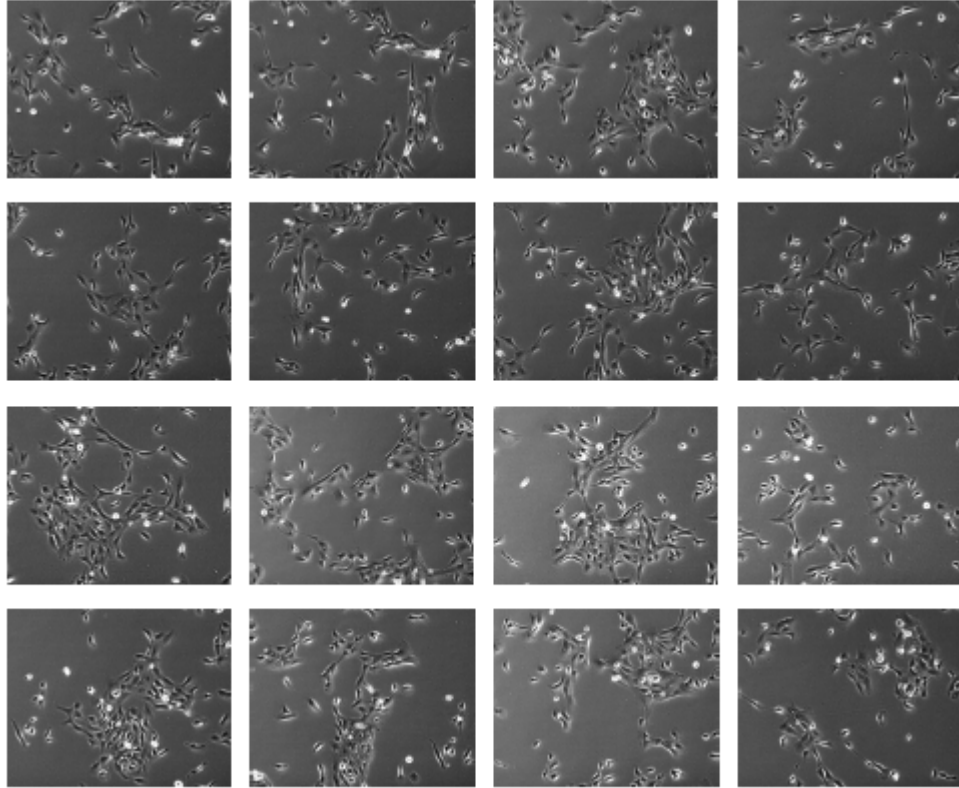


Figure III-6. Phase contrast images of MCF-10A cell aggregates treated with EGF + CT.

MCF-10A cells were maintained in SFM for 24 hours to induce the formation of multicellular aggregates. Aggregates were then treated with EGF + CT for 24 hours to induce scatter and imaged via phase contrast microscopy.

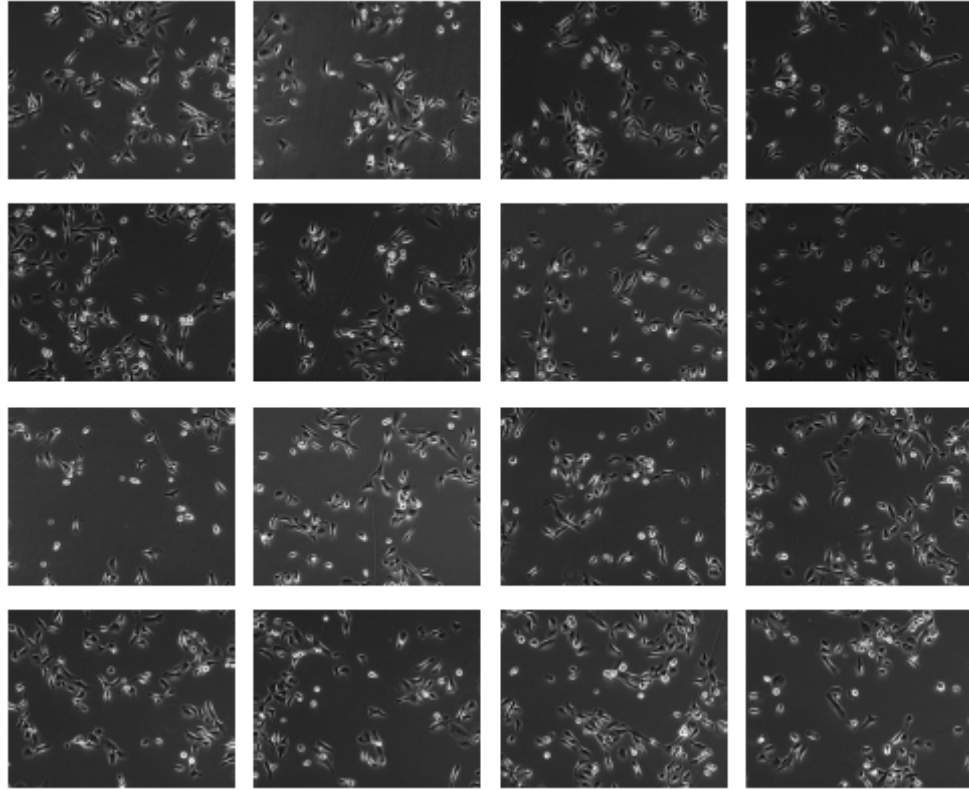


Figure III-7. Phase contrast images of MCF-10A cell aggregates treated with EGF + CT + insulin.

MCF-10A cells were maintained in SFM for 24 hours to induce the formation of multicellular aggregates. Aggregates were then treated with EGF + CT + insulin for 24 hours to induce scatter and imaged via phase contrast microscopy.

7. References

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Chapter IV. A MICROTITER ASSAY FOR QUANTIFYING PROTEIN-PROTEIN INTERACTIONS ASSOCIATED WITH CELL-CELL ADHESION

1. 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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2. 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,

these methods are low-throughput and offer 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*.

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 has been previously described for several other protein complexes.¹⁴⁻¹⁶ 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 (Figure 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 (Figure 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.

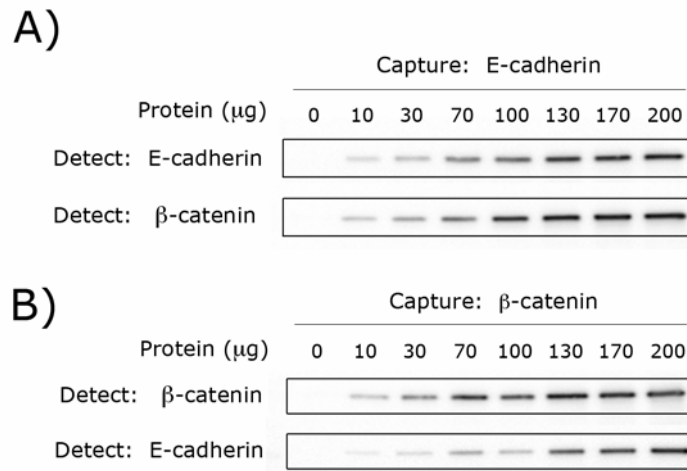


Figure IV-1. Antigen capture and protein:protein co-capture.

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) (Figure 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.

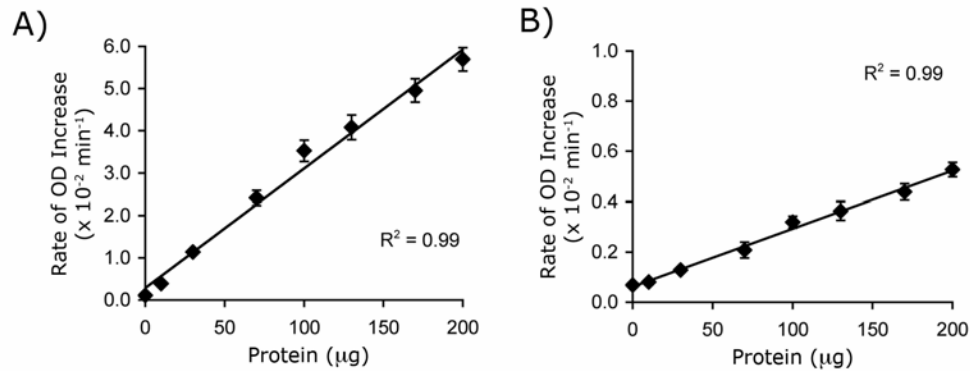


Figure IV-2. Detection of E-cadherin: β -catenin protein complexes by protein complex ELISA.

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 (Figure 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.¹⁷ 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

^aCalculated 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,¹⁸ 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. 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 °C and 37 °C showed no difference in assay sensitivity (data not shown). All results reported here are from assays conducted at 37 °C.

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 (Figure 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 (Figure IV-1), this demonstrates that the protein complex ELISA is specifically measuring the interaction of E-cadherin and β -catenin.

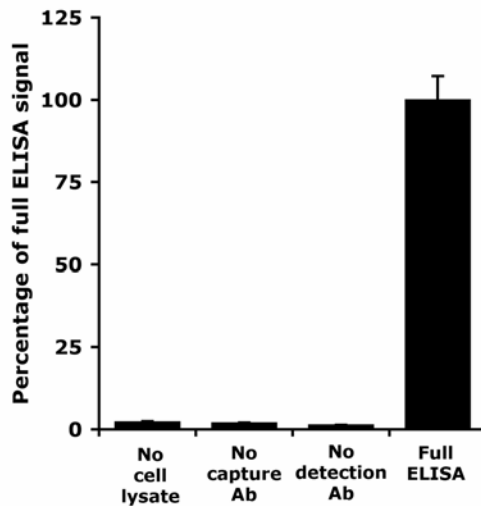


Figure IV-3. Specificity test for E-cadherin: β -catenin ELISA.

The E-cadherin: β -catenin ELISA was performed on 100 μ g 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 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.

Having demonstrated that both E-cadherin and β -catenin are captured effectively in microtiter wells (Figure 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 μ g) (Figure IV-4A). Notably, the linear range of the E-cadherin sandwich ELISA extended only to 130 μ g, as opposed to the protein complex ELISAs, which demonstrated linearity to at least 200 μ g 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 μ g of total cell protein (Figure IV-4B), with a signal/noise ratio of 3.6 (Table IV-1). The β -catenin ELISA, in contrast to the E-cadherin sandwich 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.

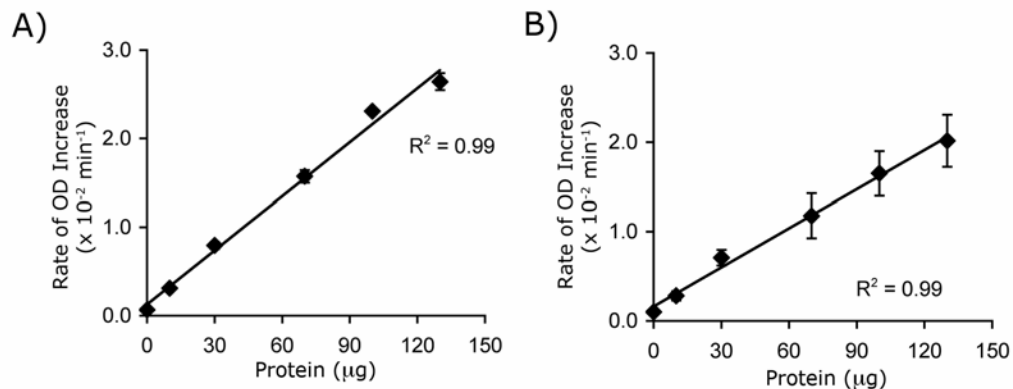


Figure IV-4. Detection of E-cadherin and β -catenin total protein levels by sandwich ELISA.

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 ($n = 3$).

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 level 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;¹⁹ meanwhile, SW480 is a colon carcinoma cell line with a dysfunctional β -catenin degradation machinery that permits β -catenin to build up to high levels in the cytoplasm.²⁰

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 load in the assay for both cell types (Supplementary Figure IV-1). We quantified the slope of the ELISA signal versus the amount of whole cell lysate as a metric of the amount of β -catenin. 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 cells.

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.

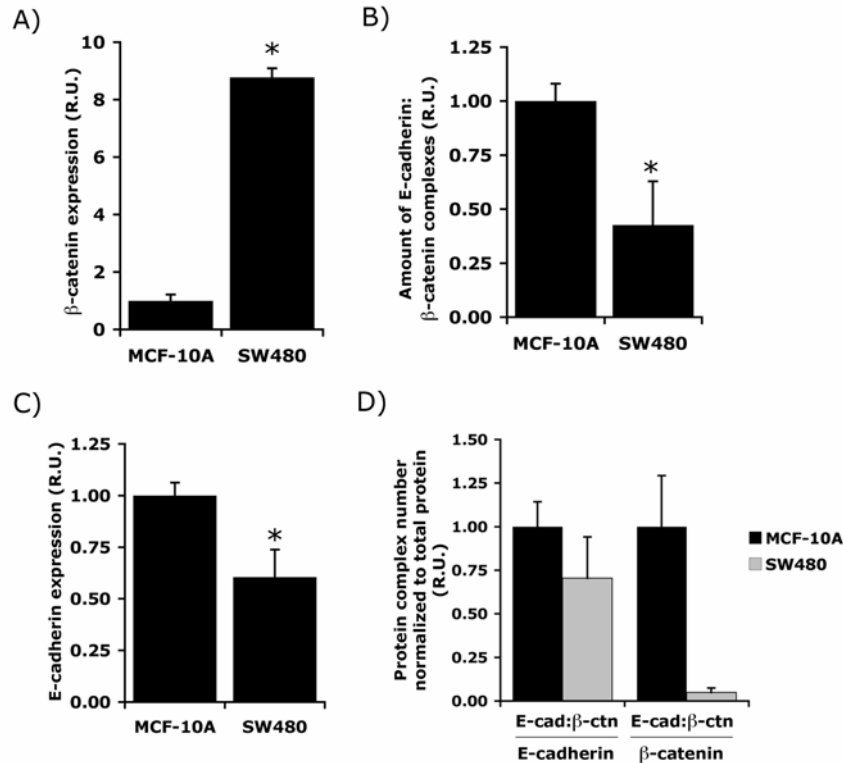


Figure IV-5. Quantitative comparison of the levels of E-cadherin:β-catenin complexes, E-cadherin and β-catenin expression in normal and tumorigenic cell lines.

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 both the sandwich ELISAs and the protein complex ELISA, the rate of change in optical density over time was plotted versus total cell lysate, as shown in Figure IV-7 (Supplemental Data). 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 were normalized to total cellular levels of E-cadherin or β-catenin, as measured by the sandwich ELISA.

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 two-

fold lower levels of E-cadherin per μg of total cell lysate than MCF-10A cells (Figure IV-5C). This two-fold reduction in E-cadherin expression mirrors the two-fold decrease in E-cadherin: β -catenin complexes in SW480 cells, suggesting that the limiting factor in 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 show nearly identical values, with SW480 cells showing only a $30 \pm 24\%$ decrease compared to MCF-10A cells (Figure 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 ten-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 (Figure 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 in the levels of total E-cadherin, 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 SW480.²¹

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 cells, since several lines of evidence have implicated Src in regulating intercellular adhesion.^{7, 8, 10, 22} 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);^{23, 24} 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 (Supplementary Figure IV-2).

Using the protein complex ELISA revealed that activated Src reduced levels of the E-cadherin: β -catenin complex by ~40% in MCF-10A cells (Figure IV-6A), corroborating reports that Src can disrupt E-cadherin mediated adhesions *in vivo*.^{7, 8, 10, 22} 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 the amount of total E-cadherin 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 (Figure 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 (Figure IV-6C).

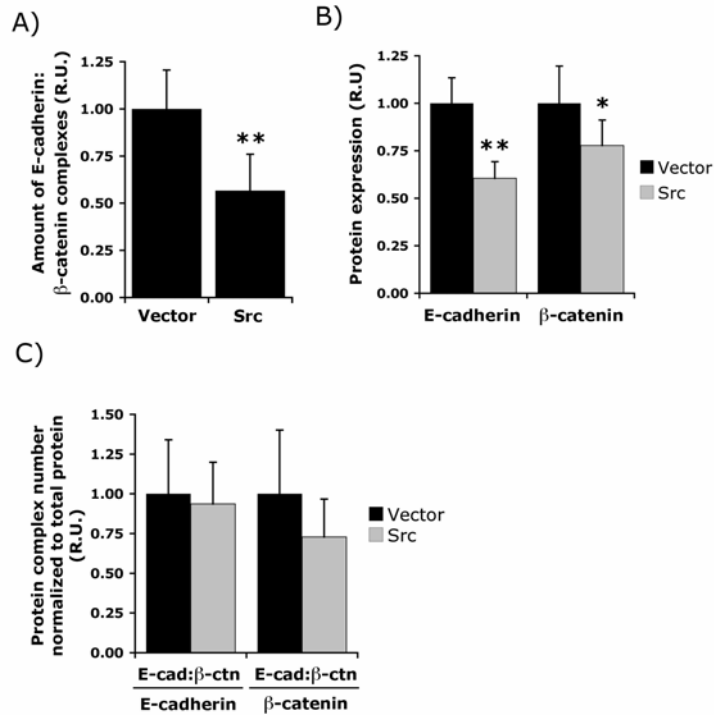


Figure IV-6. Quantifying the effect of constitutively-active Src on cellular levels of E-cadherin:β-catenin complexes and the expression of E-cadherin and β-catenin.

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-8 (Supplemental Data). 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 either total cellular levels of 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, 22} 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 six-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 μ M *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 ubiquitination 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.²⁵

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 because the degradation complex cannot bind β -catenin.²⁶ However, once released from E-cadherin, β -catenin is subject to degradation by the APC/axin/GSK3 β /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% (Figure 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 (Figure 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-mediated transcription as reported by others.^{27, 28}

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.^{14, 16} 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. Acknowledgements

We thank Joan Brugge and David Schaffer for providing reagents. This work was supported by grants from the Concern Foundation for Cancer Research and the Jacobs Institute for Molecular Engineering for Medicine. N.A.G. and M.D.P were partially supported by graduate fellowships from NDSEG and NSF, respectively.

6. Materials and Methods

6.1. 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).

6.2. 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_i, 30 mM NaF, 1 mM benzamide, 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).

6.3. 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 $\mu\text{g/ml}$ 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 $^{\circ}\text{C}$. 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 $\mu\text{g/ml}$ in blocking buffer was added to each well and incubated for 1 h at 37 $^{\circ}\text{C}$. 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 $\mu\text{g/ml}$ in blocking buffer, and 100 μl was added to each well for 1 h at 37 $^{\circ}\text{C}$. 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 $^{\circ}\text{C}$ 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).

6.4. 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 μ l 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 °C, and the contents of the well were collected, resolved by SDS-PAGE on 10% poly-acrylamide 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).

6.5. 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).

6.6. 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.²⁹ 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.

6.7. Data Analysis and Statistical Calculations

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

$$S/N = \frac{(\mu_s - \mu_b)}{\sigma_s}$$

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:

$$Z' = 1 - \frac{3 \times (\sigma_s + \sigma_b)}{|\mu_s - \mu_b|}$$

where μ_s , μ_b , and σ_s are as defined above and σ_b is the standard deviation of the blank.³⁰ The Signal/Noise ratio and the Z' factor were calculated at 200 μ g and 130 μ g 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 (Figure IV-5) or between cells infected with a dominant-active oncogene or an empty vector control (Figure 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 Figures IV-7 and IV-8 (Supplemental Data). The statistical significance of the observed difference in slopes was calculated by analysis of covariance (ANCOVA).

7. Supplemental Data

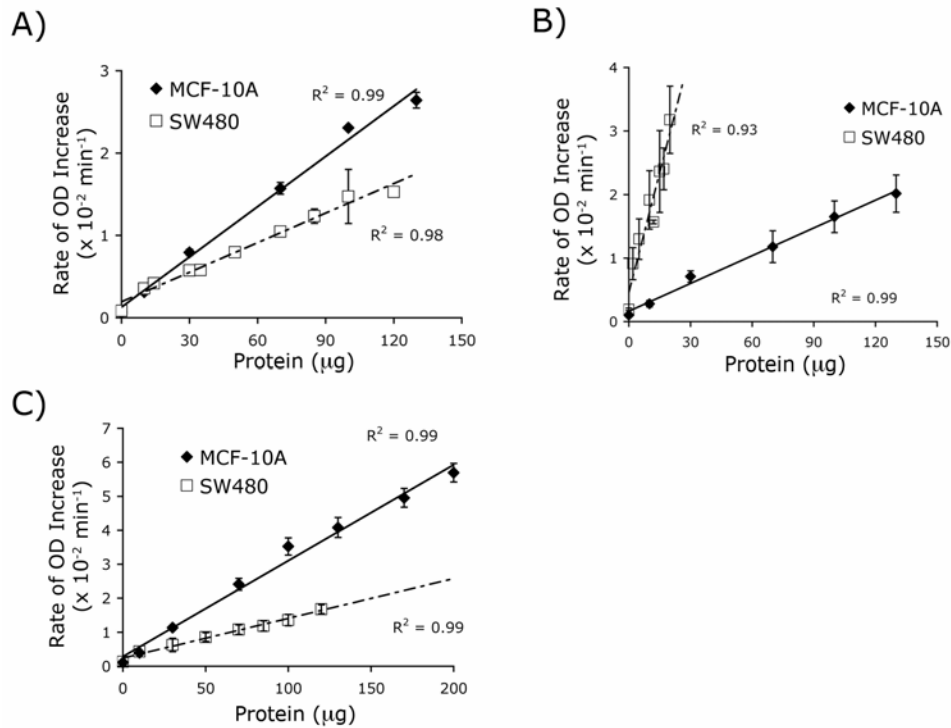


Figure IV-7. Quantification of E-cadherin, β-catenin, and E-cadherin:β-catenin complexes in normal and tumorigenic cell lines.

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).

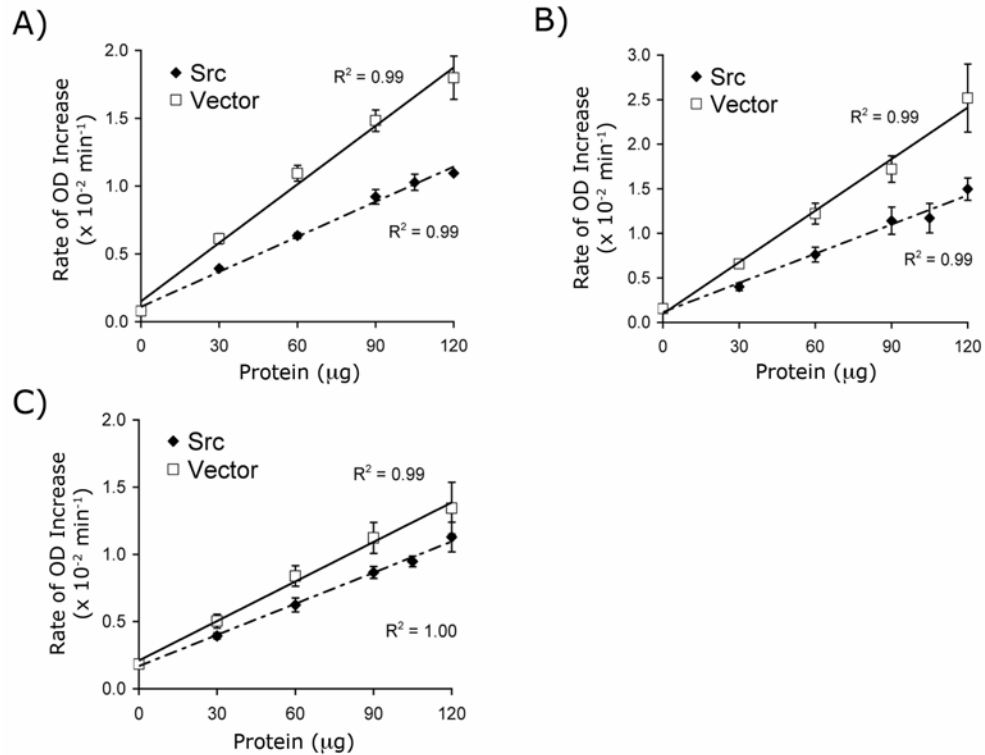


Figure IV-8. Quantifying the effect of constitutively-active Src on cellular levels of E-cadherin:β-catenin complexes and the expression of E-cadherin and β-catenin.

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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