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.

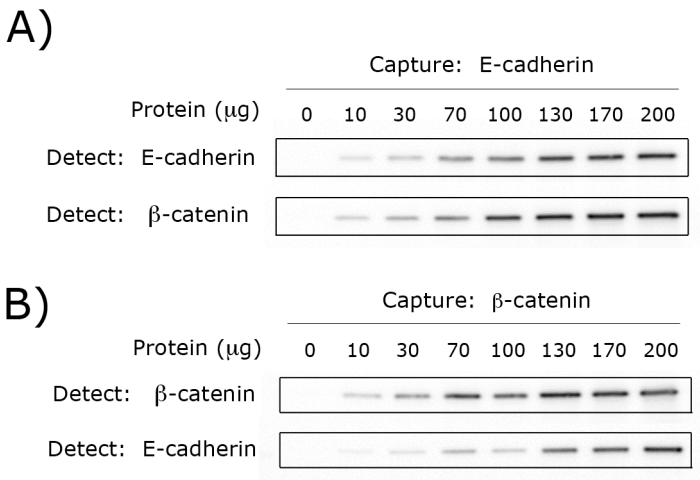

Fig. IV-1. Antigen capture and protein:protein co-capture
Mouse monoclonal antibodies against either (A) E-cadherin or (B) β-catenin were adsorbed to a microtiter plate, and varying amounts of whole cell lysates from MCF-10A cells were incubated in the wells. Protein fractions isolated by the capture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

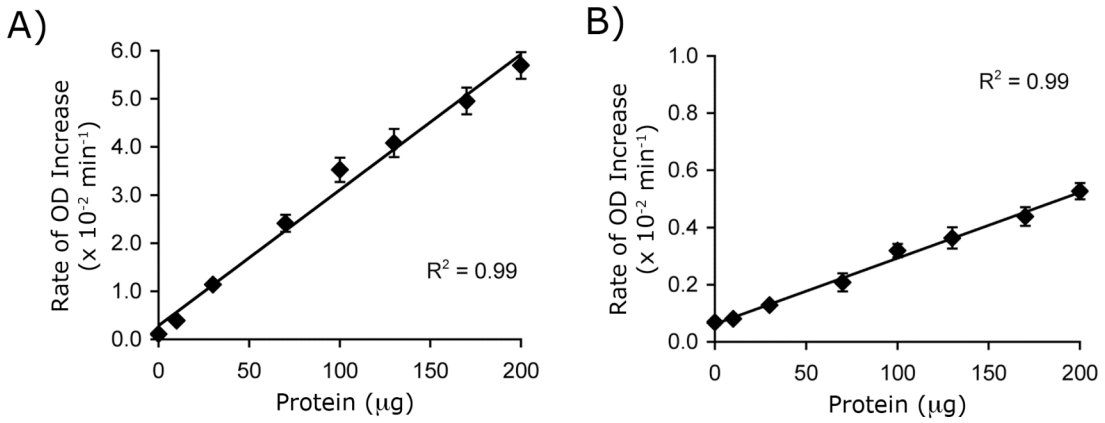

Fig. IV-2. Detection of E-cadherin:β-catenin protein complexes by protein complex ELISA
Whole cell lysate from MCF-10A cells was analyzed by protein complex ELISA for E-cadherin:β-catenin complexes by either (A) E-cadherin capture or (B) β-catenin capture. The rate of change in the optical density over 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.

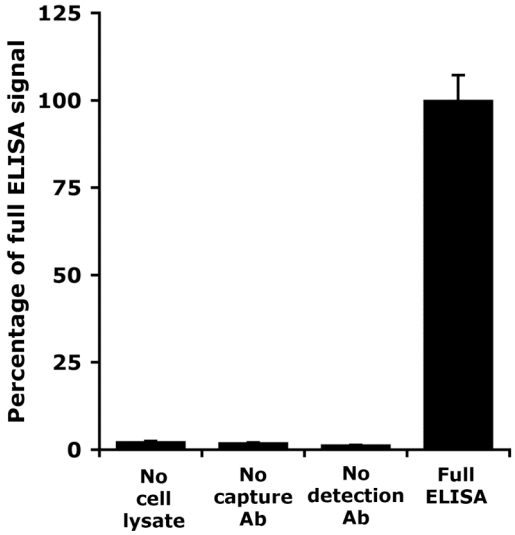

Fig. IV-3. Specificity test for E-cadherin:β-catenin ELISA
The E-cadherin:β-catenin ELISA was performed on 100 μg of MCF-10A whole cell lysate. At the appropriate step, either the whole cell lysate, the anti-E-cadherin capture antibody (Ab), or the anti-β-catenin detection antibody was omitted from the 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.

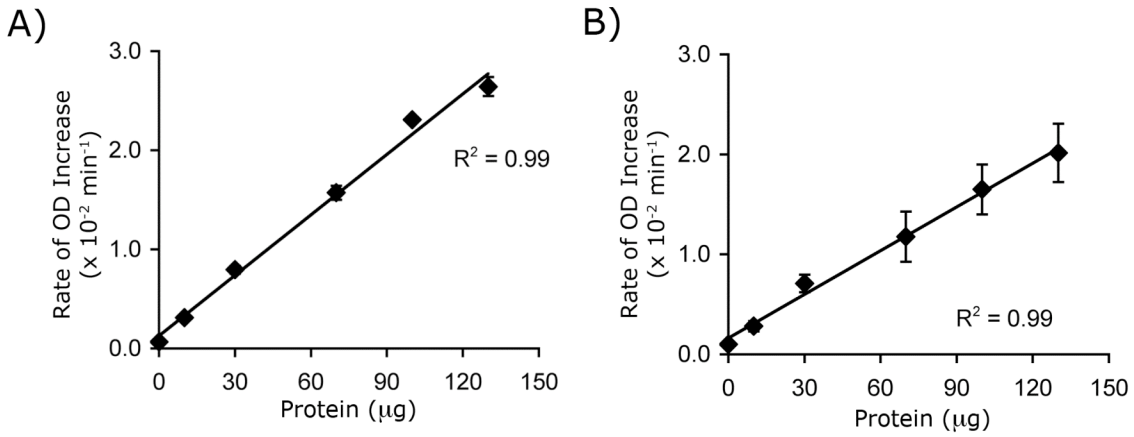

Fig. IV-4. Detection of E-cadherin and β-catenin total protein levels by sandwich ELISA
Whole cell lysate from MCF-10A cells was analyzed by sandwich ELISA for (A) total E-cadherin expression or (B) total β-catenin expression. The rate of change in optical density over time was plotted versus total cell 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

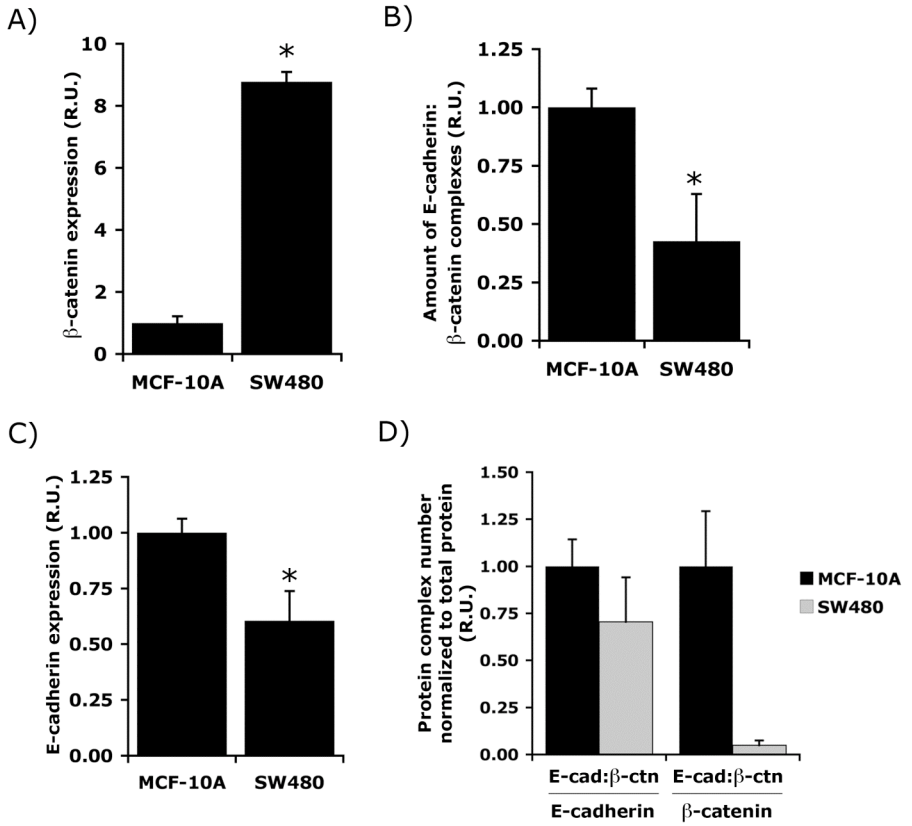

Fig. IV-5. Quantitative comparison of the levels of E-cadherin:β-catenin complexes, E-cadherin and β-catenin in normal and tumorigenic cell lines
Whole cell lysates were prepared from MCF-10A and SW480 cells, and were analyzed either by (A) sandwich ELISA for total β-catenin levels, (B) protein complex 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).

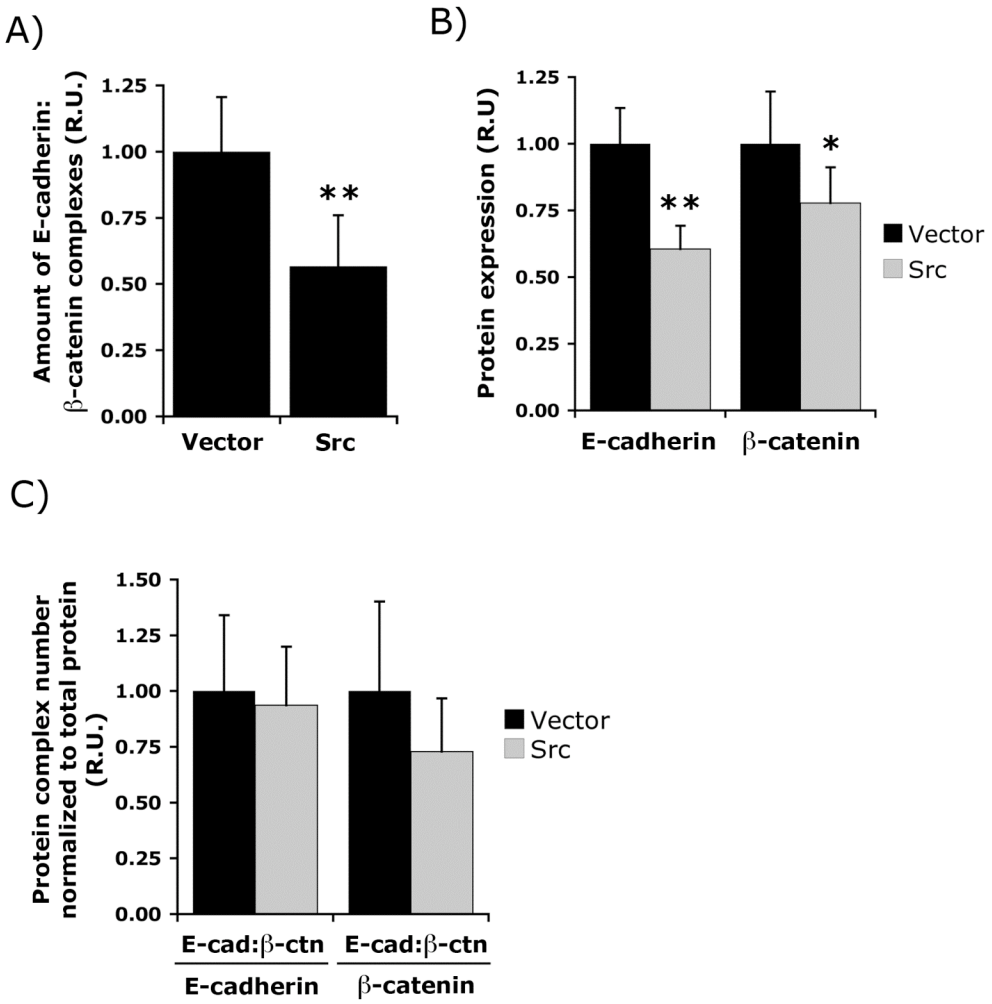

Fig. IV-6. Quantifying the effect of constitutively-active Src on cellular levels of E-cadherin: β-catenin complexes and the expression of E-cadherin and β-catenin

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.

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6. Supplemental Data

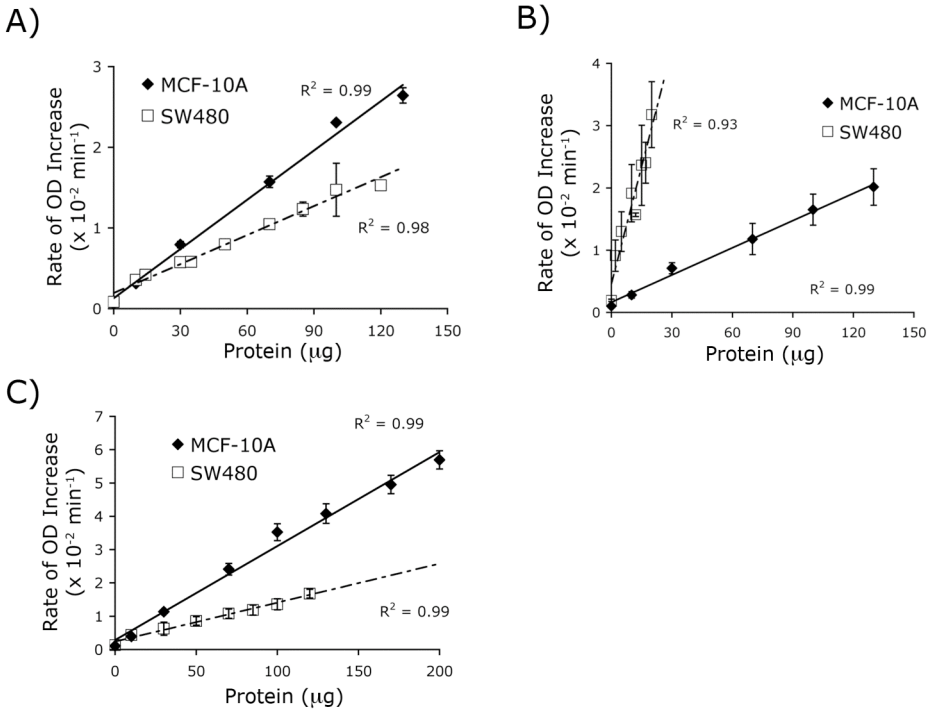

Fig. IV-S1. Validation of standard sandwich and protein complex ELISAs in normal and tumorigenic cell lines

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).

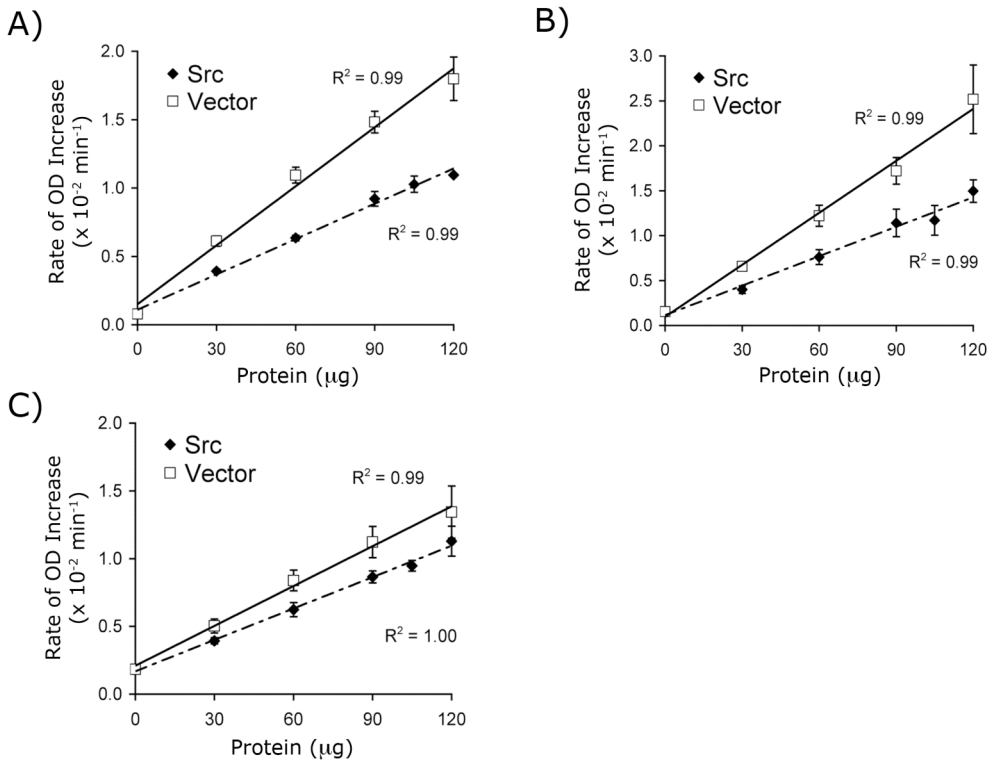

Fig. IV-S2. Validation of standard sandwich and protein complex ELISAs in cells expressing constitutively-active Src
Subconfluent cultures of MCF-10A cells infected with either Src Y527F or the empty vector were lysed and analyzed by sandwich ELISA for (A) E-cadherin or (B) β-catenin expression or by 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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