Chapter V. Mechanisms underlying growth saturation of epithelial cells

Abstract

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

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

2. Results

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

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

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

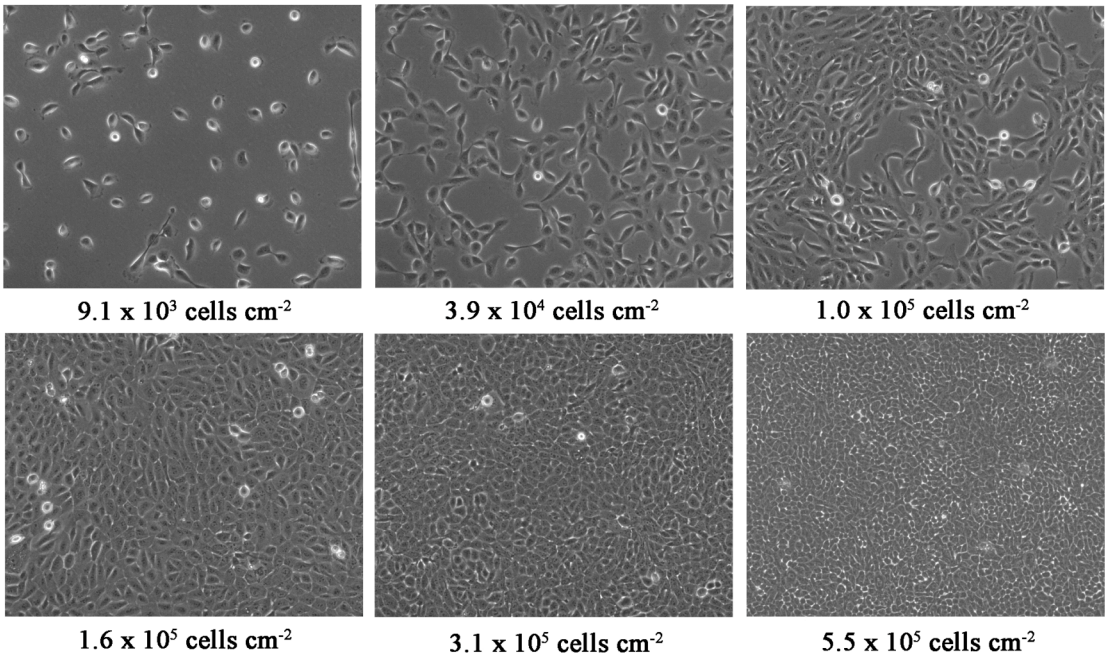

Figure V-1. Quantification of intercellular contact by measuring cell density in MCF-10A
Varying numbers of MCF-10A were plated and cultured for 72 h in GM before trypsinization and quantification of cell density.

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

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

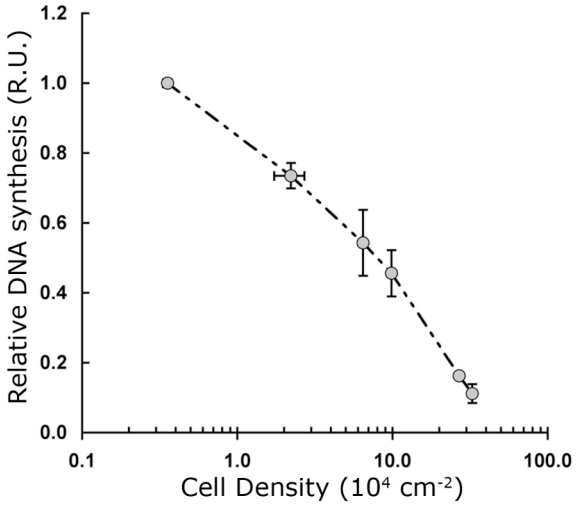

Figure V-2. DNA synthesis is inversely correlated with cell density.
Serum-starved MCF-10A were stimulated with GM, and the amount of DNA synthesis was measured by ^{3}H-thymidine incorporation as described in Experimental Procedures. DNA synthesis data was normalized to the response of cells at 3.6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

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

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

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

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

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

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

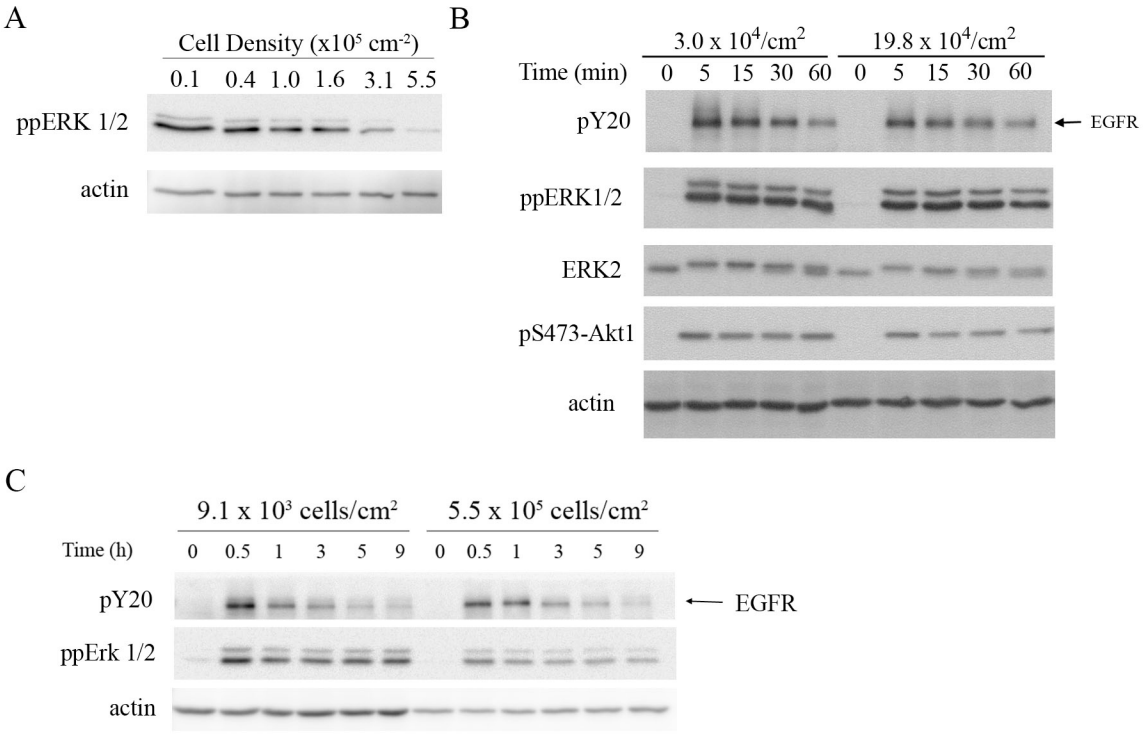

Figure V-3. EGFR, ERK, Akt signaling at various cell densities
Whole cell lysates from MCF-10A at the indicated cell densities were immunoblotted for dually-phosphorylated ERK 1/2 (ppERK 1/2), phospho-tyrosine, total ERK2, phospho-serine 473 Akt, or the equal loading control actin. (A) Cells were cultured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

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

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

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

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

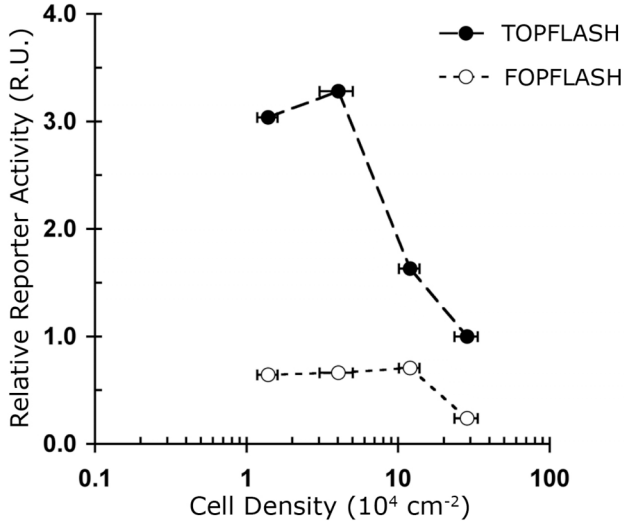

Figure V-4. β-catenin-mediated transcription is inversely correlated with cell density. MCF-10A that had been transfected with the Tcf/Lef reporter TOPFLASH or the negative control FOPFLASH were plated at various densities, serum-starved, and stimulated with GM for 9 h. All reporter activities were normalized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

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

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

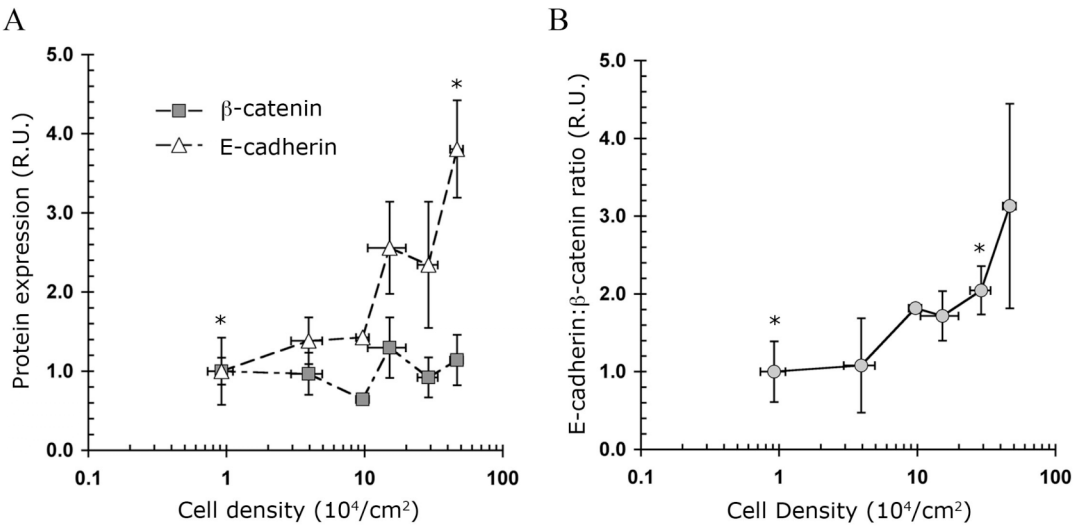

Figure V-5. Expression of endogenous E-cadherin, but not β-catenin, is cell density-dependent. MCF-10A were cultured in GM as described in Figure V-1A and levels of E-cadherin, β-catenin, and actin were measured by semi-quantitative immunoblotting as detailed in Experimental Procedures. Protein expression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

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

2.4. Exogenous E-cadherin inhibits DNA synthesis.

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

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

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

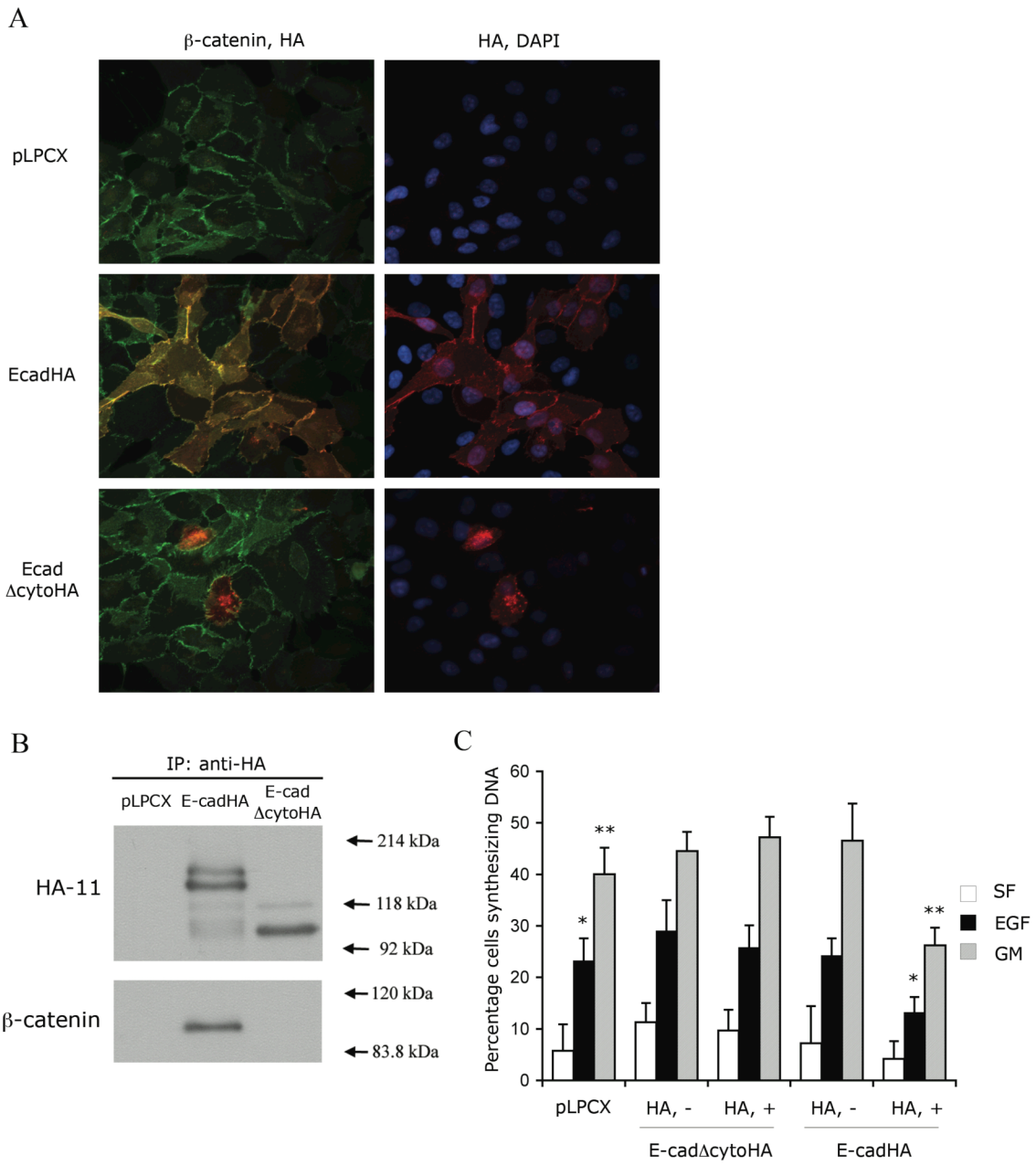

Figure V-6. Full-length E-cadherin, but not the cytoplasmic-domain truncated mutant, reduces DNA synthesis.
MCF-10A cells were retrovirally transduced with full-length E-cadherin (E-cadHA), the cytodomain-truncated E-cadherin mutant (E-cadΔcytoHA), or retrovirus generated with the empty vector pLPCX.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

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

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

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

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

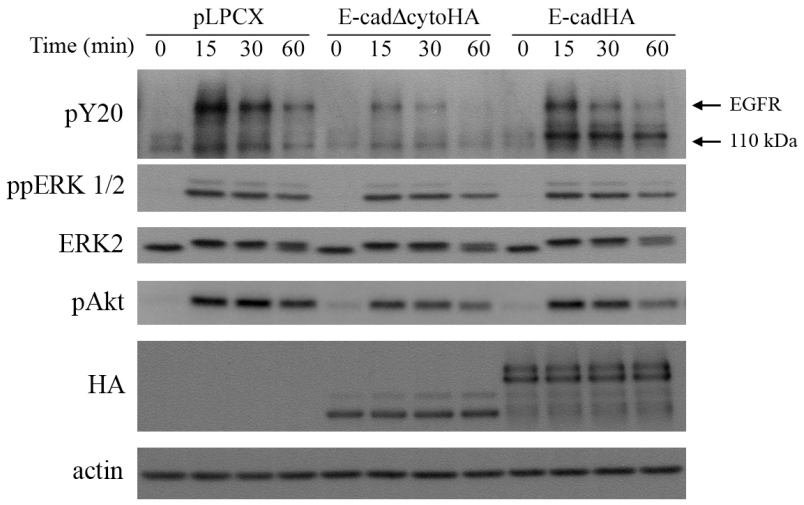

Figure V-7. Exogenous E-cadherin constructs do not affect ERK despite affecting EGFR and Akt phosphorylation.
MCF-10A cells retrovirally transduced with the empty vector pLPCX, exogenous full-length E-cadherin (E-cadHA), or the cytodomain-truncated mutant of E-cadherin (E-cadΔcytoHA). Cells were serum-starved,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

2.6. Growth factor availability contributes to growth arrest.

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

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

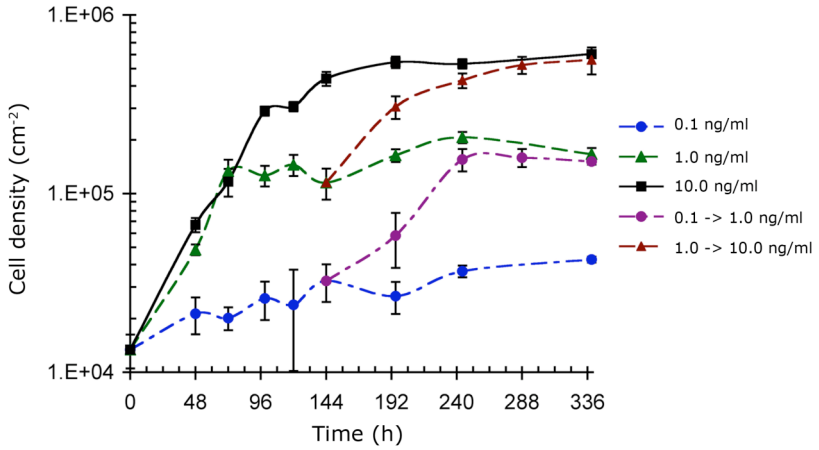

Figure V-8. Growth factor concentration-dependent saturation of cell growth
Serum-starved MCF-10A cells were stimulated with EGF (0.1, 1.0, or 10.0 ng/ml) and cell density was monitored by enzymatic lifting and counting cells approximately every 24 h. To prevent growth factor depletion, EGF was refreshed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

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

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

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

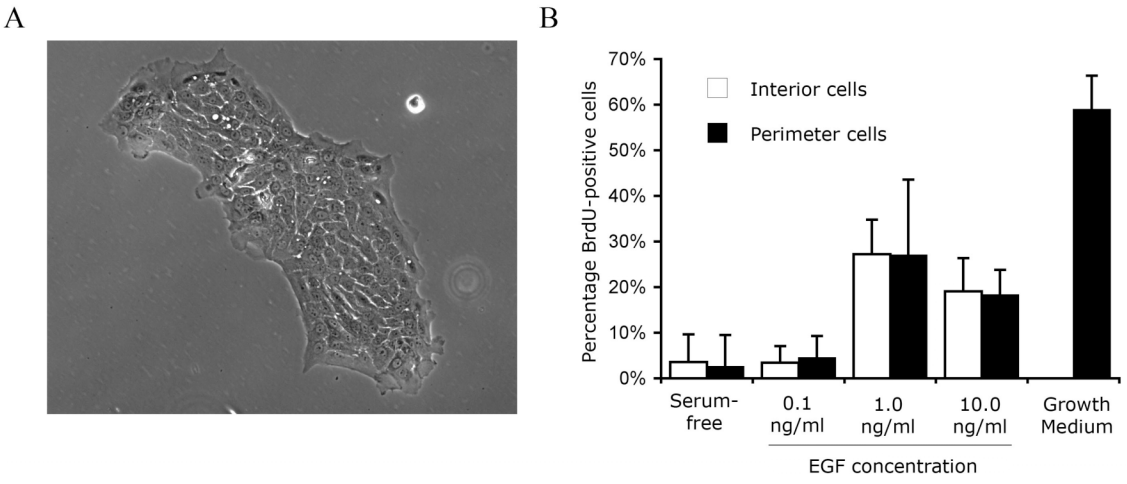

Figure V-9. Cell colony formation does not affect EGF-mediated proliferation.
(A) Phase contrast image of a cell colony formed by serum-starvation of MCF-10A. (B) Serum-starved MCF-10A, which had formed cell islands, were stimulated with serum-free medium, EGF at the indicated concentrations, or GM,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

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

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

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

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

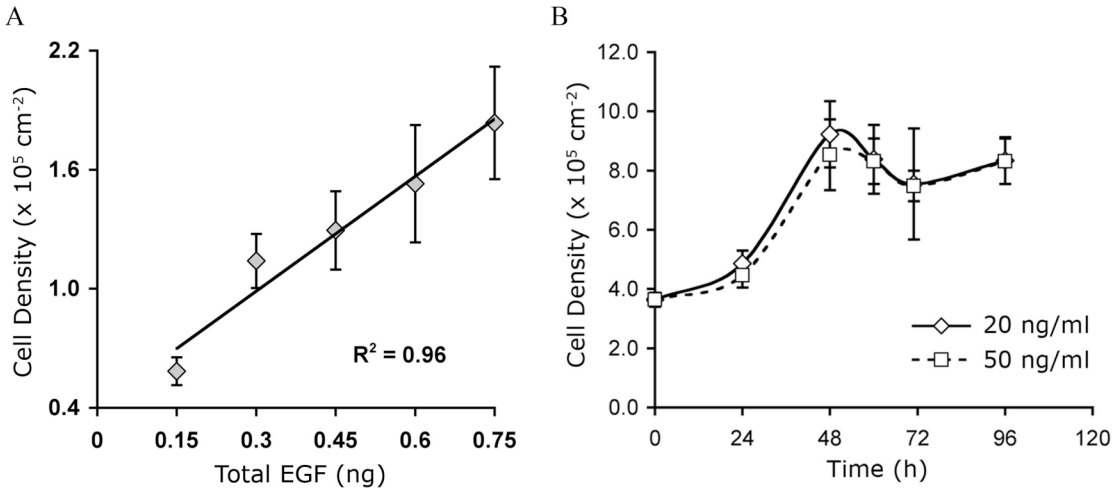

Figure V-10. Growth factor availability determines the cell density at growth saturation. (A) Serum-starved MCF-10A cells were stimulated with varied amounts of total EGF by supplying different volumes of culture medium while holding the growth factor concentration constant (0.1 ng/ml). To prevent growth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

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

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

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

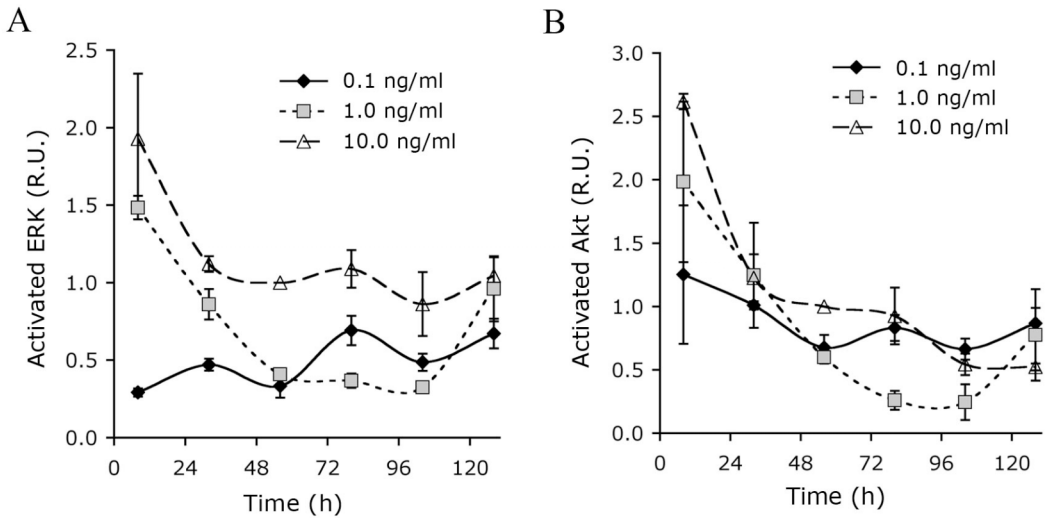

Figure V-11. Akt signaling, rather than ERK signaling, may control cell density at saturation. Serum-starved MCF-10A were stimulated with the indicated concentrations of EGF every 24 h for a total of 5 days. 8 h after every stimulation, cells were lysed and whole cell lysates were analyzed by semi-quantitative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

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

3. Discussion

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

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

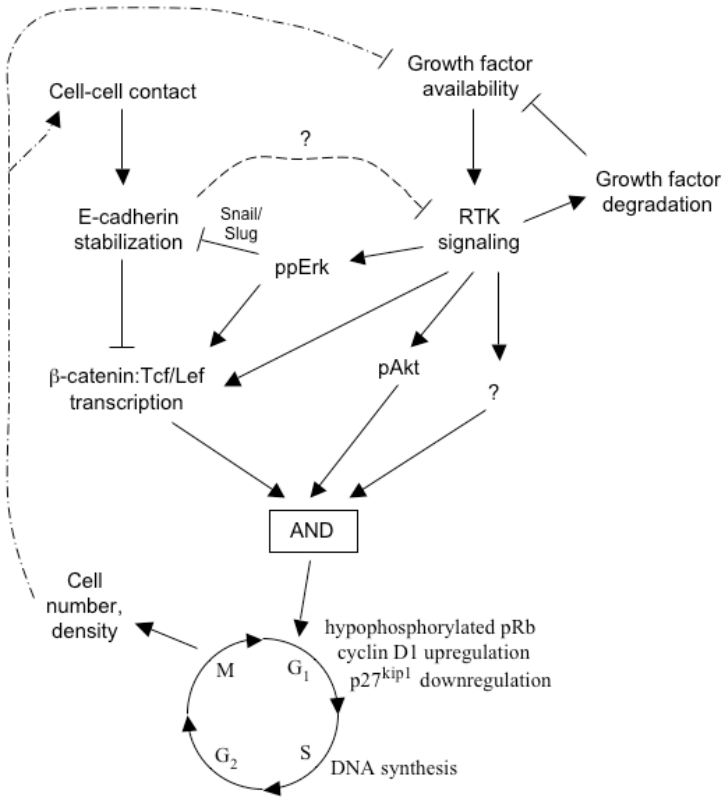

Figure V-12. Growth inhibition at high cell density in MCF-10A.
In non-tumorigenic cells, growth inhibition is mediated by two interconnected signals, one contact-dependent and one density-dependent. Contact inhibition of proliferation of MCF-10A is mediated by E-cadherin, which is stabilized by intercellular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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.4. Mitogenic ligand depletion as a mediator of growth suppression

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

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

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

4. Future Work

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

4.1. The role of ERK and Akt in growth suppression

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

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

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

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

4.2. Mechanism underlying upregulation of endogenous E-cadherin

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

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

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

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

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

5. Conclusions

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

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

Acknowledgments

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

6. Experimental Procedures

6.1. Antibodies

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

6.2. Cell Culture

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

6.3. Plasmid Constructs

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

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

6.4. Retroviral Infection

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

6.5. Cell Lysis

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

6.6. Immunoblotting

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

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

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

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

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

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

6.7. Reporter Assays

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

6.8. DNA Synthesis Measurements

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

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

6.9. Immunofluorescence

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

6.10. Immunoprecipitation

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

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