E-cadherin engagement stimulates proliferation via Rac1

Wendy F. Liu,[1,2] Celeste M. Nelson,[1] Dana M. Pirone,[2] and Christopher S. Chen[2]

[1]Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205
[2]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104

E-cadherin has been linked to the suppression of tumor growth and the inhibition of cell proliferation in culture. We observed that progressively decreasing the seeding density of normal rat kidney-52E (NRK-52E) or MCF-10A epithelial cells from confluence, indeed, released cells from growth arrest. Unexpectedly, a further decrease in seeding density so that cells were isolated from neighboring cells decreased proliferation. Experiments using microengineered substrates showed that E-cadherin engagement stimulated the peak in proliferation at intermediate seeding densities, and that the proliferation arrest at high densities did not involve E-cadherin, but rather resulted from a crowding-dependent decrease in cell spreading against the underlying substrate. Rac1 activity, which was induced by E-cadherin engagement specifically at intermediate seeding densities, was required for the cadherin-stimulated proliferation, and the control of Rac1 activation by E-cadherin was mediated by p120-catenin. Together, these findings demonstrate a stimulatory role for E-cadherin in proliferative regulation, and identify a simple mechanism by which cell–cell contact may trigger or inhibit epithelial cell proliferation in different settings.

Introduction

Classical cadherins interact homophilically with cadherins of neighboring cells to form adherens junctions, which serve both as mechanical linkages between cells and as signaling hubs that relay information from the extracellular environment. Epithelial cadherin, or E-cadherin, is thought to be a tumor suppressor molecule largely because it is frequently down-regulated in carcinomas (Birchmeier and Behrens, 1994; Berx et al., 1995; Hirohashi, 1998). E-cadherin has also been shown to directly suppress metastasis in the late stages of tumor progression using a transgenic mouse model (Perl et al., 1998). Loss of contact inhibition of proliferation is a hallmark of cancer cells lacking E-cadherin, and transfection of E-cadherin into several such cancer cell lines causes a decrease in proliferation (Navarro et al., 1991; St. Croix et al., 1998; Gottardi et al., 2001). Despite the abundance of literature supporting an antiproliferative role for E-cadherin, there is also evidence that E-cadherin is associated with increased cell proliferation. In colon carcinomas, proliferation is associated with the localization of E-cadherin to the cell periphery (Brabletz et al., 2001). Ovarian cancers up-regulate E-cadherin, the suppression of which inhibits their proliferation (Sundfeldt, 2003; Reddy et al., 2005). In nontumorigenic contexts, E-cadherin levels are maintained in proliferating tissues (Perez-Moreno et al., 2003). In fact, loss of E-cadherin in these physiological settings does not lead to uncontrolled growth, but instead prevents proliferation and causes tissue degeneration during development (Ohsugi et al., 1997), in lactating mammary glands (Boussadia et al., 2002), and in hair follicles (Tinkle et al., 2004). Thus, the effects of E-cadherin on proliferation appear to be multifaceted, dependent on context, and poorly defined.

Cross talk between cell–cell and cell–substrate interactions may contribute to the effects of cadherins on proliferation. The introduction of E-cadherin into cells cultured on a nonadhesive surface not only decreases proliferation but also causes cells to aggregate into large clusters (St. Croix et al., 1998). When cultured on an adhesive substrate, cells expressing E-cadherin exhibit increased cell attachment to the substrate when compared with their nonexpressing counterparts (Watabe et al., 1994; Gottardi et al., 2001). Because such cadherin-induced changes in aggregation or adhesion to ECM can directly affect cell proliferation, the adhesive context in which cadherin engagement is manipulated may contribute to the different proliferative responses that have been observed. In studies of VE-cadherin, which is the major cadherin in endothelial cells, paradoxical effects on proliferation appear to depend on cross talk with cellular adhesion to the ECM. Engagement of VE-cadherin causes growth arrest with increasing cell densities, in part, by causing cells to decrease their adhesion and spreading against the underlying substrate (Nelson and Chen, 2002).

Correspondence to Christopher S. Chen: chrischen@seas.upenn.edu
Abbreviation used in this paper: NRK, normal rat kidney.
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In a setting where cell spreading is held constant, engagement of VE-cadherin causes an increase in proliferation (Nelson and Chen, 2003). It appears that various adhesive contexts need to be explored to fully appreciate the mechanisms by which cadherins regulate proliferation.

E-cadherin engagement influences several intracellular signaling pathways that are involved in the regulation of proliferation, including the canonical Wnt pathway, receptor tyrosine kinases, and Rho GTPase signaling (Wheelock and Johnson, 2003). Signaling to Rho GTPases has been of particular interest because of their involvement in regulating the stability of junctions and associated cytoskeletal structures (Braga, 2000; Yap and Kovacs, 2003). Specifically, E-cadherin activation of Rac1 has been observed by several groups (Nakagawa et al., 2001; Noren et al., 2001), and appears to lead to actin recruitment and physical strengthening of adherens junctions (Ehrlich et al., 2002; Chu et al., 2004). Rac1 is also involved in regulating progression through the G_1 phase of the cell cycle (Olson et al., 1995; Coleman et al., 2004) by modulating p21 levels and cyclin D_1 transcription (Mettouchi et al., 2001; Bao et al., 2002). However, because Rac1 activity appears to provide different functions in response to different stimuli (Ehrlich et al., 2002; del Pozo et al., 2004), Rac1 signaling induced by E-cadherin engagement may not be related to Rac1 signaling in proliferative regulation. Indeed, a link from E-cadherin engagement to proliferation through Rac1 has not been previously reported.

We examined the effects of E-cadherin engagement on proliferation of normal rat kidney epithelial cells (NRK-52E) and nontumorigenic human mammary epithelial cells (MCF-10A) under a variety of adhesive contexts. Limited degrees of cell–cell contact, which were introduced at intermediate cell seeding densities or by forming pairs or small clusters of cells, stimulated cell proliferation, but further increasing cell–cell contact by seeding to confluence inhibited proliferation. The proliferative stimulus was mediated by E-cadherin engagement and coordinated through Rac1 and p120-catenin, whereas the cell–cell contact inhibition of proliferation was driven by a decrease in cell adhesion and spreading on the underlying ECM. These findings demonstrate that cell–cell contact can either enhance or inhibit proliferation via distinct mechanisms, and suggest a novel pathway by which E-cadherin can locally modulate tissue growth in contexts such as development, tissue mass homeostasis, and wound healing.

Results

Epithelial cells exhibit density-dependent biphasic proliferation and cell spreading

To explore the role of cell–cell contact in the proliferation of epithelial cells, we varied the degree to which cells contacted neighboring cells by seeding at different densities. We G_0-synchronized NRK-52E cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200510087/DC1) and seeded them from densities at which cells were completely isolated from each other (2×10^3 cells/cm^2) to confluence (4×10^5 cells/cm^2) overnight in the presence of BrdU, and analyzed for entry into S phase (Fig. 1, A and B). At confluence, cell proliferation was low. Decreasing the plating density from confluence increased the percentage of cells entering S phase, with maximal proliferation at intermediate seeding densities (2–6 $\times$ 10^4 cells/cm^2). Interestingly, further decreasing cell density so that cells did not contact their neighbors caused an unexpected decrease in proliferation. We also noted that cells at the lowest seeding density were often rounded in shape, whereas cells at intermediate densities appeared to be well spread. Directly measuring the spread area of cells revealed that the degree of cell spreading against the substrate was also biphasic with seeding density, and correlated with the levels of proliferation (Fig. 1 C).

These findings were confirmed in a second epithelial cell type, MCF-10A nontumorigenic human mammary epithelial cells. Progressively increasing MCF-10A cell–cell contact by seeding G_0-synchronized cells (Fig. S1) from sparse (10^3 cells/cm^2) to confluent (2×10^5 cells/cm^2) densities also resulted in the density-dependent biphasic proliferative and cell- spreading

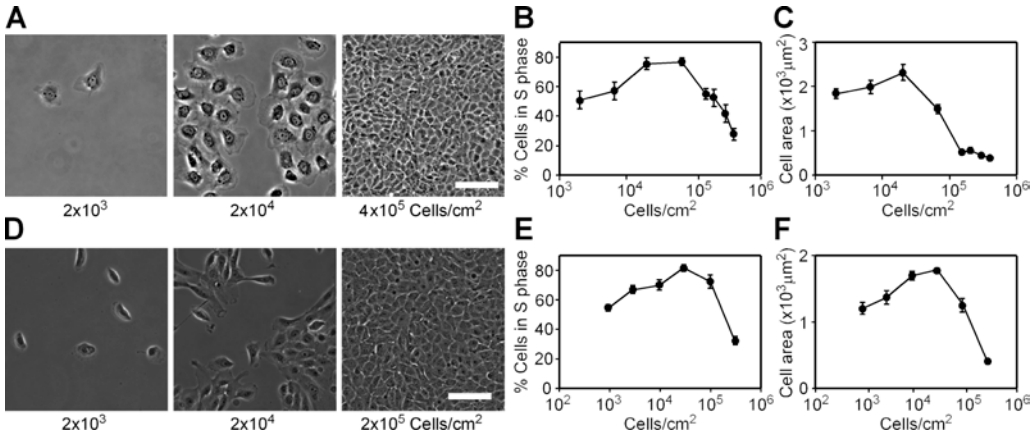

Figure 1. **Epithelial cell proliferation is dependent on seeding density and cell spreading.** (A) Phase-contrast images of NRK-52E cells seeded at the indicated densities. (B) Graph of the percentage of NRK-52E cells entering S phase after seeding of synchronized cells at different densities, measured by BrdU incorporation. (C) Graph of cell spreading of NRK-52E seeded at different densities. (D–F) The same experiments as in A–C, but performed with MCF-10A cells. Error bars indicate the SEM of three independent experiments. Bars, 50 μm.

response, with peak levels at intermediate densities (2×10^4 cells/cm^2), and a precipitous inhibition of proliferation and spreading at high seeding densities (Fig. 1, D–F).

Cell–cell contact–induced proliferation is independent of adhesive context

It has previously been observed that increasing the degree of cell spreading increases proliferation (Folkman and Moscona, 1978; Chen et al., 1997). Because changes in cell spreading correlated with changes in proliferation in both NRK-52E and MCF-10A cells that were seeded at different densities, we examined whether changes in cell spreading were required for contact-induced proliferation. To control cell spreading, we seeded cells onto substrates patterned with microscale agarose wells of varying sizes (Nelson and Chen, 2002). The microwells were fabricated with walls of nonadhesive agarose on top of a glass substrate that was coated with ECM protein. Cells were seeded onto substrates with bowtie-shaped microwells such that two cells would settle into each bowtie. Each cell in the pair adhered to the base of the well and spread to fill half the well, making contact with each other through the center of the bowtie (Fig. 2 A). These contacts were stable over time, as the microwells prevented cells from migrating apart. As a control, single cells were seeded into triangular-shaped wells with areas equal to one half of a bowtie. We found that given the same degree of cell spreading, pairs of cells proliferated at a dramatically higher rate compared with single cells for both NRK-52E and MCF-10A cells (Fig. 2, B and C). This effect was observed for several different microwell sizes, demonstrating that the changes in cell spreading induced by cell–cell contact were not necessary for contact-dependent up-regulation of proliferation.

The ability for cell–cell contact to induce proliferation independent of changes in cell spreading against the underlying ECM suggested that contact-induced proliferation may be a more general phenomenon that is not specific to the two-dimensional culture context. To address this possibility, we examined whether the biphasic proliferative response to cell–cell contact also occurs in three-dimensional culture. MCF-10A cells were seeded at a density of 5–10 $\times$ 10^6 cells/cm^3 within collagen gels overnight. Under these conditions, both single, isolated cells, as well as clusters with varying numbers of cells, developed. Immunostaining revealed no striking differences in individual cell morphology or their junctions among the different sized clusters (Fig. 2 D). To examine the proliferation of cells within the clusters, we seeded G_0-synchronized cells into collagen gels in the presence of BrdU overnight. Single cells proliferated at a low rate, whereas cells within small clusters (two to five cells per cluster) exhibited high rates of proliferation. Proliferation was progressively inhibited when cells were aggregated in increasingly larger clusters (Fig. 2, E and F). Interestingly, we observed that proliferation in the large clusters was predominantly limited to cells on the surface of the cluster where fewer cell–cell contacts are formed, and that most cells within the interior of the cluster remained quiescent.

In summary, proliferation appears to respond biphasically to the amount of cell–cell contact in both two- and three-dimensional culture contexts. Cells without any cell–cell contact exhibit the lowest rate of proliferation. Low degrees of cell–cell contact, such as those experienced by cells within small clusters, on the periphery of large clusters, in bowtie-shaped microwells, or in intermediate seeding densities, stimulate proliferation. But high degrees of contact, such as those experienced by cells within the interior of large clusters or at high densities, appear to inhibit proliferation.

E-cadherin is required for cell–cell contact–induced proliferation

We then examined whether cadherins were involved in cell–cell contact–mediated changes in proliferation. We constructed an adenovirus containing a mutant of E-cadherin lacking the

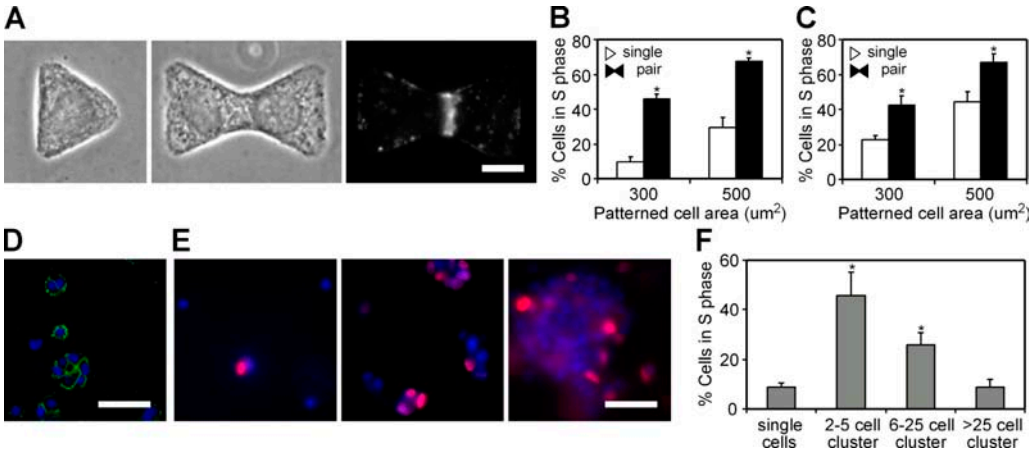

Figure 2. **Contact-stimulated proliferation is spreading independent.** (A) Phase-contrast images of MCF-10A cells patterned in triangular (left) and bowtie-shaped (middle) microwells and then immunostained for E-cadherin (right). Graph of percentage of single and pairs of NRK-52E (B) and MCF-10A (C) cells entering S phase after being synchronized and seeded onto microwell substrates of indicated sizes. (D) MCF-10A cells embedded in collagen gels and immunostained for E-cadherin (green) and (E) BrdU (red), both of which were counterstained with Hoechst (blue). (F) Graph of percentage of MCF-10A cells within different sized clusters entering S phase after seeding of synchronized cells into collagen gels. Error bars indicate the SEM of three independent experiments. *, $P < 0.05$, calculated by *t* test and compared with single cells. Bars: (A) 10 μm; (D and E) 20 μm.

β-catenin–binding domain (Ad-EΔ), which has previously been shown to act as a dominant negative by blocking E-cadherin–mediated intercellular adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). Immunostaining of E-cadherin in Ad-EΔ–infected cells confirmed the loss of cadherin localization at the cell–cell junctions in both NRK-52E and MCF-10A cells (Fig. 3, A and D). Infection with Ad-EΔ eliminated the contact-induced peak in proliferation seen at intermediate densities, when compared with Ad-GFP–infected control cells in both cell lines (Fig. 3, B and E). Interestingly, expression of EΔ did not affect proliferation of cells seeded at very high densities, which have many cell–cell contacts, suggesting that E-cadherin is not required for the reduced levels of proliferation at confluence observed in this setting.

We also examined whether expression of EΔ blocked the proliferation stimulated by cell–cell contact within microwell cultures in both epithelial cell types. Ad-EΔ reduced the proliferation of pairs of cells to the levels of single cells that were spread to the same degree (Fig. 3, C and F).

We confirmed these results by using a blocking antibody against E-cadherin that prevented E-cadherin engagement in MCF-10A cells (Fig. 3 G). Inhibition of cadherin engagement with the blocking antibody abrogated the contact-induced peak in proliferation at intermediate seeding densities and the increase in proliferation of pairs of cells compared with single cells (Fig. 3, H and I). Similarly, knockdown of E-cadherin expression using siRNA also eliminated contact-induced proliferation (Fig. 3, J–L). Together, these data suggest that E-cadherin is required for stimulation of proliferation induced by cell–cell contact.

Interestingly, in all three methods of eliminating E-cadherin engagement and in both cell lines, E-cadherin engagement appears not to be required for the cell–cell contact–induced proliferation arrest at high cell densities. An alternative possibility is that cell–cell contact nonspecifically crowds cells to spread less

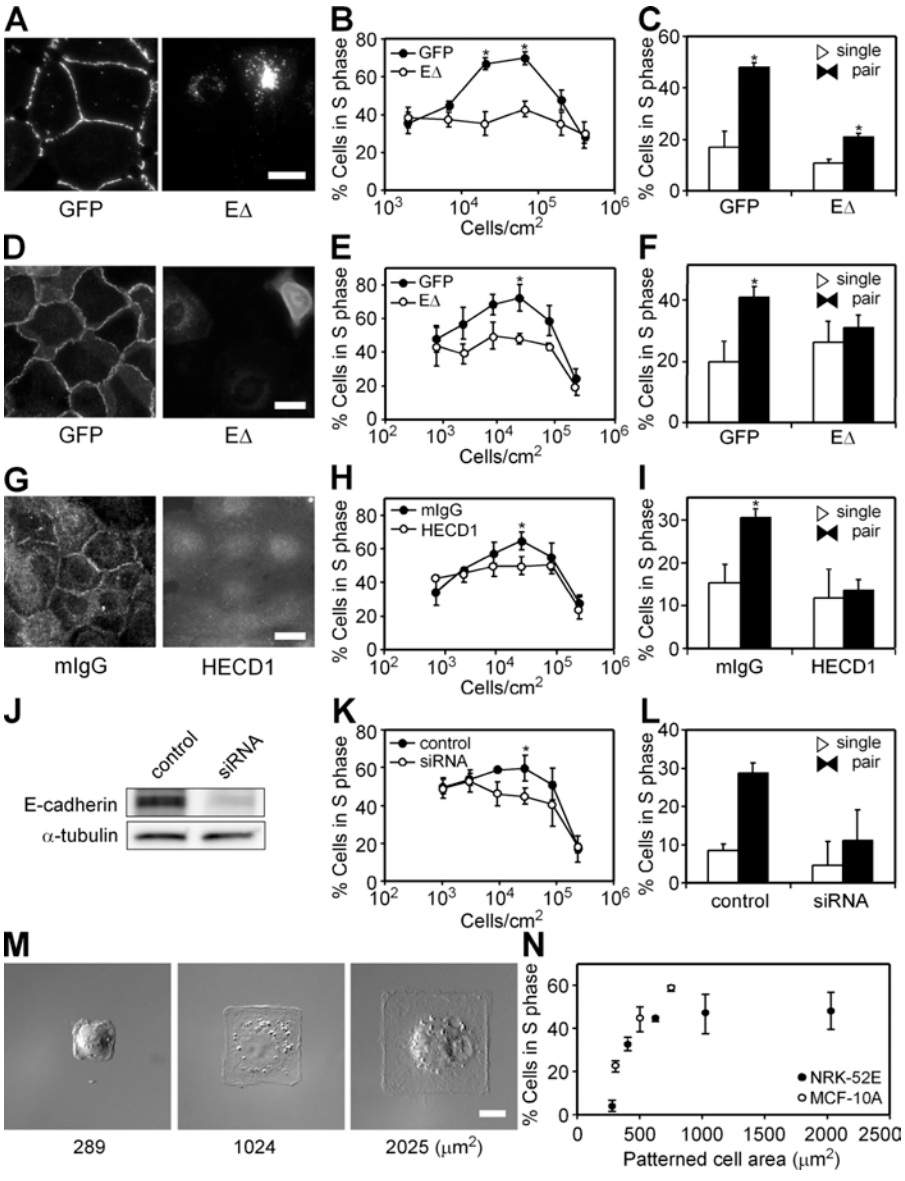

Figure 3. **E-cadherin is required for contact-mediated proliferation.** Fluorescence images of E-cadherin in Ad-GFP– (left) and Ad-EΔ–infected (right) NRK-52E (A) and MCF-10A (D) 24 h after seeding. Graph of percentage of NRK-52E (B) and MCF-10A (E) cells synchronized and infected with Ad-GFP and Ad-EΔ entering S phase seeded at different densities. Graph of percentage of pairs and single Ad-EΔ− or Ad-GFP–infected NRK-52E (C) and MCF-10A (F) cells entering S phase. (G–I) The same experiments in MCF-10A cells as in D–F, except the cells were treated with HECD-1 blocking antibody to E-cadherin or mouse IgG (mIgG) control. (J) Western blots of a 120-kD E-cadherin and a 50-kD α-tubulin for control-transfected and E-cadherin siRNA–transfected MCF-10A cells. Graph of percentage of MCF-10A cells transfected with control or E-cadherin siRNA entering S phase when seeded at different densities (K) or in triangular or bowtie-shaped microwells (L). Differential interference contrast images of NRK-52E cells micropatterned onto different sized squares and (M) graph of percentage of synchronized NRK-52E and MCF-10A single cells entering S phase after seeding in different size microwells (N). Error bars indicate the SEM of at least three experiments, and a range of two independent experiments in L. *, $P < 0.05$, calculated by *t* test between control (Ad-GFP–infected, mIgG-treated, or control-transfected) and experimental (Ad-EΔ–infected, HECD-1–treated, or siRNA-transfected) cells in the same seeding condition. Bars: (A, D, and G) 20 μm; (M) 10 μm.

against the underlying substrate, and this decrease in cell–ECM interaction arrests cells. To address this, G_0-synchronized NRK-52E and MCF-10A cells were seeded overnight into microwells of different sizes, such that single cells attached in each microwell, and analyzed for S phase entry (Fig. 3 M). In both cell lines, the micropatterned islands decreased proliferation with decreased cell spreading, even in the absence of cell–cell contact (Fig. 3 N). This inhibition of proliferation on micropatterns was not affected by infection of Ad-EΔ, confirming that E-cadherin is not involved in this regulation of proliferation by cell spreading, and that Ad-EΔ does not nonspecifically disrupt proliferation in these cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200510087/DC1). These data suggest that cell–cell contact inhibits proliferation by decreasing cell spreading, and stimulates proliferation through E-cadherin engagement.

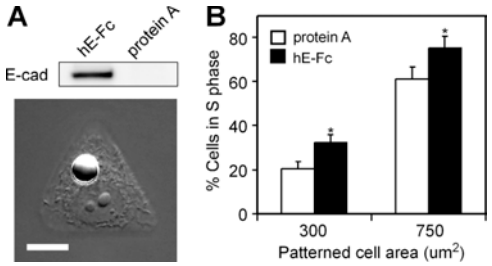

Figure 4. **Engagement of E-cadherin receptors stimulates proliferation.** (A) Western blot of a 120-kD E-cadherin on hE-Fc–coated or control protein A–coated beads (top) and differential interference contrast image of patterned cell in a 750 μm² microwell with bead attached (bottom). (B) Graph of percentage of cells entering S phase of cells seeded in the indicated microwell sizes, with hE-Fc–coated beads or control protein A–coated beads. Error bars indicate the SEM of at least three experiments. *, $P < 0.05$, compared with control. P was calculated by *t* test. Bar, 10 μm.

Engagement of E-cadherin is sufficient for stimulation of proliferation

Although the cadherin-blocking studies demonstrated that E-cadherin was required for the stimulation of proliferation observed at intermediate densities and in pairs of cells in bowtie-shaped microwells, it was unclear whether cadherins were inducing proliferation through juxtacrine influences or by acting as receptors themselves. To explore this further, we engaged cadherins of single, isolated, patterned MCF-10A cells using beads coated with a chimera of the ectodomain of human E-cadherin fused to the immunoglobulin Fc domain (hE-Fc; Fig. 4 A). In both unspread (300 μm²) and spread (750 μm²) conditions, cells that were bound to hE-Fc–coated beads exhibited higher proliferation compared with cells that were bound to protein A–coated control beads (Fig. 4 B). These data demonstrate that the engagement of E-cadherin alone can stimulate proliferation independently of juxtacrine influences.

Rac1 activity is required for contact-mediated proliferation

We next explored the role of Rac1 as a potential downstream mediator of the E-cadherin–induced proliferation because cells at intermediate densities exhibited morphological characteristics of high Rac1 activity, such as increased cell spreading (Ridley et al., 1992). First, we examined the timing of Rac1 activation with respect to contact formation. G_0-synchronized MCF-10A cells were seeded at an intermediate density (2×10^4 cells/cm²)

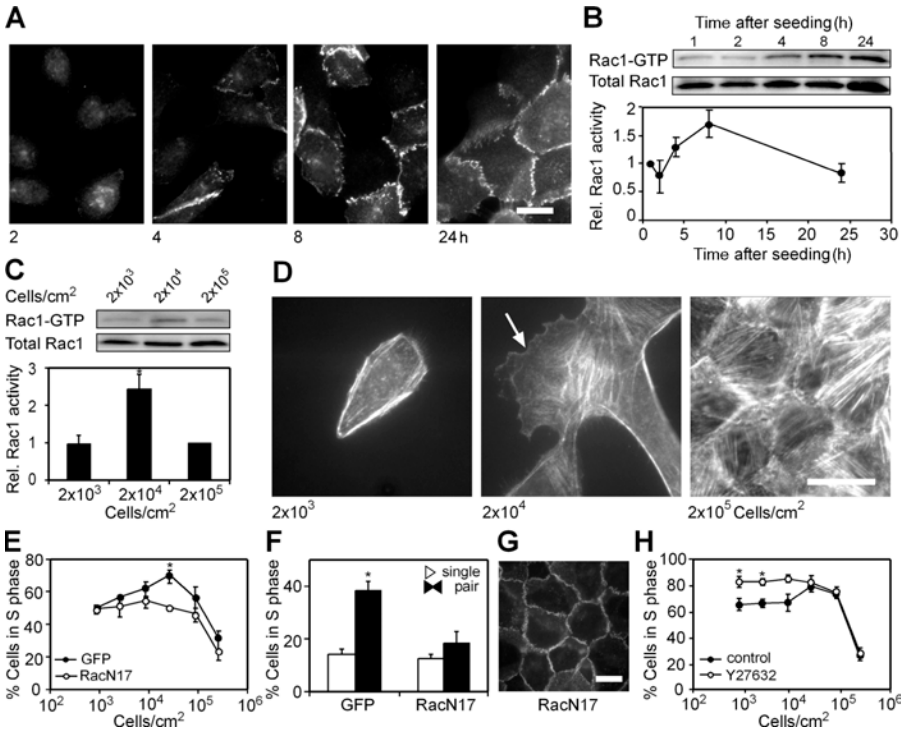

Figure 5. **Rac1 is involved in cell–cell contact–stimulated proliferation.** (A) Fluorescence images of E-cadherin in MCF-10A cells seeded for the indicated number of hours. (B) Western blot of Rac-GTP and total Rac1 levels (21 kD) in synchronized MCF-10A cells seeded at 2×10^4 cells/cm² for the indicated time (top), and a graph of averaged relative Rac1 activity across three separate experiments (bottom). (C) Western blot of Rac1-GTP and total Rac1 levels (21 kD) of synchronized MCF-10A cells seeded at the indicated densities 8 h after seeding (top), and graph of averaged relative Rac1 activity across three separate experiments (bottom). (D) Fluorescence images of phalloidin-stained cells seeded at indicated densities. Arrow indicates the presence of lamellipodia. (E) Graph of percentage of synchronized MCF-10A cells infected with Ad-RacN17 entering S phase seeded at different densities. (F) Graph of percentage of pairs and single Ad-RacN17– or Ad-GFP–infected MCF-10A cells entering S phase. (G) Fluorescence image of E-cadherin in Ad-RacN17–infected cells 24 h after seeding. (H) Graph of percentage of MCF-10A cells synchronized and seeded at different densities in the presence of Y27632 entering S phase. Error bars indicate the SEM of at least three experiments. *, $P < 0.05$, between intermediate density compared with low or high density seeded cells (C), or between control (Ad-GFP or no treatment) and experimental (Ad-RacN17– or Y27632-treated) cells (E, F, and H). P was calculated by *t* test. Bars, 20 μm.

and assayed for the formation of cell–cell contact by immunofluorescence staining and for Rac1 activity by pulldown assay over the course of 24 h. Initial formation of cadherin-containing contacts occurred at 4 h after seeding. By 8 h, most cells had formed contacts with neighbors, and the intensity of staining at junctions continued to increase over the subsequent 16 h (Fig. 5 A). Relative Rac1 activity was initially low, gradually increased to a peak at ~8 h after seeding, and then decreased to baseline levels by 24 h (Fig. 5 B). The correlation between E-cadherin staining and Rac1 activity at 8 h after seeding supported the possibility that Rac1 was activated by cell–cell contact.

To directly examine the role of cell–cell contact on Rac1 activity levels, we seeded G_0-synchronized MCF-10A cells at high (2×10^5 cells/cm^2), intermediate (2×10^4 cells/cm^2), and low (2×10^3 cells/cm^2) densities and assayed for Rac1 activity 8 h after seeding. Rac1 activity was twofold higher at intermediate cell densities when compared with either low or high cell densities (Fig. 5 C). Phalloidin staining of MCF-10A cells 8 h after seeding at different densities also demonstrated that cells at the intermediate density exhibited increased membrane ruffling, a phenotype of elevated Rac1 activity (Fig. 5 D).

To determine whether the Rac1 activity induced by cell–cell contact was involved in the proliferative response, G_0-synchronized MCF-10A cells were infected with an adenovirus containing dominant-negative Rac1 (Ad-RacN17) and examined for S phase entry at different seeding densities. Infection of Ad-RacN17, like Ad-EΔ, eliminated the peak in proliferation at intermediate densities, and did not affect proliferation at high and low densities (Fig. 5 E). In the microwell system, where changes in cell spreading were prevented, expression of RacN17 reduced the proliferation of pairs of cells to that of single cells (Fig. 5 F). Immunostaining for E-cadherin in Ad-RacN17–treated cells confirmed that dominant-negative Rac1 did not affect localization of E-cadherin at the cell–cell junction (Fig. 5 G).

This inhibitory effect appeared to be specific to a Rac1-mediated pathway, as inhibition of RhoA signaling through its effector Rho kinase by exposure to 50 μM Y27632 did not inhibit proliferation at any density, but, interestingly, increased proliferation at the low densities (Fig. 5 H). Y27632 also had a stimulatory effect on proliferation of single and pairs of cells patterned in microwells, and did not appear to mediate these effects by altering E-cadherin localization to the cell–cell contacts (unpublished data). Y27632 has been shown to activate Rac1 and cyclin D_1 signaling in fibroblasts (Welsh et al., 2001). Supporting this possibility, infection of cells with Ad-RacN17 inhibited the increase in proliferation with Y27632 at low densities (unpublished data). These data demonstrated that cell–cell contact–induced proliferation is mediated through Rac1, and not RhoA.

Rac1 activity lies downstream of E-cadherin engagement

Our findings indicated that E-cadherin and Rac1 activity are both required for the proliferation induced by cell–cell contact, but the causal relationship between E-cadherin engagement and Rac1 activity remained unclear. To address whether E-cadherin is responsible for Rac1 activation at intermediate densities, we infected G_0-synchronized MCF-10A cells with Ad-EΔ or Ad-GFP, and assayed for Rac1 activity 8 h after seeding. The increase in Rac1 activity observed at intermediate densities was abrogated by Ad-EΔ (Fig. 6 A), but baseline Rac1 activity at high and low densities were not significantly affected. Phalloidin-stained cells at intermediate densities revealed that Ad-EΔ diminished the previously observed membrane ruffling (Fig. 6 B). Furthermore, the peak of cell spreading at intermediate densities was abolished by Ad-EΔ (Fig. 6 C). These data confirm that Rac1 and its functional effects on cytoskeletal processes lie downstream of E-cadherin at intermediate seeding densities, and suggest that E-cadherin stimulates proliferation through a Rac1-mediated pathway.

Because the Rac1 effector Pak has been implicated in Rac1-mediated proliferation, we explored its role in E-cadherin–mediated proliferation. Similar to Ad-EΔ– and Ad-RacN17–infected cells, MCF-10A cells infected with an adenovirus that expressed a kinase-dead mutant of Pak (Ad-PakR299) also lost the biphasic proliferative response (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200510087/DC1). However, this full-length dominant-negative Pak has been shown to interact with and inactivate Rac1. Expressing the more specific

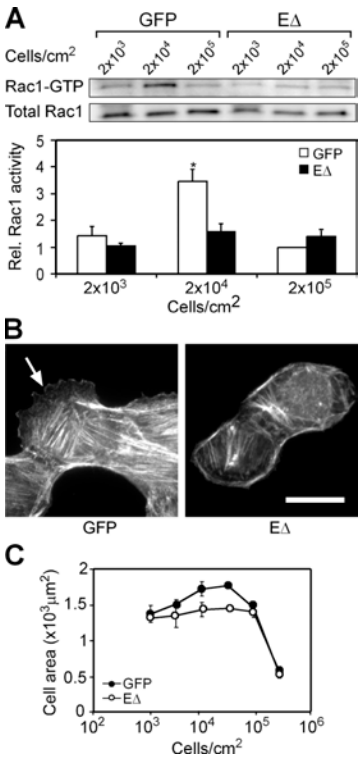

Figure 6. **Rac1 activity is downstream of E-cadherin.** (A) Western blot of Rac-GTP and total Rac1 levels (21 kD) of synchronized MCF-10A cells infected with Ad-EΔ or Ad-GFP control and seeded for 8 h (top), and graph of averaged relative Rac1 levels across three separate experiments (bottom). (B) Fluorescence images of phalloidin-stained Ad-GFP– (left) and Ad-EΔ–infected (right) synchronized MCF-10A cells seeded at 2×10^4 cells/cm^2 for 8 h. Arrow indicates the presence of lamellipodia. (C) Graph of cell area of synchronized MCF-10A cell infected with Ad-GFP or Ad-EΔ and seeded at varying densities for 24 h. Error bars indicate the SEM of at least three experiments in A, or the range between two experiments in C. *, $P < 0.05$, between Ad-GFP– or Ad-EΔ–infected cells at the indicated density. P was calculated by *t* test. Bar, 20 μm.

Pak-PID mutant, in contrast, showed no effect on the proliferative response to cell density (Fig. S3 B). Lastly, endogenous total Pak levels and Pak phosphorylation monotonically decreased with cell-seeding density (Fig. S3 C). Together, these data suggest that Pak signaling is not involved in the cadherin- and Rac1-induced proliferation.

p120-catenin is involved in E-cadherin–induced proliferation and Rac1 activation

Cadherin engagement has been observed to stimulate Rho GTPases, in part by binding p120-catenin (p120) and abrogating the ability of p120 to inhibit Rho (Anastasiadis et al., 2000). To explore the role of p120 in E-cadherin–induced proliferation via Rac1, we generated MCF-10A cell lines stably expressing p120-siRNA or empty vector alone as a control using the pRetroSuper retroviral system (Ireton et al., 2002). Cells with p120-siRNA expressed <30% of control levels of p120 (Fig. 7 A). G_0-synchronized cells were seeded at different densities and assayed for proliferation. p120 knockdown abolished the biphasic proliferative response to cell seeding density, and cells exhibited a higher level of proliferation at the lowest seeding densities, as compared with control cells (Fig. 7 B). To examine whether Rac1 was involved in this up-regulation of proliferation, we seeded control and siRNA-treated cells at 9×10^3 cells/cm^2, and measured Rac1 activity. Cells lacking p120 exhibited increased Rac1 activity (Fig. 7 C). Furthermore, Rac1 was required for the proliferation observed in siRNA-treated cells, as Ad-RacN17 abolished the knockdown-induced proliferation (Fig. 7 D).

We next examined whether reexpression of p120 could inhibit the Rac activity observed in knockdown cells. siRNA-treated cells were infected with a second retroviral vector (LZRS) expressing the murine form of the p120 found in MCF-10A cells (isoform 3A). Knockdown cells stably expressing murine p120 had significantly lower Rac activity when compared with knockdown cells expressing the empty second retroviral vector (Fig. 7 E). Furthermore, reexpression of p120 suppressed the high proliferative levels observed in knockdown cells (Fig. 7 F). This inhibitory effect did not require the binding of p120 to cadherins because expression of a mutant p120, which lacks ARM repeat 1 and therefore cannot bind to cadherins (p120Δ; Reynolds and Roczniak-Ferguson, 2004), also exhibited low levels of Rac1 activity and proliferation at intermediate seeding densities. These data suggest that p120 may be involved in cadherin-induced Rac1 signaling and proliferation, and support a model in which p120 normally suppresses Rac1 and proliferation until engagement of E-cadherin sequesters p120, disinhibiting Rac1 activity.

Discussion

E-cadherin engagement at cell–cell contacts has a known function in the suppression of proliferation, which is best described in the context of tumorigenesis (Hirohashi, 1998). We observed a biphasic proliferative response to cell–cell contact. Blocking E-cadherin engagement abrogated the elevated proliferation levels observed at intermediate seeding densities, but not the inhibition of proliferation observed at confluence. Instead, mimicking the reduction in cell adhesion and spreading induced at confluence by culturing isolated cells on micropatterned substrates resulted in proliferation arrest, suggesting that decreased cell spreading may be responsible for contact inhibition in these cells. These findings contrast with previous conclusions that transformed cells exhibit uncontrolled growth after down-regulation of E-cadherin (Navarro et al., 1991), possibly because the tools to uncouple the confounding effects of cell–cell contact, cadherin engagement, and cell spreading have only recently become available. Nonetheless, reconciling responses between tumorigenic and nontumorigenic cells may be inappropriate because cancerous cell lines may have already lost

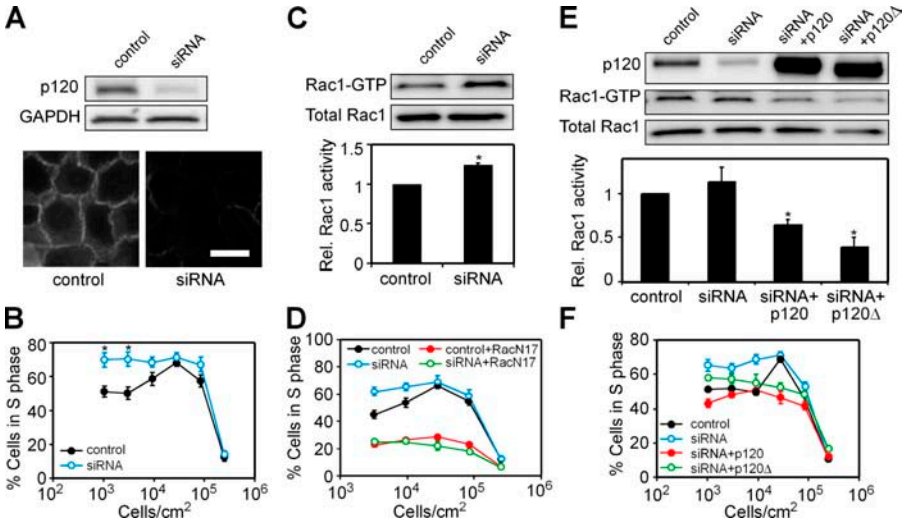

Figure 7. **p120-catenin is involved in E-cadherin stimulation of Rac1 and proliferation.** (A) Western blot of p120 levels (120 kD) and GAPDH (38 kD) in control- or p120-siRNA–infected (top) and fluorescence images of p120 in control- (bottom left) and siRNA-infected (bottom right) MCF-10A cells. (B) Graph of percentage of synchronized control or p120-siRNA MCF-10A cells entering S phase when seeded at different densities. (C) Western blot of Rac1-GTP and total Rac1 levels (21 kD) in control- or siRNA-infected MCF-10A cells synchronized and then seeded at 10^4 cells/cm^2 (top), and a graph of averaged relative Rac1 activity across three separate experiments (bottom). (D) Graph of percentage of synchronized control- or p120-siRNA–infected MCF-10A cells, which were also infected with Ad-GFP or Ad-RacN17 entering S phase when seeded at different densities. (E) Western blot of p120 levels (120 kD), Rac1-GTP, and total Rac1 levels (21 kD) in cells infected with control, p120-siRNA, and p120-siRNA with murine p120 or p120Δ, synchronized, and seeded at 2×10^4 cells/cm^2 (top), and graph of averaged relative Rac1 activity across three experiments (bottom). (F) Graph of percentage of cells infected with control, p120-siRNA, and p120-siRNA with murine p120 or p120Δ, synchronized, and seeded at different densities entering S phase. Bar, 20 μm. Error bars indicate the SEM of at least three experiments. *, $P < 0.05$, compared with control. P was calculated by *t* test.

their adhesion-regulated controls (Wittelsberger et al., 1981). The finding that degrees of contact may differentially regulate cells through numerous mechanisms highlights the need for better approaches to tease out the various environmental cues that may be affected by cell–cell adhesion.

In propagating a proliferative signal, cadherins may act directly as receptors that cause intracellular signaling, or they may function primarily to bring cells into contact with each other to signal via other juxtacrine receptors. For example, E-cadherin has been shown to initiate signaling in an EGF receptor–dependent manner (Pece and Gutkind, 2000; Betson et al., 2002). Using E-cadherin-Fc–coated beads to ligate E-cadherin, we found that the engagement of E-cadherin alone is sufficient to stimulate proliferation. Although these findings do not eliminate the possibility that juxtacrine signals can also contribute to the cadherin-mediated proliferative response, they add to the growing body of evidence that cadherins can provide direct, functionally relevant signaling beyond their structural role.

We demonstrate that E-cadherin–activated Rac1 and downstream effects on cell spreading and membrane ruffling only occurred with limited cell–cell contact. Several mechanisms may be responsible for the activation of Rac1 in these limited cell–cell contact settings. First, the dynamics of the cadherin contacts in a cell with only a few bordering cells may be distinct from those in a cell within a confluent monolayer. As in studies of integrin activation of Rho GTPases (Ren et al., 1999; del Pozo et al., 2000), such receptor dynamics may be important for cadherin activation of Rac1. Second, the mechanism for up- and down-regulation of Rac1 signaling may be distinct; for example, E-cadherin engagement at intermediate densities might activate Rac1 and the decrease in cell spreading at high densities might inhibit Rac1. This biphasic response demonstrates how multiple inputs are likely integrated by the Rac1 signaling pathway to produce a decisive response within the cell.

E-cadherin activation of Rac1 appears to involve p120. p120 has also been implicated in the regulation of other Rho GTPases by cadherins. In the case of RhoA, cadherin binding of p120 appears to compete with the ability of p120 to inhibit RhoA signaling (Anastasiadis et al., 2000). Although the role of p120 in mediating E-cadherin–induced Rac1 activity has been less well characterized, our findings suggest that p120 may function analogously, whereby E-cadherin engagement shifts p120 between cadherin-bound and Rac-inhibitory roles. This model is also consistent with reports that Rac1 activation by E-cadherin engagement is inhibited by a mutation that prevents p120 from binding to E-cadherin (Goodwin et al., 2003).

E-cadherin–mediated Rac1 activity stimulated proliferation. Rac1 activity has been shown to regulate cell cycle progression via MAPK signaling (Minden et al., 1995), as well as the NFκB pathway (Joyce et al., 1999). The Rac1 effector Pak has also been shown to activate numerous mitogenic pathways (Brown et al., 1996; Frost et al., 1996). However, our data suggest no role for Pak in E-cadherin–mediated proliferation via Rac1. A viable alternative is that Rac1 signaling may feed back to affect cell–matrix interactions through changes in actin and integrin dynamics (Kiosses et al., 2001)—a possibility that will require further study. Although Rac1-induced proliferation was evident before the current study, it has been unclear what physiologic situation might invoke the Rac1 proliferative pathway. Our results now suggest that E-cadherin engagement provides a physiologic stimulus for Rac1-mediated proliferation (Fig. 8).

Major differences are revealed when comparing cellular response to cadherin engagement among different cell types. E-cadherin engagement in epithelial cells stimulated proliferation via Rac1. In endothelial cells, engagement of VE-cadherin induces RhoA signaling (Nelson and Chen, 2003), and cell–cell contact in fibroblasts stimulates a contractile response (Abercrombie, 1970). Interestingly, both endothelial cells and fibroblasts have been previously reported to exhibit a RhoA- and tension-dependent pathway to proliferation (Huang et al., 1998; Welsh et al., 2001; Nelson and Chen, 2003), whereas we demonstrate that epithelial cells proliferate through a RhoA-independent and Rac1-dependent mechanism. In fibroblasts, Rac1 also induces cyclin D_1 expression, but this pathway is cryptic under normal conditions, and is revealed only upon artificial inhibition of RhoA or Rho kinase (Welsh et al., 2001). Together, these data suggest that the links between specific cadherin subtypes, Rho GTPase signaling, and cell proliferation may vary for different cell types. Subtle shifts in such regulatory pathways could have far-reaching consequences on the canonical relationships between multicellular organization, cell structure, and cell function.

Although cadherins were originally discovered to serve as a mechanical linkage between adjacent cells, it has become apparent that these receptors also regulate cell function via biochemical signaling pathways. The biphasic increase in Rac1 activity, cell ruffling and spreading, and proliferation via cell–cell contact observed might be important in several

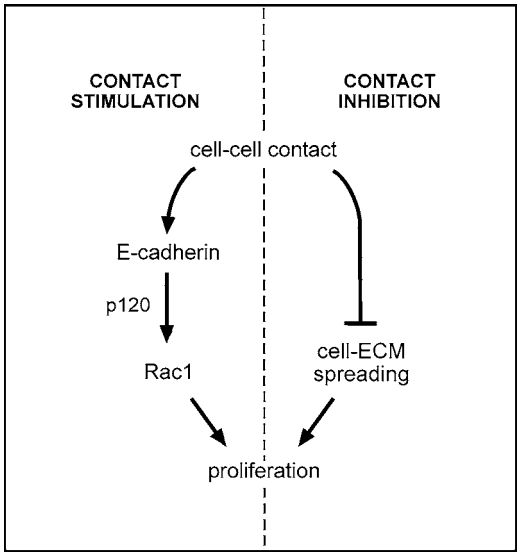

Figure 8. **Schematic of signaling pathway leading cell–cell contact to inhibition or stimulation of proliferation.** Cell–cell contact stimulates proliferation through activation of Rac1 that depends on E-cadherin engagement and p120. Inhibition of proliferation by cell–cell contact results from a crowding-dependent, cadherin-independent decrease in cell–ECM spreading.

physiological contexts. During both development and adult tissue homeostasis, the link between cell–cell contact, Rac1, and proliferation may be in place to ensure that cells at the edges of epithelial sheets or masses ruffle, spread, and proliferate, whereas those fully constrained within these structures remain quiescent. In the context of loosely associated cells coming together to form new tissue, this system also would encourage tissue growth and rearrangement only when enough cells of the same type are associated with each other, but not when single cells are mislocalized or when cells have formed a sufficient mass. Thus, the ability of cells to sense varying degrees of cell–cell contact through this biphasic cadherin–Rac1 pathway may provide a key element in focusing cellular activity to the appropriate coordinates within a multicellular tissue, and underscores the importance of the numerous transduction mechanisms that are regulated by cadherins and used by cells to navigate their complex, structured microenvironment.

Materials and methods

Cell culture and reagents

NRK-52E and MCF-10A cells were obtained from the American Type Culture Collection and cultured according to their recommendations. Phoenix cells (a gift from A. Reynolds, Vanderbilt University, Nashville, TN) were cultured as previously described (Ireton et al., 2002). hE-Fc–producing CHO cells (a gift from A. Yap, University of Queensland, Brisbane, Australia) were cultured as previously described (Kovacs et al., 2002). Reagents were obtained as follows: anti–E-cadherin (36 [BD Biosciences]; HECD-1 and SHE78-7 [Zymed Laboratories]; DECMA-1 [Sigma-Aldrich]); anti-p120 (98; BD Biosciences); anti–α-tubulin (Sigma-Aldrich); anti-GAPDH (Ambion); anti-Pak1/2/3 and anti–phospho-Pak1(Thr423)/Pak2(Thr402) (Cell Signaling Technology); and Y27632 (Calbiochem). Myc-tagged RacN17 adenovirus, GFP-tagged PakR299, and GFP-tagged Pak-PID adenoviruses were gifts from A. Ridley (University College London, London, UK), W. Gerthoffer (University of Nevada, Reno, NV), and J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA) and V. Weaver (University of Pennsylvania, Philadelphia, PA), respectively.

Proliferation assays

Cells were G_0-synchronized by replacing growth medium with starvation medium (1% serum for NRK-52E or 0% serum for MCF-10A) for 24 h. Synchronization was confirmed in >90% of cells in G_0/G_1 by FACS analysis of propidium iodide–stained (Invitrogen) cells (Fig. S1, A and C). To determine a time point for proliferation assays, cells were seeded onto 25 μg/ml fibronectin- or 50 μg/ml collagen-coated glass substrates, pulsed with BrdU, and analyzed for BrdU incorporation (GE Healthcare). In all proliferation experiments, cells were cultured in the presence of BrdU for a time period during which cells had entered S phase, but had not begun mitosis (as determined by examination of mitotic figures); 18 h for NRK-52E and 22 h for MCF-10A (Fig. S1, B and D), and then fixed and assayed for BrdU incorporation. Unless otherwise noted, at least 300 cells were examined across a minimum of three experiments for all conditions reported.

Three-dimensional culture

Collagen gels (2.5 mg/ml) were generated with a solution of acidic collagen (BD Biosciences), sodium bicarbonate (Sigma-Aldrich), Hepes buffer and 10× M199 (both from Invitrogen), which was neutralized with sodium hydroxide (Sigma-Aldrich). Cells were pelleted and resuspended within the collagen solution, and incubated at 37°C until the collagen solidified. Full serum media was added on top of the gel and proliferation was assayed as described in the previous section.

Micropatterned substrates

Microwell substrates were prepared as previously described (Nelson and Chen, 2002). In brief, stamps of polydimethylsiloxane (Dow Corning), which were cast from photolithographically generated master patterns, were treated with UV/ozone for 5 min before use. A 0.6% agarose/40% ethanol solution in water was flowed between the stamp sealed against a glass slide. Upon stamp removal, substrates were coated with fibronectin or collagen for at least 1 h. Cells were seeded and assayed for proliferation as described in the Proliferation assays section.

hE-Fc–coated beads

hE-Fc was purified from conditioned media of CHO cells stably expressing a secreted human E-cadherin fused to the Fc region of IgG, as previously described (Kovacs et al., 2002), and was used at 100 μg/ml in 0.1% BSA for binding to protein A-coated latex beads (Bangs Laboratories, Inc.). Beads were applied to cells 2 h after seeding, and cells were fixed and analyzed for proliferation as described in the Proliferation assays section.

Microscopy, immunofluorescence, and image acquisition

Images of fixed samples were acquired at room temperature using an epifluorescence microscope (model TE200; Nikon) equipped with Plan Fluor 10×, 0.3 NA, and Plan Apo 60×, 1.4 NA, oil immersion lenses, Spot camera and software (Diagnostic Instruments), or an epifluorescence microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with 40× Plan-Neofluar, 1.3 NA, oil immersion, 63× Plan-Apochromat, 1.4 NA, oil immersion objectives, an Axiocam camera, and Axiovision software. For measurements of projected cell area, cells were outlined in 10× phase-contrast images and analyzed using Spot software. For immunostaining, cells were fixed in 1:1 methanol/acetone for 20 min (for E-cadherin) or formaldehyde followed by 0.05% Triton X-100 (for p120-catenin), blocked with goat serum (Invitrogen) in PBS, and incubated in primary and Alexa Fluor 594– or 488–conjugated secondary antibodies (Invitrogen). Apotome and AxioVision software (Carl Zeiss MicroImaging, Inc.) were used to capture images in three-dimensional cultures. Some image levels were adjusted using Photoshop (Adobe).

Recombinant adenovirus construction

The cDNA fragment encoding human E-cadherin lacking 105 bps at the COOH-terminus (the β-catenin–binding domain) was amplified by PCR from hEcad/pcDNA3 vector (a gift from C.J. Gottardi and B.M. Gumbiner, University of Virginia, Charlottesville, VA) using 5′ (5′-GAGGCGGCCGCACCATGGGCCCTTGGAGCCGC-3′) and 3′ (5′-GAGCTCGAGTCAGGAGCTCAGACTAGCAGC-3′) oligonucleotide primers. Recombinant adenoviruses encoding human E-cadherin bicistronic to GFP were prepared using the AdEasy XL system (Stratagene) as previously described (Nelson et al., 2004).

Rac1 activity assays and Western blotting

GTP-loaded Rac1 was measured using a commercially available kit (Upstate Biotechnology) as previously described (Glaven et al., 1999). Pak1-PBD beads were used as supplied and also made using GST-tagged recombinant Pak1-PBD produced in BL21 cells containing the pGEX-PBD vector (a gift from L. Romer, Johns Hopkins University, Baltimore, MD). Protein levels were determined by Western blot, detected with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), developed using ECL substrate (Pierce Chemical Co.), and quantified using Versadoc imaging system (Bio-Rad Laboratories).

siRNA transfection and infections

siRNA against E-cadherin (a gift from R. Assoian, University of Pennsylvania, Philadelphia, PA) was transfected using Lipofectamine 2000 (Invitrogen) 24 h after seeding MCF-10A cells at 5×10^4 cells/cm^2. Cells were G_0-synchronized and seeded onto appropriate substrates, and proliferation was assayed as described in the Proliferation assays section.

Retroviral supernatants were produced in Phoenix cell packaging line, as previously described (Ireton et al., 2002). Stable lines of MCF-10A cells expressing p120-siRNA were generated with pRetroSuper containing p120-siRNA. Cells were selected and maintained in 4 μg/ml puromycin. To generate cell lines reexpressing murine p120, some of both control and RNAi cell lines were infected with a second retrovirus (LZRS) containing p120 or p120Δ, and selected and maintained in 800 μg/ml G418. Empty vector controls were used in all cases. All retroviral reagents were gifts from A. Reynolds.

Online supplemental material

Fig. S1 shows the synchronization and proliferation profiles for NRK-52E and MCF-10A cells. Fig. S2 shows that E-cadherin is not required for inhibition of proliferation caused by reduced cell spreading in single-patterned cells. Fig. S3 shows that the Rac1 effector Pak is not involved in cell–cell contact–mediated proliferation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200510087/DC1.

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References

Abercrombie, M. 1970. Contact inhibition in tissue culture. *In Vitro*. 6:128–142.

Anastasiadis, P.Z., S.Y. Moon, M.A. Thoreson, D.J. Mariner, H.C. Crawford, Y. Zheng, and A.B. Reynolds. 2000. Inhibition of RhoA by p120 catenin. *Nat. Cell Biol.* 2:637–644.

Bao, W., M. Thullberg, H. Zhang, A. Onischenko, and S. Stromblad. 2002. Cell attachment to the extracellular matrix induces proteasomal degradation of p21(CIP1) via Cdc42/Rac1 signaling. *Mol. Cell. Biol.* 22:4587–4597.

Berx, G., A.M. Cleton-Jansen, F. Nollet, W.J. de Leeuw, M. van de Vijver, C. Cornelisse, and F. van Roy. 1995. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J.* 14:6107–6115.

Betson, M., E. Lozano, J. Zhang, and V.M. Braga. 2002. Rac activation upon cell-cell contact formation is dependent on signaling from the epidermal growth factor receptor. *J. Biol. Chem.* 277:36962–36969; 10.1074/jbc.M207358200.

Birchmeier, W., and J. Behrens. 1994. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim. Biophys. Acta*. 1198:11–26.

Boussadia, O., S. Kutsch, A. Hierholzer, V. Delmas, and R. Kemler. 2002. E-cadherin is a survival factor for the lactating mouse mammary gland. *Mech. Dev.* 115:53–62.

Brabletz, T., A. Jung, S. Reu, M. Porzner, F. Hlubek, L.A. Kunz-Schughart, R. Knuechel, and T. Kirchner. 2001. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. USA*. 98:10356–10361.

Braga, V. 2000. Epithelial cell shape: cadherins and small GTPases. *Exp. Cell Res.* 261:83–90.

Brown, J.L., L. Stowers, M. Baer, J. Trejo, S. Coughlin, and J. Chant. 1996. Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. *Curr. Biol.* 6:598–605.

Chen, C.S., M. Mrksich, S. Huang, G.M. Whitesides, and D.E. Ingber. 1997. Geometric control of cell life and death. *Science*. 276:1425–1428.

Chu, Y.S., W.A. Thomas, O. Eder, F. Pincet, E. Perez, J.P. Thiery, and S. Dufour. 2004. Force measurements in E-cadherin–mediated cell doublets reveal rapid adhesion strengthened by actin cytoskeleton remodeling through Rac and Cdc42. *J. Cell Biol.* 167:1183–1194.

Coleman, M.L., C.J. Marshall, and M.F. Olson. 2004. RAS and RHO GTPases in G1-phase cell-cycle regulation. *Nat. Rev. Mol. Cell Biol.* 5:355–366.

del Pozo, M.A., L.S. Price, N.B. Alderson, X.D. Ren, and M.A. Schwartz. 2000. Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J.* 19:2008–2014.

del Pozo, M.A., N.B. Alderson, W.B. Kiosses, H.H. Chiang, R.G. Anderson, and M.A. Schwartz. 2004. Integrins regulate Rac targeting by internalization of membrane domains. *Science*. 303:839–842.

Ehrlich, J.S., M.D. Hansen, and W.J. Nelson. 2002. Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell-cell adhesion. *Dev. Cell*. 3:259–270.

Folkman, J., and A. Moscona. 1978. Role of cell shape in growth control. *Nature*. 273:345–349.

Frost, J.A., S. Xu, M.R. Hutchison, S. Marcus, and M.H. Cobb. 1996. Actions of Rho family small G proteins and p21-activated protein kinases on mitogen-activated protein kinase family members. *Mol. Cell. Biol.* 16:3707–3713.

Glaven, J.A., I. Whitehead, S. Bagrodia, R. Kay, and R.A. Cerione. 1999. The Dbl-related protein, Lfc, localizes to microtubules and mediates the activation of Rac signaling pathways in cells. *J. Biol. Chem.* 274:2279–2285.

Goodwin, M., E.M. Kovacs, M.A. Thoreson, A.B. Reynolds, and A.S. Yap. 2003. Minimal mutation of the cytoplasmic tail inhibits the ability of E-cadherin to activate Rac but not phosphatidylinositol 3-kinase: direct evidence of a role for cadherin-activated Rac signaling in adhesion and contact formation. *J. Biol. Chem.* 278:20533–20539.

Gottardi, C.J., E. Wong, and B.M. Gumbiner. 2001. E-cadherin suppresses cellular transformation by inhibiting β-catenin signaling in an adhesion-independent manner. *J. Cell Biol.* 153:1049–1060.

Hirohashi, S. 1998. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am. J. Pathol.* 153:333–339.

Huang, S., C.S. Chen, and D.E. Ingber. 1998. Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol. Biol. Cell*. 9:3179–3193.

Ireton, R.C., M.A. Davis, J. van Hengel, D.J. Mariner, K. Barnes, M.A. Thoreson, P.Z. Anastasiadis, L. Matrisian, L.M. Bundy, L. Sealy, et al. 2002. A novel role for p120 catenin in E-cadherin function. *J. Cell Biol.* 159:465–476.

Joyce, D., B. Bouzahzah, M. Fu, C. Albanese, M. D'Amico, J. Steer, J.U. Klein, R.J. Lee, J.E. Segall, J.K. Westwick, et al. 1999. Integration of Rac-dependent regulation of cyclin D1 transcription through a nuclear factor-kappaB-dependent pathway. *J. Biol. Chem.* 274:25245–25249.

Kiosses, W.B., S.J. Shattil, N. Pampori, and M.A. Schwartz. 2001. Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration. *Nat. Cell Biol.* 3:316–320.

Kovacs, E.M., R.G. Ali, A.J. McCormack, and A.S. Yap. 2002. E-cadherin homophilic ligation directly signals through Rac and phosphatidylinositol 3-kinase to regulate adhesive contacts. *J. Biol. Chem.* 277:6708–6718.

Mettouchi, A., S. Klein, W. Guo, M. Lopez-Lago, E. Lemichez, J.K. Westwick, and F.G. Giancotti. 2001. Integrin-specific activation of Rac controls progression through the G(1) phase of the cell cycle. *Mol. Cell*. 8:115–127.

Minden, A., A. Lin, F.X. Claret, A. Abo, and M. Karin. 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*. 81:1147–1157.

Nagafuchi, A., and M. Takeichi. 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO J.* 7:3679–3684.

Nakagawa, M., M. Fukata, M. Yamaga, N. Itoh, and K. Kaibuchi. 2001. Recruitment and activation of Rac1 by the formation of E-cadherin-mediated cell-cell adhesion sites. *J. Cell Sci.* 114:1829–1838.

Navarro, P., M. Gomez, A. Pizarro, C. Gamallo, M. Quintanilla, and A. Cano. 1991. A role for the E-cadherin cell–cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. *J. Cell Biol.* 115:517–533.

Nelson, C.M., and C.S. Chen. 2002. Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal. *FEBS Lett.* 514:238–242.

Nelson, C.M., and C.S. Chen. 2003. VE-cadherin simultaneously stimulates and inhibits cell proliferation by altering cytoskeletal structure and tension. *J. Cell Sci.* 116:3571–3581; 10.1242/jcs.00680.

Nelson, C.M., D.M. Pirone, J.L. Tan, and C.S. Chen. 2004. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. *Mol. Biol. Cell*. 15:2943–2953; 10.1091/mbc.E03-10-0745.

Noren, N.K., C.M. Niessen, B.M. Gumbiner, and K. Burridge. 2001. Cadherin engagement regulates Rho family GTPases. *J. Biol. Chem.* 276:33305–33308; 10.1074/jbc.C100306200.

Ohsugi, M., L. Larue, H. Schwarz, and R. Kemler. 1997. Cell-junctional and cytoskeletal organization in mouse blastocysts lacking E-cadherin. *Dev. Biol.* 185:261–271.

Olson, M.F., A. Ashworth, and A. Hall. 1995. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science*. 269:1270–1272.

Ozawa, M., M. Ringwald, and R. Kemler. 1990. Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc. Natl. Acad. Sci. USA*. 87:4246–4250.

Pece, S., and J.S. Gutkind. 2000. Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J. Biol. Chem.* 275:41227–41233.

Perez-Moreno, M., C. Jamora, and E. Fuchs. 2003. Sticky business: orchestrating cellular signals at adherens junctions. *Cell*. 112:535–548.

Perl, A.K., P. Wilgenbus, U. Dahl, H. Semb, and G. Christofori. 1998. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature*. 392:190–193.

Reddy, P., L. Liu, C. Ren, P. Lindgren, K. Boman, Y. Shen, E. Lundin, U. Ottander, M. Rytinky, and K. Liu. 2005. Formation of E-cadherin mediated cell-cell adhesion activates Akt and mitogen activated protein kinase (MAPK) via phosphatidylinositol 3 kinase and ligand-independent activation of epidermal growth factor (EGF) receptor in ovarian cancer cells. *Mol. Endocrinol.* 19:2564–2578.

Ren, X.D., W.B. Kiosses, and M.A. Schwartz. 1999. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* 18:578–585.

Reynolds, A.B., and A. Roczniak-Ferguson. 2004. Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene*. 23:7947–7956.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 70:401–410.

St. Croix, B., C. Sheehan, J.W. Rak, V.A. Florenes, J.M. Slingerland, and R.S. Kerbel. 1998. E-cadherin–dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor $p27^{KIP1}$. *J. Cell Biol.* 142:557–571.

Sundfeldt, K. 2003. Cell-cell adhesion in the normal ovary and ovarian tumors of epithelial origin; an exception to the rule. *Mol. Cell. Endocrinol.* 202:89–96.

Tinkle, C.L., T. Lechler, H.A. Pasolli, and E. Fuchs. 2004. Conditional targeting of E-cadherin in skin: insights into hyperproliferative and degenerative responses. *Proc. Natl. Acad. Sci. USA*. 101:552–557.

Watabe, M., A. Nagafuchi, S. Tsukita, and M. Takeichi. 1994. Induction of polarized cell–cell association and retardation of growth by activation of the E-cadherin–catenin adhesion system in a dispersed carcinoma line. *J. Cell Biol.* 127:247–256.

Welsh, C.F., K. Roovers, J. Villanueva, Y. Liu, M.A. Schwartz, and R.K. Assoian. 2001. Timing of cyclin D1 expression within G1 phase is controlled by Rho. *Nat. Cell Biol.* 3:950–957.

Wheelock, M.J., and K.R. Johnson. 2003. Cadherin-mediated cellular signaling. *Curr. Opin. Cell Biol.* 15:509–514.

Wittelsberger, S.C., K. Kleene, and S. Penman. 1981. Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype. *Cell*. 24:859–866.

Yap, A.S., and E.M. Kovacs. 2003. Direct cadherin-activated cell signaling: a view from the plasma membrane. *J. Cell Biol.* 160:11–16.