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Self-contact elimination by membrane fusion

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Mutual, homophilic cell–cell adhesion between epithelial cells is required for proper maintenance of epithelial barrier function. Whereas opposing membranes from neighboring cells rapidly assemble junctional complexes, self-contacting membranes curiously do not, suggesting that cells have the ability to prevent the maturation of self-junctions. Using a self-contact–inducing microfabricated substrate, we show that self-contacts of normal epithelial cells are rapidly eliminated by membrane fusion between two opposing plasma membranes of a single cell. This membrane fusion is most frequently observed in E-cadherin–expressing epithelial cells, but not in fibroblasts. The efficiency of self-contact elimination depends on extracellular calcium concentration and the level of E-cadherin, suggesting that E-cadherin, although not required, enhances membrane fusion efficiency by bringing opposing membranes into close apposition to one another. Additionally, Rho-associated protein kinase inhibition decreases self-contact–induced membrane fusion of epithelial cells, suggesting that this fusion may be mechanically regulated through the actin–myosin network. This self-contact–induced membrane fusion is a key elimination mechanism for unwanted self-junctions and may be a feature of cell self-recognition.

seamless tubule | contractility | soft lithography

The formation of intimate cell–cell adhesion is the first critical step in the development of multicellular organisms. Many cell types, particularly epithelial cells, form and maintain cell–cell junctions with neighboring cells through homophilic interactions of cell adhesion proteins. When the plasma membranes of neighboring cells physically interact, cell–cell adhesion proteins engage and autonomously and rapidly develop into mature cell junctions. Interestingly, in native environments, epithelial cells extend thin finger-like protrusions along the basal–lateral surface (1, 2). These flexible membrane extensions are capable of touching different regions of their own membrane surface, yet, mature cell junctional complexes have not been observed at self-contacts. The curious absence of cell junctions at self-contacts suggests that cells are able to either prevent the autonomous assembly of self-junctions or rapidly eliminate nascent self-junctions.

The fate of self-contacts is not entirely clear. During neuronal network formation, thin flexible neurites recognize self-contacts through homophilic interactions of surface receptors and repel one another, whereas heterophilic interactions between neighboring cells are more favorable (3, 4). This type of self-avoidance is based on surface chemistry defined by the receptors on plasma membrane. Other adhesive cells that rely on homophilic interactions of adhesion proteins need an alternative strategy to avoid or eliminate self-contacts.

Such a self-recognition pathway is essential as many other cell types have the capacity to generate self-contacts. For example, dynamic lamellipodia and membrane ruffles often fold onto adjacent membrane regions in fibroblasts (5) and muscle cells (6). These subsequent self-contacts are eliminated, and the potential elimination mechanisms were speculated as membrane fusion (5) or macropinocytosis (6). Whereas both membrane fusion (in exocytosis or cell fusion) and fission (in endocytic processes) require initial membrane apposition and membrane dissolution, membrane fusion and fission are mechanistically and molecularly distinct processes. Due to the lack of a reproducible experimental system to systematically analyze self-contact formation, the detailed mechanisms of self-contact elimination remain ambiguous.

In the current study, we designed self-contact–inducing microfabricated substrates to investigate the molecular mechanisms underlying the elimination of self-contacts. We show that self-contacts are rapidly eliminated by membrane fusion, which depends on E-cadherin and Rho-associated protein kinase (ROCK) activity. Our report demonstrates that mammalian cell fusion is a possible mechanism to prevent self-junction maturation following cell self-recognition.

Results

Micropillar Array Promotes Cell Self-Contact. To study the fate of self-contacts, we designed and microfabricated a substrate to promote cell self-contact. We used a micropillar array where each individual pillar would serve as a physical barrier for the extending plasma membrane to wrap around and contact its own membrane. The hexagon arrangement (18 μm per side) and pillar dimensions (20 μm in height and 5 μm in diameter) were chosen to maximize the cell entrapment within the confines of a hexagon and formation of self-contacts upon spreading around individual pillars (Fig. 1 *A* and *B*).

We assessed the effectiveness of the pillar array design in promoting and maximizing self-contact by growing normal epithelial Madin-Darby canine kidney (MDCK) cells to confluence. Although a few cells crawled or adhered to the pillar tops, most cells surrounded the pillars at the base (Fig. 1*C*). Some pillars were located between neighboring cells at cell junctions (Fig. 1*C*, arrow), whereas many pillars were surrounded by only single cells (Fig. 1*C*, arrowheads). Surprisingly, we did not observe clear self-junctions in bright-field imaging or phalloidin staining, although the ring of phalloidin-labeled actin often surrounded some pillars (Fig. 1*C*). Through scanning electron microscopy, adhered cells wrapped around the base of, and not over, individual pillars (Fig. 1*D*). Furthermore, whereas cell–cell junctions were clearly visible between neighboring cells (Fig. 1*D*,

Significance

While contacting membranes from neighboring epithelial cells rapidly assemble and maintain junctional complexes, it is unclear why self-contacting membranes from a single cell, despite similar surface binding conditions, do not. Using a self-contact–inducing micropillar array, we demonstrate that self-contacts of normal epithelial cells are rapidly eliminated by fusion between the two opposing plasma membrane regions of a single cell. The efficiency of fusion depends on the expression of the cell–cell adhesion molecule E-cadherin and the contractile state of the cell regulated through Rho-associated protein kinase. This is the first demonstration of self-contact–induced membrane fusion in mammalian cells and that membrane fusion may be an underlying mechanism for seamless tubule formation.

Author contributions: G.M.S. and S.Y. designed research, performed research, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

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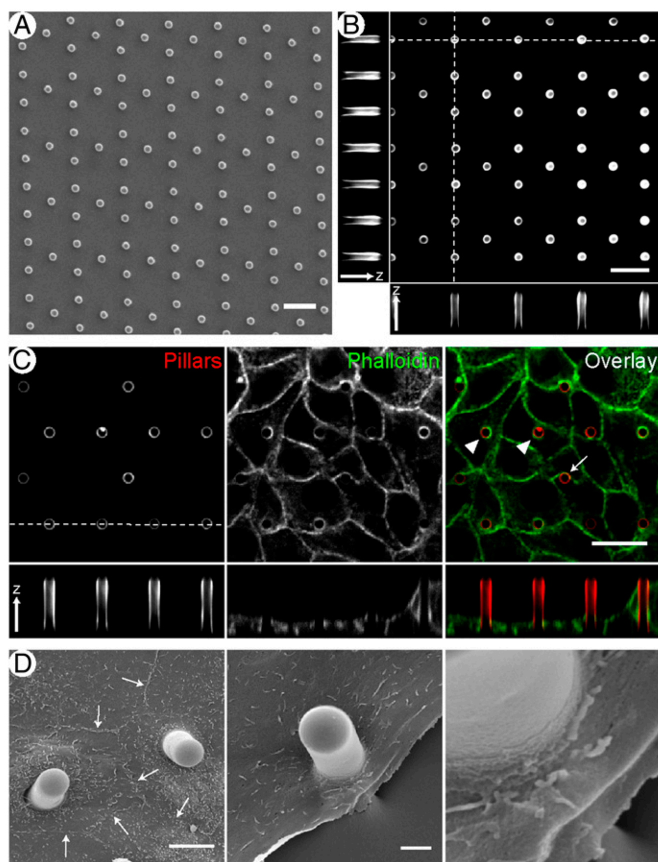

Fig. 1. Micropillar array to promote cell self-contact. (*A*) Hexagon pillar array arrangement with 18-μm pillar-to-pillar pitch along hexagon sides viewed through a scanning electron microscope. (Scale bar, 20 μm.) (*B*) The specified pillar dimensions (5-μm diameter, 20-μm height) were visualized and confirmed through DiI staining. Dotted lines indicate the corresponding Z sections (*Lower Left*) along the *x* and *y* planes. (Scale bar, 20 μm.) (*C*) MDCK cells within a confluent monolayer grown for 24 h on the pillar array wrapped around pillars (arrowheads) without apparent self-junctions identified through phalloidin staining. Pillars were also located along cell–cell contacts (arrow). Dotted line indicates the corresponding Z sections (*Lower*). (Scale bar, 20 μm.) (*D*) Scanning electron micrographs of MDCK cells grown for 24 h wrapped around pillars. (*Left*) Cells wrapped around pillars without apparent self-junctions, whereas cell–cell junctions are readily apparent (arrows). (Scale bar, 5 μm.) (*Center*) Pillar at the edge of a cell colony with a cell wrapped around the pillar base. (Scale bar, 2 μm.) (*Right*) Close-up image of pillar base with a continuous cell wrapped around it.

arrows), again, no cell self-junctions were observed (Fig. 1*D*). These results suggest that the adhered cells wrap around the base of pillars to form self-contacts, but such nonneighboring cell junctions are rapidly eliminated.

Membrane Fusion Following Cell Self-Contact. Using live-cell imaging of GFP-tagged actin-expressing cells plated on the pillar substrates, we sought to follow the fate of self-contacts. Similar to cell–cell junctions between neighboring cells, actin rapidly accumulated at the initial sites of self-contact (Fig. 2*A*, arrow, and Movie S1), but unlike cell–cell junctions, actin quickly dissipated from the sites of self-contact (Fig. 2*A*, 50 min). Furthermore, in confluent monolayers of GFP–actin-expressing cells, the movement of individual cells wrapped around pillars was highly restricted (Fig. 2*B*, arrowhead, and Movie S2), whereas neighboring cells moved freely between the pillars (Fig. 2*B*, asterisk). Interestingly, cell movement away from the pillar stretched the plasma membrane around the pillar, but the migrating cell did not separate from the pillar (Fig. 2*B*, 5 and 7 h), suggesting that once cells wrap around the pillars, the interaction is strong enough to withstand forces exerted by migrating cells even in the absence of cell junctional complexes.

We hypothesize that self-contacts are followed by membrane fusion that interconnects two plasma membrane regions of single cells. To test the cytoplasmic continuity of cells surrounding pillars and therefore membrane fusion at self-contacts, we analyzed diffusion of photoactivatable GFP along cell extensions wrapping around the pillars. In cells that have made self-contact but have not undergone membrane fusion, photoactivated GFP rapidly diffused into the membrane extension, but the diffusion was impeded by the plasma membrane separating two distinct membrane extensions of the single cell (Fig. 2*C*, self-contact, and Movie S3). In contrast, photoactivated GFP diffused around the pillars in the cytoplasm of cells that were completely wrapped around pillars (Fig. 2*C*, fused, and Movie S4), thus providing conclusive evidence that the wrapped cytoplasm is continuous without cell junctions and self-contacts lead to membrane fusion.

To readily identify self-contact–induced membrane fusion, we used trypsin to digest membrane proteins to separate cell junctions and self-contacts that have not undergone membrane fusion (Fig. 2*D*). This trypsin treatment isolates fused membrane around the pillars by eliminating cell–cell junctions and self-contacts, whereas fused membranes remain wrapped around pillars (see examples in Fig. 2*E*). We only analyzed cells around the base and not the top of pillars to ensure that self-contact–induced membrane fusion has taken place (Fig. 2*E*).

Due to the similarity between self-contact–induced membrane fusion and endocytic processes, we suspected that self-contact–induced membrane fusion may be a variant of an endocytic pathway where complete vesicles fail to form. We targeted dynamin and C-terminal binding protein 1 (CtBP1), molecules essential for membrane fissions in endocytosis (7) and macropinocytosis (8), respectively. Dynamin inhibition or CtBP1 knockdown reduced membrane (Fig. S1*A*) or fluid (Fig. S2 *A*–*C*) uptake, but did not reduce self-contact–induced membrane fusion quantified using trypsin treatment (Figs. S1*E* and S2 *D* and *E*; see *SI Methods* for details). These data suggest that self-contact–induced membrane fusion is independent of dynamin or CtBP1-dependent membrane turnover, and that the mechanisms of membrane dissolution at self-contacts likely differ from that of typical endocytic processes.

Identifying Self-Contact–Induced Membrane Fusion in Other Cell Lines. In addition to normal epithelial cells, we tested epithelial-derived prostate (DU 145) and breast (MCF7) cancer cell lines for self-contact–induced membrane fusion. In normal epithelial cells (MDCK), GFP-tagged actin at self-contacts dissipated quickly (Fig. 2*A*), whereas in the prostate cancer cell line, phalloidin-labeled actin remained at all self-contacts even after 2 h (Fig. S3*A*). However, after 6 h, self-contacts were removed in the prostate cancer cell line as indicated by the absence of a self-junctional actin network (Fig. S3*A*) and fused membrane around the pillars after trypsin treatment (Fig. S3*A*). Similar to the prostate cancer cell line, the breast cancer cell line also self-contacted with strong self-junctional actin accumulation followed by membrane fusion (Fig. S3*B*). Both epithelial-derived prostate and breast cancer cell lines are capable of self-contact removal and membrane fusion (Fig. S3 *A* and *B*), albeit with lower efficiencies than normal epithelial cells.

Interestingly, fibroblasts (3T3 and L cells), although capable of forming self-contacts, were unable to fuse self-contacting membrane (Fig. S3 *C* and *D*). We suspected that this is in part due to the lack of cell adhesion molecules, which is required to bring two contacting membranes into a close proximity that is necessary for membrane fusion. Consistent with this notion, the fusion-capable cell lines expressed E-cadherin, whereas the fibroblast cell lines did not (Fig. S3*E*), although the expression level varied among fusion-capable cell lines. These data suggested that E-cadherin may play a role in promoting membrane fusion and removing self-contacts.

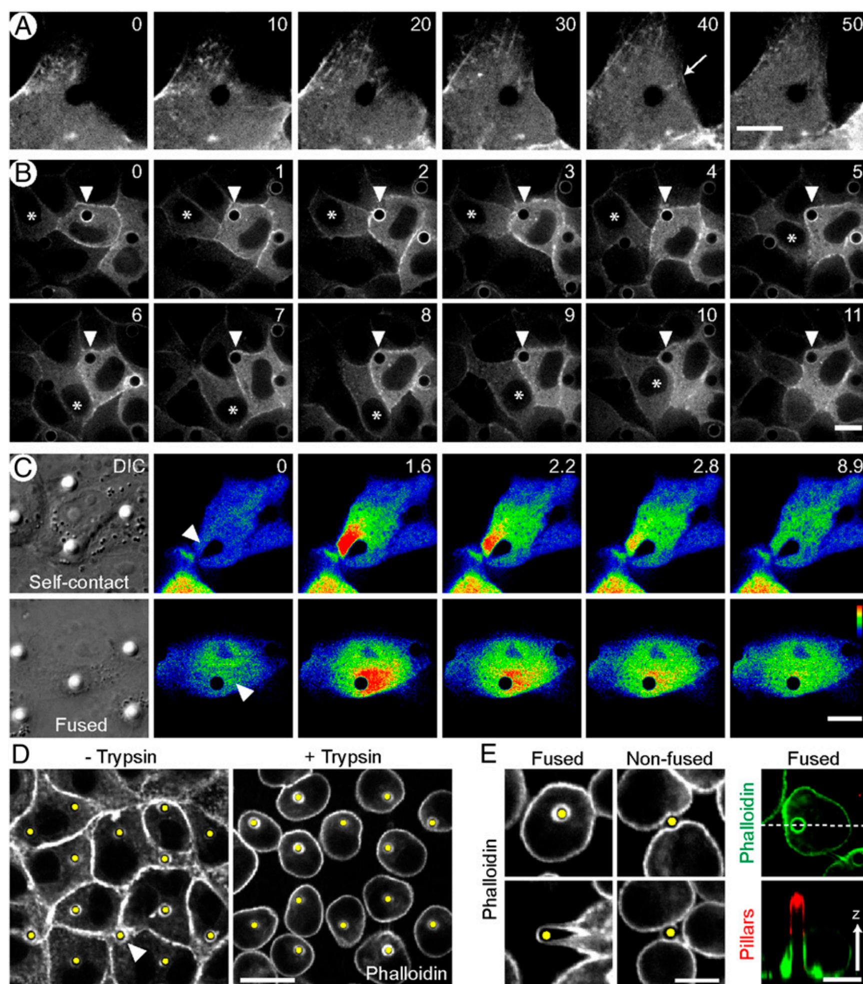

Fig. 2. Self-contact–induced membrane fusion. (*A*) Time-lapse images of a GFP–actin- expressing MDCK cell forming self-contact around a pillar. Cells were seeded onto the pillar array 24 h before imaging. GFP–actin accumulates (arrow) and dissipates over time. Time is given in minutes (Scale bar, 10 μm.) (*B*) Cell movement of GFP–actin-expressing MDCK cells within a confluent monolayer. Cells were seeded onto the pillar array 24 h before imaging. The motion of cells wrapped around pillars (arrowhead) were restricted, but not for other cells (asterisk). Time is given in hours. (Scale bar, 10 μm.) (*C*) Time-lapse images of photoactivated GFP diffusion in MDCK cells following laser activation. Cells were seeded onto the pillar array 24 h before imaging. Diffusion was impeded by membrane in nonfused cells (self-contact) but not in fused cells. Arrowheads indicate sites of photoactivation. Time is given in seconds. Pseudocolored heat map indicates photoactivated GFP intensity. (Scale bar, 10 μm.) (*D*) Because identifying membrane fusion was difficult for pillars located at cell–cell contacts (−trypsin, arrowhead), cell junctions were disrupted by trypsin treatment (+trypsin) before fixing and phalloidin staining. Yellow dots indicate pillar locations. (Scale bar, 20 μm.) (*E*, *Left*) Examples of fused and nonfused cells identified through trypsin digestion and phalloidin staining. Yellow dots indicate pillar locations. (*Right*) Fused cells remained wrapped around the base of pillars. Dotted line indicates the location of the corresponding Z section. (Scale bar, 10 μm.)

Efficient Self-Contact–Induced Membrane Fusion Depends on E-Cadherin. We followed the formation of self-contacts using GFP-tagged E-cadherin–expressing normal epithelial (MDCK) cells. Similar to the formation of cell–cell contacts (9), E-cadherin rapidly accumulated at cell self-contacts, but unlike the formation of cell–cell contacts, E-cadherin disappeared from the sites of self-contacts (Fig. 3*A* and Movie S5). In addition, α-catenin, an actin binding protein in the cadherin complex, also localized to the initial self-contacts (Fig. 3*A* and Movie S6). These data suggest that the E-cadherin complex is present at an early stage of self-junction formation and then dissipates from self-contacts as membranes fuse.

Using a low calcium condition or E-cadherin shRNA to minimize calcium-dependent cell–cell adhesion or E-cadherin-mediated cell–cell adhesion, we analyzed whether self-contact–induced membrane fusion is mediated by E-cadherin in normal epithelial cells. In all conditions, the epithelial cells adhered to the pillar substrates and formed closely packed cell monolayers (Fig. 3*B*). Interestingly, in the absence of calcium or E-cadherin (Fig. 3*C*, *Inset* for E-cadherin levels), the pillars were often located between or in close proximity to cell–cell contacts (Fig. 3*B*, −trypsin). Trypsin treatment revealed that, in comparison with the control (WT +calcium), cells did not fuse around the pillars in the low calcium or E-cadherin shRNA 1 samples (Fig. 3*B*, +trypsin). Quantifying the number of cells fused around pillars in each condition confirmed the reduced level of membrane fusion in the low calcium condition and by E-cadherin–deficient cells (Fig. 3*C*).

Interestingly, under all three conditions, cells increasingly fused around the pillars over time (Fig. 3*C*). After 48 h, E-cadherin–deficient cells (E-cadherin shRNA 1) were just as capable of membrane fusion around the pillars as the wild-type cells (Fig. 3*D*), suggesting that given sufficient time, these cells overcome E-cadherin deficiency and eliminate self-contacts by membrane fusion. Confirming these results, a second E-cadherin–deficient cell line, generated using a different targeting sequence (E-cadherin shRNA 2), also had reduced membrane fusion compared with wild type after 6 h, whereas having comparable membrane fusion after 48 h (Fig. S4). These results suggest that E-cadherin, although not required, promotes efficient membrane fusion.

Whereas reduced E-cadherin expression levels decreased membrane fusion efficiency, it is not clear whether E-cadherin is required for adhesive property or signal-inducing capacity. To test whether E-cadherin engagement along opposing membranes at cell self-contacts is needed to promote membrane fusion, MDCK cells were treated with the function-blocking E-cadherin antibody DECMA-1, specific for the extracellular domain of E-cadherin. Initially, cells were seeded on the pillar arrays in serum-free media to promote cell self-contacts, but not membrane fusion (Fig. S1 *C* and *D*). Then cells were switched to serum-containing media with either DECMA-1, or an antibody specific for the intracellular domain of E-cadherin (clone 36). After 2 and 6 h of antibody treatment, membrane fusion was significantly reduced with DECMA-1 compared with the control antibody (Fig. S5). These results demonstrate that E-cadherin engagement at cell self-contacts is required to promote efficient membrane fusion.

To determine whether E-cadherin expression alone is sufficient to promote self-contact–induced membrane fusion, we

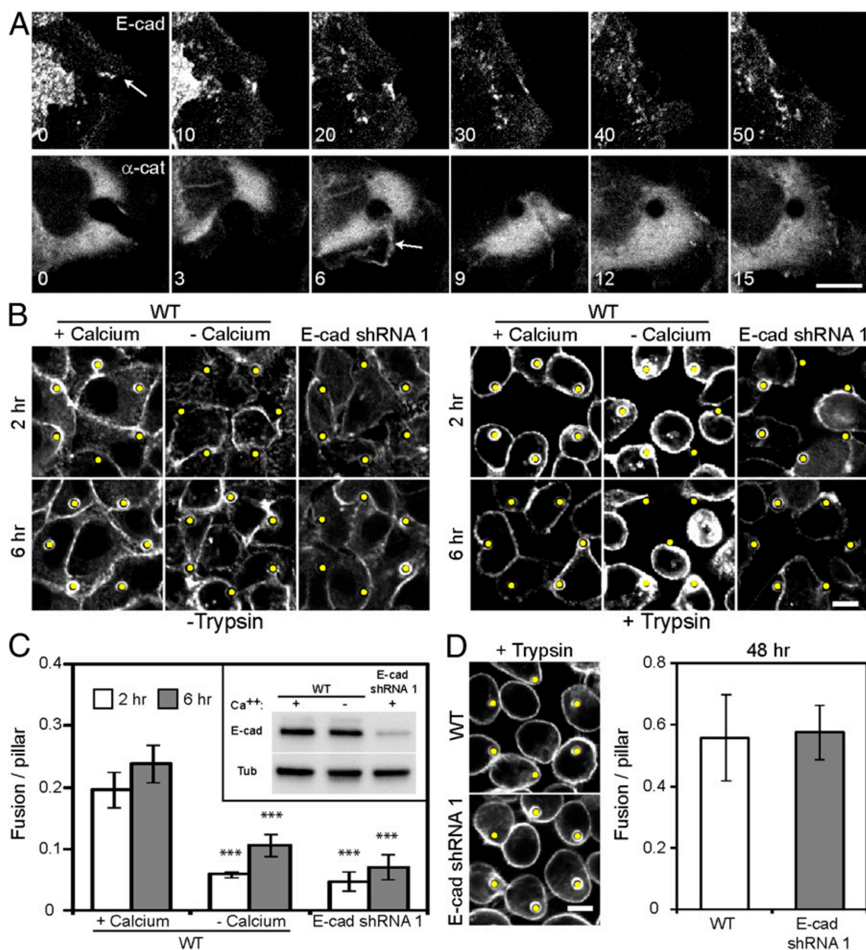

Fig. 3. Efficiency of self-contact–induced membrane fusion depends on E-cadherin. (*A*) Time-lapse images of GFP-tagged E-cadherin (E-cad)- and α-catenin (α-cat)-expressing MDCK cells forming self-contacts around pillars. Cells were seeded onto the pillar array 24 h before imaging. Both E-cadherin and α-catenin accumulate (arrows) and dissipate over time. Time is given in minutes. (*B*) Comparison of membrane fusion of MDCK wild-type (WT) and E-cadherin–deficient (E-cad shRNA 1) cells. Cells seeded at confluence for 2 and 6 h on pillar substrates were phalloidin stained. The wild-type cells were seeded in either high (+calcium) or low (−calcium) extracellular calcium conditions. (*C*) Quantitative analysis of fusion from cells seeded for 2 and 6 h on pillars (Fig. 3*B*, +trypsin) displayed as mean fusion/pillar ± SD. Data analyzed with two-factor ANOVA with Bonferroni correction [number of pillars analyzed: +calcium 2 h (438), −calcium 2 h (444), E-cad shRNA1 2 h (426), +calcium 6 h (445), −calcium 6 h (445), and E-cad shRNA1 6 h (440); $^{***}P < 0.001$, WT −calcium and E-cad shRNA 1 were compared separately to WT +calcium]. *Inset* displays immunoblot of E-cadherin levels (E-cad) of cells seeded for 6 h in high (+) or low (−) calcium conditions. Tubulin (Tub) was used as loading control. (*D*) Wild-type and E-cadherin shRNA 1 transfected cells grown to confluence for 48 h on pillars and phalloidin stained. Both cell lines had a comparable number of membrane fusion. Quantitative analysis of fusion displayed as mean fusion/pillar ± SD. Data analyzed by Student *t* test assuming equal variance [number of pillars analyzed: WT (426) and E-cad shRNA 1 (445); $P = 0.82$]. Yellow dots indicate pillar locations. (All scale bars, 10 μm.)

tested fibroblast L cells expressing E-cadherin. In comparison with wild-type L cells, these E-cadherin–expressing cells formed monolayers with tight cell–cell contacts (Fig. S6). On the pillar array, membrane fusion was not observed in the trypsinized samples of confluent E-cadherin–expressing fibroblasts (Fig. S6). This indicates that E-cadherin expression alone is not sufficient to promote self-contact–induced membrane fusion and that other molecular components are required.

ROCK Inhibition Prevents Self-Contact–Induced Membrane Fusion. Because mechanical forces play an essential role in cell–cell adhesion and cell–cell fusion, we sought to test the roles of cell contractility during self-contact–induced membrane fusion. Normal epithelial cells were seeded at confluence in the presence of phosphatase inhibitor (calyculin-A or tautomycin) and ROCK inhibitor (Y-27632 or Fasudil) to enhance and decrease cell contractility, respectively. Qualitatively, Y-27632–or Fasudil-treated cells did not fuse around the majority of the pillars compared with control, calyculin-A, or tautomycin-treated cells (Fig. 4 *A* and *D*). Note that even with the reduced cell contractility, the Y-27632–treated cells were still capable of spreading around pillars and forming self-contacts (Fig. 4*B*). Quantitative analysis revealed that indeed, Y-27632– or Fasudil-treated epithelial cells had decreased membrane fusion compared with control, whereas enhanced cell contractility with calyculin-A or tautomycin treatment did not affect membrane fusion (Fig. 4 *C* and *E*).

As an alternative approach to the ROCK inhibitors, we generated stable ROCK-deficient MDCK cells to further test ROCK as a key regulator in self-contact–induced membrane fusion (Fig. 4*F*). Interestingly, ROCK-deficient cells appeared morphologically similar (Fig. 4*G*, −trypsin) to both Y-27632– (Fig. 4*A*) and Fasudil-treated cells (Fig. 4*D*) with pillars either along cell–cell contacts or wrapped around by cells forming self-contacts, suggesting that ROCK knockdown does not promote membrane fusion. Indeed, quantitative analysis revealed that ROCK-deficient cells had decreased membrane fusion compared with scrambled control (Fig. 4*H*). Taken together, these data suggest that ROCK activity is required for efficient self-contact–induced membrane fusion.

Discussion

Using a self-contact–inducing microfabricated substrate, we demonstrate that normal epithelial cells efficiently remove self-contacts through fusion between two membrane regions of a single cell. The primary function of self-contact–induced membrane fusion is best illustrated in the development of seamless tubules in *Caenorhabditis elegans* where the lumen of single epithelial cell tubules is established by removal of self-junctions along the longitudinal axis of the tubules (10–12). In addition to *C. elegans*, seamless tubules are also found in the *Drosophila* tracheal system (13, 14), and endothelial cell capillaries in zebrafish (15, 16) and mammalian tissues (17, 18). While there are various mechanisms proposed for the formation of seamless capillaries in zebrafish (15, 16), the mechanism underlying the formation of mammalian seamless capillaries remains unclear (19, 20). Interestingly, we found that human microvascular endothelial cells were also capable of self-contact–induced membrane fusion (Fig. S7). Given the high efficiency and rapid fusion at self-contacts, self-contact elimination by membrane fusion may play a key role in seamless capillary formation.

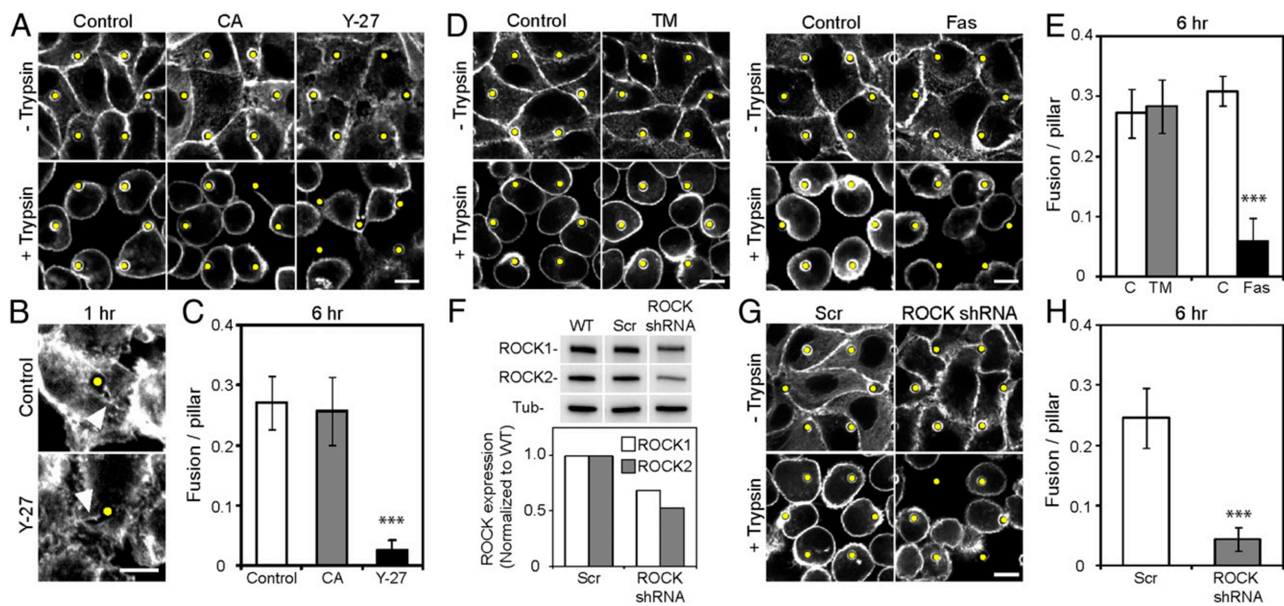

Fig. 4. ROCK activity is required for self-contact–induced membrane fusion. (*A*) MDCK cells were seeded at confluence on pillars in the presence of 1 nM calyculin-A (CA), 50 μM Y-27632 (Y-27), or media alone (control) for 6 h and phalloidin stained. Control and CA-treated cells were fused around pillars, whereas Y-27–treated cells were not (+trypsin). (*B*) After 1 h, MDCK cells seeded in 50 μM Y-27 were able to wrap around pillars to form self-contacts (arrowheads) similar to control cells. (*C*) Quantitative analysis of membrane fusion from control, CA-, and Y-27– treatment groups displayed as mean fusion/pillar ± SD. Data analyzed with single-factor ANOVA with Bonferroni correction [number of pillars analyzed: control (435), Y-27 (438), and CA (436); ***$P < 0.001$ compared with control]. (*D*) MDCK cells seeded at confluence on pillars in the presence of 100 nM tautomycin (TM), 50 μM Fasudil (Fas), or media alone (control) for 6 h and phalloidin stained. Control and TM-treated cells were fused around pillars, whereas Fasudil-treated cells were not (+trypsin). (*E*) Quantitative analysis of membrane fusion from TM- and Fas- treatment groups along with the corresponding control groups displayed as mean fusion/pillar ± SD. Data were analyzed with Student *t* test assuming equal variance [number of pillars analyzed: TM control (391), TM (397), Fas control (444), and Fas (430); ***$P < 0.001$]. (*F*) Immunoblot comparing ROCK1 and ROCK2 expression between wild-type (WT), scrambled control (Scr), and ROCK-deficient (ROCK shRNA) MDCK cells. Tubulin (Tub) used as loading control. ROCK1 and ROCK2 levels were normalized to WT. (*G*) Comparison of membrane fusion around pillars for Scr and ROCK shRNA cells seeded for 6 h and phalloidin stained. (*H*) Quantitative analysis of membrane fusion from Scr and ROCK shRNA groups displayed as mean fusion/pillar ± SD. Data were analyzed with Student *t* test assuming unequal variance [number of pillars analyzed: Scr (373) and ROCK shRNA (387); ***$P < 0.001$). Yellow dots indicate pillar locations. (All scale bars, 10 μm.)

The major difference between the fusion-competent epithelial cells and the fusion-incompetent fibroblasts is the presence of E-cadherin. In the absence of dedicated fusogens found in plasma membrane fusions in *C. elegans* [EFF-1 (21) and AFF-1 (22)] and intracellular vesicle fusions (e.g., SNARE complex), cadherins appear to play a key role in promoting fusion. In fact, cadherins have been shown to regulate cell-to-cell fusion during the formation of multinucleated cells. For example, cadherin-11 is up-regulated during trophoblast differentiation and fusion (23), E-cadherin promotes macrophage fusion to form osteoclasts or multinucleated giant cells (24, 25), and M-cadherin is localized to cell–cell contacts of fusion-competent myoblasts (26). Interestingly, fusion-competent microvascular endothelial cells also demonstrated strong cadherin accumulation (VE-cadherin) at sites of self-contact (Fig. S7) and thus further supports the role of cadherins in membrane fusion.

One potential role of E-cadherin in eliminating self-contacts is through enhancing membrane-fusion efficiency by bringing opposing membranes into close apposition to one another. This is consistent with the observation that cell-cell adhesion between these epithelial cells is strongly dependent on extracellular calcium concentration (Fig. S8). Interestingly, E-cadherin deficiency alone does not prevent the formation of cell clusters (Fig. S8), suggesting that other cell–cell adhesion molecules can compensate for the absence of E-cadherin in promoting cell clustering. The E-cadherin inhibition by function-blocking antibody (Fig. S5), however, suggests that the initial E-cadherin ligation at self-contacts cannot be compensated for by other adhesion molecules and is essential for rapid elimination of self-contacts.

Interestingly, the role of cadherins in bridging self-contacting membranes is reminiscent of the cadherin requirement in macropinocytosis (6). During endocytic processes such as macropinocytosis, close apposition of membranes is required to initiate vesicle formation. Similar to nascent cadherin junctions at self-contacts, cadherins are thought to bring together adjacent, actin-driven membrane protrusions to form macropinosomes (6). However, unlike macropinocytosis, the opposing cytoplasms of self-contacting membranes in fusion are separated by the extracellular space and prevent molecules like dynamin to pinch membranes off, thus requiring different molecular machinery than membrane fission. However, it remains possible that the last step of macropinocytosis may depend on membrane fusion instead of membrane fission. While initial E-cadherin interactions are essential in both self-contacts and macropinocytosis, our results provide clear evidence that fusion at self-contacts is independent of dynamin (Fig. S1) or CtBP1 (Fig. S2).

Because the surface chemistry at self-contacts is identical to neighboring cell–cell contacts, how cells recognize self from neighboring membrane is unclear. The distinction between self and neighboring cell contacts may be mechanically based signaling. In support of this idea, cells are capable of sensing external mechanical forces through E-cadherin (27) and this is in part due to the mechanosensitive conformation change of α-catenin, which in turn recruits vinculin (28). Interestingly, however, vinculin localization was minimal at self-contacts (Fig. S9 and Movie S7), suggesting that self-contacts are relatively force-free, although vinculin accumulation does not necessarily correlate with myosin activity in MDCK cells (29).

In the current study, blocking ROCK activity reduced self-contact–induced membrane fusion (Fig. 4). This is supported by the transient accumulation of RhoA, an activator of ROCK, at self-contacts (Fig. S9 and Movie S8). However, hyperactivation

of actin contractility does not induce membrane fusion at self-contacts or cell–cell contacts, therefore, the magnitude of overall cell contractility alone is not a discriminating factor of self-contacts. Interestingly, during the formation of cell–cell contacts, active RhoA is localized to the contact edges, locally activating actomyosin contractility, to drive contact expansion (9). The local spatiotemporal regulation of RhoA activity and subsequent ROCK signaling are likely different at self and cell–cell contacts and may be a key discriminatory factor in self-recognition processes.

Although E-cadherin deficiency and blocking ROCK activity decreases fusion, the fusogen responsible for self-contact–induced membrane fusion is, thus far, unidentified. Whereas EFF-1 and AFF-1 are fusogens identified in *C. elegans* (21, 22), strong candidates for fusogens directly responsible for mammalian cell–cell fusion have yet to emerge. Syncytin-1 and syncytin-2 are possible fusogens involved in trophoblast fusion, but their expression is limited to the placenta (30). Thus, a ubiquitous mammalian fusogen remains elusive. However, an alternative, fusogen-independent model has been proposed. In the absence of a mammalian fusogen, fusion pore formation and expansion is due to the force generated by actin polymerization (31). For instance, cell–cell membrane fusion between myoblasts requires nonmuscle myosin IIA activity and actin polymerization (32, 33). Interestingly, actin-driven membrane protrusions also promote the engagement of fusogenic proteins and subsequent fusion (34).

While we do not know the precise molecular mechanisms of membrane fusion at self-contacts, this report conclusively demonstrates remarkably efficient self-contact–induced membrane fusion in mammalian cells. Previously, the absence of cell–cell fusion has been (wrongly) interpreted as the lack of fusion machinery expressed only in specialized cells. Instead, our data suggest the presence and exceedingly tight regulation of fusion machinery in normal epithelial cells. This self-contact elimination may be an underlying mechanism for the formation of seamless capillaries and may play an important role as a unique, self-recognition signaling pathway.

Methods

Cell culture, constructs, and reagents are described in detail in *SI Methods*. Self-contact inducing pillar arrays were fabricated using standard soft lithography technique (*SI Methods*). Cells were imaged using Zeiss AxioObserver equipped with a Yokogawa spinning disk confocal system (*SI Methods*).

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