Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal

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Abstract We report a novel mechanism of cellular growth control. Increasing the density of endothelial or smooth muscle cells in culture increased cell-cell contact and decreased cell spreading, leading to growth arrest. Using a new method to independently control cell-cell contact and cell spreading, we found that introducing cell-cell contact positively regulates proliferation, but that contact-mediated proliferation can be masked by changes in cell spreading: Round cells with many contacts proliferated less than spread cells with none. Physically blocking cell-cell contact or inhibiting PI3K signaling abrogated cell-cell induced proliferation, but inhibiting diffusible paracrine signaling did not. Thus, direct cell-cell contact induces proliferation in these cells. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cell shape; Microcontact printing; Intercellular adhesion; Extracellular matrix

1. Introduction

The cell cycle is cooperatively regulated by signals from soluble factors, extracellular matrix (ECM) proteins, cell spreading, and junctional cell-cell interactions [1]. Co-stimulation by growth factors and integrin-mediated adhesion leads to the activation of numerous intracellular signaling pathways critical for cell cycle progression, including those involving ERK and PI3K [2]. The degree to which cells spread against a substrate acts as an additional regulator of proliferation in certain cell types [3]. For example, in capillary endothelial cells that are attached but prevented from spreading, ERK activation by growth factors is normal but the cells fail to enter S phase [4]. The effects of cell-cell contact on proliferation are less well understood.

In existing experimental systems, cell-cell contacts are manipulated in ways that alter other potential modulators of proliferation, making it difficult to clearly demonstrate the contribution of cell-cell contact to the observed responses. Exogenous expression of connexins [5] and cadherins [6] in different cell lines lacking these proteins retards growth, but usually results in simultaneous morphological changes and a decreased growth rate independent of cell-cell contact formation. Disruption of adherens junctions with cadherin function-blocking antibodies in endothelial [7] and epithelial [8] cells also increases the production and secretion of growth factors

[9]. Reducing Ca^{2+} destabilizes cadherins, but also affects integrins and cell spreading. In comparisons of confluent monolayers to sparse cultures, one not only observes changes in cell-cell contact, but increased cell density may also decrease cell spreading, and the increased proximity of cells may increase the effectiveness of soluble paracrine signals. Thus, traditional methods to manipulate cell-cell contact have been unable to distinguish between effects on proliferation due to cell-cell contact, paracrine signaling through soluble factors, and cell spreading.

We describe a new approach to isolate the effects of homotypic cell-cell paracrine signaling, direct cell-cell contact, and cell spreading, and use this system to study their relative contribution to the regulation of proliferation in endothelial and smooth muscle cells. We fabricated micropatterned substrates that simultaneously control cell spreading and the amount of contact between neighboring cells. Our results indicate that direct cell-cell interactions increase proliferation through a paracrine-independent, PI3K-dependent pathway.

2. Materials and methods

2.1. Cell culture and reagents

Bovine pulmonary artery endothelial cells (BPAECs, VEC Technologies, Rensselaer, NY, USA) and smooth muscle cells (BPASMCs, gift from Dr. Donald Ingber, Harvard University) were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM), 100 U/ml penicillin, and 100 μg/ml streptomycin containing 5% and 10% calf serum, respectively (Life Technologies). The following materials were purchased from the given suppliers: human fibronectin (Collaborative Biomedical Products); brefeldin A, monensin, and fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma); LY294002 (Calbiochem).

2.2. Patterning of cells with self-assembled monolayers (SAMs)

Substrates containing adhesive islands of defined size, shape, and position were fabricated using a previously described microcontact printing method [3]. Briefly, SAMs of hexadecanethiol (Sigma-Aldrich) were printed onto gold-coated glass with a flexible stamp made of polydimethylsiloxane (PDMS Sylgard 184, Essex Brownell, Fort Wayne, IN, USA) that contained a relief of the desired pattern. The unstamped regions of the gold were coated with a non-adhesive SAM by immersing the substrate in hexa(ethylene glycol)-terminated alkanethiol (2 mM in ethanol). Substrates were incubated in 25 μg/ml of fibronectin in PBS; the protein adsorbed only onto the stamped regions. Synchronized cells were plated on these substrates for 24 h, fixed, stained for analysis, and photographed with a Spot CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). Outlining cells using the Spot software determined extent of cell spreading and number of cell-cell contacts.

2.3. Patterning of cells with agarose

Substrates containing microwells were made as follows: A PDMS

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stamp containing bowtie-shaped posts was oxidized under UV/ozone (UVO Cleaner, Jelight Company, Inc., Irvine, CA, USA) and sealed against a SuperFrost slide (Fisher Scientific); the bowtie-shaped posts protected corresponding regions of glass, leaving other regions exposed. A solution of 0.6% agarose (Life Technologies)/40% ethanol in water was wicked under the stamp and dried under vacuum. The agarose coated the glass in the channels formed between the stamp and the slide. Peeling off the slide left glass-bottomed bowtie-shaped wells on the substrate. Substrates were sterilized in ethanol, washed with PBS, and incubated in a 25 μg/ml solution of fibronectin in PBS.

2.4. BrdU incorporation

Entry into S phase was quantified by measuring the percentage of cells that incorporated 5-bromo-2′-deoxyuridine (BrdU) using a commercial assay (Amersham). Cells were G_0-synchronized by holding cultures at confluence for 2 days, then plated onto substrates in full culture media. BrdU was added to the medium at 2 h after plating. At 24 h, cells were fixed and stained according to the manufacturer's instructions. BrdU-positive fluorescent cells were visualized and scored using a Nikon epifluorescence microscope (Nikon). The DNA-binding dye Hoechst 33258 (Molecular Probes) was used as a counterstain (1 μg/ml).

2.5. Statistical analysis

Statistical significance was determined using the Student's *t*-test.

3. Results

We first examined the effect of cell density on cell spreading, cell-cell contact, and cell proliferation. G_0-synchronized endothelial and smooth muscle cells were plated at densities ranging from 300 to 90 000 cells/cm^2, where the highest density was comparable to that of a confluent monolayer (Fig. 1A,B). After 24 h, cells were fixed and analyzed for quantity of cell-cell contact, cell spreading, and cell proliferation. Cell-

Fig. 1. Proliferation decreases with increasing cell density. A,B: Phase contrast images of BPAECs (EC) and BPASMCs (SMC) at seeding densities of 9000 cells/cm^2 and 900 cells/cm^2. Bar = 50 μm. C,D: Plots of mean cell area as a function of seeding density calculated from a representative experiment. E,F: Plots of the percentage of cells that incorporate BrdU as a function of cell density. Error bars indicate S.D. across three experiments. Data are shown for both BPAECs (A,C,E) and BPASMCs (B,D,F).
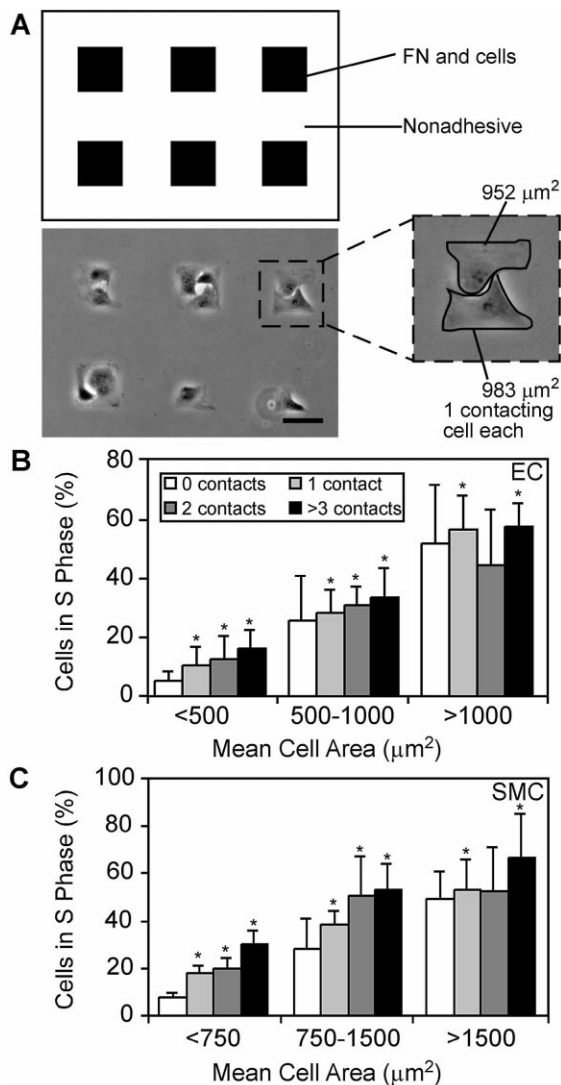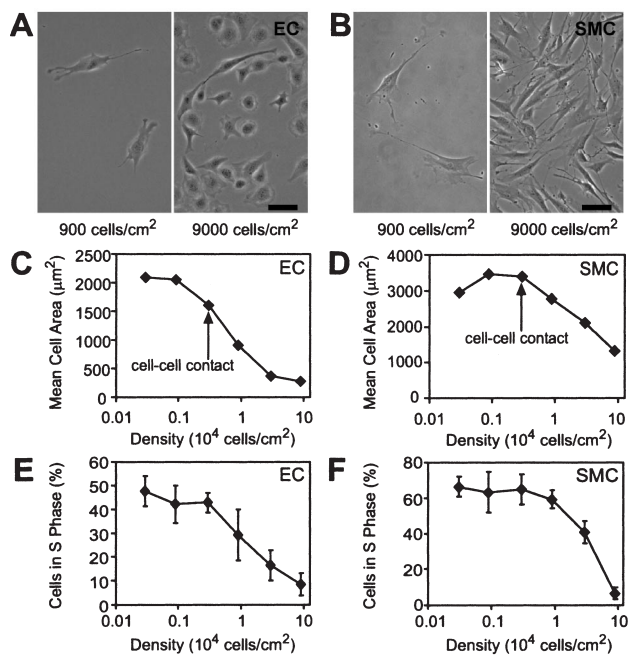

Fig. 2. Proliferation increases with both increasing cell area and increasing cell-cell interactions. A: Phase contrast micrograph of BPAECs cultured on square islands (50 μm on a side). The inset shows how cells are outlined, cell area calculated, and number of neighboring cells specified for each cell. Bar = 50 μm. B: Graph of percentage of cells incorporating BrdU as a function of mean cell area and number of cell-cell contacts in BPAEC (EC). C: Graph of BrdU incorporation as a function of mean cell area and number of cell-cell contacts in BPASMC (SMC). The data represent the mean ± S.D. of three experiments, with (*) indicating $P < 0.05$ between indicated bar and both its control and neighboring bar.

cell contact and spreading both changed with plating density (Fig. 1C,D). As cell density was increased, cell-cell contact increased, with cells first contacting each other at a seeding density of 3000 cells/cm^2, and being fully surrounded at 30 000 cells/cm^2. Cell spreading, as measured by projected cellular area, decreased with increasing cell density, with the most dramatic decrease in the 3000–30 000 cells/cm^2 range. Cell proliferation, as measured by the percentage of G_0-synchronized cells incorporating BrdU after plating, decreased with increasing cell density (Fig. 1E,F), suggesting that either cell-cell interactions or cell spreading may be involved in growth regulation. However, it was not clear what the relative contributions of cell spreading and cell-cell interactions are to cell proliferation.
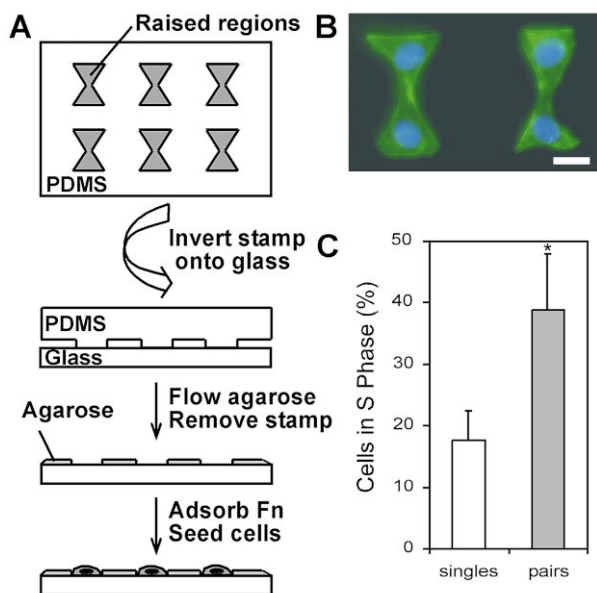
Fig. 3. A new patterning method reveals that pairs of contacting endothelial cells proliferate more than single cells. A: Schematic outline of agarose patterning. B: Fluorescence image of cells on agarose patterns stained with FITC-phalloidin and Hoechst 33258. Bar = 20 μm. C: BrdU incorporation of pairs and single cells grown on bowties pattern, where 'singles' indicates cells that attach and spread primarily in half of the bowtie-shaped well, leaving the other half empty. The data represent the mean ± S.D. of four experiments, with (*) indicating $P < 0.005$.

To decouple cell-cell interactions from cell spreading, we used a micropatterning technique to fabricate arrays of microcultures on a substrate [3]. We microcontact printed self-assembled monolayers (SAMs) of alkanethiolates to generate arrays of micrometer-scale islands coated with fibronectin and separated by non-adhesive regions. When plated on SAM substrates, cell attachment and spreading are restricted

to the area of the adhesive islands. By plating cells on islands ranging from 100 μm² to 10 000 μm², and varying the number of cells on each island (Fig. 2A), we could vary the number of cell-cell interactions independently of the extent of cell spreading. For example, single cells on 625 μm² islands have equal spreading but fewer contacts than four cells on 2500 μm² islands do. Similarly, this approach can be used to vary cell spreading without varying the number of cell-cell contacts.

We plated G_0-synchronized cells on fibronectin-coated islands and measured cell spreading, number of cell-cell contacts, and proliferation on a cell-by-cell basis at 24 h after plating for thousands of cells. Proliferation increased significantly ($P < 0.005$) with increasing cell spreading in isolated cells (Fig. 2B,C, white bars), in agreement with previous reports [3,4]. At each degree of cell spreading, increasing cell-cell interactions resulted in an additional, statistically significant increase in proliferation ($P < 0.05$; Fig. 2B,C). This effect was most dramatic in the least spread cells: Cell-cell contact increased proliferation by as much as three-fold in round cells but by only 20% in highly spread cells.

For a given island size and number of cells per island, we found that the degree of cell spreading and cell-cell contact varied from cell to cell and likely contributed to the large variation in proliferation seen in Fig. 2. To circumvent this variability, we patterned substrates with a 10-μm-thick layer of non-adhesive agarose surrounding bowtie-shaped patterns on glass coated with fibronectin (Fig. 3A). When plated on these substrates, pairs of cells spread to fill the bowtie-shaped microwells (750 μm² per half), with one cell on each side of the central constriction, while single cells attached in a bowtie were primarily confined to half of the microwell (Fig. 3B). Intercellular adhesion molecules expressed in these cells, including connexin 43, N-cadherin, VE-cadherin, and beta-catenin, were found to localize to cell-cell contacts within 4 h after plating onto the patterned substrates (data not shown). The proliferation rate of single cells in the microwells was comparable to that seen in cells without contacts on the
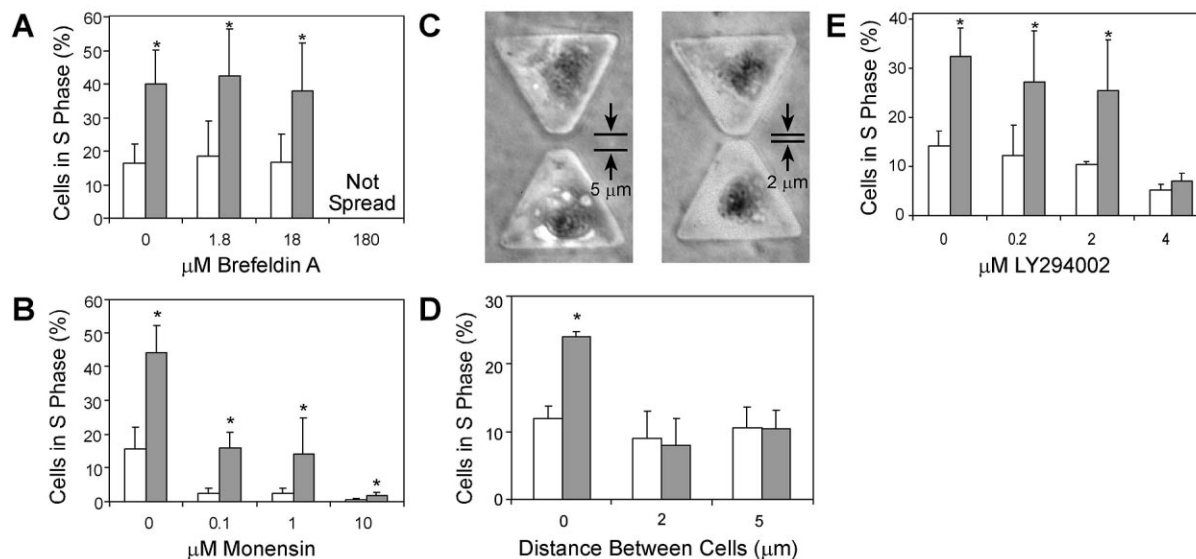

Fig. 4. A diffusible factor is not responsible for the increased proliferation seen with increasing cell-cell interactions. Graphs of BrdU incorporation in BPAEC treated with different concentrations of (A) brefeldin A and (B) monensin. C: Phase contrast image of pairs of BPAECs separated by 2 μm (right) and 5 μm (left) gaps. D: Graph of BrdU incorporation in cells grown with gaps. White bars represent single cells; gray bars represent cells grown in pairs. E: Graph of BrdU incorporation in BPAEC treated with different concentrations of LY294002. The data represent the mean ± S.D. of at least three independent experiments, with *$P < 0.005$.

SAM patterns. The proliferation rate nearly doubled for cells grown in pairs (Fig. 3C).

To determine if a soluble factor was responsible for the cell-cell-mediated increase in proliferation, we compared the proliferation rates of pairs of cells with those of single cells on patterns that were treated with brefeldin A (Fig. 4A) and monensin (Fig. 4B) to disrupt protein secretion. Both drugs caused an overall decrease in proliferation with increasing dose, but neither drug preferentially blocked the enhanced proliferation seen in cells grown as pairs. Treatment with the growth factor receptor inhibitor suramin also did not abrogate the increase in proliferation in cells grown in pairs (data not shown).

Conversely, to test whether diffusible paracrine signals mediated cell-cell induced proliferation in the absence of physical contact, we plated cells on substrates where the bowtie-shaped pattern was bisected with a strip of non-adhesive agarose, leaving 2 and 5 µm-sized gaps between neighboring cells (Fig. 4C). The gaps were sufficiently narrow to allow proteins secreted by one cell in a pair to diffuse across to the other cell, based on a simple diffusion model (data not shown). The proliferation rates of cells grown in these separated pairs dropped to the levels of single cells (Fig. 4D), confirming that direct contact between cells and not diffusible signaling is responsible for the increase in proliferation due to cell-cell interactions.

To begin to characterize the intracellular basis for direct cell-cell contact-mediated proliferation, we explored whether any traditional signal transduction pathways associated with growth factor-mediated proliferation were involved. To determine if PI3K was involved in the increase in proliferation, we treated cells grown on bowtie-shaped islands with increasing concentrations of LY294002 (Fig. 4E). Blocking PI3K completely inhibited the increase in proliferation of pairs over single cell controls at specific concentrations of the inhibitor. Further increasing LY294002 concentration inhibited cell spreading (data not shown). These results indicate that signaling through PI3K is necessary for the increase in proliferation with cell-cell contact, and that cell-cell signaling is even more sensitive to PI3K inhibition than traditional growth factor signaling.

4. Discussion

Cell-cell contact positively regulates proliferation. Our results demonstrate that cells contacting one or more neighbors have a significant growth advantage over single cells without contacts, contradicting the previously held notion that cell-cell contact inhibits cell proliferation [10,11]. Our data suggest that past studies may have been confounded by an inability to separate effects of cell-cell contact and cell spreading. Decreasing cell spreading appears to inhibit proliferative signals and may mask positive signals from cell-cell contact: Round cells with maximal contact (representing 'confluent' cells) still proliferate much less than spread cells with no contacts (representing 'sparse' cells). In the geometry of feeder layer-dependent cultures, which allows for contact-mediated signaling without constraining cell spreading, contact has been shown to improve proliferation of various cell types [12,13]. Thus, 'contact inhibition' of growth of cells cultured in monolayers may be less a result of cell-cell contact and more a result of the decreased spreading as cells become crowded. The ob-

served increase in proliferation with cell-cell contact, only apparent when spreading was controlled, suggests that future studies of contact-mediated proliferation should use methods to control confounding factors such as cell spreading. The technique to achieve this control described in the present study is relatively simple, relies on reagents available in typical biological laboratories, and appears to preserve the normal structure of cell-cell contacts.

The positive effect of cell-cell contact is due to molecules engaged when the cells physically touch and not to a diffusible factor. A number of molecular players may be involved, including gap junctions, cadherins, and CAMs. Each of these has been shown to regulate proliferation in other systems, and although the majority of studies conclude that cell-cell contact via these molecules inhibits proliferation, ours is not the first study to find that cell-cell contact stimulates growth. For example, although overexpressing connexins in some cancer cell lines leads to decreased growth [5], enhancement of gap junctional communication by connexin 43 expression increases proliferation in others [14]. Similarly, although cadherins are thought to negatively affect proliferation by sequestering beta-catenin [15], recent findings have shown that cadherins can bind to and activate growth factor receptors [16], and thereby positively regulate cell cycle progression. N-CAM, a member of the Ig-like cell adhesion molecule family, can induce FGF receptor signaling [17]. Aside from cell-cell adhesion molecules, immobilized growth factors in membrane- or ECM-bound forms, such as bFGF [18] or HB-EGF [19], could also be responsible for the observed phenomenon, since the membrane-bound form is likely to be substantially more potent as a signal due to its partial immobilization [20].

We show that PI3K is necessary for the increase in proliferation due to cell-cell contact (Fig. 4). PI3K mediates proliferation through several players, including protein kinase C, phosphoinositide-dependent kinases and MAPK, and is activated in classical mitogenic pathways such as those through growth factor receptors and integrins [21,22]. Surprisingly, the cell-cell pathway is far more dependent on PI3K signaling than the more traditional pathways, implying that cell-cell contact has fewer redundant, parallel pathways leading to proliferation. The mechanism by which cell-cell contact leads to PI3K stimulation is not known, but given the number of known upstream stimulators (growth factor receptors, non-receptor (p60-src) tyrosine kinases, Ras, and members of the Rho family of GTPases [22]), it is reasonable to conjecture that several pathways are involved. Cadherin engagement has recently been shown to upregulate both Rac and Cdc42 activity in epithelial cells [23]. Because it is unlikely that cell-cell-mediated mitogenic effects would be limited to signaling through one pathway, other independent mechanisms must be considered, including the Wnt signal transduction pathway. Engagement of the Wnt receptor inactivates GSK-3β, stabilizing cytoplasmic beta-catenin, an essential coactivator of Lef/Tcf transcription factors [15]. Since cadherins bind and sequester beta-catenin [24], and GSK-3β has recently been shown to be inhibited by PI3K signaling [25], it will be important to determine what roles beta-catenin and GSK-3β might play in cell-cell contact-stimulated proliferation.

The finding that cell-cell contact stimulates proliferation is consistent with many physiological processes. During angiogenesis, endothelial cells must proliferate in a directed manner and still maintain cell-cell contacts to ensure the integrity of

the vasculature. During wound healing, it is conceivable that enhanced proliferation of cells at the leading edge over isolated cells scattered in the wound would lead to a faster, more directed mode of wound closure. Indeed, bFGF has been found to increase connexin 43 expression and intercellular communication in endothelial cells and fibroblasts, suggesting that increased cell coupling might be necessary for the coordination of these cells in wound healing and angiogenesis [26]. Thus, cell-cell contact might not only increase survival by protecting cells from dying, but also by stimulating them to divide.

Finally, given that we and others have seen opposing effects of cell-cell contact on proliferation, cell-cell contact may have numerous molecular players that each act as positive and negative regulators. Thus, as in growth factor and integrin signaling, several receptors may be simultaneously engaged to signal the cell, and the specific conditions surrounding individual cells may alter the panoply of receptors that ultimately dominate to control a particular cell's fate.

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