Evidence for ERK 1/2 Phosphorylation Controlling Contact Inhibition of Proliferation In Madin-Darby Canine Kidney Epithelial Cells

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Running head: Contact Inhibition of Epithelial Cell Proliferation

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Abstract

Increasing cell density arrests epithelial cell proliferation by a process termed contact inhibition. We investigated mechanisms of contact inhibition using a model of contact inhibited epithelial cells. Hepatocyte growth factor (HGF) treatment of contact-inhibited MDCK cells stimulated cell proliferation and increased levels of phosphorylated ERK 1/2 (phospho-ERK 1/2) and cyclin D1. MEK inhibitors, PD98059 and UO126, inhibited these HGF-dependent changes indicating the dependence on phosphorylation of ERK 1/2 during HGF-induced loss of contact inhibition. In relation to contact inhibited high-density cells, low-density MDCK cells proliferate and had higher levels of phospho-ERK 1/2 and cyclin D1. PD98059 and UO126 inhibited low-density MDCK cell proliferation. Trypsinization of high-density MDCK cells immediately increased phospho-ERK 1/2 and was followed by a transient increase in cyclin D1 levels. Reformation of cell junctions after trypsinization led to decreases in phospho-ERK 1/2 and cyclin D1 levels. High-density MDCK cells express low levels of both cyclin D1 and phospho-ERK 1/2 and treatment of these cells with fresh medium containing HGF but not fresh medium alone for 6 h increased phospho-ERK 1/2 and cyclin D1 levels as compared to cells without medium change. These data provide evidence that HGF abrogates MDCK cell contact inhibition by increasing ERK 1/2 phosphorylation and levels of cyclin D1. These results suggest that in MDCK cells contact inhibition of cell proliferation in the presence of serum occurs by cell density dependent regulation of ERK 1/2 phosphorylation.

Keywords: Cell Density, Cyclin D1, Hepatocyte growth factor, and cell cycle
Introduction

The cell cycle is a complicated but universal process through which all cells must travel in order to proliferate and grow. Cell cycle regulation relies on a number of critical genetic and enzymatic pathways that control activation and direct progress\cite{7, 31}. When untransformed cells, unlike cancer cells, are cultured in a dish in the presence of serum they proliferate until a confluent monolayer is formed and the cells are in contact with neighboring cells on all sides. This phenomenon, known as density-dependent inhibition of cell division or contact inhibition, was thought to reflect, in part at least, the ability of a cell to deplete the medium locally of extracellular mitogens, thereby depriving its neighbors\cite{9, 15, 29}. More recently, contact inhibited endothelial cells have been shown to exhibit a reduced proliferative response to specific growth factors when they reach confluence\cite{14, 32}.

Within the cell cycle signal transduction cascade, type D cyclins play a crucial role. Cyclin D1 is thought to be a critical regulator protein of the progression of cells into the proliferative stage of the cell cycle\cite{16, 25, 26}. Cyclin D1 accumulation has also been used as a marker for entry of MDCK cells into the cell cycle\cite{21}. Expression abundance of cyclin D1 is largely dependent on extracellular signals and serves as an early checkpoint for cells entering the cell cycle\cite{7}. Equally important in the progression of the cell cycle is the role played by extracellular-signal-regulated kinases (ERKs)\cite{30}. ERKs, members of the mitogen-activated protein kinase (MAPK) family, exist as two isoforms (ERK1/ERK2 or p44/p42 MAPK respectively) and connect different types of membrane receptors to the nucleus after mitogenic stimulation\cite{6, 24}. ERK 1 and 2 are activated by phosphorylation of threonine and tyrosine residues via ERK kinases (known
as MEK1 and MEK2)(13, 20). ERK signaling can be blocked by the MEK inhibitors PD98059 and UO126(11). The ERK cascade is commonly activated by growth factors and is also believed to play a role in cyclin D1 expression and cell proliferation(23).

The Madin-Darby canine kidney (MDCK) cell line has been the most widely used system for studying important and fundamental issues in epithelial cell biology(28). These cells were derived from the kidney tubules of a cocker spaniel in 1958 and display contact inhibition in culture(12). Depending on culture conditions, MDCK cells respond to hepatocyte growth factor (HGF) through the tyrosine kinase receptor c-met receptor by scattering, forming branching tubules, or dedifferentiating(3, 4). In addition to these morphogenic changes, HGF also abrogates contact inhibition of mitosis in high-density MDCK cells(2).

While the capacity of cells to exhibit contact inhibition is widely recognized, the cellular signaling pathways controlling this process in epithelial cells remain largely unknown. The present study systematically examined the role of ERK phosphorylation, cyclin D1 accumulation, and cell proliferation using the MDCK cell model of contact inhibition.

**Experimental Procedures**

*MDCK Culture, HGF Treatment and MEK Inhibitor Treatment*

Low-passage type II MDCK cells were obtained from K. Mostov (University of California San Francisco, San Francisco, CA) and used between passages 2 and 10 as
previously described\( (4, 5) \). Cells were cultured in modified Eagle’s minimum essential medium (MEM) containing Earl’s balanced salt solution and glutamine supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 µg/ml amphotericin B. For high-density experiments MDCK cells were seeded at confluency on Transwell filter units (Costar, Cambridge, MA). Pore size on all filters was 0.4 µm. Cell monolayers were used for experiments after 3-5 days of culture with changes in medium every 1-2 days. For low-density conditions, cells were plated in plastic wells at ~10% confluency. In some experiments, HGF at 100 ng/ml was added to the basolateral compartment of MDCK cell monolayers on filters or the medium of low-density cells for periods extending to 72 hr. R. Schwall (Genentech, San Francisco, CA) generously provided recombinant human HGF. In some experiments, cells were treated with PD98059 (a MEK1 inhibitor compound) or UO126 (a MEK1/2 inhibitor compound) (Cell Signaling Technology®) or DMSO vehicle alone in various experiments. Both MEK inhibitors were used at a concentration of 50 µM, which has previously been shown to maximally inhibit MEKs in MDCK cells\( (33) \). The cells were pretreated with MEK inhibitors 30 min prior to treatment with HGF containing medium or fresh medium alone. Experiments were performed 3-5 times.

**Mitogenesis Assay**

DNA synthesis was determined by measuring thymidine incorporation with minor modifications of a previously described protocol\( (2) \). Briefly, control and HGF-treated MDCK cells at low- and high-density were pulsed for 1 h at 37° C with 5 µCi/ml \([\text{methyl-}^3\text{H}]\text{thymidine}\) (76.0 Ci/mmol) (placed on the basolateral surface of filter grown cells). After the pulse, cells were rinsed twice in phosphate-buffered saline containing
Mg\(^{2+}\) and Ca\(^{2+}\) (PBS+), fixed in methanol/acetic acid/water (50:10:40, vol/vol) for 1 h at 4º C and rinsed twice more with PBS+. Filters were cut from the support with a scalpel and methanol/acetic acid/water insoluble radioactivity was measured by liquid scintillation. Cells grown on plastic were solubilized in 1% SDS for 1 h at 37º C and methanol/acetic acid/water insoluble radioactivity was measured by liquid scintillation. Experiments were done in triplicate.

**Cell Counts**

Cells were gently rinsed with PBS- (PBS without Ca\(^{2+}\) and Mg\(^{2+}\)). Cells were then trypsinized using 0.5 ml of trypsin (0.25%)-EDTA (0.1%) solution in HBSS (Mediatech, Cellgro™) for 15 minutes at 37ºC, 5% CO\(_2\). One-half ml of trypsinized cells was then added to separate solutions of 4.5 ml of PBS- and cells were counted using a hemocytometer. Cell count experiments were done in triplicate.

**Cell Lysate Preparation**

MDCK II cells grown on filters or plastic wells were used for preparation of cell lysates for Western blot analysis. Nonadherent cells were removed by rinsing the cell monolayer once with ice-cold PBS+. Adherent cells were then exposed to 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TX-100, 1% deoxycholic acid, and 5 mM ethylenediaminetetraacetic acid (radioimmunoprecipitation assay [RIPA] buffer) containing inhibitors of proteases (2 mM phenylmethylsulfonyl fluoride, 50 µg/ml pepstatin, 50 µg/ml chymostatin, and 10 µg/ml antipain) for 20 min on ice. Cells were scraped from the filter or plastic well with a rubber policeman to generate total cell lysates. Total cell lysates were sedimented in a 4ºC microfuge at 14,000 rpm x 10 min. The protein concentration of each cell lysate was determined by using the bicinchoninic
acid determination assay (Pierce Chemical Co., Rockford, IL). Samples were diluted as necessary with varying amounts of RIPA buffer to obtain equal protein concentrations of 600 µl of each lysate sample. Lysate preparation was completed by the addition of 200 µl of 4X Laemmli buffer containing 100 mM dithiothreitol and boiling for 5 min.

**Electrophoresis and Western Blot analysis**

Western blot analysis was performed as described previously(4). Briefly, equal amounts of protein in lysates of MDCK cells were run on acrylamide gels at 160 v for 45 min. Proteins were transferred to Immobilon P membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 30 min with PBS- 5% milk, 0.1% Tween 20 (block solution). For Cyclin D1 analysis, filters were probed with Anti-Cyclin D1 (C-terminal) Antibody/Affinity purified rabbit IgG (1:200) (Medical and Biological Laboratories) for 1 h and then washed with PBS- 0.1% Tween 20 (4 x 5 min). Filters were then probed with horseradish peroxidase-labeled goat anti-rabbit at 1:10,000 dilution in block solution for 1 h. For total ERK analysis, filters were probed with Anti-ERK1 Goat polyclonal IgG (1:200) (Santa Cruz Biotechnology) for 1 h and then washed with PBS- 0.1% Tween 20 (4 x 5 min). This antibody recognizes both ERK 1 and 2 in MDCK cell lysates. Filters were then probed with horseradish peroxidase-labeled donkey anti-goat at 1:5000 dilution in block solution for 1 h. For active ERK (MAPK) analysis, filters were probed with Phospho-p44/42(ERK1/2) MAPK Antibody (1:10,000) (Cell Signaling Technologies) for 1 h and then washed with PBS- 0.1% Tween 20 (4 x 5 min). Filters were then probed with horseradish peroxidase-labeled goat anti-rabbit at 1:5000 dilution in block solution for 1 h. After secondary antibody, all filters were again washed (4 x 5 min) with PBS- 0.1% Tween 20. Filters were developed by using the enhanced chemiluminescence kit.
(ECL; Amersham Corp., Piscataway, NJ) and visualized on Kodak X-OMAT film (Eastman Kodak, Rochester, NY). Band densitometry was performed using NIH Image Version 1.60 (NIH Image is in the public domain).

**Results**

*HGF Treatment Transiently Increases Cyclin D1 Levels in Previously Contact Inhibited MDCK Cells*

MDCK cells grown at confluency on permeable filter supports in the presence of serum exhibit low proliferative activity(2). However, treatment of these MDCK cell monolayers with HGF abrogates contact inhibition and stimulates the cells to enter the cell cycle. Because cyclin D1 is an important element for entry into the cell cycle, we performed a time course analysis of the effect of HGF on MDCK cell cyclin D1 levels. Under these conditions, HGF treatment induced an increase in cyclin D1 after 3 h (Fig. 1A). The increase in cyclin D1 peaked at 6 h and was followed by a decrease to baseline after 24 h of HGF treatment. This increase in cyclin D1 levels in previously contact inhibited MDCK cell monolayers correlated with the HGF-induced stimulation of thymidine incorporation in MDCK cell monolayers by HGF(2).

*HGF Induced Cyclin D1 Increases In Contact Inhibited MDCK Cell Monolayers Requires ERK 1/2 Phosphorylation*

We examined the role of ERK phosphorylation during HGF induced increases of cyclin D1 levels in contact inhibited MDCK cell monolayers. Figures 1A and 1B show that HGF treatment transiently increases phospho-ERK 1/2 levels in contact inhibited MDCK cell monolayers. The increase of phospho-ERK 1/2 levels preceded the increase of cyclin D1 protein. HGF treatment increases phospho-ERK 1/2 as early as after 15 m
(Fig. 1B) and the levels gradually return to base line levels after 24 h. Fassett et al showed that a second peak of ERK activation was necessary for cyclin D1 up regulation and cell cycle entry (10). In the present case HGF induced phosphorylation exhibits only one peak that is followed by a transient increase of cyclin D1. Time points extending to 48 also do not show a second peak of ERK phosphorylation (see below, Fig. 3A). These data also provide evidence that HGF treatment of contact inhibited MDCK cells stimulates the cells to enter only one round of the cell cycle.

To test the role of ERK phosphorylation on cyclin D1 levels during loss of contact inhibition, we examined the effects of PD98059 and UO126 on HGF-induced increases of cyclin D1 and ERK phosphorylation in high-density MDCK cells. At 50 µM, UO126 more effectively inhibited HGF-induced increases of cyclin D1 than the PD98059 (Fig. 2A). In the absence of HGF treatment, total levels of cyclin D1 were low in the presence and absence of MEK inhibitors. In the absence of HGF treatment (Control), levels of phospho-ERK 1/2 were low in the presence and absence of MEK inhibitors. However, HGF-induced ERK phosphorylation, as monitored by western blot analysis using antibodies against phosphorylated ERK 1/2, was inhibited by PD98059 and UO126 (Fig. 2A). UO126 is also more effective at inhibiting the HGF-induced ERK 1/2 phosphorylation than PD98059. In order to gain better insight into the sensitivities of MDCK cells to the MEK inhibitors UO126 and PD98059, we performed a dose response analysis of HGF-induced ERK phosphorylation to both inhibitors at concentrations of 1, 10 and 100 µM (Fig. 2B). The results demonstrate that UO126 is a more potent inhibitor of HGF-induced ERK activation. Based on these data, one would predict a greater inhibition of ERK by UO126 than PD98059 at a concentration of 50 µM.
Effects of HGF on Cyclin D1 Levels and ERK 1/2 Phosphorylation in MDCK Cells at Low-Density and High-Density

When plated at low-density on plastic, MDCK cells actively proliferate until they reach confluency and then exhibit contact inhibition(12). MDCK cell proliferation rates at low-density are not further stimulated by treatment with HGF(2). We performed a simultaneous analysis of the effects of HGF on cyclin D1 levels and ERK 1/2 activation in MDCK cells plated at 10% confluency (low-density) and contact inhibited cell monolayers (high-density). Protein concentrations in all cell lysates were adjusted so that equal amounts from each sample were analyzed by Western blot. Western blots of proteins in high- and low-density cells were exposed to film for identical time periods. This allowed for the comparison of amounts of cyclin D1 and phospho-ERK 1/2 in low-density versus high-density cells per total cell lysate protein. The data are presented in figure 3A and 3B.

Before exposure to HGF, Cyclin D1 levels were higher in low-density cells as compared to high-density cells (Fig. 3A). Following exposure to HGF, cyclin D1 levels increased in high-density cells and returned to the baseline control levels after 48 h of HGF treatment. The levels of cyclin D1 in low-density cells also increased with HGF treatment and remained elevated up to 48 h of HGF treatment. Measurement of amounts of cyclin D1 by band densitometry in low-density versus high-density showed higher levels of cyclin D1 in low-density cells than the high-density cells at all time points except 3 h when the amounts were approximately equal (Fig. 3B). These results demonstrate that cyclin D1 levels were higher in low-density cells than high-density cells
in the absence of HGF-treatment and provide an explanation for the lack of proliferation in high-density cells in the absence of HGF.

Prior to exposure to HGF, phospho-ERK 1/2 levels were higher in low-density than high-density cells (despite lower amounts of total ERK 1/2 in low-density cells). Levels of phospho-ERK 1/2 increased with HGF treatment in both low-density and high-density cells (Fig. 3A). In the high-density cells, phospho-ERK 1/2 levels returned to baseline 24-48 h after HGF treatment while phospho-ERK 1/2 levels remained elevated in the HGF treated low-density cells. Measurement of the ratio of phospho-ERK/total ERK band densitometry in low-density versus high-density showed higher levels of phospho-ERK 1/2 in low-density cells than the high-density cells at all time points (Fig. 3B). The data provide evidence supporting the hypothesis that low-density MDCK cells are proliferating, in part, due to increased levels of phospho-ERK 1/2.

**Effect of PD98059 and UO126 on HGF Induced Cell Proliferation of MDCK Cells at High-Density and Low-Density**

HGF treatment of confluent monolayers of MDCK cells stimulates DNA synthesis and cell division(2). To determine the role of ERK-1/2 phosphorylation and cyclin D1 accumulation on HGF-induced loss of contact inhibition in high-density MDCK cells, we examined the effect of 50 μM PD98059 or UO126 on HGF-induced cell division and DNA synthesis in high-density cells. We also examined the effects of 50 μM PD98059 and UO126 on cell division and DNA synthesis in low-density cells in the presence and absence of HGF. The number of cells in confluent monolayers of filter-grown MDCK cells treated with fresh medium or fresh medium with HGF in the presence and absence of PD98059 or UO126 after 48 h was determined by trypsinization
and counting. Figure 4A (upper panel) shows that cell numbers increased in the presence of HGF as compared to control cells and that PD98059 did not significantly block the proliferative effect of HGF. In contrast, the results in figure 4A (bottom panel) demonstrate that UO126 blocked the HGF-induced stimulation of cell division in MDCK cell monolayers. We also examined the effects of PD98059 and UO126 on HGF-induced stimulation of thymidine incorporation into MDCK cell monolayers. In the case of PD98059, there was minimal inhibition of the HGF induced stimulation of thymidine incorporation (Fig. 4B, upper panel). However, UO126 more effectively inhibited the HGF induced stimulation of thymidine incorporation (Fig. 4B, bottom panel).

In cells plated at low-density, HGF treatment did not further stimulate the rates of cell division as compared to low-density cells growing in the presence of medium alone as determined by cell counts after trypsinization (Fig. 5A). PD98059 did significantly inhibit cell proliferation of low-density cells in the presence and absence of HGF at 72 h time point (P=0.0003 for controls and P=0.0016 for HGF treated cells). UO126 dramatically inhibited cell proliferation of low-density cells in presence and absence of HGF (P=0.000002 for controls and P=0.00000007 for HGF treated cells). These data demonstrated that proliferation rates of cells at low-density is dependent on activation of ERK 1/2.

Thymidine incorporation rates in MDCK cells at low-density in the presence and absence of HGF were also determined (Fig. 5B). The differences in thymidine incorporation rates in low-density MDCK cells in the presence and absence of HGF was not significant (P=0.222). PD98059 inhibited thymidine incorporation into low-density cells in the presence and absence of HGF. UO126 inhibited thymidine incorporation in
low-density cells in the presence and absence of HGF more effectively. Low-density and high-density MDCK cells exposed to UO126 and PD98059 at concentrations up to 100 µM for 72 h remain viable as determined using the LIVE/DEAD viability/cytotoxicity assay (Molecular Probes, Inc.) (Data not shown).

Maturation of Cell-Cell Contacts Inversely Correlates with ERK 1/2 Phosphorylation and Cyclin D1 Levels

We hypothesized that the establishment of cell contacts correlates with contact inhibition of mitosis as well as responsiveness to mitogenic stimuli by a mechanism of inhibiting ERK 1/2 phosphorylation and subsequent cyclin D1 accumulation. MDCK cells plated at confluency require approximately 72 h of culture to reach a fully polarized phenotype as manifested by tight junction development, adherens junction development and cilia formation(34). After 72 h in culture MDCK cell monolayers are contact inhibited in response to fresh medium(2). MDCK cells grown at low-density (no contact inhibition) actively divide and are not polarized with regard to adherens and tight junction formation. HGF also causes disruption of cell-cell contacts in MDCK cell monolayers as manifested by an increase in triton solubility of adherens junction components (suggesting dissociation from the actin cytoskeleton) and increased inulin diffusion across MDCK cell monolayers(5). We performed an analysis of cyclin D1 expression and ERK 1/2 phosphorylation in MDCK cells at different stages of cell-cell junction formation with regard to responsiveness to fresh medium and fresh medium containing HGF. Figure 6A shows a cartoon illustrating the different stages of formation of tight and adherent junctions in MDCK cells following plating the cells at confluence on permeable supports. Cell lysates from an aliquot of freshly trypsinized cells were collected and represented
unplated cells. MDCK cells were plated at confluence. At time periods of 24, 48 and 72 h after plating the cells were then exposed to no medium change (control), fresh medium (FM), or fresh medium containing HGF (HGF) for a period of 6 h. The time 0 cells were cultured for 6 h in medium ± HGF and the medium was not changed as they had just been plated in fresh medium. After the 6 h treatment period, cell lysates were prepared. Western blot analysis showed that levels of cyclin D1 in trypsinized cells were low (Fig. 6B). However, phospho-ERK 1/2 levels in freshly trypsinized cells were higher than seen in contact inhibited monolayers (see 72 h time point for control). Six h after plating at confluence on filters, the levels of cyclin D1 are dramatically increased and levels of phospho-ERK 1/2 remain elevated. The increases of cyclin D1 and phospho-ERK 1/2 in 6 h plated cells in presence of HGF were greatest. In the cells which had been plated for 24, 48, and 72 h and exposed to nothing or fresh medium for 6 h, the levels of cyclin D1 and phospho-ERK 1/2 were found to be similar to that seen in contact inhibited high-density cells. However, the cells plated for 24, 48, and 72 h and then treated with HGF for 6 h all exhibited increased levels of cyclin D1 and phospho-ERK 1/2. These data show that disruption of cell-cell contacts by trypsinization led to increased phospho-ERK 1/2 and cyclin D1 within 6 h after disruption. The levels of these proteins decreased with time as cell-cell contacts reformed. These data also show that HGF reversed the decreases of cyclin D1 and phospho-ERK 1/2 as cell-cell contacts formed. Fresh medium alone was not sufficient to reverse the decreases of cyclin D1 and active ERK 1/2 as cell contacts form. This observation argues against the explanation that contact inhibition of mitosis of cells is due to the depletion of growth factors(9, 15, 29). These data provide
evidence that the formation of cell-cell contacts decrease phospho-ERK 1/2 levels and cyclin D1 accumulation.

*The Increased Levels of Phospho-ERK 1/2 and Cyclin D1 Seen 6h after Trypsinization and Plating are Sensitive to MEK Inhibitors*

We examined the sensitivity of the increases of phospho-ERK 1/2 and cyclin D1 levels seen in cells that have been cultured for 6 h in the presence and absence of HGF following trypsinization to the MEK inhibitors PD98059 and UO126 (Fig. 7). The data show that increases of phospho-ERK 1/2 and cyclin D1 in cells cultured for 6 h in the presence and absence of HGF was more sensitive to UO126 than the PD98059.

**Discussion**

Cell cycle regulation is a complex process involving a number of intrinsic and extrinsic factors. Upon activation, a series of regulatory proteins proceed in a predictable cascade leading to entry into the cell cycle and subsequent cell proliferation. Among these regulatory proteins, cyclin D1 and the ERK family proteins are of undeniable importance. Cyclin D1 regulation has been demonstrated to occur at the transcriptional, translational, and post-translational levels. Transcription factors such as LEF-1 and AP-1 enhance cyclin D1 promoter activity(1, 27). ERK 1/2 positively regulates cyclin D1 transcription(19) and Akt phosphorylation(35). Cyclin D1 is also regulated at the level of mRNA translation by activation of phosphatidylinositol 3-kinase (PI-3 kinase)(22). At the post-translational level, modification of the protein half-life occurs and protein phosphorylation of cyclin D1 leads to its ubiquitination and proteasomal degradation(8). In this study, we explore cell-cell contact inhibition and its effect on phospho-ERK 1/2 levels and cyclin D1 accumulation as an additional regulatory mechanism.
At high-density, contact inhibited MDCK cells are not proliferating and accordingly exhibit very low levels of phospho-ERK 1/2 and cyclin D1. This contact inhibited state is abrogated by treatment with HGF by a mechanism of ERK 1/2 phosphorylation and cyclin D1 accumulation. When plated at low-density, MDCK cells have fewer cell contacts and actively proliferate. As they are in various phases of the cell cycle, they demonstrate higher levels of phospho-ERK 1/2 and cyclin D1. Interestingly, HGF treatment of low-density further increases the levels of phospho-ERK 1/2 and cyclin D1 without further stimulating cell proliferation rates. We interpret this finding as evidence that low-density cells have adequate levels of phospho-ERK 1/2 and cyclin D1 to promote maximal proliferation rates and that further increases in phospho-ERK 1/2 and cyclin D1 cannot increase the proliferation rates. The MEK inhibitor, UO126, blocks proliferation of low-density cells, suggesting that the proliferation in low-density cells is dependent on phospho-ERK 1/2 and subsequently elevated cyclin D1 levels. Trypsinization of monolayers, dissociates both cell-cell contacts (e.g., E-cadherin mediated adhesion) and cell-matrix contacts (e.g., integrin mediated adhesion) and in this setting, levels of phospho-ERK 1/2 rapidly rise and cyclin D1 levels subsequently increase. Phospho-ERK 1/2 and cyclin D1 levels rapidly diminish with re-establishment of cell contact formation over time. Collectively, these data support our hypothesis that the establishment of cell contacts causes contact inhibition of ERK signaling pathways and cyclin D1 accumulation. In addition, HGF increases phospho-ERK 1/2 and cyclin D1 as cell-cell contacts form. Fresh medium alone is not sufficient to reverse the decreases of phospho-ERK 1/2 and cyclin D1 as cell contacts form, challenging the traditional concept that contact inhibition of cell proliferation reflected, at least in part,
the ability of a cell to deplete the medium locally of growth factors, thereby depriving its neighbors(9, 15, 29).

Vinals et al. reported cell confluency induces cell cycle exit by inhibiting ERK 1/2 activity in vascular endothelial cells(32). In their study, both confluent and low-density endothelial cells expressed low levels of phospho-ERK 1/2 and cyclin D1. However, cells in both conditions had been serum starved for 24 h. Both cell culture conditions responded to stimulation with serum, but the low-density cells exhibited a more robust response with regard to ERK phosphorylation and cyclin D1 accumulation. In the our study, the MDCK cell model of contact inhibition does not require serum starvation to lower both phospho-ERK 1/2 and cyclin D1 levels and is more representative of the in vivo situation in which cells are continuously in contact with serum factors.

Cadherin mediated cell-cell adhesion has be receiving attention for a possible role in contact inhibition. Vascular endothelial cell cadherin (VE-cadherin) and β-catenin have been implicated in the contact inhibition VEGF-induced proliferation(14). The β-catenin binding site of VE-cadherin appears to be necessary to block VEGF-induced proliferation by cell-cell contact by inactivation of the phosphatase DEP-1. Another recent publication has provided evidence for E-cadherin mediated down-regulation of ERK signaling in differentiating intestinal epithelial cells(18). A continued systematic analysis of the role of E-cadherin and cellular phosphatases in contact inhibition of cell proliferation in MDCK cells is underway in our laboratory.

In conclusion, we demonstrate that HGF reverses contact inhibition in high-density MDCK cells through phospho-ERK-dependent cyclin D1 accumulation and
propose a mechanism by which the formation of cell-cell contacts in MDCK epithelial cells inhibits cell proliferation, reducing phospho-ERK 1/2 levels and subsequent cyclin D1 accumulation. In low-density, non-confluent, MDCK cells levels of phospho-ERK 1/2 and cyclin D1 remain higher and the cells continue in a proliferative state. This modulation of epithelial cell ERK 1/2 phosphorylation and proliferation rates by cell contact may play an important role in complex disease-related processes such as epithelial organ regeneration and/or epithelial cell transformation to carcinoma.
REFERENCES


35. Yu CF, Roshan B, Liu ZX, and Cantley LG. ERK regulates the hepatocyte growth
factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. *J Biol Chem*
Acknowledgements

The authors thank Drs. Paul W. Sanders and Stuart Frank for critical review of the manuscript. Support for this work was provided by a Merit Review Grant from the Medical Research Service of the Department of Veterans Affairs to D.F.B.
Figure Legends

Figure 1. Effect of HGF Treatment of Contact Inhibited Monolayers of MDCK Cells on Cyclin D1, ERK 1/2, and Phospho-ERK 1/2 Expression. MDCK cells were plated at confluency on 24-mm filters and cultured for 3-4 days with medium change every two days. Cells were treated with HGF (100 ng/ml) for indicated time points. Cell lysates were prepared and equal amounts of cell lysate protein were analyzed for cyclin D1, ERK 1/2, and phospho-ERK 1/2 expression (Panel A). Panel B shows the effect of HGF treatment on contact inhibited monolayers of MDCK cells on phospho-ERK 1/2 expression at earlier time points.

Figure 2. Effect of PD98059 and UO126 on HGF-Induced Increases In Cyclin D1, ERK 1/2, and Phospho-ERK 1/2. Panel A: Monolayers of contact inhibited MDCK cell monolayers were pretreated for 30 m with fresh medium alone or fresh medium containing either PD98059 or UO126 at a concentration of 50 µM. Following pretreatment, cell monolayers were treated for 6 h at 37°C with fresh medium or fresh medium containing HGF (100 ng/ml) in the presence and absence of PD98059 or UO126 at a concentration of 50 µM. Following 6 h treatment, cell lysates were prepared and equal amounts of cell lysate protein were analyzed by Western blot for cyclin D1, ERK 1/2, and phospho-ERK1/2. Panel B shows a dose response of HGF-induced activation of ERK-phosphorylation to UO126 and PD98059. Monolayers of contact inhibited MDCK cell monolayers were pretreated for 30 m with fresh medium alone or fresh medium containing either PD98059 or UO126 at the indicated concentrations. Following pretreatment, cell monolayers were treated for 6 h at 37°C with fresh medium or fresh
medium containing HGF (100 ng/ml) in the presence and absence of PD98059 or UO126 at the indicated concentrations. Following 6 h treatment, cell lysates were prepared and equal amounts of cell lysate protein were analyzed by Western blot for ERK 1/2 and phospho-ERK1/2.

Figure 3. Effects of HGF Treatment of MDCK Cells at Confluence (High-Density) or Low-Density on Levels of Cyclin D1, ERK 1/2, and Phospho-ERK 1/2. For High-Density conditions, MDCK cells were plated at confluency and cultured for 3-4 days with medium change every two days. For Low-Density conditions, MDCK cells were plated at 10% confluency 24 h prior to HGF treatment. High-density and low-density cells were simultaneously treated with HGF (100 ng/ml) for the indicated time points. After HGF treatment, cell lysates were prepared, protein concentration in lysates measured, and protein concentration in lysates were adjusted to equality. Equal amounts of cell lysate protein were simultaneously analyzed for cyclin D1, ERK 1/2, and phospho-ERK 1/2 by Western blot with equal film exposure times (panel A). Panel B shows cyclin D1 band densities and the ratio of phospho-ERK/total ERK band densities in control and HGF treated MDCK cells growing at high- and low-density. Band densities were determined using NIH Image on Western blots from three separate experiments.

Figure 4. Effects of PD98059 and UO126 on High-Density MDCK Cell Proliferation Response to HGF. Panel A shows the effect of 50 µM PD98059 and UO126 on MDCK cell proliferation in contact inhibited cell monolayers in presence and absence of HGF. Cell monolayers were pretreated with PD98059 or UO126 at 50 µM for 30 m. After
pretreatment, cell monolayers were exposed to fresh medium or fresh medium containing HGF (100 ng/ml) in the presence and absence of 50 µM PD98059 or UO126. Numbers of cells on filters were determined at time 0 and 48 h by trypsinization and cell counting using a hemocytometer. Cell numbers on filters at 48 h are presented in bar graph. Cell numbers on control filters at time 0 and 48 h were not significantly different. Panel B shows the effect of 50 µM PD98059 or UO126 on thymidine incorporation rates into confluent MDCK cell monolayers in presence and absence of HGF. MDCK cell monolayers were pretreated with PD98059 or UO126 at 50 µM for 30 m at 37°C. Following pretreatment cells were exposed to fresh medium or fresh medium with HGF (100 ng/ml) in the presence and absence of 50 µM PD98059 or UO126 for a period of 12 h. Thymidine incorporation rates were then determined as described in materials and methods.

Figure 5. Effects of PD98059 and UO126 on Low-Density MDCK Cell Proliferation in the Presence and Absence of HGF. Panel A shows the effect of 50 µM PD98059 and UO126 on MDCK cell proliferation at 10% confluency (low-density) in presence and absence of HGF. Cells were pretreated with PD98059 or UO126 at 50 µM for 30 m. After pretreatment, cell monolayers were exposed to fresh medium or fresh medium containing HGF (100 ng/ml) in the presence and absence of 50 µM PD98059 or UO126. Numbers of cells were determined at time 0, 24, 48, and 72 h by trypsinization and cell counting using a hemocytometer. Panel B shows the effect of 50 µM PD98059 or UO126 on thymidine incorporation rates into MDCK cells growing at 10% confluency (low-density) in presence and absence of HGF. MDCK cell monolayers were pretreated
with PD98059 or UO126 at 50 µM for 30 m at 37°C. Following pretreatment cells were exposed to fresh medium or fresh medium with HGF (100 ng/ml) in the presence and absence of 50 µM PD98059 or UO126 for a period of 12 h. Thymidine incorporation rates were then determined as described in materials and methods.

Figure 6. Effect of MDCK Cell Junctional Development on Modulation of Cyclin D1, ERK 1/2, and Phospho-ERK 1/2 Levels In Response to Fresh Medium or Fresh Medium with HGF. Confluent monolayers of MDCK cells were separated by trypsinization and plated at confluency on filters. Cell lysate was prepared from an aliquot of freshly trypsinized cells. Cells were cultured on filters for 0, 24, 48, and 72 h and then exposed to fresh medium, fresh medium containing HGF (100 ng/ml), or no change in medium for 6 h at 37°C. Cell lysates were prepared after the 6 h incubation. Cell lysates were analyzed for cyclin D1, ERK 1/2, and phospho-ERK 1/2 by Western blot. Cartoon in panel A illustrates the development of cell junctions in MDCK cells and provide reference time points for western blots. The freshly trypsinized cells were not treated with fresh medium or HGF. Panel B shows the Western blot data of the cell’s responses to control (no medium change), fresh medium, or fresh medium containing HGF.

Figure 7. Effect of PD98059 and UO126 on Changes in Cyclin D1 and Phospho-ERK 1/2 Levels in Cells After Trypsinization in the Presence and Absence of HGF. MDCK cell monolayers were subjected to trypsinization and plated at confluency of filters. Immediately after plating cells were exposed to medium alone or medium containing HGF in the presence or absence of 50 µM PD98059 or UO126 for 6 h. After 6 h
treatment period, cell lysates were prepared for Western blot analysis of levels of cyclin D1, ERK 1/2, and phospho-ERK 1/2.
Figure 1
Figure 2

A

Cyclin D1

ERK 1/2

Phospho-ERK 1/2

<table>
<thead>
<tr>
<th>PD</th>
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<tbody>
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<td>HGF</td>
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B

ERK 1/2

Phospho-ERK 1/2

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</thead>
<tbody>
<tr>
<td>HGF + PD (μM)</td>
<td>HGF + UO (μM)</td>
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</table>
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7