Loss of active MEK1-ERK1/2 restores epithelial phenotype and morphogenesis in transdifferentiated MDCK cells

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Schramek, Herbert, Elisabeth Feifel, Ingrid Marschitz, Nadejda Golochtchapova, Gerhard Gstraunthaler, and Roberto Montesano. Loss of active MEK1-ERK1/2 restores epithelial phenotype and morphogenesis in transdifferentiated MDCK cells. Am J Physiol Cell Physiol 285:C652–C661, 2003; 10.1152/ajpcell.00463.2002.—Constitutive activation of the MAPK/ERK kinase (MEK1-ERK2) signaling module in Madin-Darby canine kidney (MDCK)-C7 cells disrupts their ability to form cystlike structures in collagen gels and induces an invasive, myofibroblastlike phenotype. However, the reversibility of these cellular events, as well as the relative role of both MEK isoforms (MEK1 and MEK2) and both ERK isoforms (ERK1 and ERK2) during these processes, has not yet been investigated. We now report that loss of constitutively active MEK1 (caMEK1) and, thus, loss of active ERK1/2 in C7caMEK1 cells is associated with increased MEK2 protein expression, reexpression of ERK1 protein, and epithelial redifferentiation of these cells. The morphological changes toward an epithelial phenotype in these revertant cell lines (C7rev4, C7rev5, C7rev7) are reflected by the upregulation of epithelial marker proteins, such as E-cadherin, β-catenin, and cytokeratin, by the loss of α-smooth muscle actin expression, and by the ability of these epithelial revertants to form well-organized spherical cysts when grown in three-dimensional collagen gels. Further evidence for a role of the MEK1-ERK1/2 module in epithelial-mesenchymal transition was obtained from the analysis of two novel, spontaneously transdifferentiated MDCK-C7 cell clones (C7e1 and C7e2 cells). In these clones, increased MEK1/2-ERK1/2 phosphorylation, reduced MEK2 protein expression, and loss of ERK1 protein expression is associated with phenotypic alterations similar to those observed in transdifferentiated C7caMEK1 cells. C7e1 cells at least partially regained some of their epithelial characteristics at higher passages. In contrast, C7e2 cells maintained a transdifferentiated phenotype at high passage, were unable to generate cystlike epithelial structures, and retained invasive properties when grown on a three-dimensional collagen matrix. We conclude that in renal epithelial MDCK-C7 cells, stable epithelial-to-mesenchymal transition (EMT) is associated with loss of ERK1 protein expression, reduced MEK2 protein expression, and increased basal ERK2 phosphorylation. In contrast, loss of active MEK1-ERK1/2 results in increased MEK2 protein expression and reexpression of ERK1 protein, concomitant with the restoration of epithelial phenotype and the ability to form cystic structures.

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UNDER PHYSIOLOGICAL CONDITIONS, differentiated cells will usually maintain their specialized character, although, during adaptation or reparative regeneration, modifications of cellular phenotype can be observed. In pathological conditions, such as inflammation and carcinogenesis, even highly specialized cells can alter their properties, leading to a deranged control of cell differentiation and/or proliferation.

Normal epithelia possess cell-cell contacts that account for both proper development of these tissues during embryonic life and the maintenance of homeostasis and architecture of epithelial structure during adult life. The process by which adult, polarized epithelial cells are converted into motile, myofibroblastlike cells is called epithelial-mesenchymal transition (EMT) or transdifferentiation (7, 29). Although EMT represents a highly conserved and fundamental process that governs morphogenesis and organogenesis in multicellular organisms, there is good evidence that it might also be involved in the dedifferentiation program that leads to malignant carcinomas (2, 29). Furthermore, in many organ systems, epithelial cells are targets for inflammatory mediators. Renal tubular epithelial cells, for example, change phenotype once they are activated by inflammatory cells, inflammatory mediators, or nonspecific inducers of interstitial fibrosis such as glomerular proteinuria (2, 13, 18). In kidneys, both proteinuria and inflammatory infiltrates can generate a local cytokine environment that promotes the conversion of tubular epithelium into myofibroblastlike cells, induces their proliferation, and activates matrix synthesis as well as proteolysis.

Mitogen-activated protein kinases (MAPK), especially the intracellular signaling molecules MAPK/ERK kinase (MEK)1 and MEK2, as well as their substrates ERK1 and ERK2, are activated by many different stimuli (e.g., mitogens, differentiation factors, stress...
signals) and participate in a diverse array of cellular programs, including cell proliferation, cell differentiation, cell movement, cellular senescence, and cell death (14, 23, 29, 30). Recently accumulated evidence from several independent experimental approaches suggests that sustained activation of the MEK1-ERK2 signaling module in polarized renal epithelial cells leads to EMT and the expression of a highly invasive phenotype. In dedifferentiated Madin-Darby canine kidney (MDCK)-C7Focus (C7F) cells, reduction of ERK1 protein expression and an increase in basal and serum-stimulated ERK2 activity is associated with a stable switch toward a mesenchymal-like cell phenotype (25). Long-term incubation in the presence of ochratoxin A, a nephrotoxic fungal metabolite that has been reported to increase the incidence of Balkan endemic nephropathy, of renal carcinomas, as well as adenomas in rats, induces a dedifferentiation of epithelial MDCK-C7 cells associated with sustained ERK1/2 activation (26). In addition, administration of hepatocyte growth factor (HGF) has been reported to induce MDCK cell scattering via activation of ERK1/2 (28). This HGF-induced cell scattering was inhibited by pretreatment of MDCK cells with the MEK1/2 inhibitor PD-098059 but only partially blocked by transient expression of a dominant interfering mutant of human MEK1 (28). Stable expression of a constitutively active MEK1 mutant in MDCK-C7 cells (C7caMEK1) results in pronounced phenotypic changes similar to those obtained in dedifferentiated C7F cells, including the acquisition of a fibroblastoid morphology, reduced cytokeratin expression, increased vimentin expression, and assembly of α-smooth muscle actin-containing stress fibers (16, 24). Furthermore, in collagen gels, dedifferentiated cell lines (C7F as well as C7caMEK1) are unable to generate cystlike epithelial structures and behave as highly invasive cells (12). Their invasive properties are associated with the expression of activated matrix metalloproteinase (MMP)-2 and with elevated levels of membrane-type-1 MMP (MT1-MMP). Moreover, MEK inhibition by the synthetic MEK1/2 inhibitor U0126 induces a pronounced flattening of C7caMEK1 cells and markedly reduces their invasion into the collagen matrix (12). Finally, the transdifferentiated phenotype and the invasive properties of these caMEK1-transfected MDCK-C7 cells are reflected by the downregulation of proteins that are crucial for the assembly of actin-based adherens junctions, such as E-cadherin, β-catenin, and α-catenin (11).

All together, these results suggest that long-term activation of the MEK1-ERK1/2 module represents an important signaling mechanism involved in EMT of renal tubular epithelial cells. However, with the exception of two pharmacological approaches utilizing the synthetic MEK1/2 inhibitors U0126 and PD-098059 as cited above (12, 26), no data are available directly showing a reversibility of EMT associated with decreased or lost MEK1-ERK1/2 activity. Thus it was the aim of the present study to find direct evidence for a mesenchymal-epithelial redifferentiation associated with a reduced MEK1-ERK1/2 activity in transdifferentiated renal epithelial cells. To this end, we investigated the effect of long-term hygromycin B depletion in transdifferentiated C7caMEK1 cells, which stably express a constitutively active MEK1 mutant. Here, we report the isolation of three revertant cell lines (C7rev4, C7rev5, C7rev7) from one batch of C7caMEK1 cells. The revertant cell lines are characterized by their typical epithelial morphology, their ability to form spherical cysts composed of polarized epithelial cells when grown in three-dimensional collagen gels, and reexpression of the epithelial marker proteins E-cadherin, β-catenin, and cytokeratin. Moreover, this epithelial redifferentiation of myofibroblastoid C7caMEK1 cells was associated with loss of constitutively active MEK1, reduction of ERK2 phosphorylation, and reexpression of ERK1 protein. In addition to C7caMEK1 cells, which have been transdifferentiated by stable expression of constitutively active MEK1, we now report the establishment of a second independent cell model (C7e2 cells) in which EMT is associated with loss of ERK1 protein expression but increased ERK1/2 phosphorylation and ERK1/2 activity. All together, these results strongly support the idea that, when permanently active, the MAP kinase kinase MEK1 represents an intracellular signal that induces EMT in renal epithelial MDCK-C7 cells.

**MATERIAL AND METHODS**

**Reagents and antibodies.** Cell culture media and all other reagents were obtained from Sigma Chemicals (St. Louis, MO).

The following antibodies were applied: mouse anti-E-cadherin (Transduction Laboratories, San Diego, CA), mouse anti-β-catenin (Transduction Laboratories), mouse anti-cytokeratin (Progen, Heidelberg, Germany), mouse anti-α-smooth muscle actin (Sigma), rabbit anti-MEK1 (1:2000 dilution; a generous gift from M. J. Pouyssegur, Université de Nice, Nice, France), rabbit anti-MEK2 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-MEK1/2, which detects phosphorylated Tyr204 of both ERK1 and ERK2 (Cell Signaling Technology, Beverly, MA), rabbit anti-ERK1/2, which recognizes both ERK isoforms (a generous gift from M. J. Dunn, Medical College of Wisconsin, Milwaukee, Wl), rabbit anti-phospho-ERK1/2, which detects phosphorylated Tyr204 of both ERK1 and ERK2 (Cell Signaling Technology), and rabbit anti-phospho Elk-1, which detects Elk-1 protein only when phosphorylated at Ser383 (Cell Signaling Technology).

**Cell culture.** MDCK-C7 cell lines and their derivatives (C7Mock1, C7caMEK1, C7rev4, C7rev5, C7rev7, C7e1, C7e2) were grown in minimal essential medium (MEM) with Earle's salts, nonessential amino acids, and L-glutamine at a pH of 7.4 (24). MEM was supplemented with 10% fetal calf serum (FCS), 26 mM NaHCO3, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in 5% CO2-95% air, humidified atmosphere. The MDCK-C7 cell line is a high transepithelial resistance subclone of the heterogeneous MDCK strain from American Type Culture Collection (CCL 34) (9, 20, 31). Stable transfection of wild-type MDCK-C7 cells with caMEK1 has been described previously (24). caMEK1 was generated by substitution of the Raf1-dependent regulatory phosphorylation sites (Ser218 and Ser222) with aspartic acid (S218D/S222D mutant) as described previously (4). Once a week, cells were split in a ratio of 1:15 (C7Mock1, C7rev4, C7rev5, C7rev7) or 1:2 (C7caMEK1, C7e1,
C7e2). Experiments were performed either with cells cultured in the presence of 10% FCS or with cells made quiescent by 24 h of incubation in FCS-free medium.

Isolation of C7rev4, C7rev5, and C7rev7 subclones. C7caMEK1 cells were split once a week as described above and seeded in six-well tissue culture plates. Starting with passage 11, hygromycin B was omitted from C7caMEK1 cell culture media and the cell phenotype was subsequently studied over time. After nine passages, small islands of epithelial-like cells were detected among transdifferentiated, fibroblast-like C7caMEK1 cells (Fig. 1A). Three colonies were isolated from epithelial islands at passage 20 using 5-mm sterile filter pads soaked in trypsin. The cells thus obtained were subsequently expanded, resulting in the establishment of the revertant clones C7rev4, C7rev5, and C7rev7.

Isolation of C7e1 and C7e2 subclones. Parental C7Mock1 cells were seeded in 35-mm dishes at a concentration of 100 per dish. After 6 and 8 days of culture, variant colonies composed by elongated cells with a fibroblast-like morphology were trypsinized by using cloning rings. The cells thus obtained were subsequently expanded, resulting in the establishment of the transdifferentiated clones C7e1 and C7e2.

Western blot analysis. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold RIPA lysis buffer [50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.5% deoxycholic acid, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% protease inhibitor cocktail for mammalian tissues (Sigma), 0.1% SDS, 1% Triton X-100] for 25 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. The protein content was determined by using a microcinchoninic acid assay (Pierce) with BSA as the standard. Cell lysates were matched for protein, separated on 10% SDS-PAGE, and transferred to a polyvinylidene difluoride microporous membrane (Millipore). Subsequently membranes were blotted with one of the specific antibodies as listed above. The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (for polyclonal antibodies) or horseradish peroxidase-conjugated rabbit anti-mouse IgG (for monoclonal antibodies) visualized by LumiGLO chemiluminescent Western detection system (Cell Signaling Technology). Quantitation of ERK1/2 expression and ERK1/2 phosphorylation was accomplished by using a Personal Densitometer SI-Scanner (Molecular Dynamics) and Image Quant 1.2 Software (Molecular Dynamics).

ERK1/2 activity assay. Soluble lysates (300 μg, prepared as described above) were incubated in the presence of 15 μl of resuspended immobilized phospho-ERK1/2 monoclonal antibody (Cell Signaling Technology) at 4°C overnight. Thereafter, pellets were washed twice with lysis buffer, twice with kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM MgCl₂], and finally resuspended in 50 μl of kinase buffer containing 200 μM ATP and 2 μg Elk-1 fusion protein (Cell Signaling Technology). The reaction was initiated by incubation of the samples at 30°C for 30 min. To terminate the reaction, 25 μl × 3 SDS sample buffer [187.5 mM Tris·HCl (pH 6.8 at 25°C), 6% wt/vol SDS, 30% glycerol, 150 mM DTT, 0.3% wt/vol bromphenol blue] was added. After centrifugation, samples were boiled for 5 min, subjected to SDS-10% PAGE, and blotted with a rabbit anti-phospho Elk-1 antibody, which detects Elk-1 protein only when phosphorylated at Ser383 (Cell Signaling Technology).

Morphogenesis assay. The cyst morphogenesis assay was carried out as described previously (12). In brief, wild-type MDCK-C7 cells and their derivatives were harvested by using trypsin-EDTA, centrifuged, and resuspended at concentrations ranging from 2 × 10⁴ to 2 × 10⁵ cells/ml in a 8:1 mixture of rat tail tendon collagen solution (1.5 mg/ml), 10× concentrated MEM, and sodium bicarbonate (11.76 mg/ml) in a sterile flask kept on ice to prevent premature collagen gelation. Aliquots of the cell suspension were then dispensed in either 22-mm wells or 35-mm dishes and incubated at 37°C for at least 10 min. After collagen gelation, complete medium supplemented with 10 ng/ml basic fibroblast growth
factor (bFGF) was added and changed every 2–3 days. Collagen gel cultures were fixed after 7 days in 2.5% glutaraldehyde in sodium cacodylate buffer (100 mM) and examined using a Nikon Diaphot TMD inverted photomicroscope. Processing of the cultures for semi-thin sectioning was carried out as described previously (12).

Collagen gel invasion assay. Wild-type MDCK-C7 cells and their derivatives were harvested by trypsinization and seeded at concentrations ranging from $5 \times 10^4$ to $2 \times 10^5$ cells/ml onto the surface of three-dimensional collagen gels (1500 μl), which were cast in 35-mm dishes as described above. After a 7- to 10-day incubation in complete medium supplemented with 10 ng/ml bFGF, invading cells were identified by focusing at different levels below the surface of the gel, using the ×20 phase contrast objective of a Nikon Diaphot TMD inverted photomicroscope.

RESULTS

Formation of epithelial islands in caMEK1-transfected, transdifferentiated MDCK-C7 cell cultures after removal of hygromycin B. Recently accumulated evidence from three independent experimental approaches in polarized epithelial MDCK-C7 cells suggests that sustained activation of the MEK1-ERK2 signaling module leads to EMT and expression of a highly invasive phenotype (16, 24, 25, 26). However, a possible reversibility of these phenotypic alterations in C7caMEK1 cells associated with diminished expression of constitutively active MEK1 (caMEK1) or with reduced MEK1-ERK2 activity has not yet been investigated. To address this issue, we set out to isolate subpopulations of C7caMEK1 cells having lost constitutively active MEK1. For this purpose, starting at passage 11, we omitted hygromycin B from culture media, thereby relieving the selection pressure for caMEK1-expressing cells (22). After nine passages, small islands of epithelia-like cells were detected among transdifferentiated, fibroblastlike C7caMEK1 cells (Fig. 1A). Three colonies were isolated from epithelial islands at passage 20 and named C7rev4, C7rev5, and C7rev7. All three revertant cell lines exhibited a typical polygonal morphology (shown in Fig. 1B for C7rev7 cells) and formed a tight cobblestonelike monolayer at confluence (Fig. 1C).

Spontaneously dedifferentiated cell clones isolated from epithelial mock-transfected MDCK-C7 cells. As a complementary approach to investigating the relationships between MEK1/2-ERK1/2 activity and EMT, we set out to isolate subpopulations of epithelial MDCK-C7 cells that may spontaneously exhibit a transdifferentiated morphology. To this end, epithelial mock-transfected MDCK-C7 cells (C7Mock1) cells were seeded at clonal density and variant colonies composed of elongated cells with a fibroblastlike shape were subsequently trypsinized and expanded. The resulting clones were designated C7e1 and C7e2. At early passages after isolation (p5 to p10), both C7e1 and C7e2 cells maintained a transdifferentiated, fibroblastoid morphology (Fig. 2, A and B). At higher passages (p30 to p40), C7e1 cells partially regained their epithelial phenotype, whereas the fibroblastlike phenotype of C7e2 cells remained largely unaltered (Fig. 2, C and D).

Epithelial redifferentiation of cell lines C7rev4, C7rev5, and C7rev7 is associated with the loss of constitutively active MEK1, reduction of ERK2 phosphorylation, and reexpression of ERK1 protein. We have previously reported that increased ERK2 phosphorylation and enzymatic activity, as well as loss of ERK1 protein expression, is associated with epithelial-to-mesenchymal transdifferentiation of MDCK-C7 cells...
In the present study, we therefore investigated the expression, phosphorylation, and activation of members of the MEK1/2-ERK1/2 signaling module in revertant cell lines C7rev4, C7rev5, and C7rev7, as well as in spontaneously dedifferentiated cell lines C7e1 and C7e2. When compared with C7caMEK1 cells, epithelial redifferentiation of C7rev4, C7rev5, and C7rev7 cells was associated with the loss of hemagglutinin (HA) epitope-tagged, constitutively active MEK1 mutant (slower migrating MEK1 band in Fig. 3, upper panel), a slight increase in MEK2 protein expression (Fig. 3, lower panel), and no major alteration in intrinsic protein expression of MEK1 (Fig. 3). In addition, revertant cell lines regained ERK1 protein expression, which was markedly reduced in both C7caMEK1 cells and in low passages of spontaneously dedifferentiated cell lines C7e1 and C7e2 (Fig. 4). ERK2 phosphorylation (Fig. 5, bottom; data not shown for C7rev5) and ERK1/2 activity (Fig. 6; data not shown for C7rev5) was reduced in all three epithelial revertants (C7rev4, C7rev5, and C7rev7) to levels comparable with epithelial C7Mock1 cells, suggesting that the loss of caMEK1 is responsible for reduction of both basal ERK2 phosphorylation levels, as well as serum-stimulated ERK1/2 activity levels, and is associated with epithelial redifferentiation.

Spontaneous epithelial-to-mesenchymal transdifferentiation of cell lines C7e1 and C7e2 is associated with an increase in basal MEK1/2 phosphorylation and ERK2 phosphorylation, as well as a loss of ERK1 protein expression. When compared with mock-transfected MDCK-C7 cells, C7caMEK1 cells show increased basal MEK1 activity and increased basal ERK2 activity (24). Interestingly, we obtained similar alterations of the MEK1/2-ERK1/2 signaling module as described for C7caMEK1 cells in the two spontaneously dedifferentiated cell lines, C7e1 and C7e2. In both cell lines, increased basal MEK1/2 phosphorylation was detected (Fig. 5, top), which was paralleled by increased basal ERK2 phosphorylation (Fig. 5, bottom; Table 1), as well as by reduced ERK1 protein expression (Fig. 4, bottom; Table 1). In addition, high passage C7e2 cells showed an increased FCS-stimulated ERK1/2 activity (Fig. 6).

Quantification of the representative results presented in Figs. 4 and 5 revealed that, when compared with epithelial C7Mock1 cells, ERK1 phosphorylation relative to ERK1 protein expression (ERK1*+/ERK1) was increased 1.8-fold in C7caMEK1 cells, 2.0-fold in C7e1 cells, and 2.5-fold in C7e2 cells (Table 1). In contrast, ERK2 phosphorylation relative to ERK2 protein expression (ERK2*/ERK2) was increased 3.7-fold in C7caMEK1 cells, 3.5-fold in C7e1 cells, and 7.3-fold in C7e2 cells compared with epithelial C7Mock1 control cells (Table 1). Because the polyclonal anti-phospho-MEK1/2 antibody detects MEK1/2 only when activated by phosphorylation at Ser217 and Ser221, high passage C7e2 cells showed an increase in basal MEK1/2 phosphorylation that could be detected in C7caMEK1 cells (Fig. 5, top). Furthermore, reduced ERK1 protein expression has been obtained by reduced ERK1/2 phosphorylation levels, as well as serum-stimulated ERK1/2 activity levels, and is associated with epithelial redifferentiation.
not only in C7e1 and C7e2 cells but also in C7caMEK1 cells (Fig. 4, bottom; Table 1). Thus, in addition to C7caMEK1 cells, which have been transdifferentiated by stable expression of a constitutively active MEK1 mutant, C7e1 and C7e2 cells represent a novel independent cell model in which EMT is associated with a loss of ERK1 protein expression but increased activity of the MEK1/2-ERK1/2 signaling module.

Epithelial marker proteins are expressed in epithelial revertants C7rev4, C7rev5, and C7rev7 and in high-passage C7e1 cells. We have previously reported that in both dedifferentiated MDCK-C7F cells and in caMEK1-transfected MDCK-C7 (C7caMEK1) cells, an increase in ERK2 activity was associated with similar phenotypic changes, including the acquisition of a fibroblastoid morphology, reduced cytokeratin, and increased vimentin expression and the assembly of α-smooth muscle actin-containing stress fibers (16, 24). Moreover, the transdifferentiated phenotype of C7caMEK1 cells is reflected by the downregulation of proteins that are crucial for the assembly of actin-based adherens junctions such as E-cadherin, β-catenin, and α-catenin (11). Thus we next investigated the expression of epithelial (E-cadherin, β-catenin, cytokeratin) and of mesenchymal (α-smooth muscle actin) marker proteins in the newly established revertant cell lines and the spontaneously dedifferentiated cell lines, respectively. In contrast to transdifferentiated C7caMEK1 cells, all three revertant cell lines express the epithelial marker proteins E-cadherin, β-catenin, and cytokeratin at levels comparable with C7Mock1 cells (Fig. 7, data for C7rev5 not shown). Dedifferentiated high passage C7e2 cells lack both E-cadherin expression and cytokeratin expression and show reduced protein levels of β-catenin, which is similar to the results obtained in C7caMEK1 cells (Fig. 7). High-passage C7e1 cells, which represent a more epithelia-like phenotype when compared with parental low-passage C7e1 cells (Fig. 2, A and C), again express all three epithelial marker proteins investigated in this study (Fig. 7). In contrast to C7caMEK1 cells and to rat glomerular mesangial cells, which both show massive expression of α-smooth muscle actin (Fig. 8), expression of this mesenchymal marker protein was lost in revertant cell lines (Fig. 8, data for C7rev5 not shown). Moreover, we detected a faint α-smooth muscle actin band in high-passage C7e2 cells but not in high-passage C7e1 cells (Fig. 8). All together, these results suggest that C7e2 cells represent an additional transdifferentiated cell line in which spontaneous EMT is associated with a loss of ERK1 protein but increased activity of the MEK1-ERK2 signaling module, whereas
the phenotype of C7e1 cells is most likely an intermediate and/or transient one.

Loss of caMEK1 in epithelial revertants restores the ability to form cystic structures. We have previously reported that the transdifferentiated C7F and C7caMEK1 cell lines have lost the ability to form cystlike structures in collagen gels (12). We therefore used this in vitro assay of morphogenesis to assess the behavior of the revertant cell lines (C7rev4, C7rev5, and C7rev7), as well as of spontaneously transdifferentiated cells. When grown in three-dimensional collagen gels, the three revertant cell lines formed well-organized spherical cysts similar to those generated by either wild-type or mock-transfected MDCK-C7 cells (Fig. 9, data for C7rev7 not shown). Under the same conditions, low-passage (p8 to p14) C7e1 cells acquired an elongated shape and did not form organized multicellular structures (Fig. 10A). In contrast, high-passage (p30 to p40) C7e1 cells were able to form lumen-containing spherical cysts (Fig. 10B). Both low- (p8 to p10) and high- (p30 to p38) passage C7e2 cells populated the collagen matrix either as individual elongated cells or as thin branching cords (Fig. 10, C and D), which further supports the notion that C7e2 cells represent a transdifferentiated cell phenotype.

Increased ERK2 phosphorylation is associated with an invasive phenotype in transdifferentiated C7e2 cells.

As we have shown previously that C7F and C7caMEK1 cells behave as highly invasive cells (12), we asked whether spontaneously transdifferentiated cells would express similar properties. When seeded atop of a collagen gel, low-passage C7e1 cells acquired an elongated shape and were unable to form a fully confluent monolayer. Invasion of the underlying matrix was first detectable after ~48 h (not shown) and became extensive after 7 days (Fig. 11A). However, at higher passage, C7e1 cells formed a confluent cobblestone-like monolayer, and elongated cells invading the underlying gel were only rarely observed, even after 10 days of culture (Fig. 11B). C7e2 cells (both at low and high passage) also invaded collagen gels, albeit less extensively than low-passage C7e1 cells (results not shown). The reduced ability of C7e2 cells to migrate into the underlying matrix compared with low-passage C7e1 cells may, however, be accounted, at least in part, by their tendency to detach from the gel surface when approaching confluence. The behavior of C7e1 cells (at low passage) and C7e2 cells (at low and high passage)
is therefore similar to that of C7F and C7caMEK1 cells but contrasts starkly with that of revertant cell lines (C7rev4, C7rev5, and C7rev7), which formed a confluent monolayer and did not invade the underlying collagen matrix (data not shown).

DISCUSSION

In this study, we have provided additional evidence that the MEK1/2-ERK1/2 signaling module is an important determinant of epithelial-to-mesenchymal phenotypic conversion. As a first approach, we have examined the expression and phosphorylation state of MEK1/MEK2 and ERK1/ERK2 in revertant cell lines derived from transdifferentiated C7caMEK1 cells and have attempted to correlate these biochemical parameters with the expression of key epithelial features. We found that upregulation of the epithelial marker proteins E-cadherin, β-catenin, and cytokeratin, as well as downregulation of the mesenchymal marker protein α-smooth muscle actin, are not only associated with loss of constitutively active MEK1 and reduction of basal ERK1/2 phosphorylation and of serum-stimulated ERK1/2 activity but also with increased MEK2 protein expression and reexpression of ERK1 protein. As a complementary approach, we have evaluated the same parameters in spontaneously transdifferentiated cells derived from mock-transfected MDCK-C7 cells. Using this experimental model, we have found that fibroblastic morphology, loss of E-cadherin and cytokeratin expression, reduced β-catenin expression, and faint induction of α-smooth muscle actin expression correlate with increased MEK1/2-ERK1/2 phosphorylation, increased ERK1/2 activity, and reduced ERK1 protein expression. These two sets of findings, obtained using two independent experimental systems, corroborate each other and strongly support the notion that the MEK1/2-ERK1/2 signaling module is a crucial regulator of EMT.

EMT allows epithelial cells to dissociate from the structure in which they were integrated, to migrate freely, and, depending on the local milieu, to partici-
pate in tumor progression or in fibrogenesis. Numerous cellular alterations are associated with EMT. Myofibroblasts that originate from epithelial cells through EMT events turn off genes coding for cell-cell adhesion molecules (11) and modify the type of intermediate filaments they express (16, 24). In some cases, they start synthesizing extracellular matrix molecules such as fibronectin and certain types of collagen (32, 33). They may also synthesize proteolytic enzymes involved in matrix degradation that contribute to cell motility and invasiveness (12). In certain cell types, EMT can be induced by specific growth factors such as TGF-β1, EGF, TGF-α, FGF-1, or HGF (3, 8, 27, 28), by extracellular matrix components binding to their cognate cellular receptors (33), or by diverse stress signals (10, 21). In light of the variety of stimuli that induce EMT in epithelial cell systems, the question arises concerning the specific intracellular signaling pathways involved in EMT. Although Src tyrosine kinase has been reported to be a positive regulator of growth factor-induced cell scattering in a bladder carcinoma cell line (21), a role for the Ras signaling pathway has been reported in both EGF- and HGF-induced epithelial cell scattering (10, 19). The small GTP-binding protein Ras has multiple effector molecules. Among them, the best characterized is the Ser/Thr kinase Raf that initiates a series of phosphorylations, resulting in the activation of the MEK1/2-ERK1/2 signaling module. A role for members of this MAPK pathway has also been demonstrated in alkali-induced EMT of MDCK-C7F cells (16, 24, 25), in ochratoxin A-differentiated MDCK-C7 cells (21), in C7caMEK1 cells, which have been transdifferentiated by stable expression of constitutively active MEK1 (11, 12, 16, 24), and in HGF-induced MDCK cell scattering (17, 28). Although the HGF-induced cell scattering was inhibited by pretreatment of MDCK cells with the MEK1/2 inhibitor PD-098059 (28), another synthetic MEK1/2 inhibitor, namely U0126, induced a pronounced flattening of C7caMEK1 cells and markedly reduced their invasion into the collagen matrix (12).

In the present study, we report for the first time more direct evidence for a reversibility of EMT in the absence of constitutively active MEK1 activity, suggesting that the MEK1/2-ERK1/2 signaling module is a dominant signaling pathway involved in EMT of renal epithelial MDCK-C7 cells. Several lines of evidence support this hypothesis. First, reversion to an epithelial phenotype, as seen in the cell lines C7rev4, C7rev5, and C7rev7, is associated with increased MEK2 protein expression (Fig. 3) and reinduction of normal levels of ERK1 protein expression (Fig. 4). Interestingly, loss of ERK1 protein expression in combination with increased ERK2 activity and EMT has previously been reported in the dedifferentiated cell line MDCK-C7F (25). In addition, it is interesting to note that in contrast to renal epithelial cell lines MDCK-C7 (dog) and HK-2 (human), which show low levels of MEK1 expression but high MEK2 expression, rat glomerular mesangial cells, which represent renal cells of mesenchymal origin, do express high MEK1 protein levels but low levels of MEK2 protein (H. Schrake, unpublished observation). Thus, depending on the cell type, the two MEK1 and MEK2 isoforms may have different roles in the regulation of the renal epithelial cell phenotype. Moreover, it is tempting to speculate that the ratio of MEK1/MEK2 and/or ERK1/ERK2 protein phosphorylation/expression could determine the phenotype of certain cells. In our renal epithelial cell model, this hypothesis is based on the results of quantitative analysis of phosphorylation levels relative to protein expression levels of both ERK1 and ERK2, respectively. The results presented in Table 1 suggest that a higher ERK2 phosphorylation/protein expression (ERK2*/ERK2) ratio when compared with ERK1*/ERK1 is associated with EMT. Comparable ratios of both ERK isoforms, on the other hand, might be a signature of the epithelial cell phenotype.

The establishment of two novel, spontaneously dedifferentiated renal epithelial cell lines represents another important outcome of the present study. Both cell lines, C7e1 and C7e2, show increased phosphorylation levels of the MEK1/2-ERK2 signaling module associated with a transient and intermediate form of dedifferentiation (C7e1 cells) or with a more stable form of EMT (C7e2 cells). The dedifferentiation status of C7e1 cells is characterized by a reduction of E-cadherin expression and a slight reduction of β-catenin expression but no alterations in cytokeratin expression (Fig. 7). Spontaneous EMT in C7e2 cells is reflected by a loss of E-cadherin and cytokeratin expression, reduction of β-catenin expression (Fig. 7), and a slight induction of α-smooth muscle actin (Fig. 8). In addition, in contrast to C7e1 cells, high-passage C7e2 cells are unable to generate cystlike epithelial structures when grown in three-dimensional collagen gels (Fig. 10) and retain their ability to penetrate into the underlying collagen matrix, which is in line with previously published results obtained with both MDCK-C7F cells and C7caMEK1 cells (12). High-passage C7e1 cells, which are likely to represent an intermediate cell phenotype, partially regained some of their epithelial characteristics, as well as their ability to form cystic structures. Moreover, in contrast to high-passage C7e2 cells, high-passage C7e1 cells are no longer invasive. We conclude that, in addition to C7caMEK1 cells, which have been transdifferentiated by stable expression of constitutively active MEK1, C7e2 cells represent a second independent cell model in which stable EMT is associated with a loss of ERK1 protein and reduced MEK2 protein but increased ERK2 phosphorylation relative to ERK2 protein expression.

Possible downstream mechanisms mediating the MEK1/ERK2-induced loss of the epithelial phenotype in MDCK-C7 cells include the transcriptional repression of E-cadherin expression. Several specific transcriptional repressors might be responsible for this effect. Thus the MEK1-ERK2 signaling module might activate the zinc-finger-containing transcription factor Snail, which has been shown to induce EMT by downregulating E-cadherin (1, 5). Likewise, the MEK1-ERK2 module may activate Slug, a transcription factor closely related to Snail that is an early target of the MAPK pathway and is involved in desmosome desta...
bilization (29). Other strong repressors of E-cadherin expression comprise Sip1, another E-box-binding zinc-finger protein, and the basic helix-loop-helix transcription factor E12/E47, the E2A gene product (6, 15).

In conclusion, the results presented in this study, taken together with the findings reported previously (24), strongly support the notion that activation of the MEK1-ERK1/2 signaling module represents an intracellular signal that induces EMT in renal epithelial MDCK-C7 cells.

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