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Migration of F9 Parietal Endoderm Cells Is Regulated by the ERK Pathway

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Abstract Cell migration is regulated by the action of many signaling pathways that are activated in specific regions of migrating cells. Extracellular regulated kinase 1/2 (ERK) signaling can modulate the migration of cells by controlling the turnover of focal adhesions and the dynamics of actin polymerization. Focal adhesion turnover is necessary for cell migration, and the formation of strong actin stress fibers and mature focal adhesions puts the brakes on cell migration. We used F9 wild-type and vinculin null ($vin^{-/-}$) parietal endoderm (PE) outgrowth to study the role of the ERK signaling pathway in cell migration. Upon plating of F9 embryoid bodies (EBs) onto laminin-coated dishes, PE cells migrate away from the EBs, providing an in vitro model for studying directed migration of this embryonic cell type. Our results suggest that the ERK pathway regulates PE cell migration by affecting the formation of focal adhesions and lamellipodia through the action of myosin light chain kinase (MLCK). J. Cell. Biochem. 97: 1339–1349, 2006.

Key words: ERK; MEK; MLCK; cell migration; parietal endoderm

Cell migration involves the assembly and disassembly of adhesion and migration structures, including focal adhesions. Focal adhesions contain many structural and signaling proteins, and form from maturing focal complexes present just behind the cell's leading edge [Carragher and Frame, 2004]. Focal complexes are transient structures and maintain weak adhesion of the cell to the substratum, whereas focal adhesions connect to the actin-based cytoskeleton and can stabilize cell-extracellular matrix (ECM) attachment. Focal adhesion turnover, or disassembly, is required for cell motility [Carragher and Frame, 2004]. Reduced turnover leads to stablization of focal adhesions [Ilic et al., 1995], providing brakes that can prevent the cell from migrating. Signaling pathways control cell migration, in part, by regulating the turnover of focal adhesions and the assembly of actin filaments. For example, focal adhesion kinase (FAK) and src both play important roles in cell migration by modulating focal adhesion turnover [Ilic et al., 1995; Volberg et al., 2001].

Lamellipodia are protrusive structures that direct the forward movement of migrating cells. They are generated by the assembly of an actin meshwork behind the plasma membrane of the leading edge of a migratory cell [Small et al., 2002]. A number of molecules play important roles in regulating lamellipodia formation, including the Arp2/3 complex and members of the Wiskott–Aldrich syndrome protein (WASP) family [Higgs and Pollard, 2001; Rosenfeldt et al., 2001]. Platelet-derived growth factor (PDGF) mediated activation of FAK and src also promotes the formation of lamellipodia [Rosenfeldt et al., 2001].

In the best studied extracellular regulated kinase (ERK) signaling pathway, phosphorylated ERK translocates to the nucleus and activates transcription of proteins that regulate cell proliferation upon activation by growth factors or integrin engagement [Renshaw et al., 1999; Aplin et al., 2001]. Besides this role in cell proliferation, there is also evidence that ERK regulates cell migration by acting downstream of integrin activation, phosphorylating the cytoplasmic protein myosin light chain kinase (MLCK) [Klemke et al., 1997; Fincham

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et al., 2000]. ERK mediated phosphorylation and activation of MLCK results in phosphorylation of serine 19 of myosin light chain (MLC). MLCK has been reported to promote focal adhesion turnover [Webb et al., 2004].

In a post-implantation mouse embryo, PE cells migrate away from the inner cell mass surface and along the inner lining of the trophectoderm. This is the first cell migration event in mammalian embryogenesis. F9 teratocarcinoma stem cells can form embryoid bodies (EBs) with an undifferentiated inner cell core surrounded by a layer of visceral endoderm. After plating on an ECM substrate, PE outgrowth migrates away from the EB, providing a convenient in vitro model system to study the migration of this embryonic cell type [Casanova and Grabel, 1988]. We are interested in defining the role of the ERK pathway in the migration of F9 parietal endoderm (PE) outgrowth.

Vinculin is an important structural component of focal adhesions. A recent study reported that ERK is more active in F9 vinculin null ($vin^{-/-}$) stem cells than in wild-type cells based upon its phosphorylation level [Subauste et al., 2004]. The upregulation of ERK phosphorylation is required for the increased motility of $vin^{-/-}$ stem cells in comparison to wild-type cells [Xu et al., 1998; Subauste et al., 2004]. Vinculin is proposed to play a role in migration by competing with FAK for the same binding site on the adaptor protein paxillin [Turner et al., 1999; Subauste et al., 2004]. The FAK–paxillin interaction activates pathways that contribute to cell migration [Parsons, 2003]. In the absence of vinculin, the interaction between paxillin and FAK is increased, as are the levels of ERK phosphorylation. Our data suggest that the ERK pathway, acting via MLCK, promotes the migration of F9 PE by inhibiting the formation of strong focal adhesions and promoting the formation of lamellipodia.

MATERIALS AND METHODS

Cell Lines and Reagents

Wild-type F9 teratocarcinoma stem cells are differentiated into EBs by culturing cells in suspension in the presence of retinoic acid 7.5×10^{-7} M for 7 days [Casanova and Grabel, 1988]. Wild-type PE outgrowth is generated by plating EBs on ECM-coated coverslips or tissue culture dishes in DMEM (Sigma) with 10% bovine serum (Hyclone). $vin^{-/-}$ cells are cultured in 5% bovine serum (Hyclone) and 5% fetal bovine serum (Atlanta Biologicals). For immunoprecipitation and Western blotting, EBs are plated on 0.1% gelatin-coated tissue culture dishes; for all other experiments, cells are plated on 30 μg/ml laminin-coated (Sigma) coverslips. Treatment of PE starts 24 h after EBs are plated. Antibodies used are the following: anti-phospho-ERK (E10, Cell Signaling), anti-ERK (Santa Cruz), anti-phosphohistone-H3 (Upstate), anti-vinculin (Sigma), anti-α-fodrin (MP Biomedicals), Alexa-green-conjugated anti-mouse IgG secondary antibody, Hoechst, and rhodamine-phalloidin (Molecular Probes). Hoechst is used to stain nuclei, and rhodamine-phalloidin is used for identifying actin filaments. Inhibitors used are as follows: U0126 (Tocris), PD98059 (Calbiochem), and ML7 (Tocris). Constitutively active Raf-1 (pUSEamp-RafY340D) and dominant negative Raf-1 (pCMV-RafS621A) constructs are generous gifts of Dr. Stanley Lin (Wesleyan University) [Lin et al., 2003]. Constitutively active MEK (MEK1 S218/222D) is from Upstate. The control vector pcDNA3.1 is from Invitrogen.

Transient Transfection

Transient transfections are performed using Lipofectamine 2000 according to the Invitrogen protocol for transfection in a 35 mm dish with 1 ml OPTI-MEM media (Gibco). Briefly, F9 PE outgrowth is transfected with different constructs 24 h after EBs are plated on 30 μg/ml laminin-coated coverslips or 0.1% gelatin-coated tissue culture dishes. Twenty four-hours post-transfection, outgrowths are allowed to recover for 24 h in normal media prior to analysis. Transfection efficiency is calculated by co-transfecting with an EGFP-expressing vector (pEGFP-N1, Clontech) and determining the percent of cells expressing EGFP using Hoechst staining of nuclei to count cells.

Cell Migration

Inhibitor (U0126, PD98059, or ML7) is added to PE outgrowths 24 h after EBs are plated on laminin-coated coverslips. Twenty-four hours after treatment, PE outgrowths are fixed in formaldehyde and phase pictures are taken. Measurement of migration distance is as described previously [Mulrooney et al., 2001].

Counting the Number of Lamellipodia

The number of lamellipodia per outer ring cell is calculated by dividing the total number of lamellipodia in the outer ring of each outgrowth by the total number of cells in the outer ring of each outgrowth. Each branch of an extension is counted as a lamellipodium. Data presented are the average from the outgrowth of five EBs. Lamellipodia are visualized in cells stained with rhodamine-phallodin and the number of cells is quantified by Hoechst staining of nuclei.

Immunofluorescence

Immunofluorescence staining is performed using methods described previously [Mulrooney et al., 2001]. Anti-vinculin antibody is used at 1:100. Rhodamine-phalloidin is used at 1:100. Anti-mouse Alexa-green-conjugated secondary antibody is used at 1:1,000. Hoechst stain is used at 1:10,000.

Immunoblotting

F9 PE cells are collected by removing EBs and then scraping PE outgrowth into an eppendorf tube. Cells are centrifuged and resuspended with lysis buffer (50 mM Tris-Hcl, pH 7.4; 150 mM NaCl; 0.5% NP40; 50 mM NaF; 10 mM $Na_4P_2O_7$; 1 mM Na_3VO_4; 10 μg/ml leupetin; 10 μg/ml pepstatin; 10 μg/ml aprotinin; 10 μg/ml benzamidine; and 1 mM PMSF). Extract preparation, gel electrophoresis, and Western blotting are performed, as previously described [Mulrooney et al., 2001]. For Western blotting, anti-phosphoERK antibody is used at 1:2,500 and anti-ERK antibody is used at 1:1,000 overnight at 4°C. Proteins bands are visualized by enhanced chemiluminescence.

RESULTS

ERK Pathway Plays an Important Role in F9 PE Outgrowth

Our previous data showed that $vin^{-/-}$ PE migrates significantly faster than wild-type PE [Mills et al., in press]. We tested whether this increase in cell migration is associated with upregulation of ERK activity, and found that the levels of ERK1/2 phosphorylation are upregulated in $vin^{-/-}$ PE compared to wild-type PE (Fig. 1A). This suggests that higher ERK activity contributes to the increased migration of $vin^{-/-}$ PE. To test this hypothesis, we treated PE outgrowth with the MEK inhibitor U0126 since MEK is the kinase directly upstream of ERK in this MAP kinase cascade. Contrary to

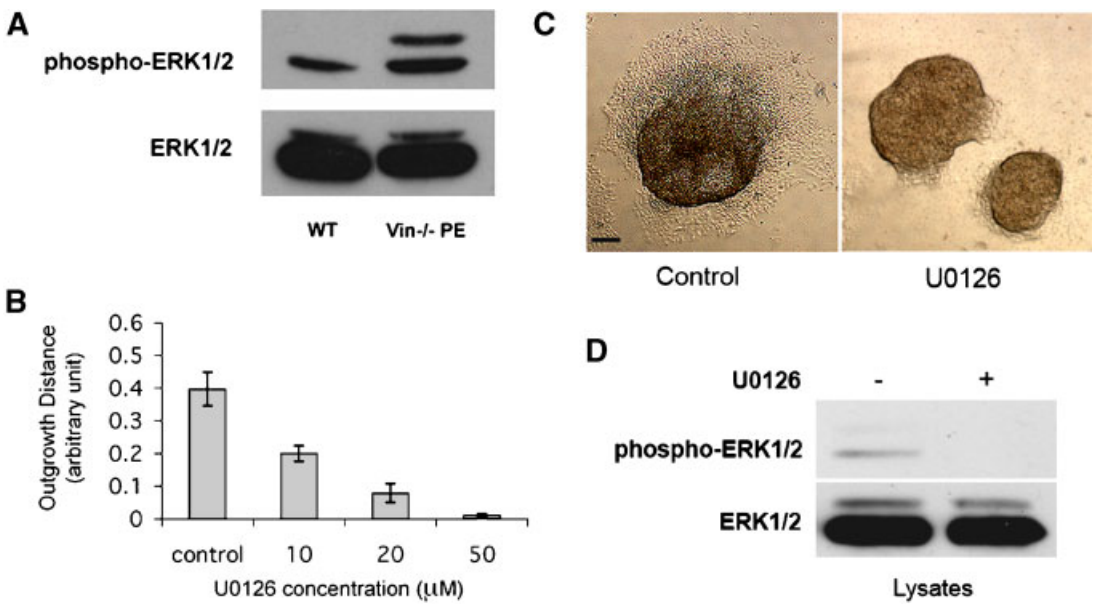


Fig. 1. ERK phosphorylation and MEK inhibitor treatment of F9 PE outgrowth. Phosphorylation of ERK1/2 is upregulated in $vin^{-/-}$ PE compared to wild-type PE. The top band in the immunoblot for phosphoERK1/2 is ERK1, and the bottom band is ERK2 (**A**). Response of migration of wild-type outgrowth to the MEK inhibitor U0126 (**B**). U0126 treatment inhibits ERK phosphorylation of wild-type PE (**C**). Phase picture of wild-type embryoid bodies (Ebs) with outgrowth under control conditions and in the presence of U0126 (20 μM). Scale bar: 10 μm. Densitometry analysis of the ratio of phospho-ERK/total ERK is 0.081 in WT cells in 1A and 0.059 in WT untreated cells in 1D, indicating consistent levels of activated ERK in PE extracts (**D**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the observation that MEK inhibition has little effect on F9 stem cell migration [Subauste et al., 2004], we find that both wild-type and vin$^{-/-}$ PE show a dose-dependent decrease in migration with U0126 treatment (Fig. 1B,C and data not shown). Consistent with higher levels of phospho-ERK in vin$^{-/-}$ PE, higher concentrations of U0126 are required to inhibit migration of these cells by 50% (15 μM vs. 10 μM for wild-type PE). This suggests that whereas stem cell migration does not depend on MEK activity, PE migration does. This treatment inhibits levels of ERK phosphorylation (Fig. 1D). We also inhibited MEK activity of wild-type PE with another potent MEK inhibitor, PD98059, and observe a dose-dependent decrease in cell migration (data not shown). In addition, we observe a recovery of cell migration after withdrawal of the MEK inhibitor (data not shown), which demonstrates that treatment is reversible and not toxic to the outgrowth cells. These data suggest that the ERK pathway promotes PE migration.

ERK also plays an important role in cell proliferation. Thus inhibition of cell migration by MEK inhibition might be caused by fewer cells present in the outgrowth due to decreased cell proliferation. To test this possibility, F9 wild-type PE outgrowth was examined for the presence of the mitotic marker phosphohistone-H3. We find no significant difference in phosphoH3 labeling between control and U0126 treated outgrowth, suggesting MEK inhibition does not affect PE migration by decreasing cell proliferation (data not shown).

To more specifically define the role of ERK in F9 PE migration, we turned to a genetic approach to block or stimulate the ERK pathway using transfection with vectors encoding loss or gain of function versions of pathway components. The transfection efficiency was approximately 80% of cells in the PE outgrowth (data not shown) based upon GFP expression in cells co-transfected with an EGFP-encoding vector. GFP immunofluorescence shows transfected cells distributed randomly throughout the outgrowth, and not preferentially in the outer region of the outgrowth (data not shown). Transfection with control empty vector does not cause significant changes in migration compared to untransfected PE (Fig. 2). F9 wild-type outgrowth migrates significantly further when transiently transfected with constructs encoding constitutively active MEK (caMEK) or Raf-1 (caRaf), the MAPKK kinases just upstream of MEK, whereas transfection with dominant negative Raf-1 (DNRaf, kinase inactive) constructs leads to a dramatic decrease in cell migration (Fig. 2). These data are consistent with a role for the ERK pathway kinases Raf-1 and MEK in promoting PE migration.

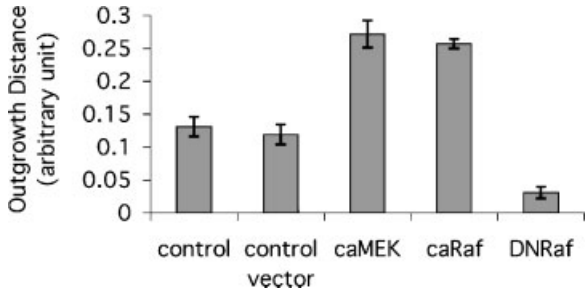
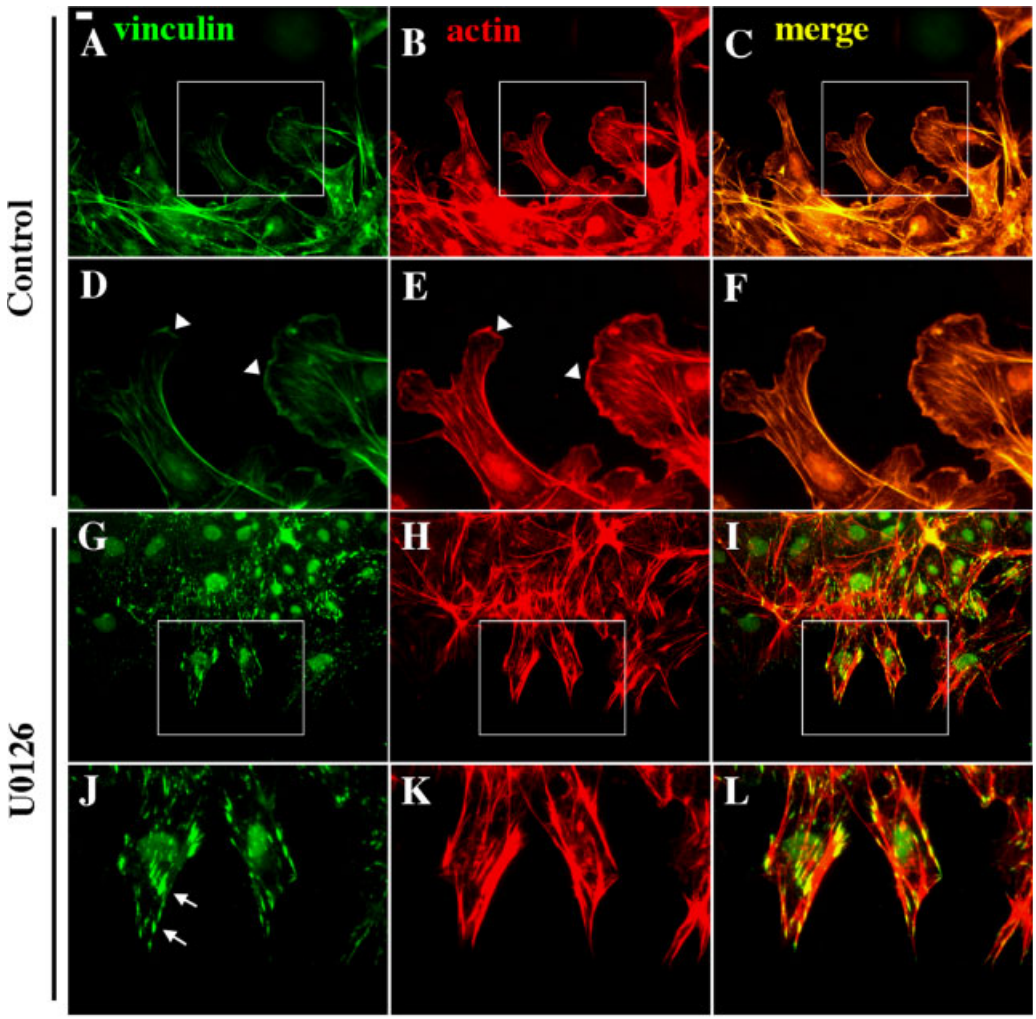


Fig. 2. Transfection with constructs encoding components in the ERK pathway. F9 wild-type EBs were plated on laminin-coated coverslips, and after 24 h the culture is transfected with different constructs. CaMEK, constitutively active MEK; caRaf, constitutively active Raf-1; DNRaf, dominant negative Raf-1. Student's *t*-test shows that the difference in outgrowth distance between control and control vector is not statistically significant. The difference between the outgrowth distances of other transfected lines and the control is statistically significant ($P < 0.01$), as determined by Student's *t*-test.

ERK Pathway Regulates PE Migration by Affecting Focal Adhesion Formation

Decreased cell motility may be attributed to a lower rate of focal adhesion turnover, producing more stabilized focal adhesions [Ilic et al., 1995]. We therefore examined focal adhesions under conditions that inhibit MEK and PE outgrowth, using vinculin to visualize focal adhesions and focal complexes. In untreated PE outgrowth, vinculin localizes to the leading edge of lamellipodia to focal complexes in the actin meshwork area (Fig. 3A,D), and to more mature focal adhesions. In contrast, in the presence of a MEK inhibitor, U0126 or PD98059, vinculin localizes predominantly to strong focal adhesions at the termini of robust actin-stress fibers (Fig. 3G,L). This suggests ERK regulates cell migration by inhibiting the formation of focal adhesions, perhaps by promoting focal adhesion turnover. MEK-inhibited PE also exhibits an apparent increase in strong actin stress fibers, which aids in stabilization of focal adhesions. To further investigate the mechanisms whereby ERK promotes migration, we determined the number of lamellipodia present in the outer ring of outgrowth under control and MEK-inhibited conditions. We observe that MEK inhibition


A vinculin
B actin
C merge
Control
D
E
F
G
H
I
U0126
J
K
L

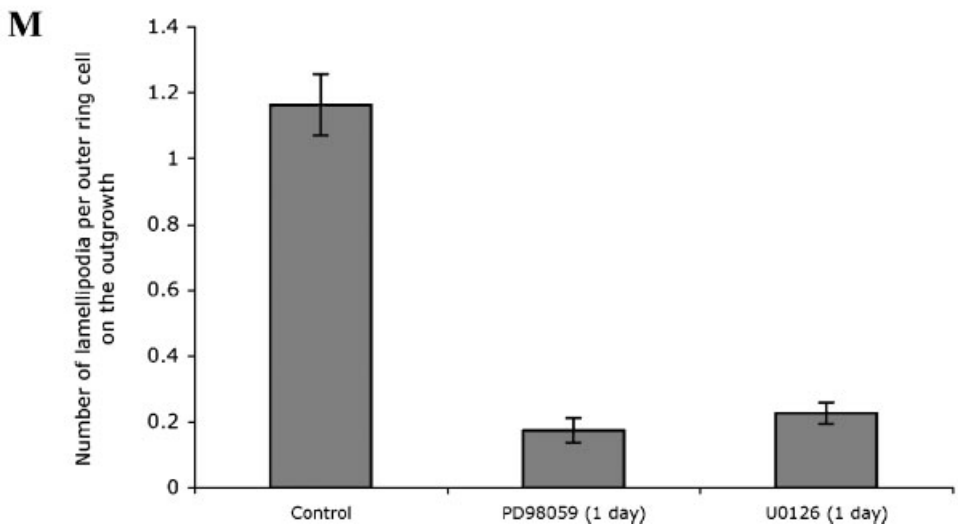

M
Number of lamellipodia per outer ring cell on the outgrowth
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
PD98059 (1 day)
U0126 (1 day)

Fig. 3.

diminished the number of lamellipodia in the outer ring of the PE outgrowth (Fig. 3M), consistent with the diminished outgrowth distance observed under this condition.

ERK Regulates Cell Migration Through MLCK

To begin to establish how ERK regulates the formation of focal adhesions and lamellipodia, we focused on MLCK, a downstream target of ERK. To determine if ERK's role in migration is mediated by MLCK, we treated outgrowth with a MLCK inhibitor, ML7. Our results show that cell migration is significantly decreased when MLCK is inhibited (Fig. 4A), suggesting MLCK does play an important role in PE migration. To test whether ERK is physically associated with MLCK in the cell, we immunoprecipitated MLCK or ERK, and analyzed the precipitate for the presence of the other kinase. Immunoprecipitation with anti-α-fodrin antibody is used as a control since α-fodrin is not reported to associate with ERK or MLCK. ERK co-immunoprecipitated with MLCK, and MLCK co-immunoprecipitated with phospho ERK (Fig. 4B). These data suggest that ERK and MLCK are associated in a complex in F9 PE.

To test whether MLCK acts downstream of MEK/ERK in promoting migration, F9 PE outgrowth was transiently transfected with constructs encoding constitutively active MEK (caMEK) and treated with the MLCK inhibitor, ML7. Without ML7 treatment, PE outgrowth shows an increase in cell migration when transfected with caMEK. However, ML7 treatment inhibits the increased cell migration of caMEK-transfected PE outgrowth to the level of those of the untransfected outgrowth (Fig. 4C). This suggests that MEK/ERK acts via MLCK to promote migration.

We tested whether MLCK inhibition affects the formation of focal adhesions and lamellipodia in the same way MEK inhibition does. As observed when MEK is inhibited, vinculin localizes predominantly to strong focal adhesions in PE treated with the MLCK inhibitor in comparison to the untreated outgrowth in which vinculin localizes to focal complexes and the leading edge of lamellipodia (Fig. 5A,L). In addition, decreased numbers of lamellipodia

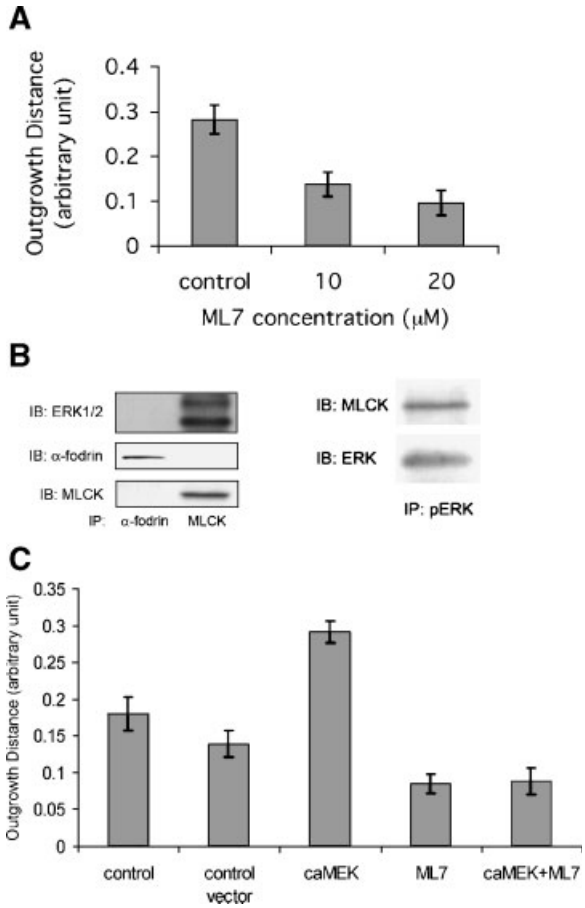
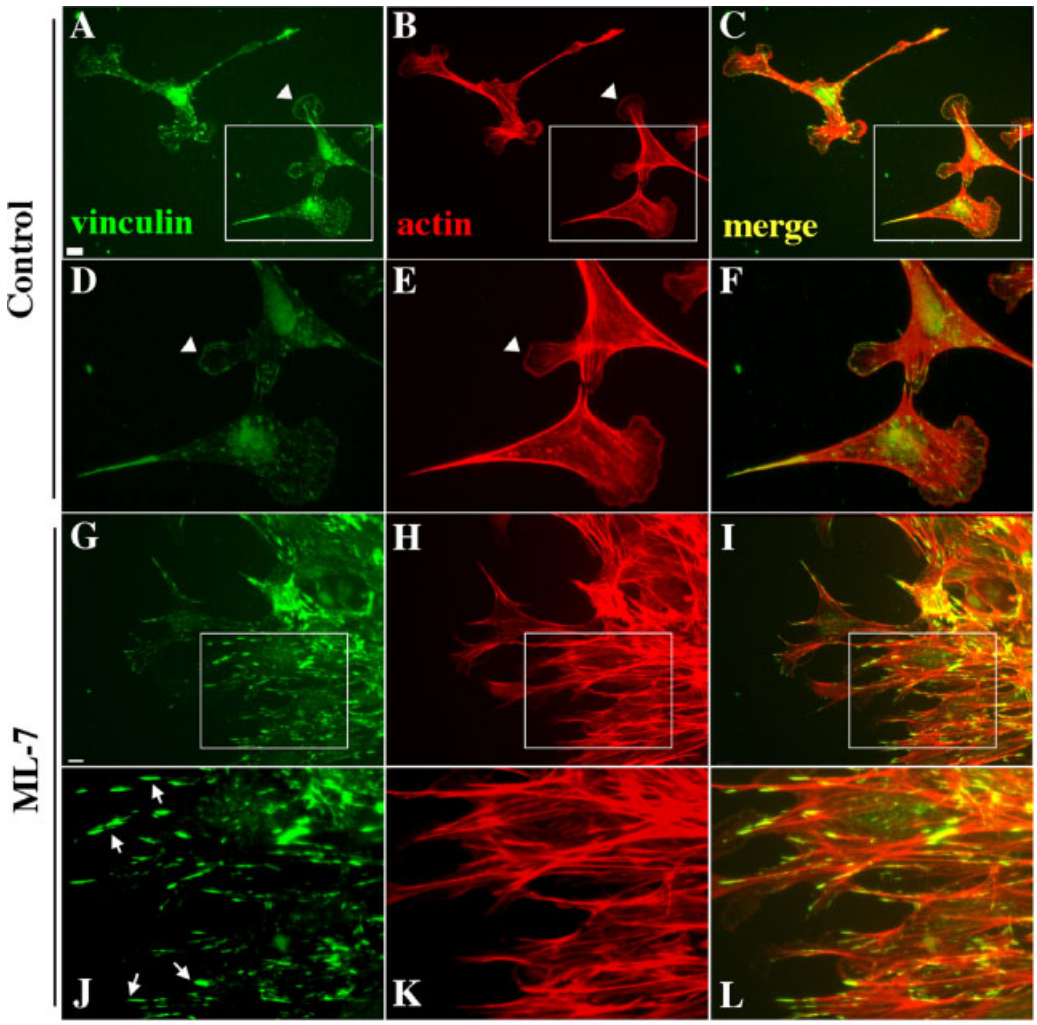


Fig. 4. MLCK functions downstream of ERK. Inhibition of MLCK function by the inhibitor ML7 decreases migration distance (**A**). Co-immunorepcipitation of ERK with MLCK, and co-immunoprecipitation of MLCK with phosphoERK. Anti α-fodrin antibody is used as a control for non-specific associations in immunoprecipitates (**B**). Effect of 10 μM ML7 on the migration of caMEK-transfected outgrowth (**C**). Student's *t*-test shows that the difference in outgrowth distance between control and control vector is not statistically significant. The difference between the outgrowth distances of other treatments and the control is statistically significant ($P < 0.01$), as determined by Student's *t*-test.

Fig. 3. (*Overleaf*) Inhibition of MEK increases prominent focal adhesions and decreases the number of lamellipodia in the outer ring of outgrowth. Immunofluorecence staining of vinculin (green, A, D, G, and J) and actin (red, B, E, H, K) in control, and U0126 treated outgrowth. Merge of vinculin and actin staining is shown in C, F, I, L. Panel D, E, F, J, K, and L are the enlarged regions of A, B, C, G, H, and I as shown. White arrowheads in D and E point to localized staining at the leading edge of lamellipodia. White arrows in J point to focal adhesions. Number of lamellipodia in the outer ring of outgrowth with MEK inhibition by 10 μM PD98059 or 10 μM U0126 (M). The cells in the control only show the very front of the large areas of outgrowth. The cells showed in MEK-inhibited outgrowth represent most of the area observed at that direction. Scale bar: 10 μm. Student's *t*-test shows that the decrease of the number of the lamellipodia in both PD98059 and U0126 treatments is statistically significant ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]


A
B
C
vinculin
actin
merge
Control
D
E
F
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ML-7
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L

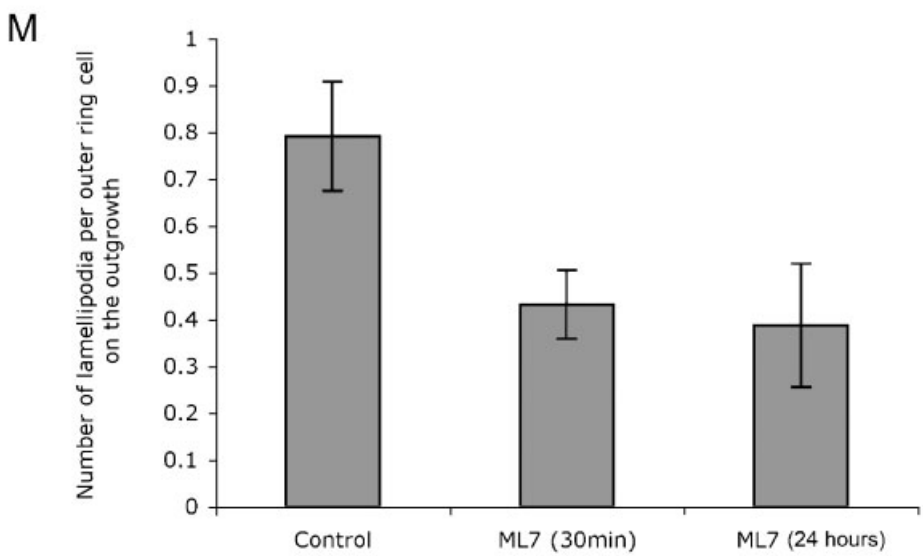
M
Number of lamellipodia per outer ring cell on the outgrowth
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
ML7 (30min)
ML7 (24 hours)

Fig. 5.

were present in the outer ring of treated outgrowth, compared to untreated controls (Fig. 5M). The similar effects of MEK and MLCK inhibitors on PE outgrowth are consistent with MEK/ERK and MLCK acting in the same pathway to regulate cell migration.

DISCUSSION

We demonstrate a role for the ERK signaling pathway in PE migration (Fig. 6). We observe a decrease in PE outgrowth in response to MEK inhibition (Fig. 1B,D), associated with a decrease in phosphorylated ERK1 and ERK2 (Fig. 1D). This role is further supported by the observation that constitutively activated MEK or constitutively active Raf promote outgrowth, whereas dominant negative Raf inhibits it (Fig. 2). ERK's effect on migration appears to be mediated by its downstream target MLCK, based on the ability of the MLCK inhibitor ML7 to block the stimulatory effect of constitutively active MEK on PE migration. In addition, MLCK and ERK1/2 co-immunoprecipitate, suggesting they are present in a complex in PE cells. MEK or MLCK inhibition results in apparent stabilization of focal adhesions and inhibition of lamellipodia formation (Figs. 3 and 5), suggesting that the ERK pathway promotes migration by favoring focal adhesion turnover and lamellipodia extension (Fig. 6).

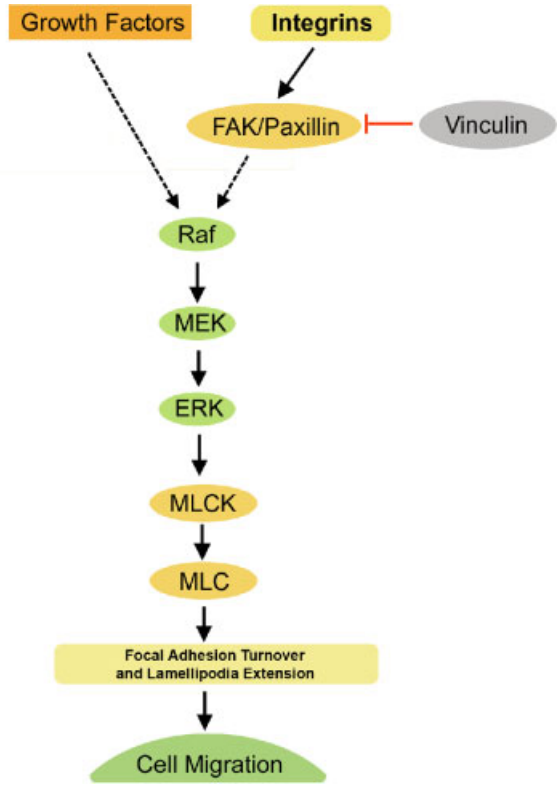

Fig. 6. The ERK pathway promotes the migration of F9 outgrowth by promoting focal adhesion turnover and lamellipodia extension, which is mediated by MLCK. Vinculin competes with FAK for binding and activating paxillin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ERK and Focal Adhesion Turnover

One mechanism whereby ERK modulates cell migration is via its effect on focal adhesion turnover. ERK has been reported to promote the turnover of paxillin-containing focal adhesions in mouse embryonic fibroblasts (MEF) [Webb et al., 2004]. Activation of ERK also mediates focal adhesion turnover stimulated by v-src transformation of CEF cells [Carragher et al., 2003]. MEK inhibition of v-src transformed cells results in reduced FAK turnover, increased formation of strong focal adhesions, and decreased cell migration [Carragher et al., 2003]. Our data also show that MEK-inhibitor-treated F9 PE exhibited more prominent, apparently stabilized focal adhesions based on vinculin staining (Fig. 3G,J).

Studies have shown that phosphorylated ERK localizes to focal adhesions [Fincham et al., 2000]. When recruited to focal adhesions, ERK may promote focal adhesion turnover by directly activating calpain-induced FAK proteolysis in fibroblasts [Carragher et al., 2003]. However, it is not known whether the regulation of focal adhesion turnover by ERK is strictly

Fig. 5. (*Overleaf*) Inhibition of MLCK increases prominent focal adhesions and decreases the number of lamellipodia in the outer ring of outgrowth. Immunofluorecence staining of vinculin (green, A, D, G, and J) and actin (red, B, E, H, K) in control, and ML7 treated outgrowth. Merge of vinculin and actin staining is shown in C, F, I, L. Panel D, E, F, J, K, and L are the enlarged regions of A, B, C, G, H, and I as shown. White arrowheads in D and E point to localized staining at the leading edge of lamellipodia. White arrows in J point to focal adhesions. Number of lamellipodia in the outer ring of outgrowth with MLCK inhibition by 10 μM ML7 (M). The cells in the control only show the very front of the large areas of outgrowth. The cells showed in MLCK-inhibited outgrowth represent most of the area observed at that direction. Scale bar: 10 μm. Student's *t*-test shows that the decrease of the number of the lamellipodia in ML7 treatment (both 30 min and 24 h) is statistically significant ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

dependent upon the localization of ERK to these adhesion sites. In migrating PE, we are unable to observe a localization of phosphoERK to pY^{31}paxillin-containing focal complexes or focal adhesions (data not shown). This suggests that association of ERK with focal adhesions may not be essential for its role in cell migration. Alternatively, the epitope recognized by anti-ERK or anti-phosphoERK antibodies may be concealed when ERK localizes to focal adhesions, preventing antibody recognition. In PE outgrowth, we observe that phosphoERK localizes to areas behind the actin meshwork of the lamellipodia, and colocalizes with actin in the center of the cell (data not shown). This suggests that ERK affects the formation of actin filaments, which indirectly affects the formation of focal adhesions in the migrating front. Using live cell imaging will help determine the subcellular localization of the components of the ERK pathway during cell migration.

Lamellipodia Formation and Cell Migration

The Arp2/3 complex catalyzes the nucleation of branched actin polymerization, which promotes formation of lamellipodia [Svitkina and Borisy, 1999]. Actin polymerization is promoted by WASP, which activates Arp2/3 [Takenawa and Miki, 2001; Martinez-Quiles et al., 2004]. Vinculin recruits Arp2/3 to the leading edge of lamellipodia, coupling focal adhesions to actin polymerization [DeMali et al., 2002]. This suggests a dynamic link between focal adhesion and lamellipodia formation. Our results demonstrate that the number of lamellipodia is significantly decreased when MEK is inhibited (Fig. 3H,K), which suggests that ERK regulates cell migration through the formation of lamellipodia, as well as focal adhesion stability.

In fibroblasts, ERK phosphorylates cortactin, an actin binding protein that plays an important role in activating Arp2/3 mediated actin polymerization [Martinez-Quiles et al., 2004]. Cortactin forms a complex with WASP and colocalizes with it in the lamellipodia [Weaver et al., 2002; Martinez-Quiles et al., 2004]. ERK phosphorylation of cortactin promotes its association with WASP, which leads to Arp2/3 activation and lamellipodia extension [Martinez-Quiles et al., 2004]. Consistent with this model, we observed that MEK inhibition diminishes lamellipodia formation in the PE outgrowth. Thus ERK may promote lamellipodia formation partly through its action on cortactin.

FAK–Paxillin Interaction and ERK Signaling

Focal adhesion molecules activate the ERK pathway, facilitating its contribution to cell motility. Vinculin and FAK bind to overlapping regions in the LD motifs of paxillin [Turner et al., 1999]. The loss of vinculin results in an upregulated association between FAK and paxillin, which is proposed to activate the ERK pathway [Subauste et al., 2004]. Ishibe et al. [2003] report that in mouse inner medullary collecting duct (mIMCD-3) cells, paxillin associates with FAK, Raf, MEK, and ERK. They proposed that paxillin is a scaffold protein that mediates the interaction between molecules in the ERK pathway and potential substrates [Ishibe et al., 2003]. Interestingly, the phosphorylation of serine 83 of paxillin by ERK can enhance the FAK-paxillin association in mIMCD-3 cells, which promotes lamellipodia protrusion [Ishibe et al., 2004].

MLCK Acts Downstream of ERK

Our data suggest that ERK's effect on cell migration is mediated through the action of MLCK. It has been demonstrated that MLCK is a substrate of ERK in vitro [Klemke et al., 1997], though kinase assays using F9 PE material need to be performed to verify this occurs in outgrowth cultures. Unfortunately, this is difficult to accomplish due to the low level of protein extracted from outgrowth. Our data also suggest that MLCK, via MLC, promotes focal adhesion turnover and lamellipodia extension. Other reports have demonstrated that ERK and MLCK positively regulate focal adhesion turnover [Xie et al., 1998; Nguyen et al., 1999; Fincham et al., 2000; Webb et al., 2004], consistent with our observations. In contrast, MLCK inhibition can prevent the formation of focal adhesions and bundling of actin stress fiber in HUVEC cells [Houle et al., 2003]. Activation of ERK promotes bundling of actin stress fibers in HUVEC cells by stimulating tropomyosin phosphorylation [Houle et al., 2003]. Therefore, whether the ERK-MLCK pathway negatively or positively regulates the formation of focal adhesion and actin stress fibers may be cell-type dependent.

ERK and Rho Family GTPases

In addition to ERK signaling, Rho family GTPases, Rho, Rac, and Cdc42 play key reg-

ulatory roles in cell migration. Rho, Rac, and Cdc42 contribute to the formation of actin stress fibers and strong focal adhesions, lamellipodia, and filopodia, respectively [Etienne-Manneville and Hall, 2002]. Treatment with the Rho GTPase kinase (ROCK) inhibitor (Y27632) disrupts focal adhesion formation and promotes PE migration [Mills et al., in press]. A recent study identifies the ERK pathway component Raf-1 as an inhibitor of ROCK [Ehrenreiter et al., 2005]. Raf-1 associates with ROCK, and Raf-1-deficient keratinocytes show enhanced actin stress fibers and decreased cell migration, mediated by ROCK hyperactivity [Ehrenreiter et al., 2005]. These data suggest that Raf-1 may also contribute to cell migration by inhibiting ROCK-induced actin stress fiber formation. The regulation of the Rho-ROCK pathway by Raf-1 is independent of the kinase activity of Raf-1 in keratinocytes [Ehrenreiter et al., 2005], although our transfection data suggest that the kinase activity of Raf-1 does promote cell migration. Interestingly, the kinase activity of MEK also mediates inactivation of the Rho-ROCK-LIM Kinase pathway by downregulating the expression of ROCK in v-src transformed rat kidney cells [Pawlak and Helfman, 2002]. They observe that deregulation of ROCK activity by MEK diminishes actin stress fibers and enhances focal adhesions. We also observe enhanced actin stress fibers accompanying stabilized focal adhesions when MEK is inhibited (Fig. 3G,J,H,K). Thus components in the ERK pathway, either dependent or independent of their kinase activities, can regulate focal adhesion turnover and actin stress fiber formation either by activating MLCK or downregulating the Rho-ROCK pathway, treatments that both promote cell migration.

The ERK pathway also interacts with the Rac GTPase pathway that promotes the formation of lamellipodia [Ridley et al., 1992]. Studies have found that Rac GTPase kinase (p21-activated kinase, PAK) family proteins phosphorylate different components in the MEK-ERK-MLCK-MLC pathway. γ-PAK phosphorylates MLC at serine 19 in bovine pulmonary artery endothelial cells (BPAE, CCL209) [Chew et al., 1998]. PAK1 phosphorylates MEK1, a direct upstream molecule of ERK1 in rat embryonic fibroblasts (REF52) [Slack-Davis et al., 2003], whereas PAK2 phosphorylates MLCK in REF52 cells [Goeckeler et al., 2000]. However, whether the action of the ERK pathway on focal adhesion and lamellipodia formation can be promoted by PAK is still not known.

Our data suggest that ERK signaling, via MLCK, destabilizes focal adhesion formation and promotes lamellipodia extension, leading to increased cell migration. Future studies will determine how this pathway interacts with Rho GTPases to control directed cell migration.

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