Roles of microtubules, cell polarity and adhesion in electric-field-mediated motility of 3T3 fibroblasts

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Summary

Direct-current electric fields mediate motility (galvanotaxis) of many cell types. In 3T3 fibroblasts, electric fields increased the proportion, speed and cathodal directionality of motile cells. Analogous to fibroblasts' spontaneous migration, we initially hypothesized that reorientation of microtubule components modulates galvanotaxis. However, cells with intact microtubules did not reorient them in the field and cells without microtubules still migrated, albeit slowly, thus disproving the hypothesis. We next proposed that, in monolayers wounded and placed in an electric field, reorientation of microtubule organizing centers and stable, detyrosinated microtubules towards the wound edge is necessary and/or sufficient for migration. This hypothesis was negated because field exposure mediated migration of unoriented, cathode-facing cells and curtailed migration of oriented, anode-facing cells. This led us to propose that ablating microtubule detyrosination would not affect galvanotaxis. Surprisingly, preventing microtubule detyrosination increased motility speed, suggesting that detyrosination inhibits galvanotaxis. Microtubules might enhance adhesion/de-adhesion remodeling during galvanotaxis; thus, electric fields might more effectively mediate motility of cells poorly or dynamically attached to substrata. Consistent with this hypothesis, incompletely spread cells migrated more rapidly than fully spread cells. Also, overexpression of PAK4, a Cdc42-activated kinase that decreases adhesion, enhanced galvanotaxis speed, whereas its lack decreased speed. Thus, electric fields mediate fibroblast migration via participation of microtubules and adhesive components, but their participation differs from that during spontaneous motility.

Movies available on-line

Key words: Electric field, Cell motility, Microtubule, Adhesion

Introduction

Cell migration plays primary roles in development, wound healing and metastasis. A better understanding of motility mechanisms would enhance understanding of instances of deficient or unwanted migration [e.g. wound healing in diabetics (Hehenberger et al., 1998)], or metastasis from the site of a primary tumor (Kassis et al., 2001). Motility models often exploit spontaneous migration of sparse cells or wound healing of monolayer cells (Abercrombie, 1970; Abercrombie, 1977). In the latter, 'wounding' confluent cell monolayers exposes a cell-free stripe, releases contact inhibition of wound-edge cells and induces migration into the cell-free space. Wounding stimulates migration of nearly all wound-edge cells, with uniform direction.

In fibroblasts, but not in epithelial cells (Yvon et al., 2002), wound-edge cells reorient cytoplasmic components to lie between their nucleus and leading edge. For example, the Golgi apparatus, microtubule-organizing center (MTOC) (Kupfer et al., 1982) and stable post-translationally detyrosinated (Glu) microtubules (MTs) all reorient (Gundersen and Bulinski, 1988). Golgi orientation polarizes membrane insertion (Bergmann et al., 1983); in turn, MTOC reorientation (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001b) presumably orients the Golgi. Despite active investigation into reorientation mechanisms and the fact that some cells carry out wound-healing migration without MTOC reorientation [e.g. epithelial cell lines (Yvon et al., 2002)], the hypothesis that reorientation affects fibroblast migration has not been tested.

Different motility systems have disparate MT requirements. For example, MTs are unnecessary for fibroblast locomotion in an oriented, three-dimensional extracellular matrix (ECM) (Schutze et al., 1991) or for rapid migration of tiny fish keratocytes (Euteneuer and Schliwa, 1984), or keratocyte fragments (Verkhovsky et al., 1999). Fibroblasts, however, require MTs (Gail and Boone, 1971; Vasiliev et al., 1970), specifically dynamic MTs (Liao et al., 1995), for spontaneous and wound-healing migration. Although reorientation of stable Glu MTs towards the leading edge makes an unknown contribution to wound-healing migration, the existence of a specific Rho signaling pathway for their selective stabilization and subsequent detyrosination argues indirectly for their importance (Pallazzo et al., 2001a; Hollenbeck, 2001). Thus far, determining the functions of stable and dynamic MTs in various cell migration systems has proved to be an elusive goal.

Cell adhesion also polarizes during wound-healing migration. Integrin-ECM interactions activate wound-edge

Cdc42 and its downstream effectors (Etienne-Manneville and Hall, 2001). Differential exposure of ECM epitopes at the cell periphery in the presence or absence of contacting cells could drive the polarization of wound-edge cells (Goldfinger et al., 1999). Cortical actin 'arcs' (Heath, 1983) along the wound-facing surface (Gotlieb et al., 1984) might associate with growth factor receptors (Crouch et al., 2000) or $p21^{Ras}$-activated kinases (PAKs) (Sells et al., 2000), and these might participate in changing cortical tension and force during motility (Sheetz et al., 1999; Geiger et al., 2001).

In addition to spontaneous migration of sparse cells and wound-healing migration, direct current (DC) electric fields (EFs) are a less prominently studied means of effecting migration. Galvanotaxis and galvanotropism (i.e. EF-mediated migration and shape change, respectively) occur in keratinocytes, epithelial cells, bone cells, chondrocytes and fibroblasts (Chao et al., 2000; Ferrier et al., 1986; Sheridan et al., 1996; Soong et al., 1990; Zhao et al., 1996a). The EF strengths commonly used (1-10 V cm^{-1}) are physiologically significant for vertebrates, because 1-2 V cm^{-1} gradients have been measured on either side of the cut surface of wounds owing to ion flux through leaky cell membranes (Soong et al., 1990) or transepithelial potential driven by Na^+ pumps (Vanable, 1989). Similarly, ~5 V cm^{-1} EFs occur during development (Altizer et al., 2001; McCaig and Zhao, 1997; Robinson, 1985). In animals, EFs introduced via implantable electrodes stimulate mitosis, recruit osteogenic cells and induce wound healing, whereas disruption of EFs during neural development causes severe abnormalities (Borgens and Shi, 1995; Hotary and Robinson, 1994). Finally, increasing evidence that tumor and non-tumor cells migrate differently in response to EFs justifies further analysis of EF-mediated migration, to further our understanding of basic tumor biology and possibly develop anti-metastatic interventions (Djamgoz et al., 2001; Siwy et al., 2003). Overall, we do not understand clearly how EFs contribute to or commandeer existing motility mechanisms during development (Robinson, 1985) or wound healing (Nishimura et al., 1996).

Although cell locomotion mechanics might be independent of whether the motility trigger is an adjacent cell or growth factor, a physical stress or an EF, the migration direction appears to be established differently with different motility triggers. Given the cell's high impedance, EFs probably work at the plasma membrane, polarizing membrane components that, in turn, scaffold the polarization of the requisite cytoskeletal elements (Zhao et al., 1999). For example, EFs might bias calcium channel activation (Cooper and Schliwa, 1985; Fang et al., 1998) or globally increase intracellular calcium concentrations (Cho et al., 2002). However, because 3T3 cells can undergo EF motility without extracellular calcium (Brown and Loew, 1994), polarized calcium channel activation seems unlikely. The EF might move other receptors or channels by electrophoresis within the membrane, as shown for various glycoproteins (e.g. conA binding proteins) (Orida and Feldman, 1982; Brown and Loew, 1994). Alternatively, EFs might signal motility by altering receptor conformation (Mosbacher et al., 1998).

Once EFs trigger migration, cells must orchestrate cytoskeletal elements and form and release adhesion structures. We have begun to use the well-studied 3T3 fibroblast to test hypotheses about the role(s) of the cytoskeleton, its reorientation and its post-translational modification during EF migration. We show here that EFs alter the speed of cells migrating spontaneously or responding to wound-healing cues, and that altering cell adhesion affects EF migration. Our results show that EFs are motility mediators whose effects depend upon the ground state of cells to which they are applied.

Materials and Methods

Tissue culture materials were from GIBCO Life Sciences or Hyclone; other chemicals were from Sigma Chemical. Antibodies were from Sigma or Organon-Teknika, except anti-tubulin (Chang et al., 2002) and anti-actin (Otey et al., 1986) antibodies. Rhodamine-conjugated phalloidin was from Molecular Probes.

NIH-3T3 cell culture is described by Gundersen and Bulinski (Gundersen and Bulinski, 1988). Depolymerization of only dynamic microfilaments was accomplished by cytochalasin-B pretreatment (1.5 hours, 2 μM) (Koukouritaki et al., 1996). Colcemid pretreatment (15 μM Colcemid for 1 hour, 0°C, then 1 hour, 37°C) depolymerized all cellular MTs. For both, a vehicle-only control (0.1% dimethylsulfoxide) was performed. Cells devoid of Glu MTs were prepared by rinsing log-phase cultures three times, incubating for 24 hours in F-10 medium with serum and plating in motility chambers in F-10 with or without 400 μM 3-nitrotyrosine (NO_2Tyr) for 14-18 hours (Eiserich et al., 1999).

For EF motility of sparse cells, 1.5×10^4-2.5×10^4 cells were plated in each 'flexiPERM-18' disk (Sigma) on microscope slides (prewashed in 1 M HCl, rinsed in water and ethanol, and ultraviolet sterilized), 14-18 hours before experiments. For wound-healing, cells maintained in sparse culture were plated 14-18 hours before wounding at a density of 2.0×10^5-2.5×10^5 cells per flexiPERM disk. Parallel wounds were made perpendicular to the EF, applied 0.5-4.5 hours after wounding.

Direct current at constant EF strength (0-6 V cm^{-1} for sparse cells, 0-2 V cm^{-1} for monolayers, 37°C) was applied to galvanotaxis chambers (Chao et al., 2000) connected to a power supply (Keithley SourceMeter 2410) through two salt bridges and Ag-AgCl electrodes. Time-lapse images (10 minute intervals) were captured and the average speed (the distance between the final and initial positions of the cell centroid divided by the total elapsed time) was analysed using a particle-tracking scheme in MetaMorph software (Universal Imaging).

We defined the migration angle, theta (θ), relative to the horizontal axis lying perpendicular to the direction of the applied EF (Fig. 1A). Using this convention, cells traveling cathodally have a negative sin(θ) because θ toward the cathode is 270° and sin(270°)=–1. Similarly, cells traveling anodally have a positive sin(θ) because θ toward the anode is 90°, and sin(90°)=+1. We calculated directional velocity as speed×sin(θ); negative quantities indicate cathodal migration and positive quantities indicate anodal migration. Graphs and statistical analysis used the absolute value of the average directional velocity, calculated in Excel, Statistica or SPSS. Analysis of variance (ANOVA) yielded *P* values for multiple data sets. Experiments were performed between two and four times, each analysing at least 15 cells.

For adhesion experiments, cells plated for various times on uncoated glass were immediately placed in the EF or centrifuged to test adhesion strength. In the centrifugation assay, reminiscent of Burdsal et al. (Burdsal et al., 1991), coverslips were centrifuged (10 minutes; Sorvall RC5-B) cell side down on a rubber stopper in a tube filled with Dulbecco's modified Eagle's medium and fixed with formaldehyde, and the remaining adherent cells were counted in four microscope fields (two experiments).

Total internal reflection fluorescence (TIRF) experiments on formaldehyde-fixed, anti-paxillin-stained cells used an Olympus IX-81 microscope with TIRF attachment. Epifluorescence and TIRF

images were matched, illuminating the latter through a single mode fiber coupled to a Coherent (Ar-Kr multimode) laser. 12-bit digital 1300×1030-pixel grayscale images (1.5-4 second exposures with a Uniblitz shutter) were captured with a Roper Scientific CoolSnap-fx cooled-CCD camera, stored and analysed. Cell surface area ('footprint') was quantified in MetaMorph ('region statistics' command). Contact area was quantified in MetaMorph by first magnifying images 4000-fold, adjusting digital contrast automatically and visualizing substratum contacts ('detect edges' command, Sobel filter). Next, contact regions were colored ('threshold image' command, state inclusive), adjusting the low setting (only) until the image closely matched the original micrograph. 'Region statistics' then quantified substratum contact area within each cell's footprint.

NIH-3T3 cells constitutively expressing PAK4 and vector-only controls are described by Abo et al. (Abo et al., 1998); PAK4-null mice are described by Qu et al. (Qu et al., 2003). Fibroblasts were prepared from embryonic-day-9.5 $PAK4^{+/-}$ and $PAK4^{-/-}$ mice according to Palmero and Serrano (Palmero and Serrano, 2001). Cells with altered PAK4 levels were assayed as sparse cultures.

Results

Changes in cell migration caused by EF exposure (galvanotaxis) had been previously studied in epithelial, endothelial or neuronal cells, or primary embryonic fibroblasts (Erickson and Nuccitelli, 1984; Sillman et al., 2003). We examined the effects of DC EFs of physiologically relevant strengths (Soong et al., 1990; Vanable, 1989) on the extensively characterized motile properties of NIH-3T3 fibroblasts. Consistent with work on primary fibroblasts, EFs of <2 V cm^{-1} did not alter basal motility speed of sparse cells, but EFs of 4-6 V cm^{-1} significantly increased cell speed, and 6 V cm^{-1} more than doubled speed relative to spontaneous motility (Fig. 1B). Because a 6V cm^{-1} EF accurately models physiological EFs without yielding the deleterious effects often observed with stronger EFs (10 V cm^{-1}) (not shown) (Onuma and Hui, 1988), we adopted this EF strength for studies of sparse fibroblasts.

As well as increasing speed, EFs also affected migration direction. Fig. 1C shows the linear relationship between directionality and EF strength. Migration was mainly cathode directed at the highest EF strength (6 V cm^{-1}); that is, the $\sin(\theta)$ value (–0.81) was similar to the sine of the cathodal angle, 270° [sin(270)=–1]. In fact, at 6 V cm^{-1}, average θ deviated from the cathodal direction by only 36° (Fig. 1A).

A 6 V cm^{-1} EF maximized the motility of sparse 3T3 cells, and so we examined individual cells' responses, both speed (Fig. 1D) and direction (Fig. 1E). First, average speed increased. Second, despite heterogeneous responses (notice the wider distribution of speeds with than without EF in Fig. 1D), nearly all cells increased speed. The EF decreased the proportion of negligibly motile cells (<2 μm $hour^{-1}$) from ~15% at 0 V cm^{-1}, to <5% at 6 V cm^{-1}. This can best be appreciated from the polar plot (Fig. 1E); at 0 V cm^{-1} but not at 6 V cm^{-1}, most cells remained within one-half cell diameter (<10 μm) of the origin throughout the recording [also compare supplementary movies SPARSE_0V_2HR and SPARSE_6V_2HR (http://jcs.biologists.com/supplemental/)]. Third, the EF specified a directional signal to which most cells responded, regardless of speed (>95%; compare 0 V cm^{-1} and 6 V cm^{-1} EF; Fig. 1D). Taken together, Fig. 1 demonstrates that EFs modulate motility direction more potently than they do speed. Finally, EFs caused cells to adopt prevalent morphologies, aligned with their long axes perpendicular to the cathodal direction [compare final frames of the movies cited above (http://jcs.biologists.com/supplemental/)].

To determine which cytoskeletal filaments fibroblasts use for EF migration, we observed EF migration after treating cells with cytoskeleton-antagonistic drugs. Cells pretreated with an actin antagonist that inhibits only dynamic microfilaments (2 μM cytochalasin B, 1.5 hours) (Koukouritaki et al., 1996) showed no detectable EF migration [Fig. 2A,C; see supplementary movie Cyto_6V_2HR (http://jcs.biologists.com/supplemental/)]. Cytochalasin-treated cells neither aligned perpendicular to the EF (Fig. 2A) nor moved spontaneously (Fig. 2C), demonstrating that microfilaments are absolutely required for migration, with or without EFs. Higher cytochalasin-B or -D concentrations gave identical results; all microfilament inhibitory treatments blocked migration (not shown). We also examined the distribution of total and polymeric actin using immunofluorescence and phalloidin labeling (Fig. 2A); only control cells showed cortical actin arcs, whereas cytochalasin-treated cells showed small processes along the cathode-facing surfaces. In the EF, neither moving (control) nor stationary (cytochalasin-treated) cells showed morphologies characteristic of spontaneously moving 3T3 cells.

In contrast to microfilament inhibition, MT inhibition did not halt EF-mediated motility. Fig. 2B documents migration of sparse cells subjected to a vigorous pretreatment that depolymerized all MTs (15 μM colcemid, 2 hours); Fig. 2C shows that cells without MTs migrated in the EF ~2.5 times slower than controls, and did not align perpendicular to the cathodal direction [Fig. 2Bb,d; also supplementary movies Colcemid_6V_2hr and SPARSE_6V_2HR (http://jcs.biologists.com/supplemental/)]. Harsh or mild MT-antagonistic treatments (i.e. high or low Colcemid, nocodazole or Taxol concentrations) gave indistinguishable results (not shown). Motility of Colcemid-treated and control cells were equivalently cathode-directed; directional velocity accounted for 81% of total speed in drug-treated cells and 74% in controls (Fig. 2C). Although EF motility occurred in the absence of MTs, significant spontaneous motility did not. In Colcemid-treated cells, spontaneous motility speed was only 19% that of control cells (2.2±0.4 μm $hour^{-1}$; directional velocity 0.7±0.4 μm $hour^{-1}$; not shown); this is negligible, because it amounts to translocation of less than one-half cell diameter during 4-hour measurements. Colcemid blockage of spontaneous motility of 3T3 fibroblasts is consistent with earlier reports (Gail and Boone, 1971; Vasiliev et al., 1970). By contrast, EF motility occurs without MTs, even though they clearly play a role, because their presence significantly enhances EF-migration speed.

MTs might enhance EF motility by polarizing cell components, perhaps the stable MT array, MTOC or Golgi apparatus, analogous to their polarization during spontaneous and wound-healing motility (Kupfer et al., 1982). We tested the hypothesis that cell orientation or polarization is required for, or enhances, EF migration. Unlike the wounding stimulus, EF exposure did not detectably alter the distribution of total or stable MT arrays or the MTOC (Fig. 3A,B). The Golgi, which in wound-healing motility lies adjacent to the oriented MTOC, also failed to polarize during EF motility. Instead, it was perinuclear and poorly grouped around the unoriented MTOC (not shown). The striking visual difference between

the fibroblasts' MT arrays during EF versus wound-healing motility (Fig. 3Ba-d) suggests that reorientation of MT components is unnecessary for EF-motility. The MTs and MTOC, which in other systems appear to confer directional persistence of motility (e.g. Ueda et al., 1997), are unlikely to play this role in the presence of an EF.

Although reorientation of MT components was unnecessary for EF motility, we hypothesized that alignment of cytoplasmic

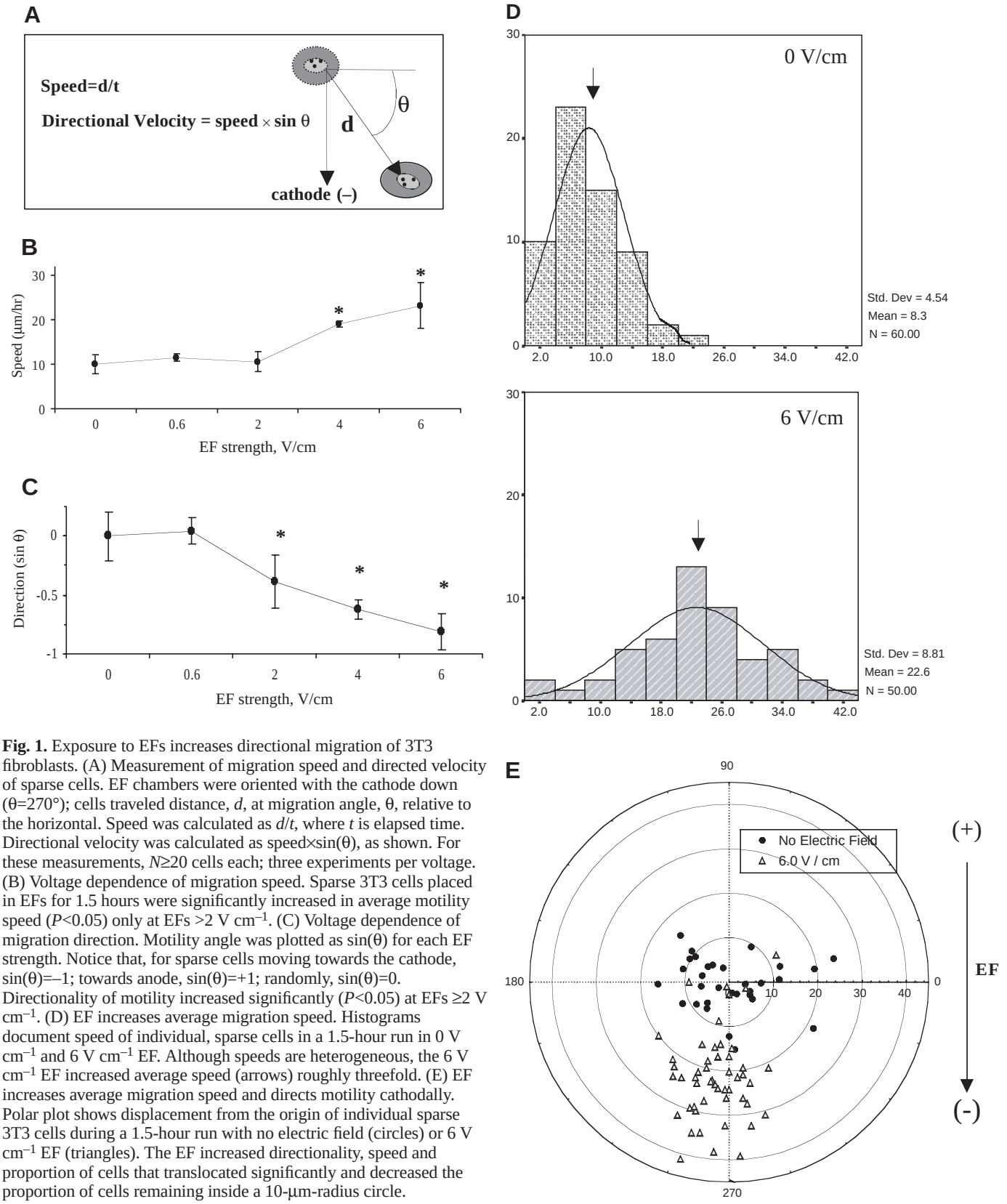


Fig. 1. Exposure to EFs increases directional migration of 3T3 fibroblasts. (A) Measurement of migration speed and directed velocity of sparse cells. EF chambers were oriented with the cathode down (θ=270°); cells traveled distance, *d*, at migration angle, θ, relative to the horizontal. Speed was calculated as *d*/*t*, where *t* is elapsed time. Directional velocity was calculated as speed×sin(θ), as shown. For these measurements, $N \geq 20$ cells each; three experiments per voltage. (B) Voltage dependence of migration speed. Sparse 3T3 cells placed in EFs for 1.5 hours were significantly increased in average motility speed ($P<0.05$) only at EFs >2 V cm^{-1}. (C) Voltage dependence of migration direction. Motility angle was plotted as sin(θ) for each EF strength. Notice that, for sparse cells moving towards the cathode, sin(θ)=–1; towards anode, sin(θ)=+1; randomly, sin(θ)=0. Directionality of motility increased significantly ($P<0.05$) at EFs ≥2 V cm^{-1}. (D) EF increases average migration speed. Histograms document speed of individual, sparse cells in a 1.5-hour run in 0 V cm^{-1} and 6 V cm^{-1} EF. Although speeds are heterogeneous, the 6 V cm^{-1} EF increased average speed (arrows) roughly threefold. (E) EF increases average migration speed and directs motility cathodally. Polar plot shows displacement from the origin of individual sparse 3T3 cells during a 1.5-hour run with no electric field (circles) or 6 V cm^{-1} EF (triangles). The EF increased directionality, speed and proportion of cells that translocated significantly and decreased the proportion of cells remaining inside a 10-μm-radius circle.

components caused by wound-healing cues is sufficient to modulate EF motility. Therefore, we observed EF motility of wound-edge cells just after wounding and at longer post-wounding intervals, to compare motility of cells that had not or had polarized their stable MTs, MTOCs and Golgi towards the wound edge (Gundersen and Bulinski, 1988). In both cases, we oriented wounded monolayers in the EF such that migration into the wound was towards the cathode or anode (that is, with or against the EF). Fig. 4A,B shows that cathode-facing cells that experienced a 0.6 V cm^{-1} or 2 V cm^{-1} EF immediately after wounding (0.5-2.0 hours) commenced migration into the wound during this interval, whereas those without EF (0 V cm^{-1}) did not move detectably. Thus, EF signals initiate cathode-directed motility before wound-healing signals have oriented cellular contents and instigated movement. We also noted that EF application induced cells to move into the wound asymmetrically [Fig. 4A,B; and supplementary movie wound_2V_3hr (http://jcs.biologists.com/supplemental/)]. In contrast to the rapid motility initiation of cathode-facing cells, anode-facing cells did not move into the wound during this EF exposure period. Their movement was indistinguishable from wound-edge cells without EF (Fig. 4A; compare anode-facing cells at 0.6 V cm^{-1} or 2 V cm^{-1} with cells facing either direction at 0 V cm^{-1} EF). Thus, EFs only initiate movement of cathode-directed cells and they do so despite the fact that these cells have not yet reoriented their MTOC and stable MTs towards the wound edge.

To test whether preorienting MT components would affect cells' EF-mediated motility, we wounded monolayers, returned them to the incubator for 2.5 hours to allow reorientation towards the wound (Gundersen and Bulinski, 1988) and then placed them in 0-2 V cm^{-1} EFs (Fig. 4C). Cathode-directed

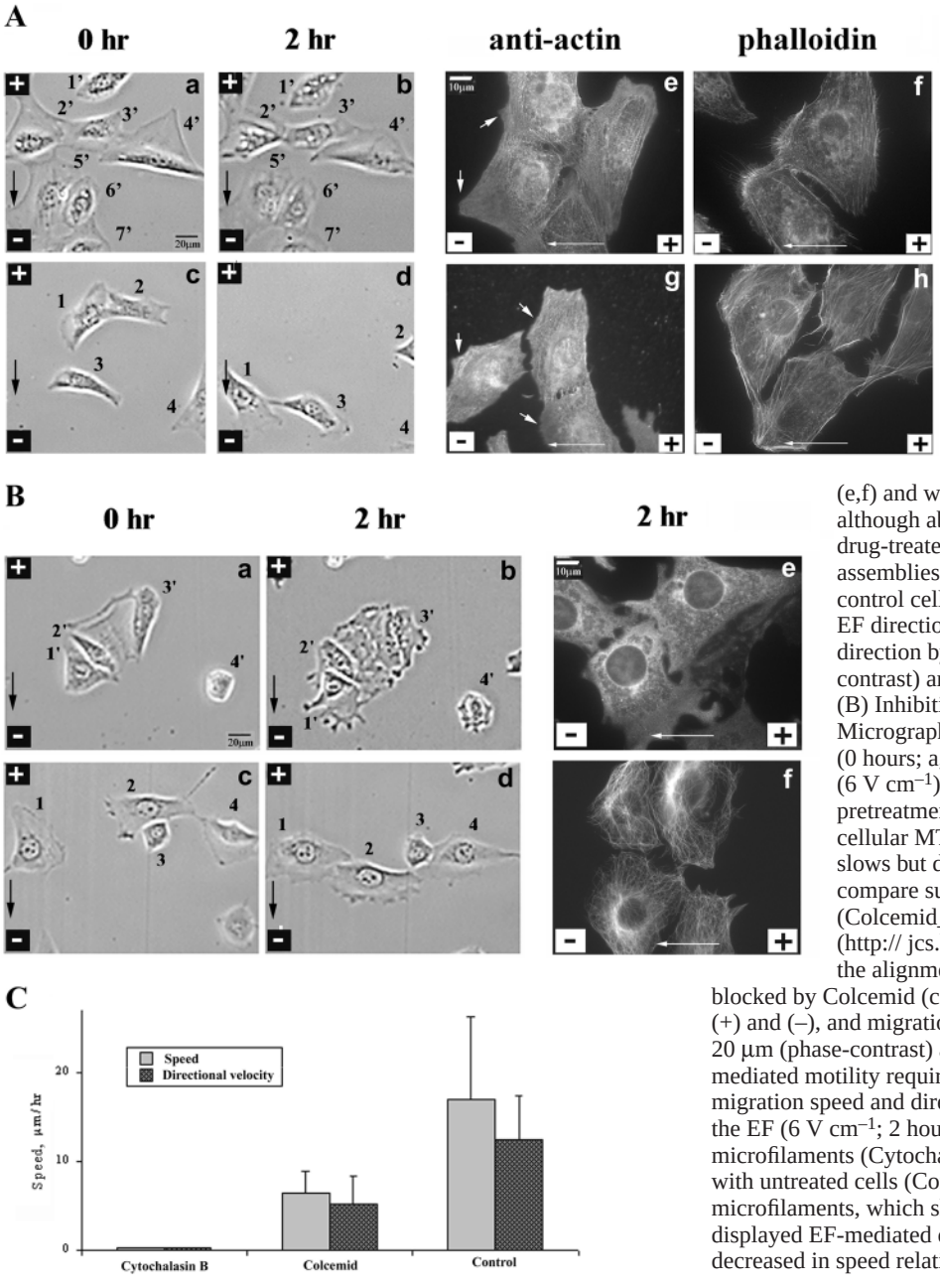


Fig. 2. Microfilaments, not microtubules, are required for EF-mediated migration. (A) Inhibiting dynamic microfilaments blocks motility. Micrographs document positions of numbered cells initially (0 hours; a,c) and after 2 hours (b,d), EF exposure (6 V cm^{-1}), with (a,b) or without (c,d) cytochalasin B (2 μM, 1.5 hours pretreatment). Cytochalasin completely abrogated motility, as quantified in (C) [see supplementary movies of drug-treated Cyto_6V_2hr and control cells Sparse_6V_2hr (http://jcs.biologists.com/supplemental/)]. The array of actin-containing structures visualized with anti-actin immunofluorescence (e,g) and rhodamine phalloidin (f,h) is shown with (e,f) and without (g,h) cytochalasin B. Notice that, although abundant microfilament structures remain in drug-treated cells (e,f), brightly labeled actin arc-like assemblies at cathode-facing edges are present only in control cells (g,h); compare thick white arrows in (e,g). EF direction shown by (+) and (–), and migration direction by thin arrows. Scale bars, 20 μm (phase-contrast) and 10 μm (immunofluorescence). (B) Inhibiting MTs slows EF-mediated motility. Micrographs show positions of numbered cells initially (0 hours; a,c) and after 2 hours (b,d), EF exposure (6 V cm^{-1}), with (a,b) or without (c,d) Colcemid pretreatment (15 μM, 2 hours). Breakdown of all cellular MTs (see anti-tubulin immunofluorescence, e,f) slows but does not stop motility [quantified in Fig. 2C; compare supplementary movies of drug-treated (Colcemid_6V_2hr) and control (Sparse_6V_2hr) cells (http:// jcs.biologists.com/supplemental/)]. Notice that the alignment perpendicular to motility direction is blocked by Colcemid (compare a,c, to b,d). EF direction shown by (+) and (–), and migration direction by thin arrows. Scale bars, 20 μm (phase-contrast) and 10 μm (immunofluorescence). (C) EF-mediated motility requires microfilaments, not MTs. Average migration speed and directional motility [speed×sin(θ); Fig. 1A] in the EF (6 V cm^{-1}; 2 hours) was quantified in cells treated to inhibit microfilaments (Cytochalasin B) or MTs (Colcemid), and compared with untreated cells (Control). Unlike cells lacking dynamic microfilaments, which showed no movement, cells lacking MTs displayed EF-mediated directional motility that was merely decreased in speed relative to controls.

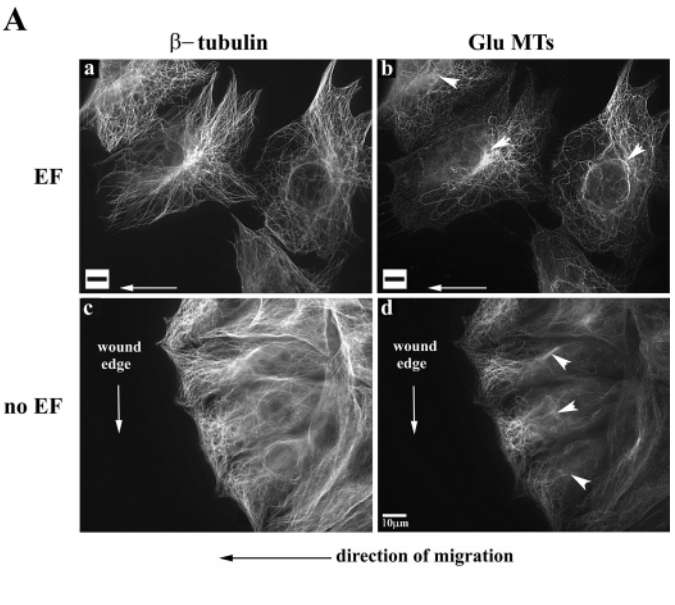


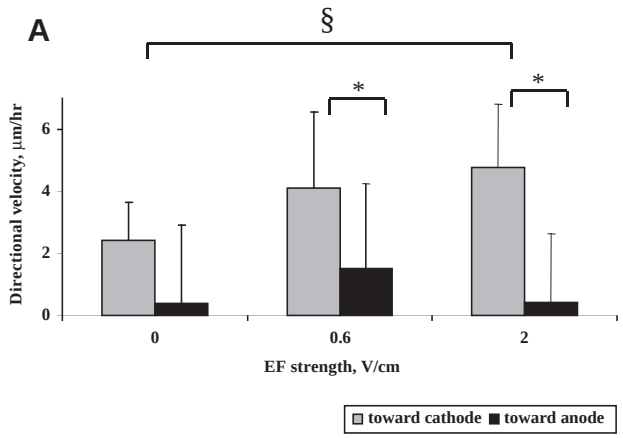
Fig. 3. The MTOC and stable MT subset do not reorient during EF motility, unlike wound-healing motility. (A) The total MT array (anti-β-tubulin; a,c) and stable MT subset (detected via enrichment in detyrosinated tubulin; Glu, b,d) did not become polarized during EF-mediated motility (a,b), in contrast to wound-healing motility without applied EF (no EF; c,d), in which Glu MTs reoriented towards the wound edge. Glu MT labeling also allowed the detection of the MTOC (arrowheads, b,d). Migration direction is to the left in all micrographs; the cathode (–) (a,b) and wound edge (c,d) are shown with thin white arrows. Scale bar (d), 10 μm. (B) The proportion of cells with an oriented MTOC [i.e. within the cathode-facing quadrant (dotted lines)] was indistinguishable from the 25% expected for a random distribution. Similarly, 5.1% of cells in the EF showed a Glu MT array oriented towards the migration direction (i.e. with >75% of Glu MTs facing the migration direction), indistinguishable from controls (8.3%). Notice that, in the EF, only a small proportion of sparse cells had Glu MTs oriented in any direction. MTs are depicted as thin lines, Glu MTs as thick lines and the MTOC as a dot.

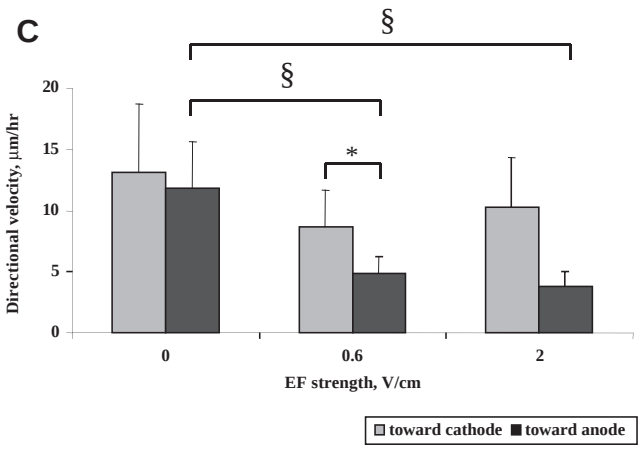
speed of pre-polarized cells was not significantly altered by the EF (Fig. 4C; cell speed was indistinguishable at 0 V cm^{-1}, 0.6 V cm^{-1} or 2 V cm^{-1}). As before (Fig. 4A), cell migration against the EF was significantly slower than with the EF. In fact, anode-facing cells assayed in an EF moved negligibly; indistinguishable from cells with no EF, tested immediately after wounding (i.e. $P>0.2$ for comparing the speed of anode-directed cells at 0.6 V cm^{-1} or 2 V cm^{-1} in Fig. 4C with cells at 0 V cm^{-1} in Fig. 4A). Cells pre-polarized for 4.5 hours behaved comparably; again, anode-directed cells showed slow migration (<5 μm $hour^{-1}$), whereas cathode-directed cells migrated significantly more rapidly, with velocities unaffected by the applied EF (not shown). Thus, contrary to our hypothesis, pre-polarizing MT components towards the wound edge was not sufficient to modify EF motility.

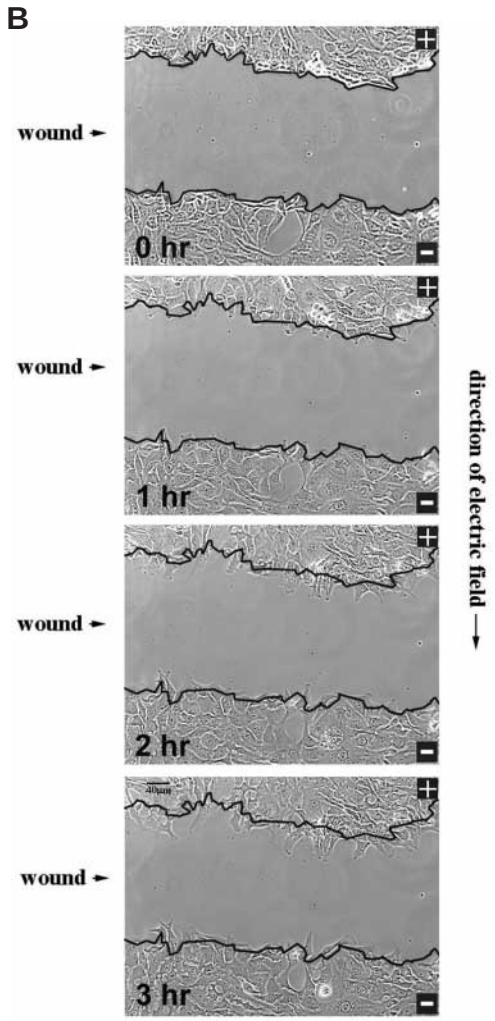
However, it remained possible that the reason the EF was able to reduce motility into the anode-facing side of a wound was that MT reorientation towards the wound had failed to occur or had occurred but then had been reversed during exposure to an oppositely polarized EF. To test this possibility, we wounded cells, immediately applied the EF for 2 hours and then asked whether MTOCs and stable Glu MTs had reoriented toward the wound. Fig. 4D shows that, whether migrating with or against the EF, MT components were reoriented towards the wound edge. In addition, cells exposed to EFs for 2 hours following a 2.5-4.5-hour pre-polarization yielded identical results; the proportion of cells with reoriented Glu MTs and MTOC's was identical whether cells faced anode or cathode (not shown). Thus, the speed of cells receiving wound-healing cues was decreased by counteracting signals from an EF, even though EF signals did not compromise the degree of wounding-induced orientation of cytoplasmic components. These data argue that, in the presence of an EF, polarization of the MT cytoskeleton is not sufficient either to induce or to bias migration in a particular direction, in contrast to other motility systems in which intact MT arrays confer directional persistence (e.g. Glasgow and Daniele, 1994).

Previous studies of wound-healing migration showed that intact MTs are required, a subset of dynamic MTs is needed to achieve maximum speed (Liao et al., 1995) and, from circumstantial evidence, a stable Glu MT subset oriented towards the leading edge is purported to perform specialized functions during wound-healing motility (e.g. Gundersen and Bulinski, 1988; Gurland and Gundersen, 1995). In marked contrast to this, in EF motility, intact MTs are not required (Fig. 2C) and, for cells with MTs, orientation of Glu MTs towards the migration direction is neither necessary nor sufficient to confer directional persistence of motility. From these results, we hypothesized that detyrosinated MTs are not required for EF motility. We tested this directly, monitoring EF motility of cells whose Glu MT formation had been abrogated with 3-nitrotyrosine (Fig. 5A), a nontoxic compound that specifically inhibits MT detyrosination (Eiserich et al., 1999) without affecting elaboration of the stable MT subset on which detyrosination would normally occur (Chang et al., 2002) (J. C. Bulinski et al., unpublished). Surprisingly, rather than blocking or decreasing motility, inhibiting Glu MT formation yielded increased speed of EF motility (Fig. 5B). This result directly tests the role of Glu MTs during cell migration; inhibition of EF migration by MT detyrosination contrasts with the positive role postulated for MT detyrosination during wound-healing motility (e.g. Gundersen and Bulinski, 1988; Pallazzo et al., 2001a). Taken together with the decrease in EF motility speed resulting from MT-depolymerizing treatments (Fig. 2C) and equivalently by lower Colcemid concentrations targeting chiefly dynamic, tyrosinated MTs (not shown), it appears that ablation of dynamic tyrosinated MTs produces a motility decrement, whereas preventing the Glu modification on stable MTs yields a motility increase.

During migration of sparse fibroblasts, one role alleged for







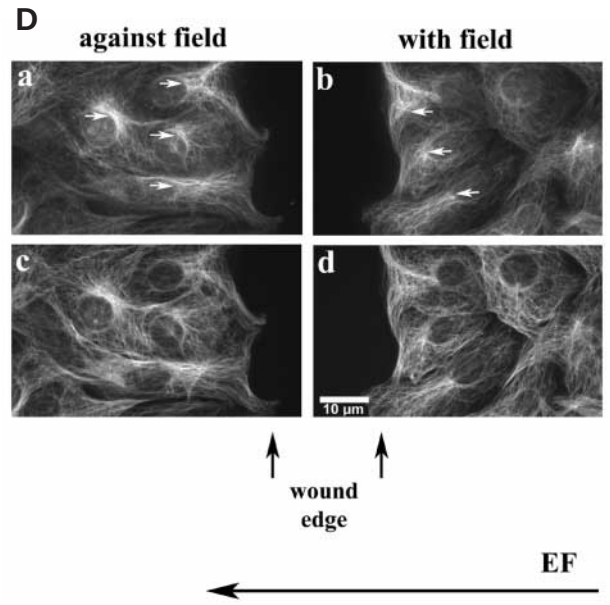


Fig. 4. Migration of monolayer cells exposed to wound-healing and EF signals. (A) EF application after wounding hastens initiation and biases motility direction of monolayer cells. Wounded 3T3 cell monolayers were placed in the indicated EFs as rapidly as practical after wounding (~30 minutes) with the length of wounds oriented perpendicular to the EF. Cell movements in a 1.5 hour interval (i.e. 0.5-2.0 hours after wounding) with the EF (towards cathode) and against the EF (towards anode) were plotted as absolute values of directional velocity. Notice that EFs increased initial speed toward the cathode significantly (P<0.05, marked with §, for comparing 2 V cm^{-1} and 0 V cm^{-1}). EFs also biased motility towards the cathode (P<0.05, marked with * for comparing cathode- and anode-directed velocities at both EF strengths). EFs >2 V cm^{-1} were not used; they caused cells to round up and motility was compromised. More than 20 cells were quantified per direction, and three experiments per condition. (B) Cells in monolayers simultaneously wounded and placed in EFs migrate preferentially towards the cathode. 3T3 cell monolayers placed in a 0.6 V cm^{-1} EF 30 minutes after wounding migrated towards the anode (+) and cathode (–) during a 3-hour recording; black line traces the initial wound edges. Notice that cells migrated farther with than against the EF (A). Scale bar, 40 μm. Also, see supplementary movie of wound-healing migration Wound_2V_3hr (http://jcs.biologists.com/supplemental/). (C) Applying EFs to pre-polarized cells directionally biases wound-healing motility. Confluent 3T3 cell monolayers were wounded and incubated for 2.5 hours to allow reorientation of cytoskeletal components toward the wound edge and were then observed for 1.5 hours (i.e. 3.0-4.5 hours after wounding) with EFs indicated. Directional velocities show that wound-healing speed of cells toward the cathode was unaffected by applied voltage (0.6 V cm^{-1} and 2.0 V cm^{-1} were equivalent to 0 V cm^{-1}, P>0.05), but speed toward the anode was decreased by the EF (P<0.05 at 0.6 V cm^{-1}). Statistical significance, § and *, shown as in (A). (D) MT cytoskeleton reorients toward wound edge with and against the EF. 3T3 cell monolayers treated as in (C) were placed in an EF (0.6 V cm^{-1}) 0.5-2.5 hours after wounding. (a,b) Glu and (c,d) total tubulin antibody staining of cells migrating against (a,c) or with (b,d) the EF shows that cells oriented MTOCs (white arrows) and stable Glu MTs towards the wound edge, regardless of their directional velocity (C) and of whether they faced the cathode or anode. Short black arrows show the wound edge for each pair of micrographs; long black arrow shows the EF direction in a-d. Scale bar, 10 μm.

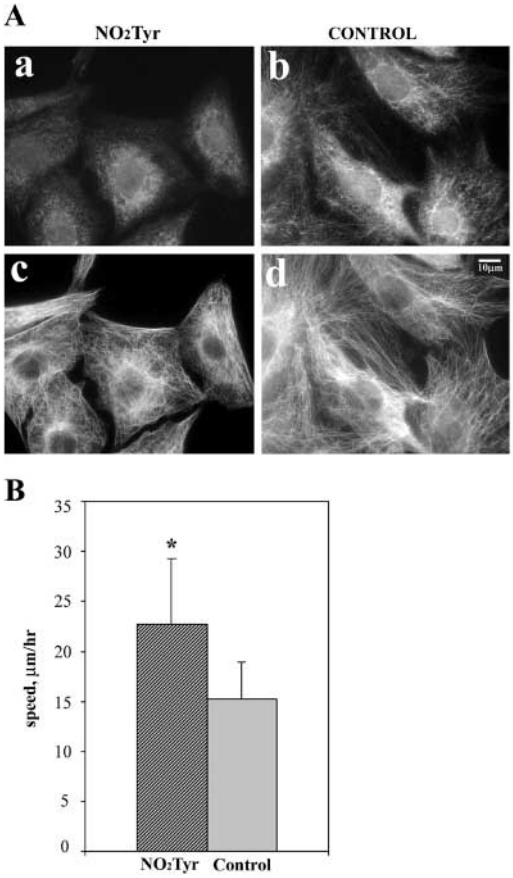


Fig. 5. Detyrosinated MTs decrease EF-mediated motility. (A) NO_2Tyr inhibits Glu MT elaboration. Incubation of 3T3 cells with (NO_2Tyr; a,c) or without (control, b,d) NO_2Tyr, which inhibits the formation of Glu MTs (a,b) without altering the total MT network (c,d) or the stable, acetylated MTs (Chang et al., 2002) (not shown). Scale bar, 10 μm. (B) Cells lacking Glu MTs migrate more rapidly in the EF. Average motility speed (μm hour^{-1}) of NO_2Tyr-treated 3T3 cells in a 6 V cm^{-1} EF (1.5 hours) was significantly greater than control cells (marked with *; $P<0.05$). At least 20 cells were quantified in each of three experiments.

MTs is promoting substratum adhesion remodeling and release (Kaverina et al., 2002a; Kaverina et al., 1999). In fact, cells lacking MTs accumulate adhesive contacts owing to their hampered turnover (Kaverina et al., 1999). It seemed likely that EFs activate mechanical events such as membrane ruffling and/or signaling that sets motility direction, but they might be less effective at inducing adhesive changes. Thus, we hypothesized that MTs play a similar role during EF motility – accelerating motility by stimulating adhesion remodeling. We tested this hypothesis by asking whether the tenacity of cell-substratum adhesions limits EF migration speed. We proposed that cells allowed to spread on a substratum for increasing times become more tightly adherent, because they display substratum contacts over a larger cell surface area or 'footprint', and/or exhibit a larger area of direct contacts with the substratum. Fig. 6Aa-c shows the morphology of cells plated onto uncoated glass for 1-24 hours. At 1 hour, >95% of cells were attached and beginning to spread, as expected (e.g. Stockton and Jacobson, 2001) but, at 3 hours and 24 hours, cells were flatter, exhibiting 1.8-times and 2.9-times, respectively, the average surface area (footprint), of 1-hour-spread cells ($P<0.5$ for comparison of each pair of spreading times). To test whether increased spreading time also increased the area in direct substratum contact, we examined TIRF and corresponding immunofluorescence images of paxillin-immunostained cells (Fig. 6Ba-c,a′-c′). The average area per cell of paxillin-stained focal contacts, quantified after 3 hours of spreading, was indistinguishable from that at 1 hour (0.91-fold, $P=0.76$) but increased 1.8-times after 24 hours of spreading ($P=0.004$).

Next, given that cell footprint, but not substratum-contact area, increased between 1 hour and 3 hours of spreading, and that both parameters increased after 24 hours of spreading, we tested adhesion strength directly. We eschewed enzyme-based assays (e.g. Chiarugi et al., 2000) in favor of a mechanical assay loosely based upon Burdsal et al. (Burdsal et al., 1991), because the EF seemed likely to provide a mechanical de-adhesion stimulus during migration. Our assay, measuring cells' centrifugal de-adhesion from upside-down coverslips, corroborated the visual impression; that is, cells that appeared flatter, with a larger footprint, better resisted de-adhesion (Fig. 6C).

Consistent with the hypothesis that adhesion tenacity limits EF migration speed, less tightly adherent 1-hour-plated cells were faster (Fig. 6D). The speed of cells plated for 3 hour and 24 hour intervals were indistinguishable, consistent with their similar resistance to being centrifugally dislodged (Fig. 6C). These data supported our hypothesis; in less tightly adherent cells, EFs promoted movement at the leading edge and/or release of adhesions at the trailing edge. In contrast to their greater EF migration, we note that 1-hour-plated cells are less likely to be spontaneously motile than 3-hour- and 24-hour-plated cells; fewer than half (44%) of the 1-hour-plated cells showed the asymmetric footprints and adhesion distributions required for spontaneous migration (M. Dubin-Thaler and B. Sheetz, personal communication), compared with 70% of 3-hour- and 77% of 24-hour-plated cells (Fig. 6Aa-c,a′-c′). We also found tighter adhesion in cells whose footprint was increased, without increase in substratum contact area. Our measurements do not address the probability that tighter cell adhesion reflects decreased dynamics of cell-substratum contacts during spreading (Geiger et al., 2001).

To address the hypothesis that dynamics of cell adhesion structures influences EF motility, we considered the range of signaling molecules known to modulate either the steady-state level or the dynamics of focal adhesions or associated microfilament structures. For example, Rho family GTPases signal remodeling of the cytoskeleton and adhesion during motility (reviewed by Evers et al., 2000; Kaverina et al., 2002b). In particular, PAK4, a serine/threonine kinase activated by the Rho GTPase Cdc42 augments actin polymerization, induces filopodia formation (Abo et al., 1998), stimulates LIM kinase and cofilin phosphorylation (Dan et al., 2001), and decreases cell adhesion (Abo et al., 1998) when overexpressed in 3T3 fibroblasts. Although less is known about their cells' adhesion or microfilament structures, mice with PAK4 genetically knocked out show an embryonic lethal phenotype at embryonic day 9.5 (Qu et al., 2003).

Owing to PAK4's roles in dynamics of microfilament assemblies and adhesion structures, we tested EF motility of

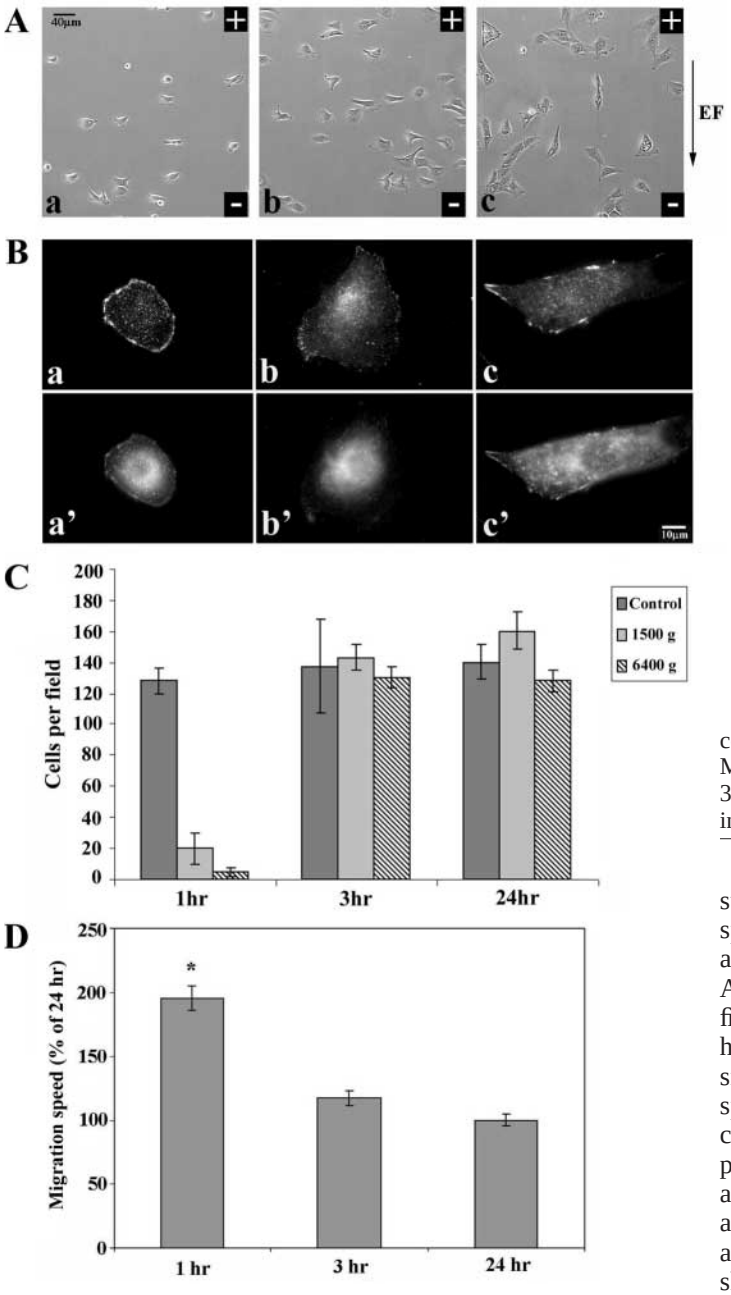


Fig. 6. Increased spreading time alters morphology and speed of cells migrating in response to an EF. 3T3 cells plated on acid-washed glass coverslips at T=0 were allowed to attach and spread for 1-24 hours. (A) Morphology of cells undergoing EF-mediated motility is altered by spreading time. Micrographs excerpted from time-lapse images of cells migrating after spreading for 1 hour (a) show that cells are less flat than after 3 hours (b) and 24 hours (c). The cathode (–) and 40 μm bar (a) are indicated. (B) Increased spreading time alters cell footprint and substratum contact area. TIRF (a-c) and corresponding immunofluorescence (a′-c′) micrographs of cells spread for 1 hour (a,a′), 3 hours (b,b′) and 24 hours (c,c′). TIRF micrographs (a-c) were used to quantify the cell surface area directly contacting substratum. Immunofluorescence micrographs (a′-c′) were used to show that cell surface area ('footprint') and proportion of cells with asymmetric lamellipodia both increased with spreading time (more than 12 cells in each of two experiments). Scale bar, 10 μm. (C) Increased spreading time alters adhesion strength. The number of cells per field remaining after centrifugation (1500 ***g*** or 6400 ***g***) was compared with the control (not centrifuged) for cells plated 1 hour, 3 hours and 24 hours before fixation. Centrifugation detached >80% of 1-hour-plated cells but did not significantly detach 3-hour- or 24-hour-plated cells. (D) EF-motility speed is altered by spreading time. Average speeds (μm hour^{-1}) of cells plated for specified times were quantified from time-lapse recordings of EF motility (6 V cm^{-1}; 1.5 hours); speeds were normalized to the 24-hour-plated cells (100%) (at least 18 cells in each of three experiments). Migration of 1-hour-plated cells (*) was significantly faster than 3-hour- and 24-hour-plated cells (P<0.05); the latter two were indistinguishable.

cells with altered PAK4 levels. These experiments might not only inform us about how EFs mediate motility but might also further elucidate PAK4 functions. An ancillary advantage of EF-motility assays of cells altered in PAK4 level is that, unlike wound-healing assays, which require ~10^6 cells in a uniform monolayer, EF motility allows a quantitative, rapid assay requiring fewer cells (~2×10^4), commensurate with small numbers of cells available from transfections or from tiny PAK4-null embryos. Also, because EFs increase motility speed, any differences are readily measurable within an interval (2-4 hours) shorter than that amenable to studies of slow-moving cells (e.g. Ronnov-Jessen and Petersen, 1996).

We examined fibroblasts with altered PAK4 expression, first comparing 3T3 cells stably overexpressing PAK4 to transfectants with plasmid alone. Fig. 7A shows that PAK4 overexpression more than doubled migration speed. This result suggested that PAK4 overexpression might enhance migration speed by acting at a step rate-limiting for motility. If so, ablating PAK4 expression would decrease migration speed. Accordingly, we compared EF-migration of primary fibroblasts from PAK4-null (–/–) mice to those from PAK4-heterozygous (+/–) littermates. PAK4-null mice showed a small but significant (~30%; P<0.05) decrement in motility speed (Fig. 7B), supporting the hypothesis that PAK4 signaling contributes to a rate-limiting step in EF-motility. PAK4 probably augments the dynamics of adhesive structures or associated microfilament structures, because PAK4-null cells appear similar in steady-state elaboration of filopodia, appearance of adhesion plaques and size of cell footprint (not shown). Although PAK4-null cells appeared slightly increased in substratum contact area over PAK4 heterozygotes, the increase was not statistically significant (16% increase, N=30 cells each; two experiments; P=0.17).

Discussion

EF effects on spontaneous and wound-healing motility are potentially relevant to normal physiology and to engineering applications. Our experiments revealed that sparse cells migrated immediately upon EF application and were enhanced in speed and directionality. Fibroblasts on the cathode-facing side of wounds in monolayers also began moving precipitously when exposed to EFs, instead of exhibiting the 1.5-2.5 hour delay characteristic of wound-healing motility in the absence of an EF (Fig. 4) (Gundersen and Bulinski, 1988). If conditions in three dimensions are similar to our two-dimensional system, EFs' enhanced induction of motility of cathode-facing cells

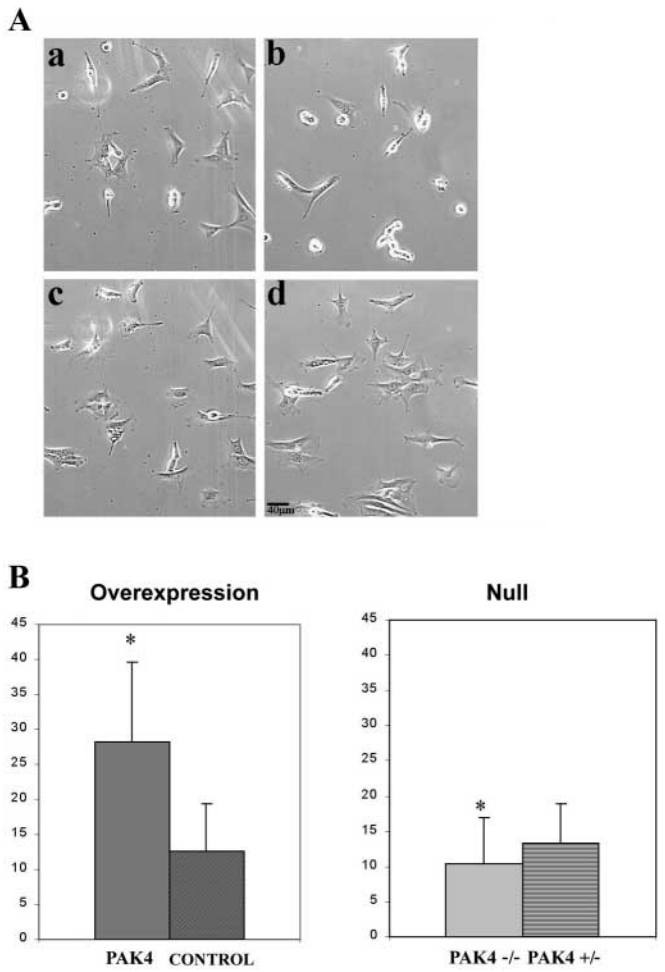


Fig. 7. Altering PAK4 expression alters EF motility. (A) Morphology of fibroblasts with altered PAK4 levels. Phase-contrast images of 3T3 cells stably transfected with PAK4 (a) or empty vector (b) show that most PAK4 transfectants were flatter and more bipolar than controls, in which 20-25% of cells appear spherical before EF application. Primary fibroblasts from mouse embryos whose PAK4 expression was knocked out by homologous recombination ($PAK4^{-/-}$; c) and PAK4-expressing heterozygotes ($PAK4^{+/-}$; d) were morphologically indistinguishable. Scale bar, 40 μm. (B) Altered PAK4 expression alters EF-motility speed. EF-motility speed of PAK4 transfectants (2 hours; 6 V cm^{-1}) was significantly greater than control 3T3 cells ($P<0.05$, marked with *). Embryonic fibroblasts from PAK4-null mice ($PAK4^{-/-}$) migrated significantly less rapidly than fibroblasts from PAK4-heterozygous littermates ($PAK4^{+/-}$) ($P<0.05$). At least 17 cells were tested in each of three experiments for each comparison.

might affect wound healing in situ, as follows. Polarized epithelia generate electrical potentials that support an 'inside-positive' EF (Barker et al., 1982). At steady state, fibroblasts in mesodermal tissue underlying the basal (anodal) epithelial surface would be blocked by the intact epithelium from receiving cathodal migration-inducing signals. However, a wound that spanned the epithelium and penetrated underlying mesoderm would release electrotactic signals from the apical (cathodal) epithelial surface. These signals would be expected to induce fibroblast migration from areas deep in the wound towards the epithelial layer on the outside surface (Barker et al., 1982). Electrotactic signals would arise concomitant with, and be limited to, the duration of wound healing, because signal from the outer, cathodal surface would halt once the wound surface healed and the epithelial layer was intact once again. Meanwhile, in vitro experiments (Fig. 4), in which the EF crippled migration speed of anode-facing wound-edge cells, suggest that EF signals present after healing, derived from the basal (anodal) surface, would stop but would not reverse the direction of cell migration during maintenance of fibroblastic tissue underlying an epithelium. Our data raise the possibility of engineering artificial EFs to promote wound healing, to generate new mesodermal tissues or to inhibit hyperactive migration into areas of forming scar tissue.

A plethora of studies have used EF migration to model epithelial wound healing (e.g. Gaffney et al., 1999; Wang et al., 2003; Zhao et al., 1996b), whereas EF responses of the fibroblasts known to underlie EF-generating epithelia have been more limited (Brown and Loew, 1994; Erickson and Nuccitelli, 1984; Sillman et al., 2003; Soong et al., 1990). One study (Soong et al., 1990b) even reported that corneal fibroblasts migrate anodally (i.e. opposite to other fibroblasts). EF-motility experiments using the familiar 3T3 fibroblast line as a model will help to elucidate the responses expected from fibroblasts to EFs generated by an organism's overlying polarized epithelia.

When applied to monolayers and sparse cells, EFs elicited intriguingly different responses. First, dose dependence differed markedly. For sparse cells, EFs <2 V cm^{-1} produced no effects, 2-6 V cm^{-1} linearly increased speed and directionality (Fig. 1A,B), and EFs ≥10 V cm^{-1} were poorly tolerated. By contrast, for wound-facing monolayer cells, EFs of only 0.6-2 V cm^{-1} yielded directional motility (Fig. 4A); these EFs yielded no time lag before cells commenced cathode-facing migration and no change in final speed (Fig. 4C). EFs >2 V cm^{-1} were deleterious. One possible explanation for dissimilar EF effects on monolayers and sparse cells is that the former experience the EF as a voltage drop across the width of several adjacent, contacting cells communicating via gap junctions (Azarnia and Russell, 1985; Saito et al., 1998), whereas the latter experience a smaller voltage drop across the width of each individual cell.

Sparse and monolayer cells also gave different morphological responses to EFs. For sparse cells, increased EF strength correlated with increased alignment perpendicular to the direction of motility. By contrast, wound-healing monolayer cells reoriented their cytoskeletons parallel, not perpendicular, to their motility direction (Fig. 4D), a wound-induced process that was independent of EFs. Thus, when cultured at different densities, the same cells showed distinctly different morphological and motility responses to applied EFs.

In contrast to other motility inducers, EFs induced fibroblasts to move directionally, whether or not they had reoriented/re-aligned relative to their migration and whether or not re-alignment was perpendicular (sparse cells) or parallel (monolayer cells) to the EF. Our experiments tested the hypothesis that directional migration requires that fibroblasts reorient in the direction of movement, as they do during spontaneous or wound-healing migration (Palazzo et al., 2001b; Ueda et al., 1997). MTOC reorientation does not occur in fibroblasts migrating within three-dimensionally oriented collagen gels (Schutze et al., 1991) or in wound-healing

migration of epithelial cells (Yvon et al., 2002), but the need for MTOC reorientation during two-dimensional migration of fibroblasts had not been assessed previously. In the presence of an EF, MTOC reorientation during migration is neither necessary (because cells do not orient their MTOC in response to EFs, and wound-healing cells exposed to the EF start migrating before MTOCs have reoriented), nor sufficient (because anode-facing cells do not migrate significantly even though their MTOC has appropriately reoriented). Perhaps directing nascent proteins from the MTOC-associated Golgi to the nearby leading edge of a motile fibroblast (Bergmann et al., 1983) is not rate limiting. Also, the EF, constantly present and dictating motility direction, might supersede the MTOC's usual role in promoting persistent direction by stabilizing new lamellipodia (Ueda et al., 1997). Thus, orientation of cytoplasmic components might signal aspects of motility direction in other systems, but these roles might be supplanted by an EF.

Testing the hypothesis that directional migration in the presence of an EF does not require Glu MTs yielded our most striking result to date. Not only did failure to detyrosinate stable MTs not compromise EF motility, it actually increased its speed, suggesting that detyrosination supplies an inhibitory signal or 'brake' for motility. Detyrosination has been postulated as a signal that a MT is a stable one; however, this signal must be unnecessary or inhibitory to MT functions during EF motility. Moreover, cells might use detyrosination of stable MTs, which accompanies many differentiative events (Bulinski and Gundersen, 1991), to signal a slowdown in cell motility. Alternatively, Glu MTs might simply be less effective in supporting processes required for adhesion/de-adhesion remodeling, which cell spreading experiments suggest are rate limiting for EF motility. Although cells with and without Glu MTs showed no significant difference in cell footprint or substratum contact areas (data not shown), a plausible hypothesis to be tested in future experiments is that adhesion/de-adhesion dynamics increase in cells lacking Glu MTs. In principle, it would be instructive to perform the reciprocal of our NO_2Tyr experiments; that is, to test whether increased Glu MT level slows EF migration. Treatment with low Taxol concentrations increases Glu MTs, concomitantly decreasing EF motility speed (data not shown), but these effects are probably attributable to Taxol-induced MT stability changes. At present, there is no known strategy for increasing the Glu MT level without concomitantly increasing MT stability.

The facts that EF motility requires intact MTs for maximum speed but requires neither their subcellular reorientation nor their post-translational modification, hints at possible MT functions during EF-mediated motility. First, MTs might coordinate intracytoplasmic signaling. Dynamic MTs, which rapidly penetrate all cytoplasmic regions, are required for wound-healing migration (Liao et al., 1995). Many signaling molecules known to interact with MTs [e.g. Rho GTPases and protein kinases (Gundersen and Cook, 1999)] effectively hitchhike via MT-based transport, passing signals from the far reaches of the cytoplasm to the nucleus and back to leading, ventral and trailing edges. Because MTs also interact dynamically with microfilaments, the contractile machinery is a logical target of MT-mediated structural organization or signaling activity (Waterman-Storer and Salmon, 1999). This communication potentially maximizes the efficiency of EF-motility. Differential binding or inefficient transport of signaling molecules on Glu MTs might explain the more rapid EF migration we observe in the absence of these MTs. Consistent with our finding that inhibition of Glu MTs accelerates EF motility, inhibition of another MT post-translational modification, acetylation, accomplished via transfection of the MT deacetylase HDAC6 was also shown to increase 3T3 cell migration speed, although no mechanism for this inhibition has been proposed (Haggarty et al., 2003; Hubbert et al., 2002).

A second possible MT function during EF motility was suggested from studies of spontaneous migration of fish fibroblasts. Kaverina et al. (Kaverina et al., 1999) showed that MTs targeted to adhesion complexes, along with activity of the MT motor kinesin (Kaverina et al., 2002b), enhance the turnover and recycling of substratum-adhesion complexes, possibly by locally activating Rac or Rho (Kaverina et al., 2002b). Neither spontaneously migrating fish fibroblasts nor EF-migrating 3T3 fibroblasts require MTs for membrane protrusion and lamellipodial locomotion. Analogously, the fact that EFs can induce motility suggests that they provide cues sufficient to mediate turnover of adhesion complexes. However, MTs might accelerate this rate-limiting step in motility by transporting molecules that augment recycling to cells' ventral or trailing edges. Kaverina et al. (Kaverina et al., 2000) showed in migrating fish fibroblasts that MTs could be obviated by externally regulating cellular contractility at the rearward cell surface, near de-adhesion sites. Thus, MTs could maximize EF-mediated movement because the EF asymmetrically modulates contractility at leading or trailing edges in a manner only partially mimicking MTs' modulation of contractility (Small and Kaverina, 2003). EF motility, a system in which fibroblasts migrate with uniform direction and fairly uniform speed, might offer a convenient system for further studies of MTs' role(s) during motility.

The EF motility of cells in which adhesion/de-adhesion was altered, independent of MT breakdown, also suggests that de-adhesion is rate limiting for motility. For example, increased activity of the Cdc42 activator PAK4 disrupts focal contacts and decreases cell adhesion (Qu et al., 2001). We found that PAK4-overexpressing transfectants showed faster migration, whereas PAK4-null cells showed a small but significant decrement in speed (Fig. 7). Kaverina et al. (Kaverina et al., 1999) suggested that Rac and Cdc42 signaling increases the turnover of adhesion complexes. Consistent with their data, we also observed rapid migration of cells allowed to spread for only 1 hour before EF application; these cells were not only less flat but also less adherent, possibly because focal contacts are rapidly remodeled or turned over in spreading cells (Geiger et al., 2001) (Fig. 6A-C). Because it is likely that alterations in steady-state level or dynamics of adhesions contribute to altered EF migration of tumor cells (Djamgoz et al., 2001; Siwy et al., 2003), our results raise the possibility of eventually using EFs for therapeutic or diagnostic purposes.

Fish keratocytes, which migrate very rapidly in the absence of MTs (Euteneuer and Schliwa, 1984), exhibit a morphology unlike that of 3T3 cells undergoing spontaneous or wound-healing migration but strikingly similar to EF-migrating 3T3 cells (Galbraith and Sheetz, 1999). In most instances, cells display a wide but shallow lamellipodium and a small trailing edge that

may be smooth or exhibit a retraction fiber. Like keratocytes, EF-migrating cells do not require MTs for migration. It is intriguing that application of an EF appears to switch some motile characteristics of a single cell type, 3T3, from those of vertebrate fibroblasts to those typical of fish keratocytes. It should be realized that cells subjected to both EF and wound-healing cues take on the morphology of the latter (e.g. Fig. 3). Clearly, applying EFs to fibroblasts, with and without other motility cues, might provide a system in which common denominators of motility mechanisms can be elucidated.

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