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Differential Effects of Matrix and Growth Factors on Endothelial and Fibroblast Motility: Application of a Modified Cell Migration Assay

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Abstract Cell migration is crucial in virtually every biological process and strongly depends on the nature of the surrounding matrix. An assay that enables real-time studies on the effects of defined matrix components and growth factors on cell migration is not available. We have set up a novel, quantitative migration assay, which enables unharmed cells to migrate along a defined matrix. Here, we used this so-called barrier-assay to define the contribution of fibronectin (FN) and Collagen-I (Col-I) to vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and lysophosphatidic acid (LPA)-induced cell migration of endothelial cells (EC) and fibroblasts. In EC, both FN and Col-I stimulated migration, but FN-induced motility was random, while net movement was inhibited. Addition of bFGF and VEGF overcame the effect of FN, with VEGF causing directional movement. In contrast, in 3T3 fibroblasts, FN stimulated motility and this effect was enhanced by bFGF. This motility was more efficient and morphologically completely different compared to LPA stimulation. Strikingly, directional migration of EC was not paralleled by higher amounts of stable microtubules (MT) or an increased reorientation of the microtubule-organizing centre (MTOC). For EC, the FN effect appeared concentration dependent; high FN was able to induce migration, while for fibroblasts both low and high concentrations of FN induced motility. Besides showing distinct responses of the different cells to the same factors, these results address contradictive reports on FN and show that the interplay between matrix components and growth factors determines both pattern and regulation of cell migration. J. Cell. Biochem. 99: 1536–1552, 2006.

Key words: cell migration; extra cellular matrix; growth factor; fibronectin

INTRODUCTION

Cell migration is essential in virtually all processes during life. Migration of cells is fundamental in both physiological and pathological processes, like embryonic development, wound healing, cancer metastasis, and angiogenesis. Migration of a cell starts with the formation of membrane protrusions in the direction of migration, resulting from actin polymerization [Lauffenburger and Horwitz, 1996; Horwitz and Parsons, 1999]. In order for a cell to migrate, it also needs to polarize itself, which may lead to the formation of triangular cell morphology, with a broad leading edge at the front of the cell and a thinning trailing edge at the back. During polarized migration, the Golgi apparatus and microtubule-organizing centre (MTOC) often are positioned in front of the nucleus. In a number of cell types, microtubules (MT) selectively stabilize in the migration direction during polarization of the cell [Gundersen and Bulinski, 1988]. To sustain polarity and migration, new adhesions between the cell and the extracellular matrix (ECM), called focal complexes, are established at the leading edge [Nobes and Hall, 1995], some of

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which develop into focal adhesions, while focal adhesions at the trailing edge of the cell are broken down. Cell migration can be subdivided into random motility and directed migration, the latter indicating whether a cell is able to maintain a single direction of migration for prolonged periods of time. The composition of the ECM, availability of growth factors and cytokines, physiological circumstances like pH and pO_2 and, of course, intracellular constituents, all together regulate cell polarity and migration.

The nature of the surrounding matrix determines to great extent the migration response of cells. For example during angiogenesis, migration of endothelial cells (EC) is crucial [Bergers and Benjamin, 2003; Carmeliet, 2003]. However, the setting in which EC migration takes place strongly differs. During tumor angiogenesis, activated EC migrate along a newly formed matrix, of which the components are synthesized by EC, tumor cells, and stromal cells [Bussolino et al., 1997]. During wound angiogenesis, in contrast, migration is stimulated by factors triggered by damage to EC and ECM. These differences affect migratory response of the EC. However, currently no assay is available to address these differences. Among angiogenic factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are well known for their regulation of proliferation, migration, and differentiation of EC [Cross and Claesson-Welsh, 2001], but how these two factors relate to each other and to ECM-components, when inducing EC migration during tumor angiogenesis, remains to be elucidated. It would be of great benefit if an assay existed in which these distinct signaling cascades leading to EC migration could be dissected [McDonald et al., 2004].

Measurement of freely moving and spreading cells (single cell movements) has provided valuable knowledge on cell migration, including that of EC [Fischer et al., 2003], but these studies do not address the properties of a layer of cells in which cells are migrating into a defined cell-free area. Moreover, the matrix-composition is not completely defined. Several migration assays have been developed to deal with this, such as the Agarose droplet [Varani et al., 1978], the Teflon ring [Pratt et al., 1984], and the Flexi perm disc [Ohtaka et al., 1996] assays. The latter two are used rarely and are not always applicable for living cell imaging. The agarose droplet assay has practical disadvantages regarding reproducibility and standardization. Using the Teflon Ring, it has been reported that fibronectin (FN) has an inhibitory effect while Collagen-I (Col-I) has a stimulatory effect on EC migration. However, only a fixed time-point of 6 days was taken [Pratt et al., 1984]. For these reasons, the Boyden Chamber assay and the scratch or wound healing assay are often used [Auerbach et al., 1991]. In the first, cells are scored for their ability to pass a filter, but cellular behavior during migration cannot be monitored. To study the role of ECM-components in this assay, either migration towards (factors in lower chamber) or into (pre-coated filters) can be evaluated. A migration-promoting role for ECM-components like FN, Col-I, and laminin has been reported for several cell types like fibroblasts, tumor cells, and EC. For EC, Col-I and FN have been suggested to be more effective than laminin [Postlethwaite et al., 1981; McCarthy and Furcht, 1984; Herbst et al., 1988]. For FN, the results seem dependent on the assay used, suggesting that either other factors are involved or that the assay does not properly address the effect of FN.

In the scratch assay, cells are grown until they reach confluence and a mechanical wound (the scratch) is made, for example with a tip or needle, after which cells can migrate into the novel cell-free area (the wound). For EC, it has been reported that no differences exist between FN and Col-I in their effect on migration [Young and Herman, 1985]. For fibroblasts and other stromal cells, FN is reported to provide a pathway during migration into the wound [Clark et al., 2003]. Strikingly in 3-D assays, the migration of fibroblasts is not induced by FN [Schor et al., 1996]. Using the scratch assay, the behavior of cells can be monitored accurately; however, cells have to be removed in order to generate a cell-free area. The remaining cells first have to recover from wounding, and the composition of ECM in the newly generated, cell-free region, cannot be controlled. The scratch assay is, therefore, a suitable in vitro model for wound healing and tissue repair, but whether it represents an authentic migration model has not yet been addressed.

We developed a novel migration assay that overcomes most of the restrictions of the other methods and enabled us to study the distinct contributions of ECM-components and growth

factors. Using this novel barrier-assay, we studied migration of EC and 3T3 fibroblasts, to enable comparison of well-established cell culture systems for motility. For EC, we document a migration promoting effect of Col-I and FN, yet a strong inhibitory effect of FN on directional movement. The addition of VEGF or bFGF mitigated the effect of FN, with VEGF being a stimulator of effective migration and bFGF a stimulator of general motility. In contrast, in 3T3 fibroblasts, FN stimulated migration in the absence of growth factors. Addition of bFGF augmented migration distance induced with FN. Lysophosphatidic acid (LPA), a known regulator of fibroblast polarity in the scratch assay [Nagasaki and Gundersen, 1996; Cook et al., 1998; Wen et al., 2004], induced comparable migration in the presence or absence of FN, while migration was less directional and morphologically different compared to bFGF. For EC, we observed a FN dose-dependent migration, while for fibroblasts FN appeared capable of inducing motility in all concentrations tested. First, together these data reveal novel interplay between growth factors and the ECM in cell motility. Second, we expect wide spread application of the barrier assay because of its ease of use. This will lead to a more detailed dissection of the molecular mechanisms and signaling pathways underlying cell migration and the specific functions of ECM components and growth factors in this fundamental process.

MATERIALS AND METHODS

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVEC) were isolated as described (Jaffe et al., 1973). Human micro vascular endothelial cells (HMVEC) and Swiss 3T3 fibroblasts were obtained commercially (Biowhittaker). EC were used between passage 3 and 7 and cultured in human endothelial-SFM (Invitrogen) supplemented with 10% new born calf serum, 5% human serum, 20 ng/ml bFGF, and 100 ng/ml EGF, in gelatine-coated flasks. Swiss 3T3 fibroblasts were cultured in DMEM/Ham's F10, 1:1 (Biowhittaker) supplemented with 10% fetal calf serum. VEGF-165 and bFGF were from PeproTech. Gelatine, Thalidomide, and LPA were from Sigma-Aldrich. Col-I was from Biowhittaker, FN from Roche Diagnostics, and SU-5416 was a gift of Dr. W. Leenders, Department of Pathology, University Medical Centre St. Radboud, Nijmegen, The Netherlands.

Migration Assays

For the novel barrier migration assay, a cover slip was placed in an Attofluor incubation chamber (Molecular Probes), which was subsequently sterilized. In this set up a removable, sterile circular migration barrier was placed (see Fig. 1D), which fits tightly in the chamber and prevents cell growth in the middle of the coated cover slip. Cells were seeded around this barrier and grown until confluence. Subsequently, the migration barrier was removed; cells were washed twice and then incubated with the appropriate medium. The incubation chamber was placed on an inverted microscope and migration of cells was measured for 24 h. The scratch or wound-healing assay was essentially performed as described by many others [Nagasaki and Gundersen, 1996], using the same Attofluor incubation chamber, but instead of the barrier, a scratch was made to generate a cell-free area.

Time-lapse imaging of both types of cell migrations was done on Axiovert 100 M microscopes, equipped with either an AxioCam HRC digital camera or an AxioCam MRC digital camera (Carl Zeiss). Microscopes were controlled by AxioVision software, version 3.1 and 4.0, respectively. Cells in the incubation chamber were maintained at 37°C in a constantly humidified atmosphere, with controlled and heated CO_2-flow. Cells were imaged every 12 min with a 10×/0.30 PLAN-NEOFLUAR objective lens or every 2 min with a 20×/0.40 LD ACHROPLAN objective lens (Carl Zeiss).

During migration of HUVEC, human endothelial SFM without standard growth factors was added to the cells, supplemented with bFGF (200 ng/ml) or VEGF-165 (10 ng/ml). In specific experiments, the inhibitors Thalidomide (40 μg/ml) or SU-5416 (30 μM) were also added. Preceding 3T3 fibroblast migration, cells were starved in serum-free culture medium (supplemented with 5 mg/ml fatty-acid free BSA) for 24–48 h [Wen et al., 2004] before the barrier was removed, or the scratch was induced. During migration, the same medium was added, supplemented with LPA (5 μM) or bFGF (200 ng/ml). Coated cover slips were made by adding FN (1, 10 or 100 μg/ml) or Col-I (1 or

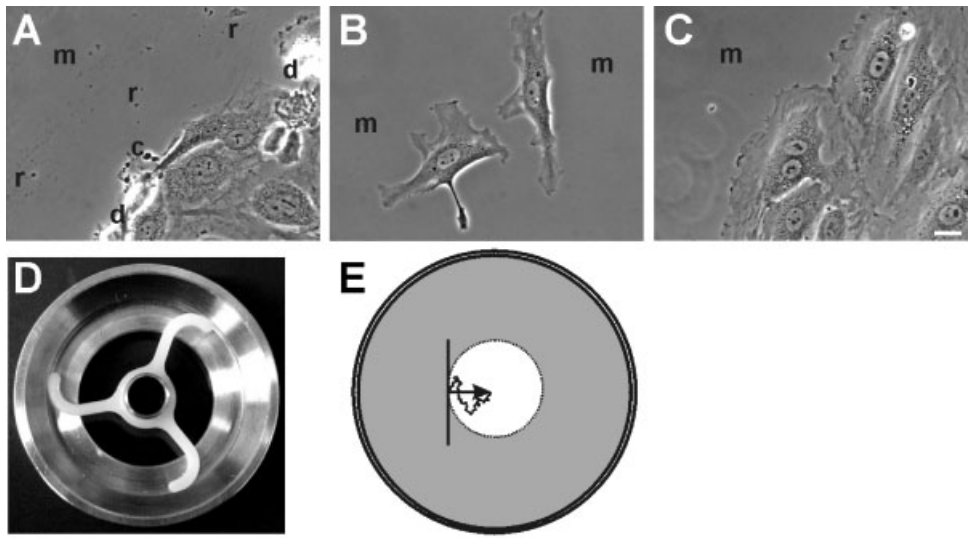


Fig. 1. Set up and rationale of the barrier migration assay. **A**: High magnification of HUVEC at migration front in the scratch assay. The cells are damaged (d) and leak cytoplasm (c). The matrix (m) in the cell-free area is contaminated with cell secreted ECM-components, growth factors and cell remnants (r). See also Movie 1. **B**: High magnification of HUVEC migrating in a single cell motility assay. During attachment, spreading and prior to the assay the cells have manipulated the matrix (m). Migrating cells follow paths of other cells where the matrix is optimal. See also Movie 2. **C**: High magnification of HUVEC at migration front in the novel assay. Cells and matrix (m) are left unharmed. The matrix can be defined and manipulated. Bar, 10 μm, See also Movie 3. **D**: Top-view photograph of the Attofluor incubation chamber with the migration barrier in place. The barrier-insert fits tightly but can be removed easily. Cells are seeded on the cover slip outside the insert and are grown until confluence, after which the barrier is removed. **E**: Schematic representation of the barrier migration assay as in vitro model for cell migration. After removal of the barrier, a cell-free area (white) is present next to a cell-dense region (gray). Cells are able to migrate into an area whose matrix composition is defined. The freehand line indicates the hypothetical path of a single cell (not in scale). The total migrated distance of this cell is tracked over time. Migration towards the center of the cover slip (arrow) is defined as effective distance reflecting the directionality of cell movements. Other parameters like velocity, cell divisions, and cell morphology changes were obtained from migration movies.

60 μg/ml) in serum-free medium to the cover slips and incubated for at least 1 h at 37°C.

Analysis of Cell Migration

Parameters of cell migration, including the total and average migrated distance, migration velocity, effective migration distance, number of cell divisions, and cell polarity, were obtained from time-lapse movies, taking the nucleus as a reference. For each treatment, at least 10 migrating cells per experiment and at least 3 independent migration assays were performed. After cell division, one of the daughter cells was followed.

Migration velocity was calculated after 2 h and 24 h, by dividing migration distance by time. Both the total distance (reflecting random motility) and effective distance (reflecting directed migration) were calculated, the latter being the distance that cells travelled towards the centre of the cover slip (see Fig. 1E). We also calculated effective migration, being the percentage of movement towards the centre of the cover slip reflecting directionality during migration with the lesser changes in direction being defined as more effective migration. We determined the cell elongation in the migration direction as extend of cell polarity (expressed as the ratio of the length of the major to the length of the minor cell axis) as described [Totsukawa et al., 2004], from at least eight migrating cells per time point and at least three independent assays. All measurements were done using AxioVision 3.0 software. Images from the time-lapse analysis were processed in Adobe Premiere to generate movies for publication.

Immunofluorescence Staining

After time-lapse microscopy, cells were washed twice with PBS and fixed in 4% paraformaldehyde at room temperature for 15 min or in ice-cold methanol at −20°C. After washing in PBS, cells were permeabilized for 45 min using 0.15% Triton-X-100 for 10 min in blocking solution (1% BSA/0.05% Tween-20/PBS). Incubations with first (1/200) and secondary (1/500)

antibody-mixtures were done for 1 h at room temperature in blocking solution. In between, incubations cells were washed three times with PBS/0.05% Tween-20. Thereafter, cells were briefly washed in 70% and 100% ethanol, respectively; air-dried and mounted onto microscope slides using 10 μl of a 1:1 solution of VectaShield (Vector Laboratories) and DAPI-DABCO (Molecular Probes). Primary antibodies used β-tubulin, acetylated MT, vinculin (Sigma-Aldrich), and f-actin (Molecular Probes). Secondary antibodies used Alexa Fluor 594 (Molecular Probes) and FITC (Nordic)-conjugated antibodies. Immunofluorescent images were taken using an Axiovert 100 M microscope with 40×/1.30 Oil-FLUAR objective lens (Carl Zeiss) and an ORCA II ER camera (C4742-98, Hamamatsu Photonics Systems). Image analysis, including MT stabilization, MTOC reorientation, and f-actin staining pattern analysis was performed using Openlab 3.1.5 software (Improvision). Cells were scored as positive for acetylated tubulin if the cell contained 10 or more brightly stained MT [Palazzo et al., 2004], MTOC were considered reoriented using the "triangle-method" as described [Palazzo et al., 2001b], f-actin staining pattern was analyzed by counting the stress fiber and dense peripheral bands containing cells and vinculin-positive adhesions were counted per cell. For all measurements, a total of 50–150 cells in 3 different experiments were counted. Images were processed using Adobe Photoshop.

Statistics

Groups were compared with the Kruskal–Wallis H test and considered significantly different when $P < 0.05$. Different groups were compared using the Mann–Whitney U-test (Bonferroni correction for multiple testing).

Supplemental Online Material

All movies, except 1–3, are phase-contrast or DIC movies of HUVEC and 3T3 cells migrating for 24 h. Movies contain 121 frames and were accelerated resulting in a 12 s movie. Movies 1–3 are phase-contrast movies of HUVEC migrating for 8 h and contain 960 frames. QuickTime movies correspond with still images in the figures: movie 1–3 (Fig. 1A–C), movie 4–6 (Fig. 2A), movie 7–11 (Fig. 4B), movie 12–23 (Fig. 6B), movie 24–27 (Fig. 8B) and movie 27–30 (Fig. 8B).

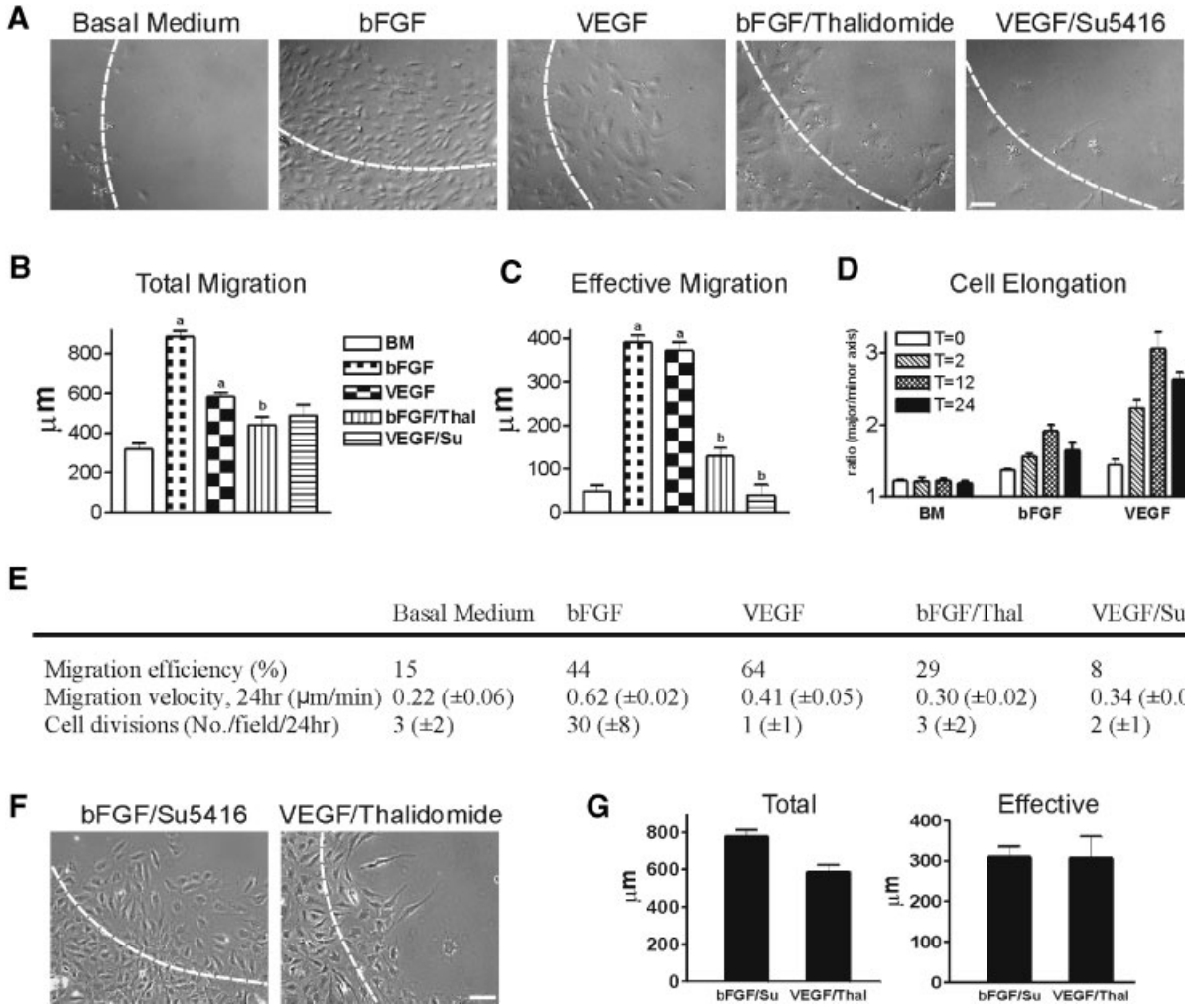
RESULTS

Novel Cell Migration Assay

To study cell migration into a defined cell-free area, often the scratch assay is used. However, by introducing the scratch, damage is inflicted to the cells (Fig. 1A, Movie 1). Damaged cells leak cytoplasm and secrete growth factors and cytokines. Most importantly, the matrix is not defined. It is made up of cell-secreted ECM-components, growth factors, and contaminated with cell-remnants. A defined matrix is also absent when single cell motility is studied. Spreading and migrating cells modify matrix composition and cells will follow patterns of other cells (Fig. 1B, Movie 2). Within the novel cell migration assay, both the cells and the matrix are intact and the matrix can be strictly controlled (Fig. 1C, Movie 3). A round, removable barrier, which prevents cell growth in the middle of the cover slip, is placed in a culture device (Fig. 1D, top view). After removal of the barrier, cells can migrate into the cell-free area, which has a matrix composition that has been defined a priori. In this barrier-assay, cells and matrix are untouched. Cell movement and morphology are tracked over time and several parameters can be determined, including (effective) migration distance (Fig. 1E), velocity, cell polarity, and number of cell divisions.

Response of EC to Growth Factors and Inhibitors Using the Novel Migration Assay

The barrier migration assay was tested by studying the response of EC to VEGF and bFGF, and inhibitors of these growth factors to verify the assay (Fig. 2). In basal medium (containing serum, without additional growth factors), HUVEC showed little motility and cells started to die at later time points, due to the lack of growth factors [Cross and Claesson-Welsh, 2001] (Fig. 2A, left panel, Movie 4). When bFGF or VEGF was added, EC started to migrate into the gelatin-coated cell-free area. Under these conditions, remarkable differences were observed between the two growth factors (Fig. 2A, see also Movie 5,6). bFGF triggered EC to migrate long distances and induced frequent cell divisions over 24 h. VEGF also induced migration but, cells migrated over less distance and sporadically divided during migration. However, the effective distance migrated (migration towards the middle of the cover slip, Fig. 2C) was the same for both growth factors, so



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	Basal Medium	bFGF	VEGF	bFGF/Thal	VEGF/Su
Migration efficiency (%)	15	44	64	29	8
Migration velocity, 24hr (μm/min)	0.22 (±0.06)	0.62 (±0.02)	0.41 (±0.05)	0.30 (±0.02)	0.34 (±0.04)
Cell divisions (No./field/24hr)	3 (±2)	30 (±8)	1 (±1)	3 (±2)	2 (±1)

Fig. 2. bFGF and VEGF induced HUVEC migration in barrier assay. Confluent HUVEC grown on gelatin were allowed to migrate for 24 h using the barrier migration assay. **A**: HUVEC migration under different treatments (basal medium (with serum, without additional growth factors), bFGF (200 ng/ml), VEGF (10 ng/ml), bFGF/Thalidomide (200 ng/ml, 40 μg/ml), VEGF/SU5416 (10 ng/ml, 30 μM)), respectively. The white dashed line indicates the migration front at T = 0 h. Pictures are at T = 24 h and obtained from migration movies available in supplemental movies 4–6, bar, 100 μm **B**: Total migrated distance (μm) per cell. At least 30 cells in at least 3 separate experiments were measured. **C**: Effective migrated distance (μm) per cell. Effective migration is the distance towards the centre of the cover slip (straight line from starting-point to end-point, perpendicular to the migration front, reflecting directionality of the movement). At least 30 cells in at least 3 separate experiments were measured. Data in B and C represent mean ± SEM from at least three independent experiments; $^{a}P < 0.05$ compared to basal medium; $^{b}P < 0.05$ compared to non-inhibited control. **D**: Cell elongation measurements as indication for cell polarity for bFGF and VEGF compared to basal medium (BM). Ratios of major and minor axis were measured in 24 cells in 3 separate experiments. **E**: Migration efficiency and velocity were calculated from the measurements shown in B and C. Cell divisions were counted over the time course of the experiment. Numbers represent the average ± SD of three independent experiments. **F**: Specificity of bFGF and VEGF inhibitors. SU5416 and Thalidomide were used in combination with their unmatched growth factors. No inhibition was observed. **G**: Total and effective distance (μm) per cell after 24 h of growth factors with their unmatched inhibitors. Data represent mean ± SEM of three independent experiments.

the migration efficiency (effective migration divided by total migration) is higher under a VEGF regime (Fig. 2E). In accordance with these results, cell elongation measurements revealed that bFGF treatment increased the ratio of major to minor axis length approximately 1.5-fold, while treatment with VEGF caused a steeper increase of approximately 2.5–3 fold (Fig. 2D). Interestingly, this steeper increase became visible only after 12 h and later.

Next to migration-induction in this novel assay, we studied inhibition of migration as well using two well-defined inhibitors. Thalidomide, known to affect bFGF [D'Amato et al.,

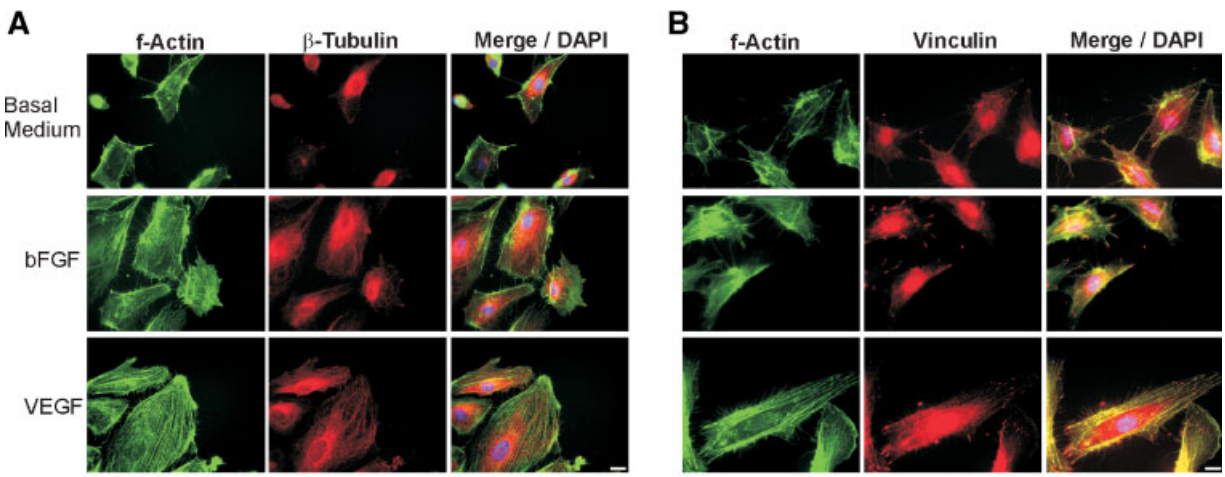
1994] and SU-5416 (a Flk1/KDR receptor-kinase inhibitor), known to inhibit VEGF [Fong et al., 1999]-induced cell activity. Thalidomide blocked the migration distance and efficiency induced by bFGF, albeit incompletely. Addition of SU-5416 only slightly affected migrated distance induced by VEGF, but migration efficiency was completely inhibited (Fig. 2B,C). These results indicate that VEGF-signaling through Flk-1 is essential for directed EC migration. Flk-1 is known to affect VEGF-induced EC migration [Bernatchez et al., 1999], but the effect on directionality is novel. Experiments done with HMVEC showed similar results (data not shown). We confirmed specificity of these two inhibitors by combining them with the non-matching growth factors. No inhibitory effects were seen when SU-5416 was used in combination with bFGF or Thalidomide with VEGF (Fig. 2F,G).

bFGF and VEGF Induce Differential Effects on Cytoskeleton and Adhesions

To examine the effect of bFGF and VEGF on cytoskeleton and adhesions, we stained fixed cells for actin, MT, and vinculin after 24 h of migration (Fig. 3). In non-stimulated cells, f-actin appeared mostly as dense peripheral bands, no stress-fibers were seen, and the MT network was poorly developed. In bFGF-induced cells, we observed stress-fibers, f-actin accumulation at the edge of cells, and a well-developed MT network. Interestingly, in most of the VEGF-induced cells f-actin is predominantly organized into stress-fibers and hardly as dense peripheral bands (Fig. 3A,C). In these cells, the MT network is well organized, with network endings in what could be focal adhesions. To test the effect on adhesions, we stained for vinculin. In addition to the cytoskeleton, adhesions are also distinct between the growth factors. We observed many vinculin-positive adhesions in VEGF-treated cells (Fig. 3B,C). When exposed to bFGF, cells displayed a rather heterogeneous expression of vinculin-positive adhesions, and were lacking in some cells. Interestingly, bFGF-treated cells displayed longer and stretched adhesions compared to VEGF. These results suggest differential organization of the EC cytoskeleton and adhesions upon stimulation with bFGF of VEGF.

Contribution of ECM Components to EC Migration Studied in Barrier and Scratch Assays

Our findings with the barrier assay are in accordance with previous reports on EC



C

f-Actin	BM	bFGF	VEGF
Stress Fibers (%)	13	45	92
Dense Peripheral Bands (%)	100	66	15
No. FA/cell (±SD)	3 (±1)	6 (±7)	14 (±7)

Fig. 3. Visualization of cytoskeleton and adhesions of HUVEC in barrier assay. HUVEC were induced to migrate for 24 h, fixed and stained for f-actin, β-tubulin, and nuclei (**A**) or f-actin, vinculin, and nuclei (**B**). A: Cytoskeleton differences in cells at migration front in a negative control with basal medium, treated with bFGF (200 ng/ml) or treated with VEGF (10 ng/ml), bar, 10 μm. B: Differences in adhesions number and structure in cells migrating in a negative control with basal medium, treated with bFGF (200 ng/ml) or treated with VEGF (10 ng/ml), bar, 10 μm. **C**: Quantification of stress fibers, dense peripheral bands, and vinculin-positive focal adhesions (FA). For actin some cells contain both types of staining resulting in more than 100% total.

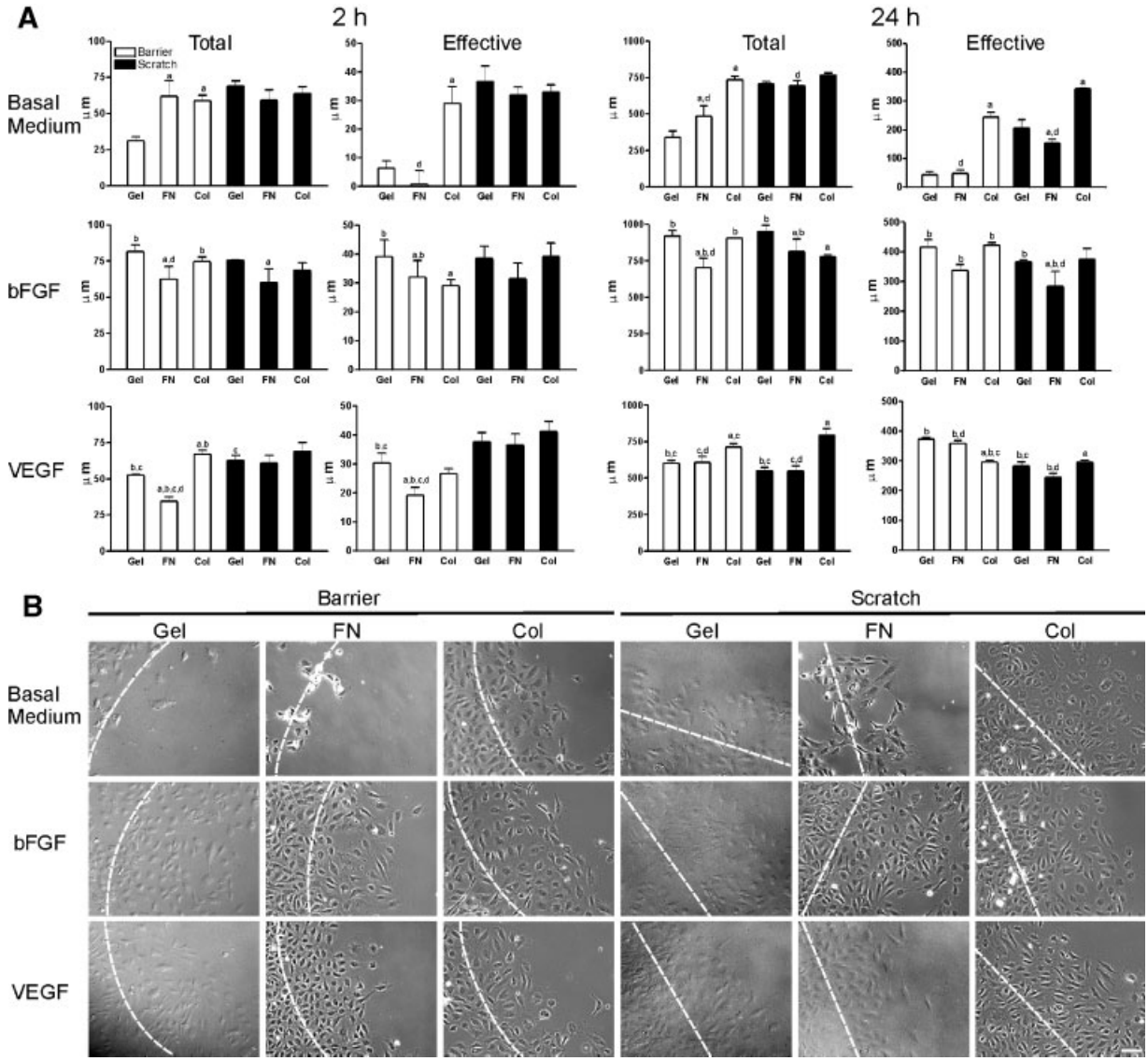


Fig. 4. Effect of ECM-components on HUVEC migration in barrier and scratch assays. HUVEC were grown on different coatings until confluence and followed for 24 h after removal of the barrier, or introduction of a scratch. **A**: Total and effective migration distances (μm) after 2 h and 24 h of HUVEC treated with basal medium (control), bFGF (200 ng/ml), or VEGF (10 ng/ml) compared between scratch and barrier assay when coated with gelatine, FN, or Col-I. At least 30 cells in at least 3 separate experiments were measured. Especially after 2 h (before cell division takes place), almost all differences found in the barrier assay are overlooked when using the scratch assay. All data represent mean ± SEM of at least three independent experiments; $^{a}P < 0.05$ FN and Col-I compared to gelatine; $^{b}P < 0.05$ bFGF and VEGF compared to basal medium (BM); $^{c}P < 0.05$ bFGF compared to VEGF on same coating; $^{d}P < 0.05$ FN compared to Col-I in same treatment. **B**: Pictures of migrating HUVEC in both assays, after 24 h obtained from migration movies (Movies 7–11 for basal medium experiments). White dashed lines indicate the migration front at T = 0 h, bar, 100 μm.

migration [D'Amato et al., 1994; Fong et al., 1999], indicating the validity of this assay. Next, we used this setup to examine contribution of growth factors and ECM components to EC migration and compared these results with the scratch assay (Fig. 4). FN coating induced migration of EC, but inhibited effective migration (Fig. 4A,B). After several hours, the cells started to die (Movie 7). FN was reported to inhibit EC migration using the Teflon ring assay [Pratt et al., 1984], while on freely moving EC, it induced migration [Chon et al., 1998]. Here, we show that FN indeed can induce migration of a layer of EC, but that individual cell movement is random resulting in an inhibition of net movement. Col-I, a known inducer of EC migration [Pratt et al., 1984], induced total and effective migration (Fig. 4A,B, Movie 8).

These data indicate that Col-I is able to promote EC migration in basal medium. Strikingly, neither the effect of FN, nor that of Col-I, as compared to gelatine-controls, was observed in the scratch assay after 2 h (Fig. 4A). After 24 h, effective migration distances showed the same trend in the scratch assay as in the barrier assay after 2 h, but the differences were much less pronounced (Fig. 4A,B and Movies 9–11). These results are not unexpected since during the days prior to wounding, EC have been able to deposit their own ECM, thereby obscuring effects of the defined coating. Interestingly, in the scratch assay less EC died after 24 h than in the barrier assay (Fig. 4B).

When bFGF was added in the two assays, EC migration was stimulated and only little variation was seen between the coatings (Fig. 4A,B). bFGF is a potent stimulator of EC migration, which is able to supersede the inhibitory effects of FN on directionality even after 2 h, as shown in the novel assay.

Addition of VEGF to EC in the barrier assay induced migration along gelatin and FN (Fig. 4A,B). Compared to bFGF, VEGF induced similar effective migration distances after 2 h and 24 h, but the total migration distance was lower, resulting in higher migration efficiency (see also Fig. 2). VEGF had no additive effect on cell migrating along Col-I. Addition of VEGF in the scratch assay had similar but smaller effects on the effective distance because this distance was already high without addition of growth factors in this assay.

Taken together, using the scratch assay, only mild differences between coatings and growth factor became apparent after 24 h. We hypothesize that molecules secreted by EC cells prior to the assay obscure migration responses. Due to the nature of the scratch assay, in particular the wounding and the presence of cell-secreted material, one cannot distinguish between the contribution of ECM components and growth factors to cell migration. Hence, crucial effects are overlooked.

MT Stabilization and MTOC Reorientation During EC Migration in Barrier and Scratch Assays

In a number of cell types, stable MT are selectively formed after wounding and subsequent addition of serum [Gundersen and Bulinski, 1988; Palazzo et al., 2004]. Because stabilization of MT and MTOC reorientation are involved in migration and directionality [Gotlieb et al., 1983; Ueda et al., 1997; Wen et al., 2004], we studied these phenomena in EC using the barrier and the scratch assay. Before induction of migration, we did not observe differences in stable MT staining in either assay and between coatings (Fig. 5A,C, $T = 0$). In the scratch assay, VEGF treatment caused a mild increase in the number of cells with stable MT, on gelatine and Col-I. Using the barrier assay, this increase was much more pronounced (Fig. 5C).

The organization of the stable MT network was different between bFGF and VEGF. VEGF induced more stable MT, presumably because EC were quite elongated and flattened (Fig. 5B). The MT staining pattern was used to estimate the orientation of the MTOC with respect to the migration direction. At $T = 0$, the percentage of MTOC oriented towards the cell-free area was approximately 33%, as expected (Fig. 5D). In the barrier assay, MTOC reorientation towards the cell-free area increased, irrespective of coating used or factor added. In the scratch assay, we found more cells with reoriented MTOC after bFGF and VEGF treatment, an effect that was dependent on the coating used (Fig. 5D). These results indicate that orientation of the MTOC was influenced by the assay used; a purely defined matrix and removal of contact inhibition reoriented the MTOC irrespective of treatment. Importantly, our results on MT stabilization and MTOC reorientation do not correlate with cell migration. For example, addition of bFGF has little effect stable MT, while migration is strongly stimulated.

Contribution of ECM Components to Fibroblast Migration Studied in Barrier and Scratch Assays

To further study the contribution of ECM and growth factors to migration, we tested the widely used 3T3 fibroblast cells. Without growth factors or FN-coating, 3T3 cells migrated similarly in barrier and scratch assays with respect to total and effective distances, both after 2 h and 24 h (Fig. 6A). However, on FN-coated glass, total and effective migration was strongly stimulated in the barrier assay (Fig. 6A,B, Movies 12–15). Thus, using the barrier assay we identified FN as a stimulant of fibroblast migration which is in accordance with previous literature [Clark et al., 2003]. LPA is a known regulator of 3T3 fibroblast polarity and migration in the scratch assay [Nagasaki and Gundersen, 1996; Cook et al., 1998]. We

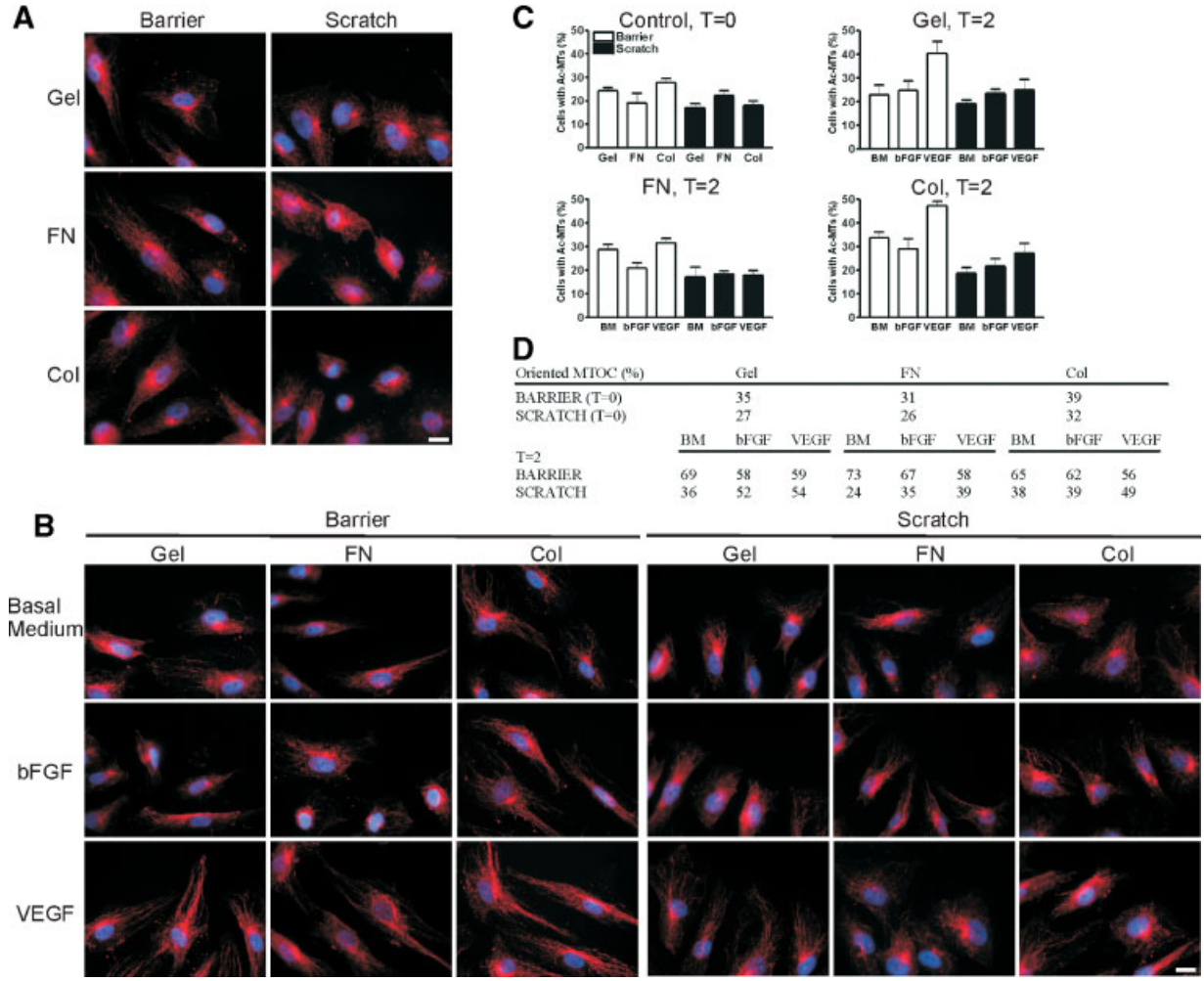


Fig. 5. MT stabilization and MTOC reorientation during HUVEC migration. HUVEC were grown on different coatings until confluence and allowed to migrate for 2 h after removal of the barrier, or introduction of a scratch. After 0 h (control) or 2 h, the cells were fixed and stained for acetylated MT (red) and nuclei (blue). Cell-free area is at upper/upper-right for all pictures. **A**: Control cells at T = 0 h. HUVEC were grown until confluence and immediately after removing the barrier or introducing the scratch, cells were fixed and stained. **B**: Migrating HUVEC in both assays, fixed at T = 2 h. Except for VEGF-treated cells in the barrier assay no differences were seen. The elongated cells bundle their stable MT **C**: Quantification of acetylated MT. Cells with stable MT are depicted as percentage of total counted cells. Data represent mean ± SEM of three independent experiments. **D**: Table represents percentage of cells with MTOC oriented towards the cell-free area after 0 h and 2 h for the different treatments in the two assays.

recapitulated these results in our scratch setup (Fig. 6). In the barrier assay without coating, LPA had a stimulatory effect on total and effective 3T3 migration (Fig. 6A). Strikingly, coating with FN did not alter migration induction by LPA (Fig. 6A,B, Movies 16–19). Thus, the barrier assay revealed that FN does not influence the migration pattern stimulated by LPA or vice versa.

Without coating, bFGF treatment resulted in similar induction of migration in both assays (Fig. 6). Noticeably, bFGF combined with FN increased total and effective migration considerably in the barrier assay. In contrast, using the scratch assay, this stimulatory effect of bFGF on FN action was not perceptible (Fig. 6A). The morphology of 3T3 cells after treatment with bFGF and FN was remarkably different, as compared to LPA-treated fibroblasts. LPA-induced cells demonstrate a triangle-like morphology, while cells exposed to bFGF are very elongated and forming long thin extensions that seem to probe the environment (Fig. 6B, Movies 20–23). Thus, for fibroblasts we showed an enhancing effect of bFGF on the FN-induced cell migration when we used the assay with defined matrix composition.

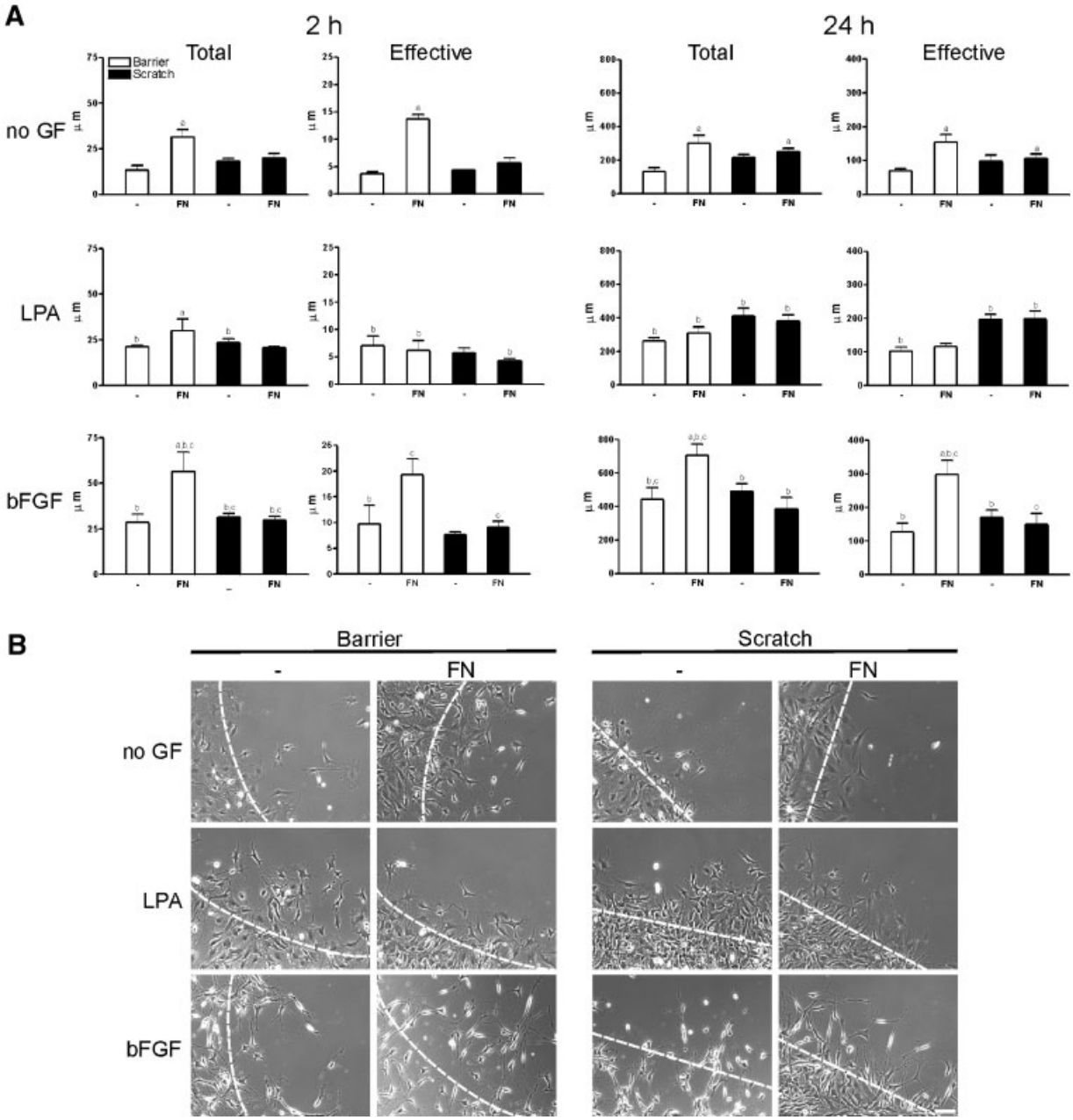
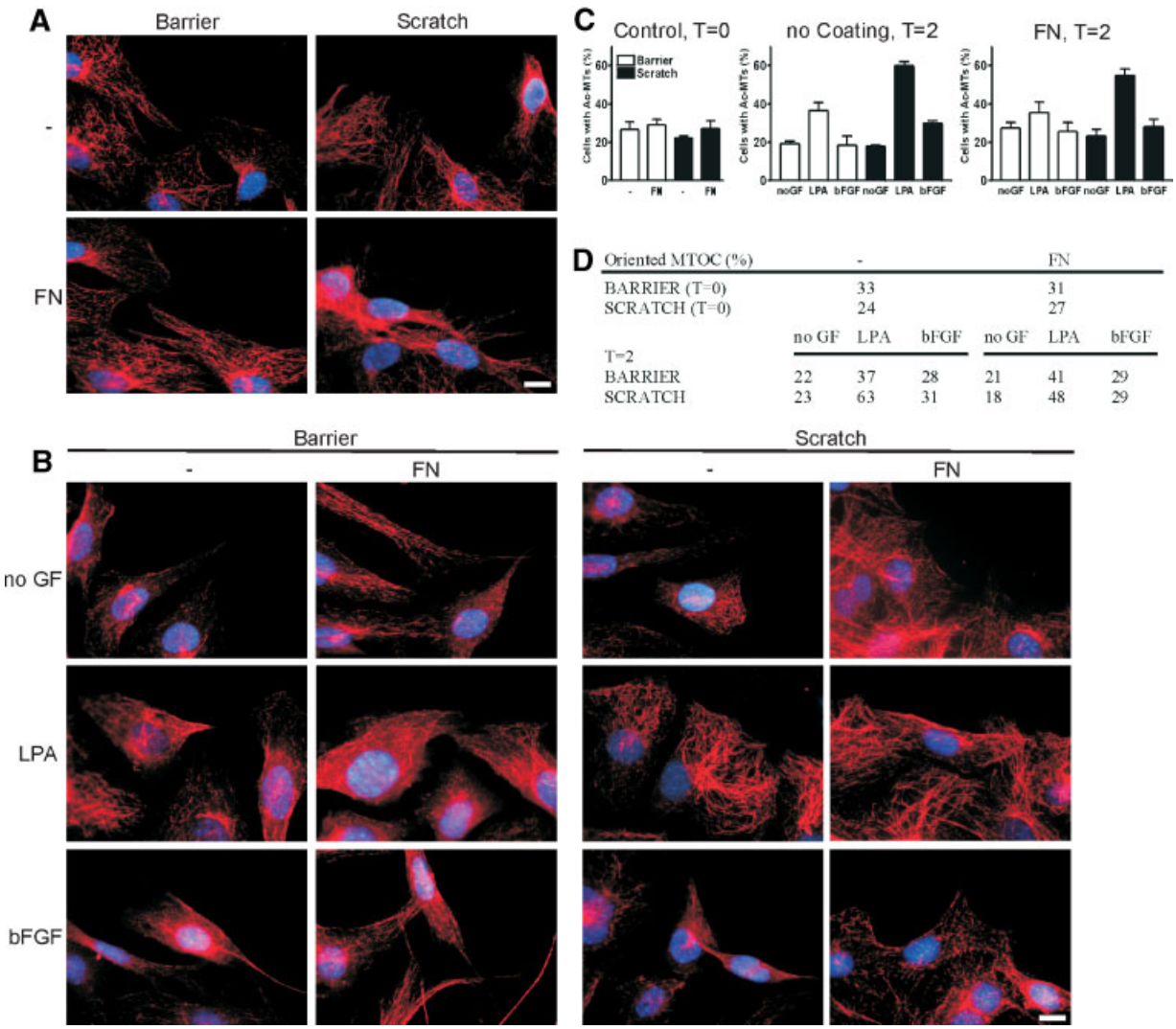


Fig. 6. Effect of FN on 3T3 migration in barrier and scratch assays. 3T3 fibroblasts were grown with and without FN coating until confluence, starved for 24–48 h and followed for 24 h after removal of the barrier, or introduction of a scratch. **A**: Total and effective migration distances (μm) after 2 h and 24 h of 3T3 cells treated with serum-free medium (no GF, control), LPA (5 μM), or bFGF (200 ng/ml) compared between scratch and barrier assay with and without FN-coating. At least 30 cells in at least 3 separate experiments were measured. In the barrier assay, FN induces migration that was strongly augmented by bFGF, a feature not observed when the scratch assay was used. All data represent mean ± SEM of at least three independent experiments; $^{a}P < 0.05$ FN compared to non-coated controls; $^{b}P < 0.05$ LPA and bFGF compared to no GF; $^{c}P < 0.05$ bFGF compared to LPA on same coating. **B**: Pictures of migrating 3T3 cells in both assays, after 24 h obtained from migration movies (Movies 12–23). The white dashed line indicates the migration front at T = 0 h. Movies clearly show major differences in migration profile and cell morphology, bar, 100 μm.

MT Stabilization and MTOC Reorientation During Fibroblast Migration in Barrier and Scratch Assays

LPA is reported to induce stable MT during fibroblast migration [Gundersen et al., 1994; Wen et al., 2004]. We investigated whether this also holds true for bFGF in the presence or absence of FN. Before induction of migration, similar numbers of fibroblasts were expressing stable MT, irrespective of coating (Fig. 7A,C).

In both assays, LPA treatment increased the number of cells with stable MT. However, the increase in stable MT was much higher in the scratch assay (Fig. 7B,C). The findings with LPA are consistent with other reports on LPA [Gundersen and Bulinski, 1988; Cook et al., 1998]. In addition, we showed that the scratch attributed strongly to the LPA-induced MT stabilization. Exposure of the cells to bFGF, however, only slightly increased the number of cells with stable MT (Fig. 7B,C). When we used the stable MT staining pattern to estimate the reorientation of the MTOC, we found that LPA is most effective in both assays, irrespective of coating. MTOC reorientation after bFGF treatment is hardly influenced (Fig. 7D). These results again indicate that increased MT stabilization and MTOC reorientation do not correlate with enhanced migration capacity, as the migration induced by bFGF and FN is higher



D

Oriented MTOC (%)	-			FN		
BARRIER (T=0)	33			31		
SCRATCH (T=0)	24			27		
	no GF	LPA	bFGF	no GF	LPA	bFGF
T=2						
BARRIER	22	37	28	21	41	29
SCRATCH	23	63	31	18	48	29

Fig. 7. MT stabilization and MTOC reorientation during 3T3 migration. 3T3 fibroblasts were grown with and without FN coating until confluence and allowed to migrate for 2 h after removal of the barrier, or introduction of a scratch. After 0 h (control) or 2 h, the cells were fixed and stained for acetylated MT (red) and nuclei (blue). Cell-free area is at upper/upper-right for all pictures. **A**: Control cells at T = 0 h. 3T3 cells were grown until confluence and immediately after removing the barrier or introducing the scratch, cells were fixed and stained. **B**: Migrating 3T3 cells in both assays, fixed at T = 2 h. Especially in the scratch assay, LPA induced MT stabilization irrespective of coating. **C**: Quantification of acetylated MT. Cells with stable MT are depicted as percentage of total counted cells. Data represent mean ± SEM of three independent experiments. **D**: Table represents percentage of cells with MTOC oriented towards the cell-free area. LPA induces MTOC reorientation in scratch assay both with and without FN. In spite of a lack in MTOC reorientation, a strong migration profile with accompanied cell morphology was seen when the cells were exposed to bFGF and FN (Fig. 6).

and more effective than LPA. The pattern of migration, rather than the induction of migration itself, seems to correlate with MT stabilization.

Effect of Matrix-Concentration on EC and Fibroblast Migration

Because it is known that the concentration of matrix components can influence migration properties [DiMilla et al., 1993; Chon et al., 1998], we tested whether our FN results were concentration dependent. To validate the effect on EC, we performed migration assays with 1, 10, and 100 μg/ml FN coating. With low FN (1 μg/ml), we found the same inhibitory effect compared to 10 μg/ml (Fig. 4A); however, high FN (100 μg/ml) induced EC migration (Fig. 8A). In addition, we used low Col-I (1 μg/ml) and migration was still strongly induced. For both the high FN and low Col-I, the cells lost contact during migration (Fig. 8B, Movies 24–26). Interestingly, when we lowered or increased FN concentration in fibroblast migration assays, we did not observe any differences. For both the low, intermediate (Fig. 6), and high FN concentration migration was stimulated and enhanced when combined with bFGF

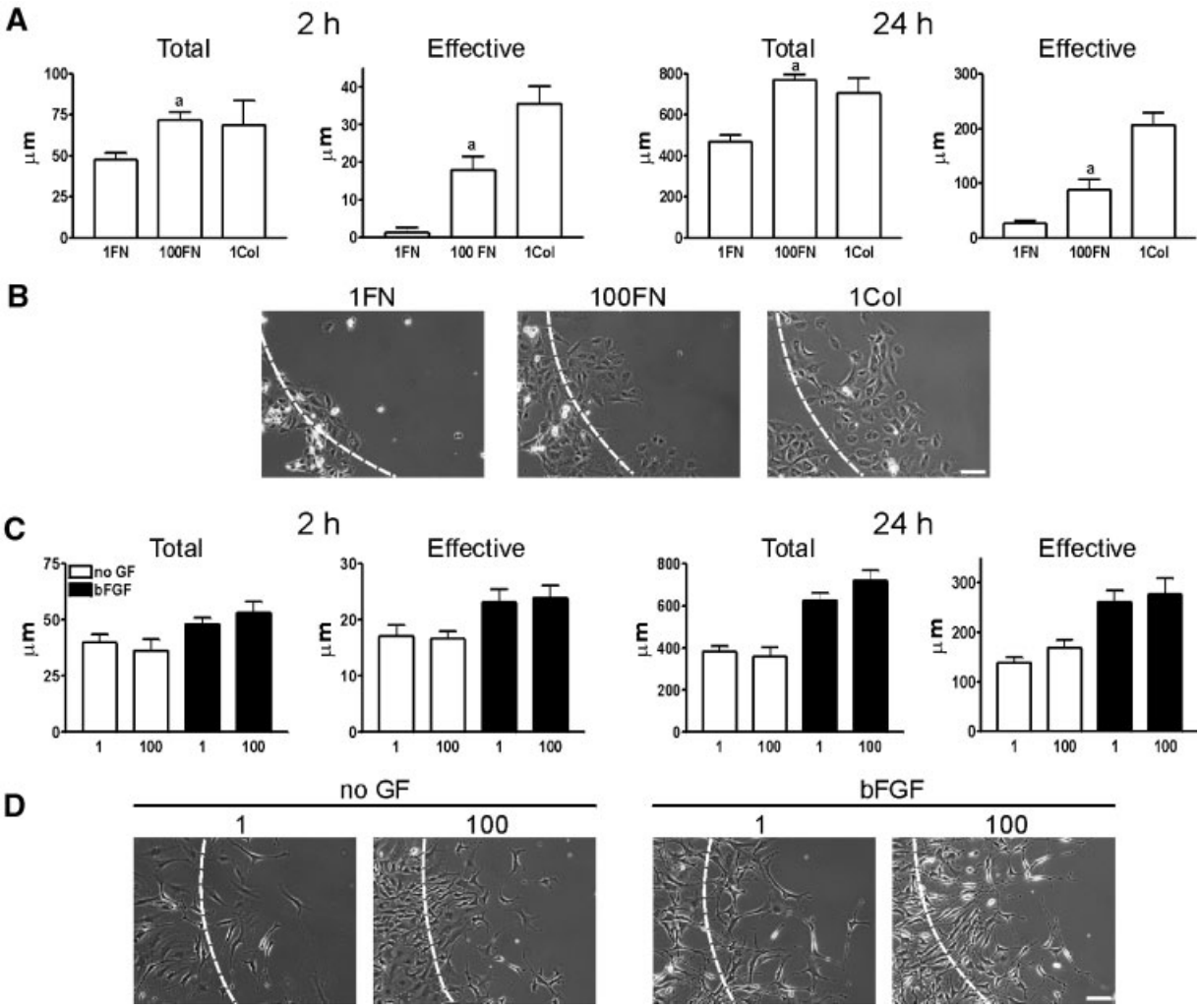


Fig. 8. Effect of matrix concentration on migration response in barrier assay. **A**: Total and effective migration distances (μm) after 2 h and 24 h of HUVEC migration along high (100 μg/ml) and low (1 μg/ml) FN and low (1 μg/ml) Col-I concentration. Data represent mean ± SEM of at least three independent experiments. [a]$P < 0.05$ 1 FN compared to 100 FN. **B**: Pictures of migrating HUVEC in basal medium after 24 h obtained from migration movies (Movies 24–26). White dashed lines indicate the migration front at T = 0 h, bar, 100 μm. **C**: Total and effective migration distances (μm) after 2 h and 24 h of 3T3 migration along high (100 μg/ml) and low (1 μg/ml) FN concentration treated with serum-free medium (no GF) of bFGF (200 ng/ml). Data represent mean ± SEM of at least three independent experiments. No statistical differences were found between low and high FN. **D**: Pictures of migrating 3T3 cells after 24 h obtained from migration movies (Movies 27–30) revealing no obvious differences when FN concentration is altered. The white dashed line indicates the migration front at T = 0 h, bar, 100 μm.

(Fig. 8C). Morphological changes during migration were also comparable between the different FN concentrations. Without GF, cells migrated into the FN-coated cell-free area and long and tiny extensions were formed that were increased when bFGF was added (Fig. 8D, Movies 27–30). These results show that along a defined FN-coating, EC migration is dependent on the concentration (low inhibitory and high stimulatory), while for fibroblasts, induction of migration seems independent on FN concentration and seems a capacity of FN itself.

DISCUSSION

During the process of tumor angiogenesis, EC are activated and migrate along a tightly controlled (provisional) matrix. During wound healing, in contrast, the EC and matrix are damaged before migration starts. An assay to overcome these differences is currently not available. In this study, we present a novel approach to study cell migration to address these differences. Using this novel barrier-based migration assay, we showed that both FN and Col-I stimulate EC migration. Col-I thereby induced directionality as well, while FN-induced migration was completely random resulting in an inhibition of the net cell movement. In earlier reports using the Teflon ring assay, FN inhibited and Col-I stimulated EC migration [Pratt et al., 1984]. However, these results were obtained after 6 days. With the Boyden Chamber assay both FN and Col-I were reported to stimulate EC migration [Herbst et al., 1988]. Our finding, that FN can induce migration but not directionality, could explain the previous opposing data. Using the previous assays, no continuous monitoring of the cells was possible; so no distinction could be made between distance and directionality, overlooking the actual effect of FN. FN is known to be an essential substrate for EC adhesion and growth [Danen and Yamada, 2001]. Possibly, turnover of adhesion sites in EC cultured on FN is too slow to facilitate net movement of the cells. Our results with the novel barrier migration assay indicate that questions in cell migration research can be addressed which are overlooked by the scratch assay. Cells can be stimulated with (secreted) factors of choice and migrate along a predetermined and controlled matrix and are not harmed at the onset of the experiment. The interplay between matrix and growth factors strongly determines migration response and pattern.

EC migration appeared to strongly depend on the FN concentration. Compared to low concentrations, high FN levels induced migration. In contrast to Col-I, cells do not migrate in sheets, but gaps appear. These gaps are absent when FN is combined with certain growth factors. Addition of bFGF or VEGF overcame the matrix effects, which was not seen when the scratch assay was used. These data indicate that in the latter set-up, EC have secreted ECM-components prior to the assay, which strongly interfere with the migratory response. The inhibitory effect of FN, as observed in the barrier assay, was overlooked in the scratch assay.

Recently, it was shown that cells can migrate without the formation of lamellipodia [Gupton et al., 2005]. Our results with EC show that a far-reaching cell body is not necessary for migration, although this increases directionality as shown by the differences in migration pattern between bFGF and VEGF. The formation of a tightly organized cell body, as induced by VEGF results in directionality, pointing at lamellipodium-forming factors as likely candidates in sustaining cell-polarity [Waterman-Storer et al., 1999; Small et al., 2002b]. The cytoskeletal distinction we found between the two growth factors is in agreement with endothelial wound healing [Lee and Gotlieb, 1999]. How these and the effects on adhesions relate to migration pattern and matrix compositions have to be studied in further experiments.

In EC, a mild MT stabilization was found for VEGF treatment in the barrier assay with gelatine and Col-I coating. In all other treatments, no change in stable MT-expressing cells was found. This may imply that MT stabilization in EC is not necessary for (directional) cell movement along defined matrix components. However, MT stabilization might be involved in determining migration pattern and morphology. Simultaneously, MTOC reorientation was not linked to migration or treatment of EC. These differences indicate that reorientation of the MTOC might be regulated by particular ECM components but also occurs when contact inhibition is obviated. The contact inhibition feature of EC might interfere with studying the role of matrix proteins on MTOC reorientation in this novel system. Other cell types will be

used in future experiments to elucidate this phenomenon. Moreover, other factors are likely involved in regulation of directionality of EC migration, as is shown for other cell types as well [Ueda et al., 1997]. MT stabilization and MTOC reorientation may be involved in other steps during EC migration, like the formation and stabilization of a widely spread cell body (strongly induced by VEGF). MT and MTOC are essential for EC migration and angiogenesis, but their functions and regulation may be more complex than assumed until now [Hotchkiss et al., 2002; Small et al., 2002a].

3T3 fibroblasts, which are slow-moving cells, have been very well documented in terms of migration in the scratch assay and the cytoskeletal rearrangements that accompany this process [Conrad et al., 1989; Berven et al., 2004]. In this setup, LPA was shown to be a unique regulator of 3T3 cell polarity [Nagasaki and Gundersen, 1996; Wen et al., 2004]. We, therefore, used 3T3 cells to compare barrier and scratch assays and examined the effect of LPA in relation to bFGF and FN. Surprisingly, when FN was used as coating, a strong induction of migration was observed with the barrier assay in the absence of LPA. With the scratch assay, we did not observe this effect. These data indicate that the scratch assay is not suited to study the role of defined ECM components in migration. FN, reported to be an inhibitor of EC migration [Madri et al., 1991], is an inducer of fibroblast migration. Different cell types respond dissimilar to the same matrix component, which shows that the composition of the matrix is essential for cell motility.

In the barrier assay, the action of bFGF is more pronounced on 3T3 cell migration compared to LPA. In addition, bFGF enhances FN-induced migration, whereas LPA does not. Besides the matrix itself, the interplay with growth factors is crucial for both migration response and morphology. The strong synergistic bFGF-FN effect was not observed using the scratch assay. The marked differences in morphology between the bFGF and LPA-induced migrating cells may imply that migration signaling is distinct as well. Additionally, bFGF and FN-induced migration was not accompanied by increased stabilization of MT or MTOC reorientation, whereas LPA in the scratch assay induced stable MT very efficiently, as reported previously [Nagasaki and Gundersen, 1996; Palazzo et al., 2001a]. The treatments, however, resulted in strongly different migration profiles and morphologies of the 3T3 cells during migration. Whereas MT stabilization and MTOC reorientation is induced in fibroblasts stimulated with LPA in the scratch assay, this does not imply that efficient migration of 3T3 cells always requires such drastic intracellular rearrangements. Recently, it has been reported that FN induces the stabilization of MT in cells that have started to adhere to glass [Palazzo et al., 2004]. We hypothesize that the FN-induced stabilization is important for cell adherence and spreading, and might occur when cells prepare to migrate and polarize. During actual movement of a cell, the regulation of MT stabilization may be altered. MT will be stabilized but for a smaller time frame when the cell is quickly moving as seen when stimulated with bFGF.

Taken together, the introduction of the novel migration assay strongly expands the possibilities for cell migration research and reveals that the interplay between matrix and growth factors determines migration response and morphology. Our results show that FN differentially affects cell migration between cell types and growth factors. The assay is easy to employ, overcomes restrictions of other assays, and reveals differences overlooked by other assays. Contribution of the cytoskeleton, cell–matrix interactions, matrix–growth factor interactions, and signaling during migration of unharmed cells are open avenues for future research. Wider application of this novel barrier-based migration assay has the potential to answer important questions related to cell migration in many physiological and pathological processes.

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