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# The position of the microtubule-organizing center in directionally migrating fibroblasts depends on the nature of the substratum

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**ABSTRACT** **Immunofluorescence and confocal microscopy were used to monitor the positioning of microtubule-organizing centers (MTOCs) during directional migration of chicken embryo fibroblasts on planar substrata and within three-dimensional collagen gels. Homologous assay conditions based on the radial emigration of cells from cell aggregates were used in both cases. Whereas ≈70% of the cells migrating directionally on glass and at least 60% on other planar substrata have their MTOCs anterior to the nucleus, MTOCs are randomly distributed around the nucleus in cells within collagen gels. The anterior location of the MTOC in cells on glass is attained gradually during the first 4 hr of directional migration. Cells on oriented planar substrata, manufactured by photolithographic etching of narrow parallel grooves into the glass surface, also have a random position of the MTOC, although the cells themselves assume a highly polarized cell shape parallel to the grooves. This environment mimics the partial orientation of the collagen fibers produced by the tractive forces of the cells within collagen networks. These findings demonstrate a difference in MTOC positioning between fibroblasts on planar substrata and within a quasi-natural environment.**

Microtubules play important roles in the life of a eukaryotic cell. Originating from the centrosome, the cell's microtubule-organizing center (MTOC), they are involved in cell division, intracellular transport, the development and maintenance of cell asymmetry, and cell migration (1). An involvement in the expression of a locomotory phenotype is revealed in the experimental observation that microtubule depolymerization impairs cell locomotion (e.g., see refs. 2 and 3), and the morphological finding that, in certain migrating cells, the MTOC is located ahead of the nucleus and behind the advancing lamellipodium (4–6). This intriguing correlation was interpreted to mean that MTOC reorientation to the front of the cell not only accompanies the onset of cell migration but actually "may play a role in determining the direction of cell movement" (ref. 4; for reviews, see refs. 7–9).

Fibroblasts cultured on glass or plastic substrata have served as important models for some of these studies (3, 5, 10). However, the advantage of two-dimensional surfaces for microscopic observation is counterbalanced by the disadvantage that these conditions are clearly unrepresentative of a fibroblast's natural environment, a three-dimensional collagen network (11, 12). Observations on fibroblasts in hydrated collagen gels *in vitro* and *in situ* demonstrate a number of differences in morphology and behavior from their counterparts on planar substrata (e.g., see refs. 11, and 13–17). It would seem important to ascertain that findings on cells cultured on planar substrata apply to cells in a more natural environment as well, particularly with respect to the question of MTOC positioning during cell migration.

We have used a convenient assay for determining directional migration of large numbers of cells that does not require constant observation of each individual cell. The assay is based on the fact that cells of small aggregates, or plaques, move radially from the plaque during the initial stages of migration. The advantage of this assay is that it can be applied to cells on both planar and three-dimensional substrata. We compared MTOC positioning during the radial migration of cells on glass and within a three-dimensional collagen environment. We find that, in contrast to two-dimensional substrata, cells in collagen gels have a random position of the MTOC relative to the nucleus during directional movement.

## MATERIALS AND METHODS

Chicken embryo fibroblasts (CEFs) of day 9–12 embryos (kindly provided by S. Martin, University of California, Berkeley) were prepared and cultured as described (18). Rat tail collagen (kindly provided by S. Nandi, University of California, Berkeley) was used as described (19). For the preparation of cell aggregates, primary cultures of CEFs were allowed to grow to confluency for ≈3 days. Regions of high cell density were separated from the rest of the monolayer with a glass needle, collected with a Pasteur pipette, and either placed directly onto coverslips or mixed with an equal amount of neutralized collagen. About 30 μl of the collagen gel/cell aggregate mixture was carefully spread onto a 12-mm coverslip with a sterilized strip of parafilm. For immunofluorescence microscopy, cells were processed as described (20). Cells in gels were lysed for 2 min with 2% Triton X-100 in PHEM buffer (21) and fixed with 1% glutaraldehyde in PHEM buffer containing 2% Triton X-100. For best results, antibody incubations were carried out overnight at room temperature. A Bio-Rad MRC 600 laser scanning microscope was used for the visualization of some cells embedded within collagen gels but turned out to be impractical for determining MTOC position in large numbers of cells. Much of the scoring of MTOC positions was done independently by three different people and was done directly on the fluorescence microscope because the ability to focus through the preparations facilitates the determination of MTOC position, particularly in gels. In some experiments, MTOC position was also determined by the regrowth of small microtubule asters after depolymerization at 0°C. For each cell migrating from a cell aggregate in a radial fashion, the position of the MTOC relative to the nucleus was recorded in a schematic drawing (for an example, see Fig. 4). In this way, a complete record of MTOC position in all the cells analyzed under all experimental conditions was compiled.

Glass slides with a series of parallel grooves of different depth and pitch (width) were prepared by a photolithographic

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Abbreviations: MTOC, microtubule-organizing center; CEF, chicken embryo fibroblast.
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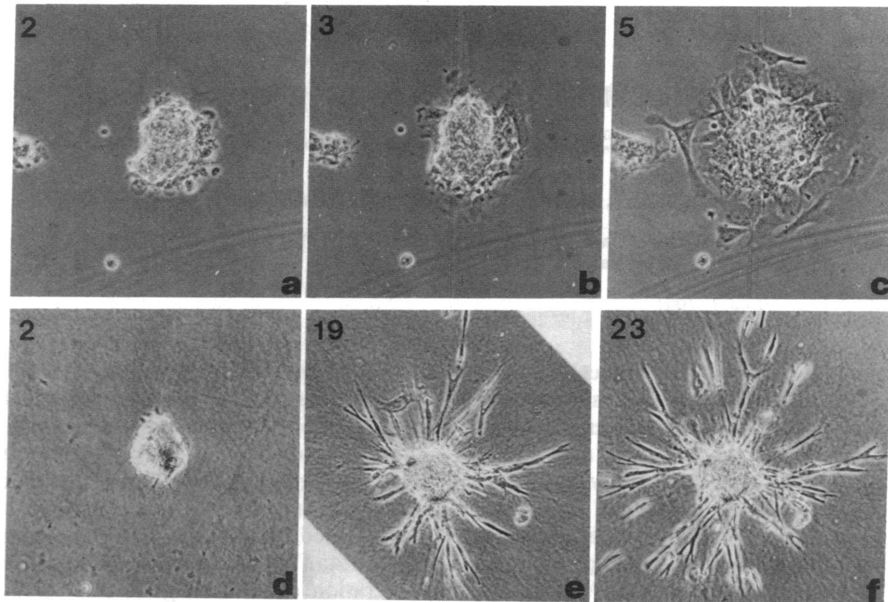


FIG. 1. Time course of CEF emigration from cell aggregates (plaques) on a glass surface (*a–c*) and within a three-dimensional collagen gel (*d–f*). The numbers in each micrograph represent time (in hr) after plating. (*a–c*, ×135; *d–f*, ×90.)

process in the Microfabrication Facility of the Department of Electrical Engineering and Computer Sciences (University of California, Berkeley). The steps in the preparation process consist of computerized design of a mask with the desired grating pattern, coating of the glass surface with photoresist, exposure to UV light through the mask, development of the photoactivated glass slide, and etching to the desired groove depth. The groove depths used here ranged from 2 to 5 μm. In most of our experiments, slides with a groove depth of 2–3 μm were used.

## RESULTS

**Comparison of Plaques on Glass and Within Collagen Gels.** The behavior and morphology of fibroblasts on glass and within three-dimensional hydrated collagen gels have been described (e.g., see refs. 11–15) and need not be repeated here. Only some features specifically pertaining to the plaque assay shall briefly be mentioned. Plaques on two-dimensional substrata adhere quickly (within 20 min). The leading edges of cells emerge from the plaque perimeter after ≈30 min, and cells move out radially with broad, flat lamellipodia typical for fibroblasts (Fig. 1 *a–c*). After 5–8 hr, cells that had lost contact with the plaque begin to move in directions other than radial. Plaques embedded within three-dimensional collagen gels usually show a refractory period of several hours before cells emerge. During this period, radial alignment of collagen fibers in the vicinity of the plaque becomes visible. Emerging cells are elongated and fusiform with filopodia extending from the leading edge (Fig. 1 *d–f*; Fig. 2). The nucleus is often found in the rear of the cell. In contrast to cells on glass, cells in gels keep moving radially for at least 50 hr, even after losing contact with the plaque. Video recordings show that the overall movement of cells is smooth and continuous in both cases, although advances of the leading edge may be interrupted by brief phases of retraction, particularly in collagen gels. The average speed of movement during emigration from a plaque is 0.21 ± 0.08 μm/min ($n$ = 128) for cells on glass and 0.16 ± 0.05 μm/min ($n$ = 241) within a collagen gel. In both conditions, an intact microtubule system is required for migration since disassembly of microtubules by nocodazole (5 μg/ml) severely reduces (glass) or completely inhibits (gels) cell movement. Immunofluorescence microscopy confirms the complete absence of microtubules in both cases (data not shown).

**MTOC Position.** Immunofluorescence and laser scanning confocal microscopy were used to determine the position of the MTOC during the peak phase of radial migration, when cells had moved approximately one cell length away from the plaque. This phase was between 4 and 6 hr after plating plaques on glass and between 15 and 25 hr after embedding plaques within a collagen gel (Fig. 3). On glass, >70% of the cells have the MTOC positioned anterior to the middle of the nucleus (Table 1). In cells moving away from gel-embedded plaques, the MTOC has a random position relative to the nucleus, with ≈45% located anterior to the nuclear midline. The MTOC distributions in cells of one population on glass and one population within a collagen gel are shown in Fig. 4.

To determine the influence of the nature of the planar substratum, plaques were placed on the surface of a dried collagen gel, on the surface of a hydrated collagen gel, or on glass with an overlying hydrated collagen gel, and MTOC

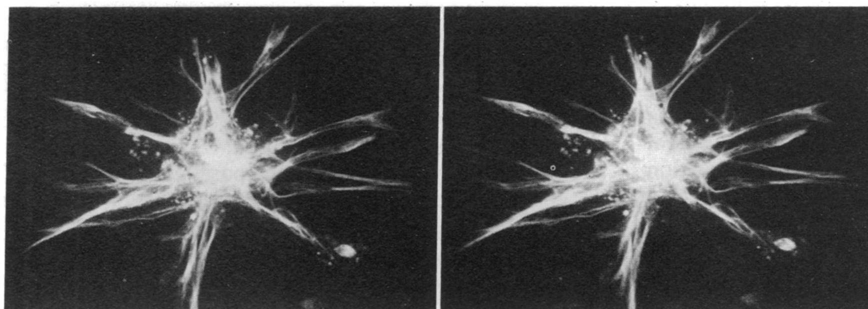

FIG. 2. Overview of a plaque 17 hr after embedding into a collagen gel. Confocal microscope image of microtubule localization. Stereo projection of 14 optical sections spaced 1 μm apart. The radial emigration of several cells and their slender, cylindrical shape are demonstrated in three dimensions. (×180.)

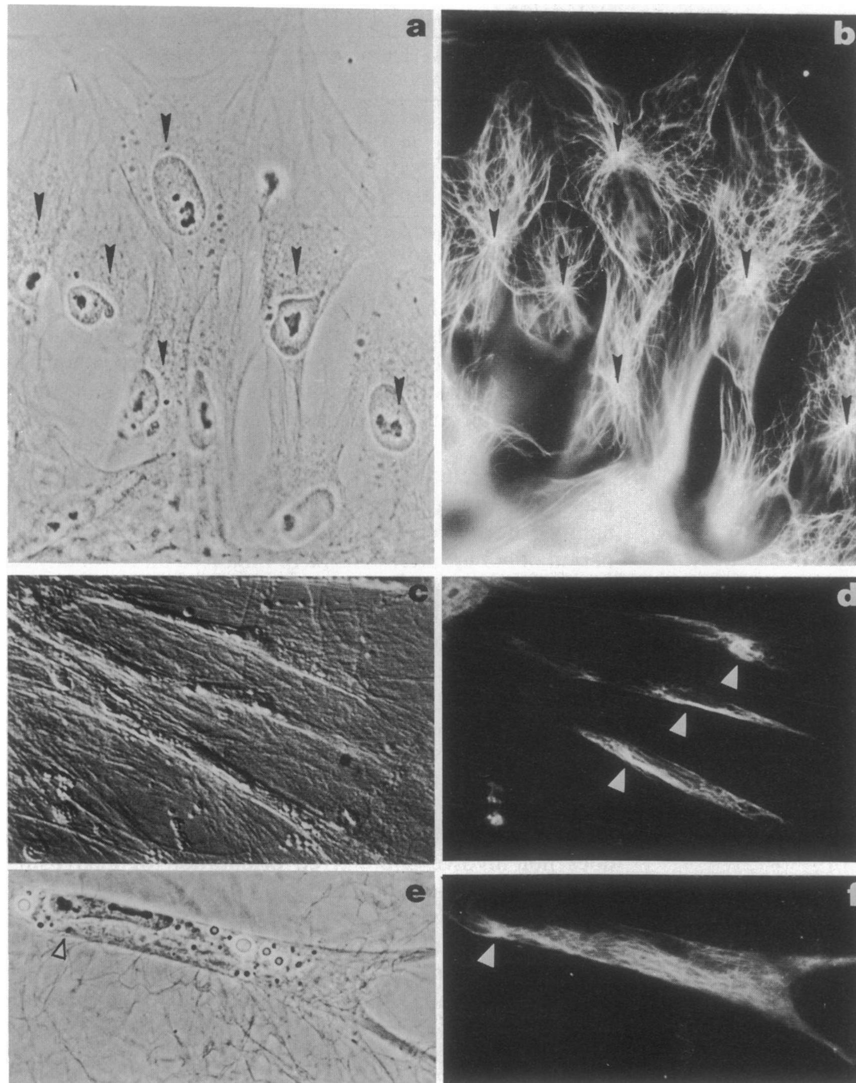


FIG. 3. Microtubule distribution and MTOC position in CEFs on glass and within a collagen gel. (*a* and *b*) Phase-contrast and immunofluorescence micrographs of a group of cells emigrating from a 6-hr-old plaque on glass. Most of the cells of this group have their MTOC (arrowheads) in front of the nucleus. A phase-dense body most likely corresponding to the centrosome is seen in the center of each microtubule aster (arrowheads). (×405.) (*c* and *d*) Confocal DIC and immunofluorescence micrographs of three cells emigrating from a 17-hr-old plaque in a three-dimensional collagen gel. The micrographs are composed of one (*c*) and two (*d*) optical sections, the latter spaced 0.5 μm apart. In these three cells, the MTOCs (arrowheads) are ahead, on the side, and behind the nucleus, respectively. (×490.) (*e* and *f*) Higher magnification of microtubule distribution and MTOC position in a single cell migrating from a plaque (left, not in view) in a three-dimensional collagen gel. This cell has a branched anterior lamellipod and a blunt, rounded tail typical of many migrating cells in collagen gels. The MTOC (arrowhead) as shown by immunofluorescence microscopy is located in the cell posterior. In phase-contrast microscopy, a phase-dense body is located in the center of the MTOC. (×735.)

position was assayed as described above. In all cases, a predominantly anterior location of the MTOC was observed, although the percentage is lower than for cells on glass (Table 1). Under these conditions, cells assume a morphology intermediate between that of cells on glass and that within gels but they resemble the former more than the latter (data not shown). Cells are flattened onto the substratum with up to five lamellipodial extensions at the leading edge, but cell width is more attenuated than on glass alone.

**Time Course of Centrosome Positioning on Glass.** Centrosome position in cells on glass was followed over a period of ≈6 hr, beginning with the time nuclei of emigrating cells

Table 1. Comparison of MTOC positioning on different substrata

| | MTOC anterior, % ± SD | No. of plaques | No. of cells |
|---|---|---|---|
| Gel* | 45.4 ± 15.9 | 31 | 445 |
| Glass† | 73.8 ± 14.9 | 58 | 713 |
| Bottom dry†‡ | 69.8 ± 9.1 | 19 | 530 |
| Bottom wet†§ | 66.6 ± 12.9 | 27 | 469 |
| Top layer†¶ | 64.7 ± 10.0 | 10 | 160 |

The difference between gel and the other experimental conditions is statistically significant ($P \leq 0.001$).
*Counts were made ≈24 hr after plating.
†Counts were made ≈6 hr after plating.
‡Cells on air-dried collagen gel.
§Cells on hydrated collagen gel.
¶Cells on glass with an overlay of hydrated collagen gel.

were fully visible (≈1.5 hr after plating). We found that centrosome position changes over time. Early stages of cell emigration are characterized by a random position of the centrosome relative to the nucleus. Later a predominantly anterior location is assumed—i.e., between 2.5 and 4 hr after plating (Table 2). Thus, the initial phase of directional cell movement takes place in the absence of a preferred position of the MTOC.

**The Importance of Substrate Features.** Cells in collagen gels exert tractional forces on the network that reorient some of the collagen fibers to produce a radial alignment ("tractional structuring of the gel"; ref. 22). This phenomenon was also observed in the assay used here, raising the question of the influence of an aligned substrate on centrosome positioning. To examine this, plaques were placed on glass coverslips with parallel grooves of various depths and widths etched into the surface. Thus, the cells were exposed to a planar yet highly aligned substrate that incorporates properties of both featureless glass surfaces and aligned collagen gels. On grooved coverslips, cells emigrate almost exclusively parallel to the grooves with an elongated morphology reminiscent of cells in collagen gels (Fig. 5). Under all conditions of pitch (groove width) tested, MTOC position is essentially random with respect to the nucleus (Table 3).

## DISCUSSION

Using a simple and effective assay for directional cell migration, we demonstrate here a lack of a preferred position of the

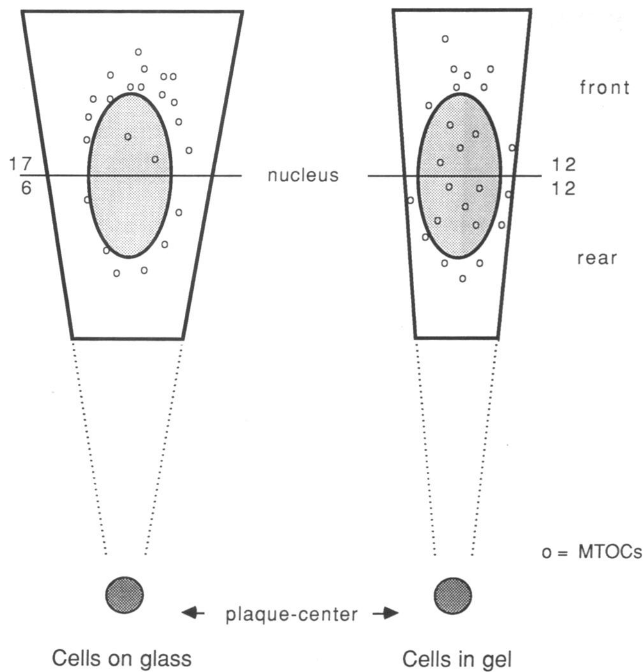


FIG. 4. Two examples of the schematic diagrams used to protocol MTOC position of cells moving radially from a plaque on glass (Exp. no. MP02689, plaque no. C2/16; *Left*) and within a three-dimensional collagen gel (Exp. no. MP072689, plaque no. B2/7; *Right*). In the plaque on glass, 17 of 23 MTOCs are ahead of a line bisecting the nucleus; the corresponding number for the plaque in the gel is 12 of 24.

MTOC relative to the nucleus in CEF cells migrating directionally within three-dimensional collagen gels, an environment that closely resembles the *in vivo* milieu (cf. ref. 12). Homologous assay conditions on planar substrata, both artificial (glass) and biological (collagen), result in a predominantly anterior location of the MTOC. The lack of a preferred position of the MTOC within three-dimensional gels

Table 2. Time course of MTOC positioning on glass

| Time, hr | MTOC anterior, % ± SD | No. of plaques | No. of cells |
|---|---|---|---|
| 1.5 | 42.0 ± 25.4 | 29 | 242 |
| 2.5 | 44.0 ± 14.7 | 103 | 1084 |
| 4.0 | 64.9 ± 17.8 | 52 | 656 |
| 7.0 | 69.9 ± 14.0 | 24 | 434 |

Both early time points are statistically different from the two later time points ($P < 0.001$).

does not imply that microtubules are not required, as their disassembly leads to cessation of migration. Since cells move directionally at comparable rates on both two- and three-dimensional substrata, a position of the MTOC anterior to the nucleus is not universally required for directional locomotion.

An unexpected finding was that even when cultured on glass, cells emigrating from explants attain their predominantly anterior location of the MTOC only slowly, over a period of ≈3 hr, after cells have migrated directionally for at least one cell length away from the explant. The simplest explanation for this finding is that the position of the MTOC ahead of the nucleus develops during directional locomotion. It is not a prerequisite for the onset of directional movement, as suggested on the basis of some observations (8). Furthermore, an anterior location of the MTOC is clearly not a strict requirement for directional movement even after this initial period of positioning, since about one-third of the cells migrate perfectly well in a directional fashion with a posterior location of the MTOC (Table 1). Directional movement also does not involve a preferred orientation of detyrosinated microtubules (K.S. and M.S., unpublished data), as suggested on the basis of studies on 3T3 cells (5).

Which factors might contribute to the observed differences between glass and gels in MTOC positioning constitutes a significant question. Aside from the obvious dissimilarities in cell shape, cells on planar substrata possess not only an anterior–posterior axis but also a dorsal–ventral polarity. This polarity finds its most prominent morphological expres-

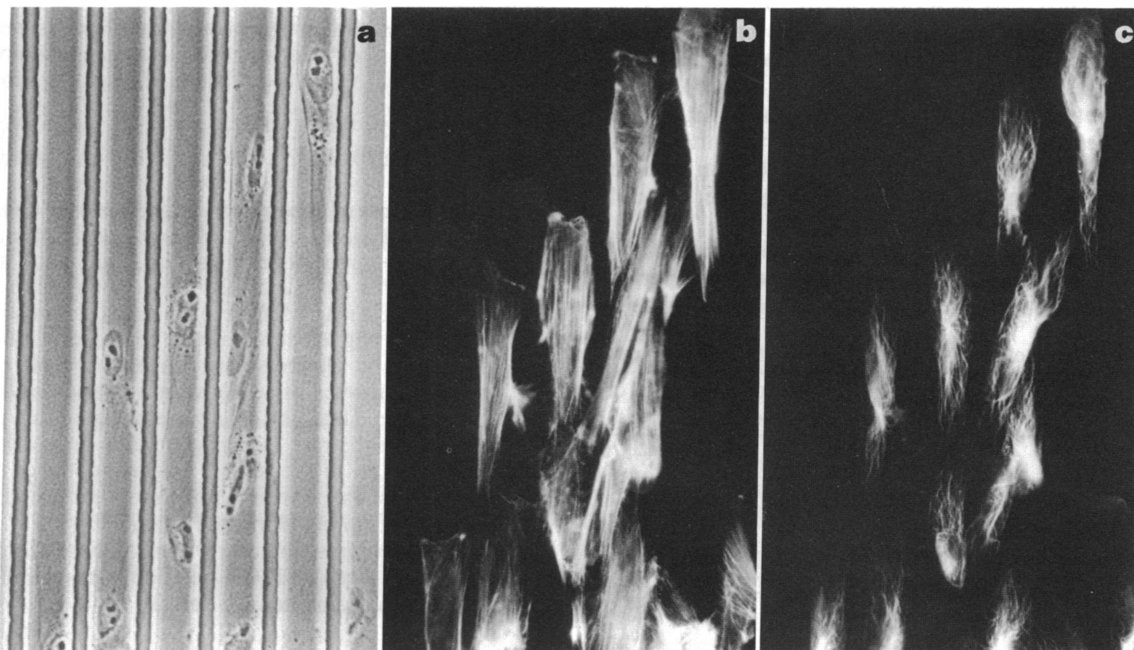


FIG. 5. Overview of microtubule and actin organization in cells emigrating from plaques placed on grooved coverslips. The plaque center is near the bottom of the micrographs. (*a*) Phase contrast. (*b*) Rhodamine-phalloidin staining (for actin). (*c*) Tubulin immunofluorescence of the same preparation. Pitch, 10 μm; groove depth, 2 μm. (×405.)

Table 3. MTOC positioning in cells emigrating from plaques on grooved glass surfaces

| | MTOC anterior, % ± SD | No. of plaques | No. of cells |
|---|---|---|---|
| Plain glass | 73.8 ± 14.9 | 58 | 713 |
| Collagen gel | 45.4 ± 15.9 | 31 | 445 |
| Grooved glass | | | |
| Pitch 5 $\mu$m | 58.4 ± 14.9 | 17 | 184 |
| Pitch 7 $\mu$m | 49.2 ± 13.5 | 19 | 188 |
| Pitch 10 $\mu$m | 51.1 ± 11.2 | 14 | 175 |
| Pitch 15 $\mu$m | 51.5 ± 10.9 | 23 | 256 |

The differences between plain glass surfaces and grooved glass are statistically significant ($P < 0.001$). Grooves were etched to a depth of $\approx 3$ $\mu$m. Counts were made 6 hr after plating onto grooved substrata.

sion in a complement of actin cables, or stress fibers, at the ventral cell surface that are anchored to focal adhesions in close apposition to the substrate (23). Stress fibers are largely absent from cells in three-dimensional collagen gels, and focal adhesions are dramatically reduced (17). It is unclear to what extent the loss of dorsoventrality might affect other aspects of internal cell architecture, including the position of the centrosome and the deployment of microtubules. Certainly, culture on a planar substratum introduces constraints that these cells do not normally experience and imposes epithelial features on these spindle-shaped fibroblastic cells.

Even though the external milieu of a CEF in a hydrated collagen gel is decidedly three-dimensional, it is not entirely isotropic. The sources of this anisotropy are the cells themselves, which stress the collagen network and align some of the collagen fibers, a process termed "tractional structuring" (22). Cells emerging from the aggregate appear to follow the partially aligned collagen fibers (see also ref. 15). Guidance cues also exist on planar substrata, but presumably they are derived from the pattern of contacts with neighboring cells, which are restricted to the lateral and posterior cell margins. This "guidance by contact inhibition of locomotion" (24) may occur in gels as well, but presumably traction-aligned collagen fibers are the predominant stimulus for directional migration. One might expect guided cells to be less dependent on intact microtubules; however, the opposite is the case since microtubule disassembly causes cessation of all movements in gels.

To test the hypothesis that extrinsic cues influence internal microtubule deployment, cells were exposed to oriented planar substrata. Recent advances in microfabrication techniques developed for the microelectronics and computer industry have found useful applications in the study of cell behavior and motility as well. Grooved substrata have been used to determine how surface topography influences tissue cell behavior *in vitro* to understand guidance factors in complex *in vivo* environments (e.g., see refs. 24–26). To mimic, on a planar substratum, the guidance cues that might exist in collagen gels, we allowed cells to emigrate from plaques on a grooved glass surface. We found that cells emigrate largely parallel to the grooves and with an elongated morphology reminiscent of cells in gels and that MTOC position is random with respect to the nucleus. Thus, extrinsic guidance cues will modify the organization of the cell's microtubule system even on a planar, though structured, substrate. In conclusion, these observations provide evidence for the importance of the substratum in the organization of the microtubule system and the position of the MTOC during directional migration. They support the view that, though essential for migration *per se*, the position of the MTOC is not under all experimental conditions correlated with, and therefore not necessarily causally related to, the direction of movement.

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