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# Microtubules and microfilaments during cell spreading and colony formation in PK 15 epithelial cells

(immunofluorescence/microfilament bundles/cell shape)

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**ABSTRACT** **We have studied the distribution of microtubules and microfilaments during cell spreading and subsequent colony formation in PK 15 pig kidney epithelial cells using indirect immunofluorescence. During cell spreading on a solid substratum, microtubules grew out from the region around the nucleus, and a collar of microfilament bundles formed around the cell periphery. Although virtually all well-spread cells showed a complex microtubular network, distinctly different patterns of stress fibers were observed. In small colonies, the most commonly observed pattern was a ring of microfilament bundles that appeared to be in register between adjacent cells and encircled the entire colony in a fashion similar to that seen in single cells. In large colonies (more than 50 cells), ≈60% of the cells displayed clearly stained microfilament bundles, either at the cell periphery or throughout their cytoplasm, whereas in the remaining 40%, no microfilament bundles were observed and only the outline of the cells was delineated by interaction with anti-actin. Such "negative" cells were seen in groups alongside "positive" cells (i.e., cells possessing extensive stress fiber networks) within the same colony. Independent of their stress fiber phenotype, all cells maintained a flattened shape and an extensive network of microtubules. We suggest that dense microfilament bundles are not a uniform feature of well-spread PK 15 cells in culture and that a loss of microfilament bundles occurs in some cells.**

Cultured cells possess a complex network of cytoplasmic fiber systems, including microfilaments, microtubules, and 10-nm filaments (1, 2) collectively known as the cytoskeleton. When cultured cells are exposed to trypsin, these fiber systems are disorganized as the cells round up and detach from their substratum. In recent years, specific antibodies against the constituent proteins of the cytoskeleton have been used to visualize these fibers at the light microscopic level (3–8), and they provide an excellent method for studying the reorganization of the cytoskeleton as trypsinized cells begin to spread out on a new substratum (9–11).

Because of the key role that spreading epithelial cells play in morphogenetic changes in developing organisms and in the metastasis of carcinomas, it is important to understand the mechanics of epithelial cell movement (12). Cytochalasin B, which causes the disorganization of microfilaments, will prevent the spreading of epithelial cells in culture (13, 14); however, the microtubule-depolymerizing drug colchicine has little effect on this process (13). Beyond the obvious implication that microfilaments are more directly involved in cell spreading than microtubules, these observations indicate that the role the cytoskeleton plays in developing and maintaining cell shape is not well understood.

PK 15 cells represent a clonally derived cell line of epithelial origin that displays growth patterns similar to those of primary epithelial cells in culture and so provides a model system for studying the development of cell shape. We examined the distribution of microfilaments and microtubules during cell spreading and subsequent colony formation in these cells, using indirect immunofluorescence and antisera to actin and tubulin. We found that although all well-spread cells possess a complex microtubular network, the distribution of microfilament bundles in the same cells can vary significantly.

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## MATERIALS AND METHODS

**Cells.** PK 15 pig kidney cells (CCL 33) were obtained from the American Type Culture Collection (Rockville, MD) and grown in Eagle's minimum essential medium supplemented with 5% (vol/vol) newborn calf serum. Before plating, the trypsinized cell suspension was passed over a glass wool column to remove nondissociated cell clumps.

**Immunofluorescence.** Cells grown on glass cover slips were fixed in 3.5% (vol/vol) paraformaldehyde (pH 6.6) in phosphate-buffered saline for 10 min, washed three times in the buffered saline, and then postfixed for 7 min in acetone at −20°C (3). In some experiments, cells were fixed directly in methanol for 4 min followed by acetone for 2 min, both being at −20°C (15). The results were identical when either fixation was used. For fluorescent staining, cells were rehydrated and treated as described (16, 17).

**Antisera.** Anti-actin antiserum was prepared against purified, heat-denatured pig muscle actin as described (18). Before use, this serum was diluted 1:5 in phosphate-buffered saline. Anti-tubulin was prepared against pig brain tubulin and also has been characterized (16, 17). Before use, this serum also was diluted in saline (1:30).

**Cinematography Studies.** For these studies, cultures were placed in a humidified chamber maintained at 37°C and 5% $CO_2$/95% air on the stage of a Nikon inverted microscope. Frames were exposed every 30 sec for periods up to 24 hr on Kodak Plus X movie film.

## RESULTS

When PK 15 cells were replated after trypsin dissociation, approximately 40% of the cells were found after 1 hr in small colonies or clumps of two or more cells (Table 1). Because of the brief incubation time, it is probable that these are aggregates of cells that were not dissociated by the trypsin treatment and not removed by the passage of the suspension through glass wool. However, as little as 4 hr after plating, more than 80% of the cells were found in colonies; after 20 hr, almost no single cells remained (Table 1). Time lapse cinematography showed considerable mitotic activity during this period and demonstrated that daughter cells did not move apart after division. Moreover, colonies were observed to increase in size by fusion with adjacent colonies or cells. Thus, the average colony size

Table 1. Cell distribution after trypsinization and replating

| Hours after plating* | Single cells, % | Cells in colonies, % |
|---|---|---|
| 1 | 56.8 | 43.2 |
| 2 | 50.8 | 49.2 |
| 3 | 30.0 | 70.0 |
| 4 | 19.8 | 80.2 |
| 20 | 1.5 | 98.5 |

* At each time point, 400 individual cells were counted.

Table 2. Actin staining in spreading cells

| Hours after plating* | % cells in different stages | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 1 | 38.0 | 40.0 | 22.0 | 0 |
| 2 | 14.5 | 40.0 | 43.5 | 2.0 |
| 3 | 18.5 | 35.5 | 45.0 | 1.0 |
| 4 | 10.5 | 19.5 | 67.0 | 3.0 |

* At each time point, 200 individual cells were counted. Stages refer to the pattern of actin staining seen in the cells. Stages 1, 2, 3, and 4 are illustrated in Fig. 1 *a*, *b*, *c*, and *d*, respectively.

increased rapidly; within 4–5 days, a continuous sheet had formed on the substratum. These observations are similar to those reported for primary epithelial cells in culture (12, 19).

We examined the distribution of microtubules and microfilaments during the spreading of single cells 1, 2, 3, and 4 hr after trypsinization and replating. At the end of 4 hr, approximately 65% of the single cell population had well-developed microtubular networks (not shown). The microtubules were first seen around the nucleus and then were observed to spread from this region towards the cell periphery. In contrast, actin staining, first seen as diffuse in small rounded cells (Fig. 1*a*), became localized as a band of fluorescence around the outer edge of the cell (Fig. 1*b*); subsequently, strongly stained bundles of microfilaments began to appear in this region (Fig. 1*c*). At the end of 4 hr, almost 70% of the cells demonstrated this prominent peripheral "collar" of microfilament bundles, with only a few cells showing strongly stained bundles throughout their cytoplasm (Fig. 1*d*; Table 2). Using electron microscopy, Kaiho and Sato (20) found similar marginal bundles of microfilaments in spreading JTC-12 cells, another cell line of epithelial origin.

Over the next 68 hr, the distribution of microtubules in the cells of the colonies remained relatively constant; regardless of colony size, more than 99% of these cells showed extensive microtubular networks in their cytoplasm (Fig. 2).

The pattern of actin staining was much more complex, however. In small colonies (those containing fewer than 10 cells), actin staining exhibited two principal patterns: either strong staining of cytoplasmic stress fibers was seen throughout the cells in the colony or, more commonly, a peripheral staining pattern was observed (Fig. 3; Table 3). In the latter case, bundles of microfilaments appeared to run in register from cell to cell at the periphery of the colony while the rest of the cytoplasm in these cells was largely devoid of stress fibers (Fig. 3 *b* and *c*). Thus, it appears that these cells are coordinately responsible for the establishment of this collar of microfilament bundles surrounding the colony (Fig. 3*c* and *d*).

With increasing colony size, this peripheral staining pattern became less prominent (Table 3), and there was a marked increase in the percentage of cells in these colonies that appeared to be devoid of cytoplasmic stress fibers (Table 3; Fig. 4). Phase-contrast microscopy of these cells confirmed this lack of stress fibers (Fig. 4 *d* and f). Only the outline of such "negative" cells was faintly delineated by the antiserum to actin (Fig. 4*e*). These negative cells were usually found in groups as opposed to in-

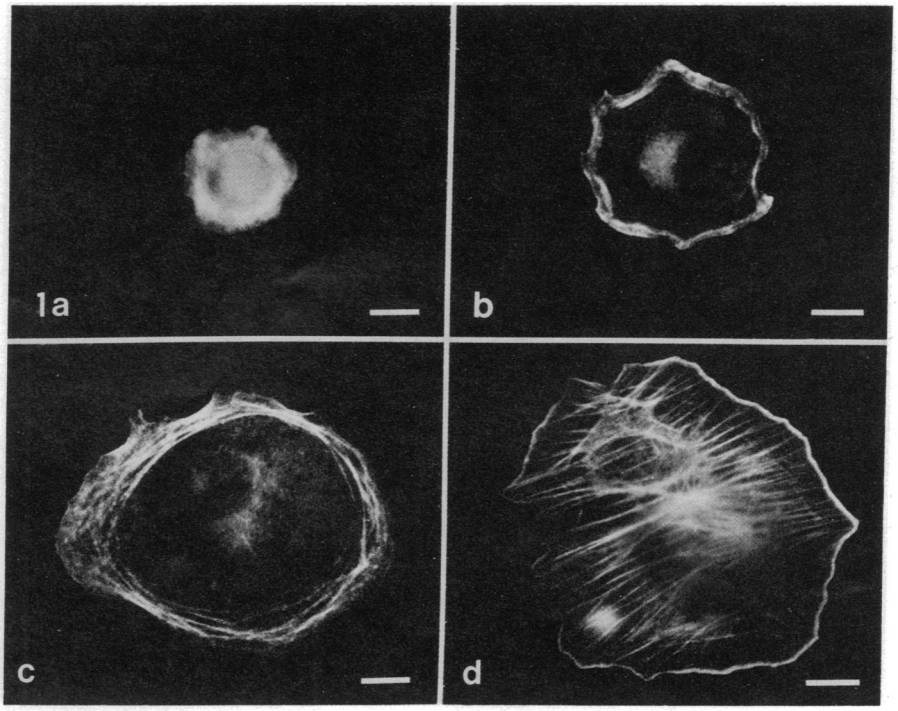


FIG. 1. The distribution of actin in PK 15 cells at different stages of spreading. Cells were sampled 1–4 hr after plating and were treated with antiserum to actin. Diffuse staining is seen in small rounded cells (*a*); in partially spread cells (*b*), this staining begins to localize along the cell margin. In more fully spread cells (*c*), stress fibers are most commonly located along the cell periphery; occasionally stress fibers in the more central regions of the cell (*d*) are strongly stained. (Bars = 10 μm.)

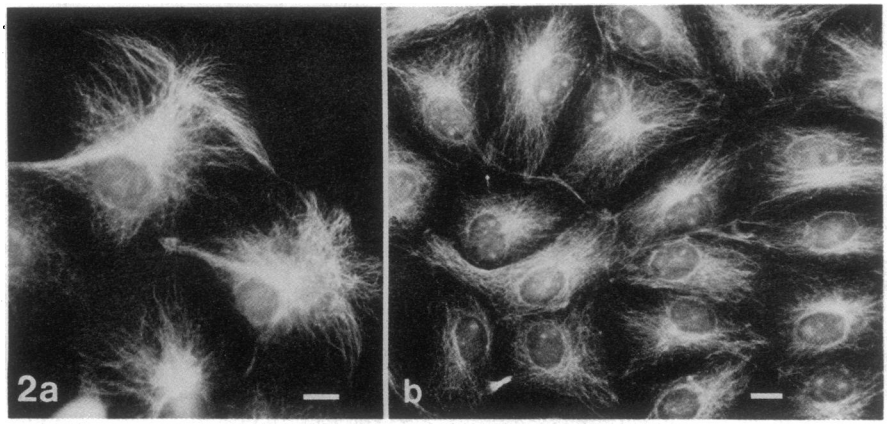


FIG. 2. Microtubule distribution in colonies of PK 15 cells. In both a small (*a*) and a large (*b*) colony of PK 15 cells treated with antiserum to tubulin, all of the cells show an extensive network of microtubules. (Bars = 10 μm.)

dividual cells dispersed through the colony; in fact, individual negative cells were almost never seen. These groups of negative cells were generally in the colony interior rather than at its edge, and several negative groups could be found in single colonies, especially the large ones.

## DISCUSSION

The distribution of microtubules observed in PK 15 cells during cell spreading and in well-spread cells is similar to that reported by other investigators in different cell types (5, 6, 11). Thus, these cells in general are not different from those of other cell lines and primary cultures as far as the distribution of microtubules is concerned. However, the distribution of actin staining appears to be unique.

From our examination of the colonies formed by these cells, two rather striking features emerged. The first of these is the observation that, in a large fraction of the smaller colonies, there exists a collar or ring of microfilament bundles that circumscribes the entire colony. The apparent continuity of this ring between adjacent cells at the periphery of the colony suggests an interaction capable of influencing the distribution of the

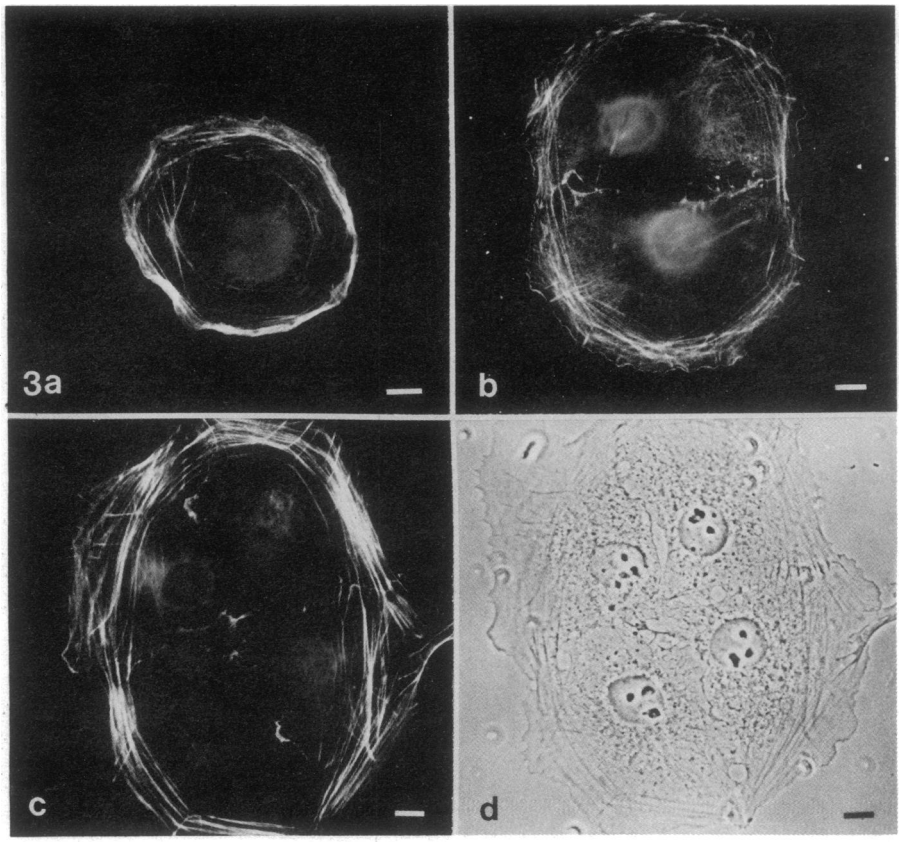


FIG. 3. Actin distribution in small colonies of PK 15 cells. A comparison of anti-actin staining patterns in a well-spread single cell with patterns seen in small colonies of cells shows that, in both the single cell (*a*) and the smaller colonies (*b* and *c*), the strongly stained microfilament bundles are located along the cell and colony outer margins and are absent from their more central parts. The marginal stress fibers in cells of the colony are often in register with those in adjacent cells. The colony in *c* is shown in phase contrast in *d*. (Bars = 10 μm.)

Table 3. Actin staining in colonies

| Cells per colony | Colonies counted | Avg. cells per colony | Cell type, % | | |
|---|---|---|---|---|---|
| | | | 1 | 2 | 3 |
| 2–9 | 51 | 4.8 | 9.8 | 57.3 | 32.9 |
| 10–25 | 20 | 14.4 | 19.0 | 50.9 | 30.1 |
| 26–50 | 16 | 35.3 | 29.6 | 39.8 | 30.6 |
| >50 | 5 | 147.4 | 39.1 | 27.9 | 33.0 |

Type 1 cells show uniform diffuse staining with virtually no stress fibers visible in the cytoplasm. Type 2 cells show prominent peripheral staining of stress fibers clearly visible at those edges of the cell that border the outside edge of the colony. Occasionally small stress fibers are seen scattered in the rest of the cytoplasm. Type 3 cells show strong staining of stress fibers throughout the cytoplasm, with no peripheral pattern distinguishable. For illustrations of these patterns, see Figs. 3 and 4.

microfilament bundles in these cells. Similar observations of microfilament bundles appearing to run in register from one cell to another have been reported in other cell types (21).

Albrecht-Buehler (22) has examined the locomotory activity of colonies of PtK1 cells, a clonal cell line of similar epithelial origin. He found that small colonies of cells moved in unison in a pattern that closely resembled the movement of a single cell, suggesting cell–cell interaction between members of locomoting cell groups. Our results indicate that small colonies of PK 15 cells also may exhibit properties characteristic of single cells. The collar of microfilament bundles around the small colonies bears a striking resemblance to the peripheral collar of microfilaments in single spreading cells.

The second surprising observation with the PK 15 cells was the clear identification of two distinct types of cells in the larger colonies by anti-actin immunofluorescence. In one cell type, an extensive array of strongly stained cytoplasmic stress fibers was present, whereas in another, such fibers were almost totally absent. However, both cell types possessed a well-developed microtubular network. Furthermore, the relationship between these two populations of cells was not static because the number of negative cells increased as the colony size increased. By assuming that the medium and the substratum experienced by all cells in the colony are the same, these results suggest that specific intercellular interactions may well be the dominant influence in regulating the degree of microfilament organization

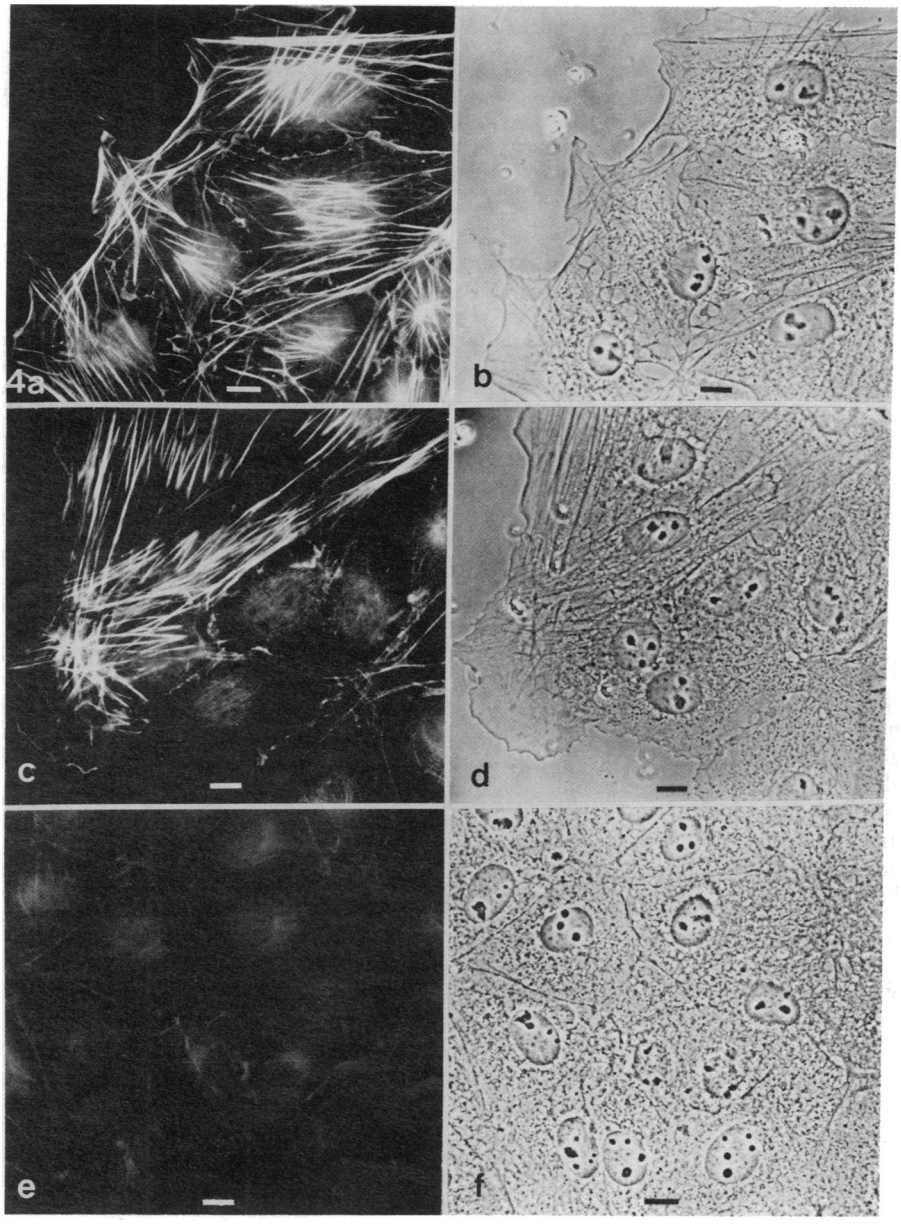


FIG. 4. Actin distribution in large colonies of PK 15 cells. Different staining patterns are seen in different parts of a large colony of PK 15 cells treated with antiserum to actin and examined by immunofluorescence (*a*, *c*, and *e*) and phase-contrast microscopy (*b*, *d*, and *f*). In some parts of the colony, cells with strongly stained stress fibers are evident (*a* and *b*), whereas in other parts of the colony, clusters of cells lacking such fibers were found (*e* and *f*). The latter show faint staining along cell margins and only a few poorly stained stress fibers in their more central parts (*e*). Both types of cells and the border between the two regions are seen in *c* and *d*. Regions containing cells without the strongly stained stress fibers (*e* and *f*) tended to occur in the more central parts of the colony. Both cell types stained equally well with antiserum to tubulin and had extensive networks of microtubules. (Bars = 10 μm.)

in individual cells in the colony. It now will be important to assess the capacity of a given cell to change from the "positive" to the "negative" state (and vice versa) with respect to its stress fiber pattern and, if possible, to identify the local environmental conditions that favor such transitions.

Precedents clearly exist for a dramatic alteration in stress fiber distribution in cultured cells, either as the result of the expression of new genetic information or the experience of particular environmental conditions. For example, both immunofluorescence (23–25) and electron microscopic (26, 27) studies have indicated that the degree of microfilament bundle formation is markedly reduced in cells transformed by oncogenic viruses. Perhaps the most convincing data in this regard are those documenting the change in stress fiber patterns during the reversible transformation of cells infected with temperature-sensitive virus mutants (28, 29). A possible clinical correlate of the relationship between neoplastic transformation and stress fiber content has been reported with the inherited malignancy, adenomatosis of the colon and rectum (30). Skin fibroblast cultures derived from persons with this malignancy show a marked reduction in the percentage of cells that expressed stress fibers, resulting in a mixed population of positive and negative cells—a phenomenon similar to that seen with PK 15 cells in the present study.

It is also known that stress fibers can be reversibly disassembled in cultured cells by the addition of exogenous proteases, such as trypsin or plasmin (31), or by the introduction of tumor-promoting phorbol esters (32). These morphological conversions, which affect virtually all cells in the culture, are invariably accompanied by corresponding cell-shape changes. In each case, the loss of stress fibers is closely correlated with a more rounded cell shape (33). Such cell rounding and loss of stress fibers can be produced also by culturing cells on substrates with poor adhesive properties (34). In the case of the negative PK 15 cells, however, dramatic changes in cell shape were not observed. These cells continued to display an extensive microtubular network and appeared, by phase-contrast microscopy, to retain a flattened cell morphology. Nonetheless, the strong correlation between cell shape and stress fiber organization in other cell types suggests that the nature of the contacts made with the substratum by both positive and negative cells needs to be examined in more detail. Such information will be necessary to address the question of whether or not, in the case of PK 15 cells, cell–cell rather than cell–substrate interactions represent the dominant influence in determining the extent and nature of stress fiber organization.

In conclusion, PK 15 cells provide a useful model for studying epithelial cell spreading and sheet formation *in vitro*. Perhaps more importantly, the changing expression of microfilament bundles in these cells may provide real insight into the function of these organelles. The fact that one can demonstrate within a single colony cells which are very similar in shape but which differ markedly in their expression of a stress fiber network offers a unique model system in which to study the mechanisms that influence microfilament assembly and organization.

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