*Proc. Natl. Acad. Sci. USA*
Vol. 76, No. 3, pp. 1256–1260, March 1979
Cell Biology

# Differentiation of a rat mammary cell line *in vitro*

(stem cells/myoepithelial cells/domes/immunofluorescence)

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*Contributed by Renato Dulbecco, December 1, 1978*

**ABSTRACT** **We have studied the development of fusiform (probably related to myoepithelial) cells in Rama 25 cultures [Bennett, D. C., Peachey, L. A., Durbin, H. & Rudland, P. S. (1978) *Cell* 15, 283–298]; we show that they are generated from special differentiated structures (projections) that contain a rapidly differentiating cell type (F-precursor cells). Clonal sublines isolated from projections develop in several directions under both environmental and genetic control. Some types of differentiation are reversible; others are irreversible. The various cell types occurring *in vitro* may correspond to specific cell types *in vivo*.**

Cultivation of cells *in vitro* from mammary cancers of mice or rats has led to the establishment of several cell lines (1–4). Many of these lines contain more than a single cell type (5–7), usually a polygonal and a fusiform type. The polygonal cells of many of these lines and of primary cultures of mammary tumors of rodents and other species produce characteristic structures (3, 5, 8–15). A fluid-filled blister formed by the local detachment of the epithelial layer from the dish, called a "dome" or "hemicyst," is found especially in primary cultures of mammary cells (12). Another structure is a solid cell cylinder sticking out into the medium, called a "mound" (6).

The accumulation of liquid in the domes is caused by active transport of ions and water (13). The liquid is electrically insulated from the culture medium (13) and is retained by tight junctions between the cells (14). Dome formation is a differentiation event involving several cellular changes (unpublished observations); it is promoted by hydrocortisone (9), prevented by Colcemid (9) or by the inhibition of synthesis of RNA or protein (13), and is induced by the same agents that induce erythroid differentiation in erythroleukemia cells (4, 16). In mammary cells, domes have been reported to produce casein and, therefore, they have been likened to mammary acini (9, 11, 13). However, similar domes are also produced by nonmammary cells. In primary cultures domes are transient; they last from 30 to 120 min, then suddenly flatten out, but later reform (11); in permanent lines they last longer and may become permanent (6).

We have studied a cell line (Rama 25), described by Bennett *et al.* (4) and derived from a dimethylbenz[*a*]anthracene-induced mammary cancer in a Sprague–Dawley rat, that is made up of polygonal cells, but regularly forms, even in clonal sublines, fusiform cells, possibly related to myoepithelial cells. We have inquired into the cellular events that lead to the emergence of the fusiform cells from the polygonal cells. We have postulated that cell differentiation tends to occur in cell complexes; hence we have studied certain cell complexes present in Rama 25 cultures as possible sites of fusiform cell formation. One cell complex is a solid fingerlike projection, probably equivalent to the previously described mounds. Usually the projections observed in Rama 25 cultures are 3–5 cells in diameter and 10–20 cells or more in length; sometimes they are branched. Another cell complex is an annular structure—i.e., a slightly elevated (on observation with a binocular microscope) area of polygonal cells with beaded edges and often with fine marginal vacuoles—surrounded by a ring of flat cells with much less distinct straight edges. The annular structures often have domes, which are otherwise absent in uninduced Rama 25 cultures.

Here we show that both projections and annular structures contain a cell type, which we call "F-precursor" cell, which can differentiate reversibly in various directions, generating domes, projections, or other structures. We also show that some cells in the projections are the direct precursors of the fusiform, myoepithelium-like cells. We will indicate possible correspondences between cell types *in vitro* and *in vivo*.

## MATERIALS AND METHODS

**Cells and Media.** The Rama 25 culture was provided by Dorothy Bennett. It was maintained by regular transfer of $3 \times 10^5$ cells to a 90-mm Nunc or Falcon dish every week in Dulbecco's modified Eagle's medium supplemented by 10% calf serum and insulin and hydrocortisone (50 ng/ml each). For transfer, cells were detached from the dish by trypsin/EDTA (250 mg of trypsin and 90 mg of EDTA per liter in a Tris-buffered saline, pH 7.5); after gentle pipetting they yield a suspension of individual cells.

For electron microscopy, cells were fixed in 2.5% gluteraldehyde and then $OsO_4$, stained with uranyl acetate, and embedded in Epon. α-Lactalbumin was measured by a competitive radioimmunoassay (unpublished data). Rat α-lactalbumin and rabbit antibody against rat α-lactalbumin were a gift of A. E. Bogden (Mason Research Institute). α-Lactalbumin was iodinated by the lactoperoxidase method; specific activity ranged from 40 to 15 Ci/g (1 Ci = $3.70 \times 10^{10}$ Bq). Antibody–antigen complexes were collected by absorption to *Staphylococcus aureus*.

Myosin was detected by indirect immunofluorescence. Cells grown on glass coverslips and fixed in ice-cold acetone for 5 sec were exposed to the gamma globulin-enriched fraction of a rabbit antiserum against purified chicken gizzard myosin (a gift of U. Gröschel-Stewart) at a concentration of 1 mg/ml for 30 min at room temperature. After four washings with phosphate-buffered saline the coverslips were incubated for 30 min with a 0.4 mg/ml solution of fluorescein-labeled goat anti-rabbit immunoglobulins (Antibodies Incorporated, Davis, CA). After four washings in phosphate-buffered saline the cells were covered with glycerol/phosphate-buffered saline, 9:1 (vol/vol) and examined by epi-illumination at 440 nm. The antibodies do not react with any other contractile protein (17) and cross-react with rat myosin.

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Table 1. Developmental potential of cells from projections

| | Stage 1 + 2 | | Stage 1 |
|---|---|---|---|
| | Rama 25 projection | Rama 25 control | LA8-C1 projection |
| Total cells examined | 13 | 15 | 46 |
| Cells generating only polygonal colonies | 3 | 13 | 4 |
| Cells generating only fusiform colonies | 6 | 0 | 37 |
| Cells generating both colonies | 4 | 2 | 5 |
| Colonies with fusiform cells | 3230 | 52 | |
| Total colonies | 6980 | 16,010 | |
| Colonies with fusiform cells/total colonies | 0.46 | 0.0012 | |

**Clonal Analysis and Isolation of Sublines.** Projections were removed from the cell layer by suction with a fine pipette, in some cases after a brief treatment with trypsin/EDTA, to decrease the chance of contamination with cells of the surrounding layer. Parts of annular structures were sucked into pipettes. Clonal analysis was carried out in two stages. In the first stage, the isolated fragments were individually placed in tubes containing trypsin/EDTA. After the cells were dissociated by pipetting, medium was added to produce single-cell suspensions containing an average of 2.5 cells per ml; 0.2 ml was introduced into each well of a 96-well microtiter plate. Part of the cell suspension was also plated in petri dishes in order to obtain colonies from individual cells. In the second stage, colonies growing in the microtiter wells were removed with trypsin/EDTA; the separated cells were plated out for colony formation in petri dishes. In this protocol the first stage characterizes individual cells present in the fragments (i.e., whether they are polygonal or fusiform), and the second stage characterizes their progeny cells.

New sublines were started from cells by transferring the whole content of a well to a 35-mm dish; some sublines were similarly started from colonies after they were trypsinized individually in wells formed by small glass cylinders secured to the dish by silicon grease. The 35-mm cultures were later expanded and finally frozen in 10% dimethyl sulfoxide/20% calf serum.

## RESULTS

In a preliminary experiment, 10 projections were isolated and each was transferred without trypsinization to a microtiter well together with medium. Each projection generated a mixed culture in which roundish islands of polygonal cells were surrounded by heavy bundles of fusiform cells. This showed that the projections contain either both cell types or their precursors.

In order to study the details of this process, we performed cloning experiments with cells from projections by the two-stage procedure described under *Materials and Methods*. As a control, the procedure was repeated with cells obtained from young Rama 25 cultures that did not contain projections.

The results, summarized in Table 1, show that a proportion of cells from a projection are fusiform, because each generates a clone entirely of fusiform cells; the others are polygonal cells that tend to generate fusiform cells at much higher frequency than ordinary Rama 25 cells. Twenty-three percent of the cells of projections were polygonal cells that did not generate fusiform cells at high frequency. Most of these cells produce spontaneous domes at high frequency; hence, they are not contaminants from the cell layer, at least in the experiment with a Rama 25 culture, which only exceptionally produces spontaneous domes. In contrast, in the control experiment, 86% of the cells generated pure clones of polygonal cells (not producing spontaneous domes), and the others generated clones in which fusiform cells were present but in low proportions. The proportion of fusiform colonies generated from projection cells was 380 times greater than from control cells in the Rama 25 experiment.

These results show that progenitors of fusiform cells and fusiform cells themselves are formed in the projections. The presence of projections in isolated colonies of polygonal cells and their absence in cultures of fusiform cells show that they are not formed by the aggregation of preexisting fusiform cells. In order to study the generation of fusiform cells in greater

Table 2. Spontaneous production of domes, projections, and F cells in clonal sublines isolated from Rama 25 cultures

| | Domes | Projections | F cells |
|---|---|---|---|
| From projections | | | |
| LB2 | ++ | ++ | ++ |
| LB11 | ++ | + | + |
| LH6 | ++ | + | + |
| LA7 | ++ | ++ | ++ |
| LA8 | ++ | ++ | ++ |
| 102E12 | ++ | + | ± |
| 106AE10 | ++ | + | + |
| 106AF10 | ++ | + | + |
| 106AH7 | ++ | + | ± |
| LA12 | − | | |
| LH12 | − | | |
| From annular structures | | | |
| 107 | ++ | ± | ± |
| 106AA10 | − | − | − |
| 106AA12 | − | − | |
| 106A10a | − | − | − |
| 106A10c | − | − | − |
| 106A10d | ± | ± | ± |

++, Abundant production; +, moderate production; ±, rare production.

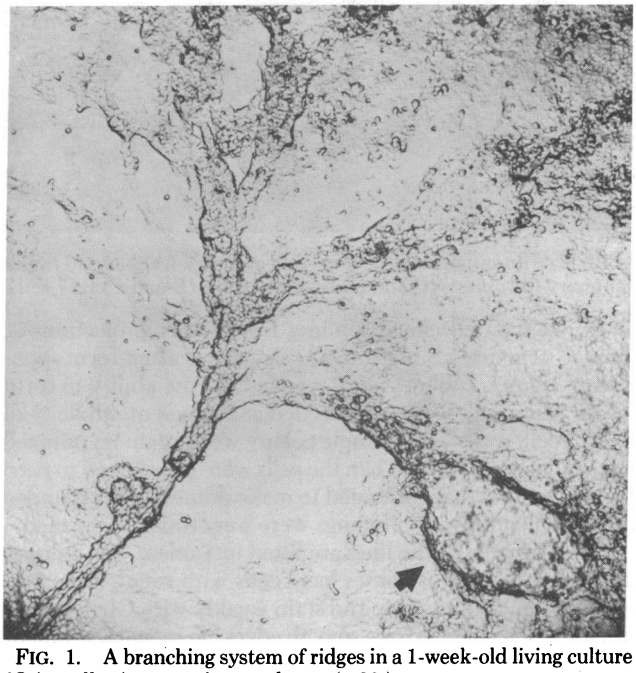

FIG. 1. A branching system of ridges in a 1-week-old living culture of LA7 cells. Arrow points to dome. (×80.)

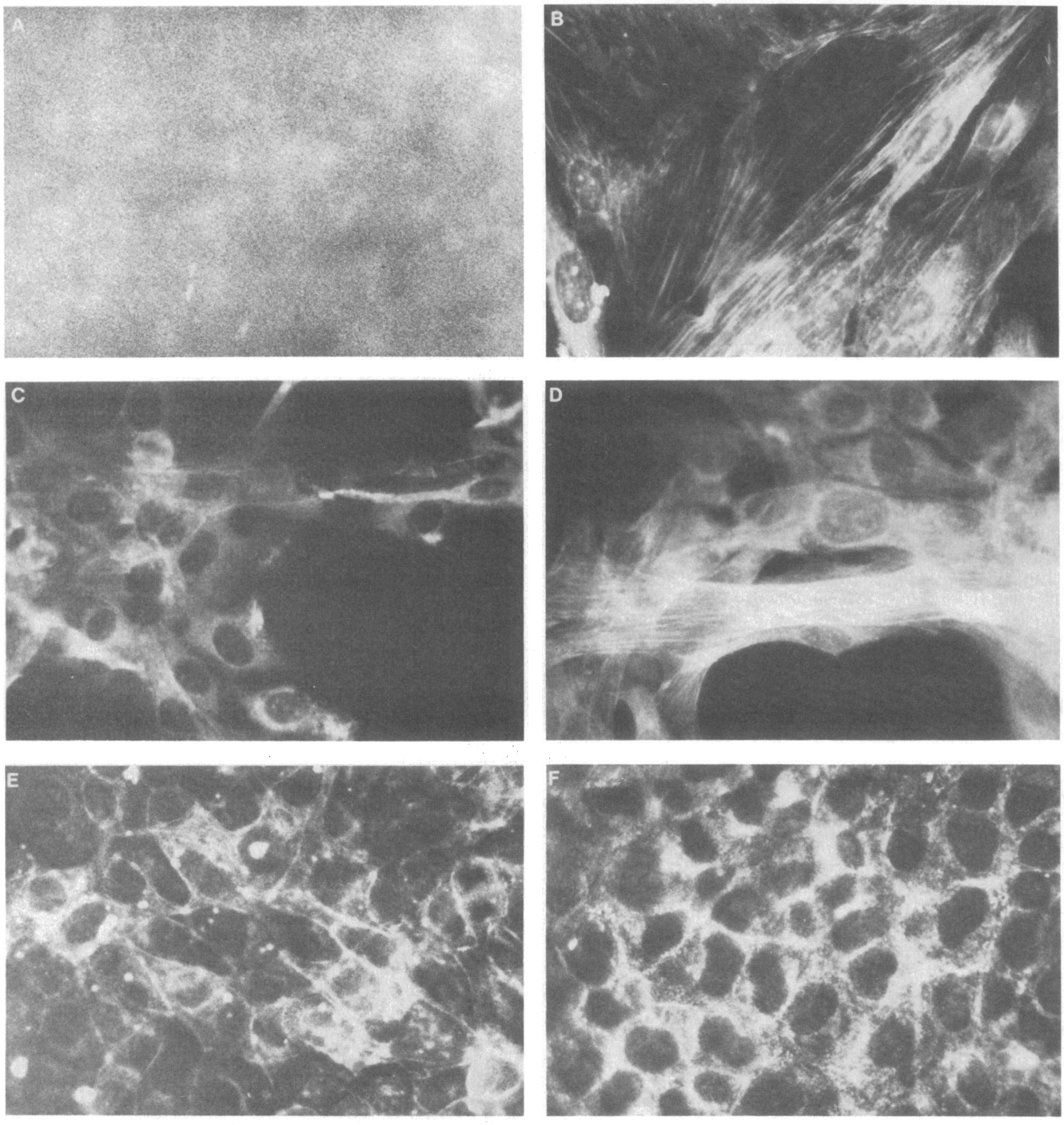


FIG. 2. Immunofluorescent myosin staining. (*A*) Normal rabbit serum; LA7 cells. (*B–F*) Antibodies to myosin. (*B*) Primary culture of rat mammary fibroblast; (*C* and *D*) fusiform cells; (*E* and *F*) LA7 cells. (×600.)

detail, we isolated clonal sublines from either projections or annular structures. Since annular structures often form spontaneous domes, sublines were selected for the ability to form domes. Microtiter wells were seeded at average multiplicity of 0.3–0.5 cell; wells with a single colony were again trypsinized and similarly recloned. Then the cells were transferred to petri dishes; the lines that continued to make domes were expanded and frozen; additional clonings were sometimes performed.

The properties of the lines are listed in Table 2. The cultures forming spontaneous domes have cells with rough or beaded edges in light microscopy and stain weakly with Giemsa; cells of nonbeaded colonies have smooth edges, no vacuoles, and stain more deeply. The doming sublines produce fusiform cells or projections, but at greatly variable frequencies. Fusiform cells arise only in cultures that make projections.

These results show that the same cell type can produce projections or domes, because the property of producing both structures persists through serial cloning in which only the dome-forming ability was selected for: four serial clonings for the LA8 line and at least two for all others.

These results identify a cell type (the *F-precursor cell*), which has the property of generating domes or projections in a reversible manner, alternating between one and the other phenotype. In addition, these sublines may also develop a system of elevated ridges, often branching (Fig. 1). When cultures heavily intersected with such ridges are trypsinized and the cells

are transferred to new cultures, they again form a typical uniform layer of polygonal cells. That the same cell can generate either domes or ridges is shown by the appearance of both structures in colonies originated from single F-precursor cells. The relatedness between domes and projections is also shown by the significant association between the two structures. In fact, of the domes having an average diameter of between 100 and 150 $\mu$m (average area, 12,300 $\mu m^2$) in a LA8-C1 culture, 252 completely overlapped one or more projections and 63 did not overlap any projection. This gives 1.61 projections per dome (Poissonian) and $1.3 \times 10^{-4}$ projections per $\mu m^2$ of dome surface. The cell layer outside the domes contained $4.0 \times 10^{-6}$ projections per $\mu m^2$. Projections could be seen forming from domes in cultures examined at frequent intervals over a period of several days.

Preliminary experiments (unpublished results) show that the medium strongly influences the type of structure produced: formation of ridges and projections is associated with a rapid proliferation rate; formation of domes, with a slow rate. In addition, the presence of clonal sublines that rarely produce projections, ridges, or fusiform cells but generate domes at high frequency also implies a genetic control of these functions.

**Other Properties of Precursor Cells.** Electron microscopy shows that the F-precursor cells have stunted microvilli at the medium (apical) surface; in domes they interact with fusiform (putative myoepithelial) cells at the opposite (basal) surface, where they are sometimes coated by material resembling basal lamina. The cells are connected by lateral junctions near the apical side, probably of the occluding type; however, the abutting sides of adjacent cells are often separated by a large space at the basal side of this junction. This feature probably explains the beaded appearance of the edges in light microscopy. The cells have well-pronounced rough endoplasmic reticulum and occasional secretory granules at the basal surface; Golgi apparatus is not prominent. Fusiform cells lack microvilli, have a similarly developed rough endoplasmic reticulum, and possess more abundant microfilaments in cortical areas.

F-precursor cells, whether or not doming, do not produce $\alpha$-lactalbumin at the level of detectability of about 3 ng/mg of total cell protein; this value must be compared with between 6 and several hundred ng/mg of protein for a number of rat mammary cancers and with 40–890 ng/mg of protein for cancers induced by dimethylbenz[*a*]anthracene (18, 19). Immunofluorescence studies of fusiform cells with antibodies to myosin (Fig. 2) revealed both diffuse staining and thin fibers, which are especially abundant in some cells. The fibers are always much less prominent than in fibroblasts cultivated from rat mammary glands. The F-precursor cells showed mostly nonfilamentous staining close to the edges of the cells, probably corresponding to the filaments detectable by electron microscopy.

## DISCUSSION

We have isolated an intermediate between Rama 25 cells and fusiform cells as a cell type (F-precursor cell) capable of spontaneously forming domes, projections, and ridges. Cells from each of these structures can revert to F-precursor cells when they are trypsinized and used to start new cultures. However, a proportion of the cells in projections then generate pure fusiform cell colonies, an irreversible process. The probability of the formation of domes, projections, or ridges in cultures of F-precursor cells depends on the medium and growth conditions. In addition, these transitions are under genetic control because they occur with different frequencies in different clonal sublines.

An important question is whether the regular evolution from doming cells to cells of projections and then to fusiform cells reflects a developmental sequence related to events occurring within the normal mammary gland. The Rama 25 line is, in all likelihood, of ductal origin, because dimethylbenz[*a*]anthracene-induced mammary carcinomas in rats arise from end buds (20), which are the growing tips of ducts. Stem cells in these buds generate epithelial and myoepithelial cells. Hence, there might be similarities between the cells of the sublines we have described and those of cells present in ducts and end buds *in vivo*.

Of the structures formed *in vitro*, ridges, because of their branching pattern, recall the ductal tree; fusiform cells are probably related to myoepithelial cells because of their relation to the basal side of the F-precursor cells and the presence of Thy-1 antigen (21), which in lactating mammary glands appears to be confined to myoepithelial cells (unpublished observations). Fusiform cells differ markedly from fibroblasts in the organization of the myosin fibers. Projections, being the sites of differentiation of fusiform (putative myoepithelial) cells, may correspond to end buds. Since the cell layer has the basal surface at the plastic, projections must be equivalent to cell clones growing from the outer to the inner surface of the bud *in vivo*. Such clones are indeed generated by stem cells at the periphery of end buds (unpublished observation) and appear to be precursors of the cells of ducts. Domes contain cells that pump ions and water from the apical to the basal cell surface; they may correspond to duct cells with the function of resorbing constituents of the milk (22–24), although there is no complete agreement that duct cells have such a function (25, 26). An equivalence of domes to alveoli seems unlikely because our cultures do not produce significant amounts of $\alpha$-lactalbumin when doming profusely.

On the basis of these assignments, the F-precursor cells, the cells of ridges and of domes, and the cells of projections not yet committed to fusiform differentiation are different states of a type of duct cell with multiple potentials (Fig. 3). Formation of ridges and projections seems to be a morphogenetic response accompanied by rapid cell multiplication; formation of domes seems to be a functional response with slow proliferation. The

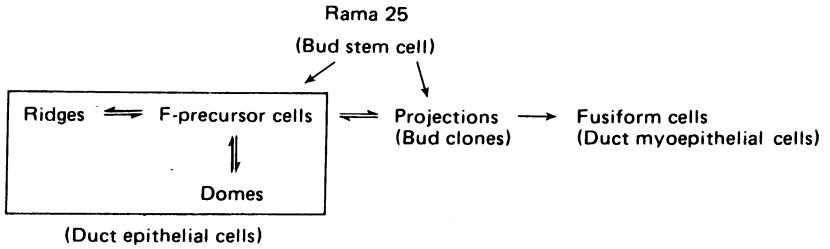


FIG. 3. Possible scheme of differentiation in the Rama 25 line and sublines. In parentheses are the possible corresponding cells in the mammary gland.

original Rama 25 cells would represent a bud stem cell derived from those present in end buds *in vivo*, which infrequently generates F-precursor cells in the form of projections and sometimes of annular structures (which in turn might have evolved from projections).

The various differentiations observed *in vitro* may be controlled by the cell environment. A role of the medium has been observed; the association of each differentiation with a specialized structure suggests a role of cellular interactions. The cell lines we have isolated may be useful for studying these two control mechanisms of differentiation of mammary cells in an *in vitro* system.

We acknowledge the excellent technical assistance of Margaret Henahan and Sharon Muir-Okada. We thank Dr. U. Gröschel-Stewart for a gift of antibodies to myosin, A. E. Bogden for a gift of rat α-lactalbumin and antiserum, and George Klier for helping with electron microscopy. This investigation was supported by Grant 1-RO1 CA21993-01, awarded by the National Cancer Institute, and by grants from the Hammer Foundation, the Educational Foundation of America, and the Pardee Foundation. M.B. is a recipient of an Educational Assistance Fellowship from Merck, Sharp & Dohme International. M.U. is an American Cancer Society Postdoctoral Fellow, Grant PF 1212.

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