

Polyploid Monolayer Ishikawa Endometrial Cells form Unicellular Hollow Spheroids Capable of Migration

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Abstract

The results in this paper demonstrate that Ishikawa endometrial monolayer cells become multinucleated by a process of nuclear “donation” from neighboring cells. As the resulting polyploid cell detaches from the colony in which it was formed, it is possible to detect mitonucleon(s) in the center of the cell. The mitonucleon is a transient mitochondrial superstructure surrounding aggregated chromatin (Fleming et al. 1998) with characteristics of the family of mitochondrial superstructures that are sometimes called spheroids or cup-shaped mitochondria (Fleming, 2016a). As was recently demonstrated gas vacuoles form within mitonucleons (Fleming, 2018). In the free-floating single cell, the retained gas creates a central vacuole, and the cell becomes a spheroid that floats above the monolayer. It resembles a “signet ring cell” in being characterized by a central vacuole and chromatin compressed against the vacuole membrane. The resulting structure is a spheroids that is hollow and unicellular, albeit polyploid. But whereas signet ring cells are assumed to be undergoing apoptosis, that is not the case for unicellular spheroids. Complete spheres with chromatin and cytosolic cell contents compressed against the cell membrane can be found floating independently above Ishikawa monolayers. When an isolated sphere settles back onto the surface of the petri dish, it is possible to observe dissipating gas bubbles within the now flattened sphere for a short period of time. When the gas is discharged the resulting cell looks like a typical giant polyploid cell.

Introduction

Almost 20 years ago, we described a transient mitochondrial superstructure formed when confluent Ishikawa (Nishida et al., 1973) endometrial cells were stimulated to differentiate into fluid-filled hemispheres (Fleming et al., 1998; Fleming, 2016a,b,c). These hemispheres can ultimately extend into gland-like structures, a frequent epithelial differentiation that takes place in organs throughout the body and in pre-menopausal women every month in the endometrial lining of the uterus.

Within the first few hours following monolayer stimulation, large structures staining for endogenous mitochondrial carboxylases can be seen lying next to nuclear aggregates in developing syncytia (Fleming, 2018). The resulting spheroidal superstructure which eventually completely envelops aggregated nuclei fosters vacuole formation. Small vacuoles form within the chromatin and a second much larger vacuole forms, contiguous with, but separate from, the enclosed chromatin mass. As the vacuole expands within the mitochondrial superstructure, it causes a significant protrusion of the syncytial apical membrane against which it presses the enveloped chromatin.

We have called this structure a mitonucleon, hoping that the name communicates the essence of this transient mitochondrial superstructure surrounding aggregated chromatin. On the basis of this structure, it seems reasonable to conclude that the mitonucleon belongs to a family of mitochondrial superstructures that others have called spheroids or cup-shaped giant mitochondria that can also surround chromatin. There are particularly relevant similarities between the mitonucleon (Fleming, 2018) and the mitochondrial superstructure that has been called nebenkern and is responsible for the elongation of grasshopper (and other insects) spermatid tails (Fleming, 2018). This paper describes the observations that in addition to forming in syncytia mitonucleons can develop in a single Ishikawa endometrial monolayer cell if it becomes multinucleated.

Results

The structure in fig. 1a is larger than the surrounding cells and stains intensely for chromatin using hematoxylin and eosin. Cells immediately around it are oriented toward, sometimes even appearing to be physically in contact with, this enlarged central cell. That orientation together with the density of staining in the receiving cell suggests nuclei are being donated from surrounding cells into the central cell. Although this is not a phenomenon frequently or perhaps even ever previously recorded in cell culture, the transfer of “nurse cell contents” into germ cells was observed decades ago in *Drosophila* (Painter, 1959).

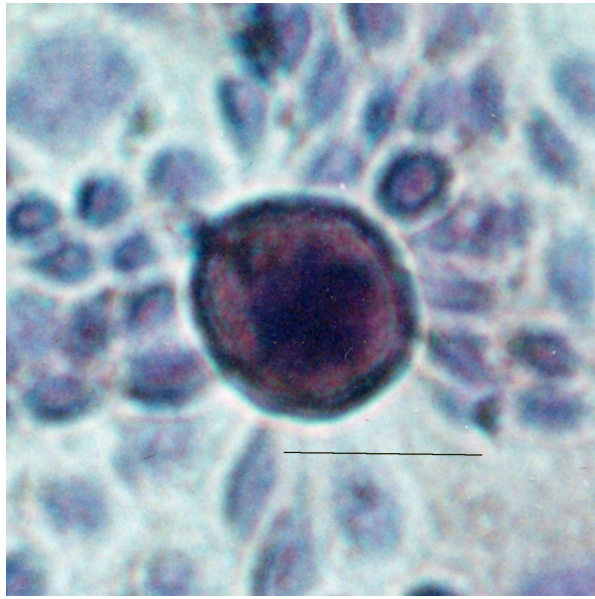


Fig. 1a Enlarged cell staining both for chromatin (dark central mass) and for biotin (pink material surrounding nuclei).
Bar=50 microns

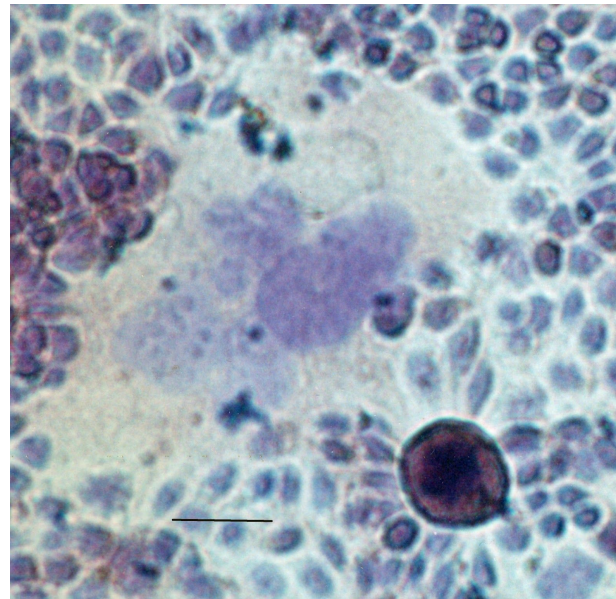


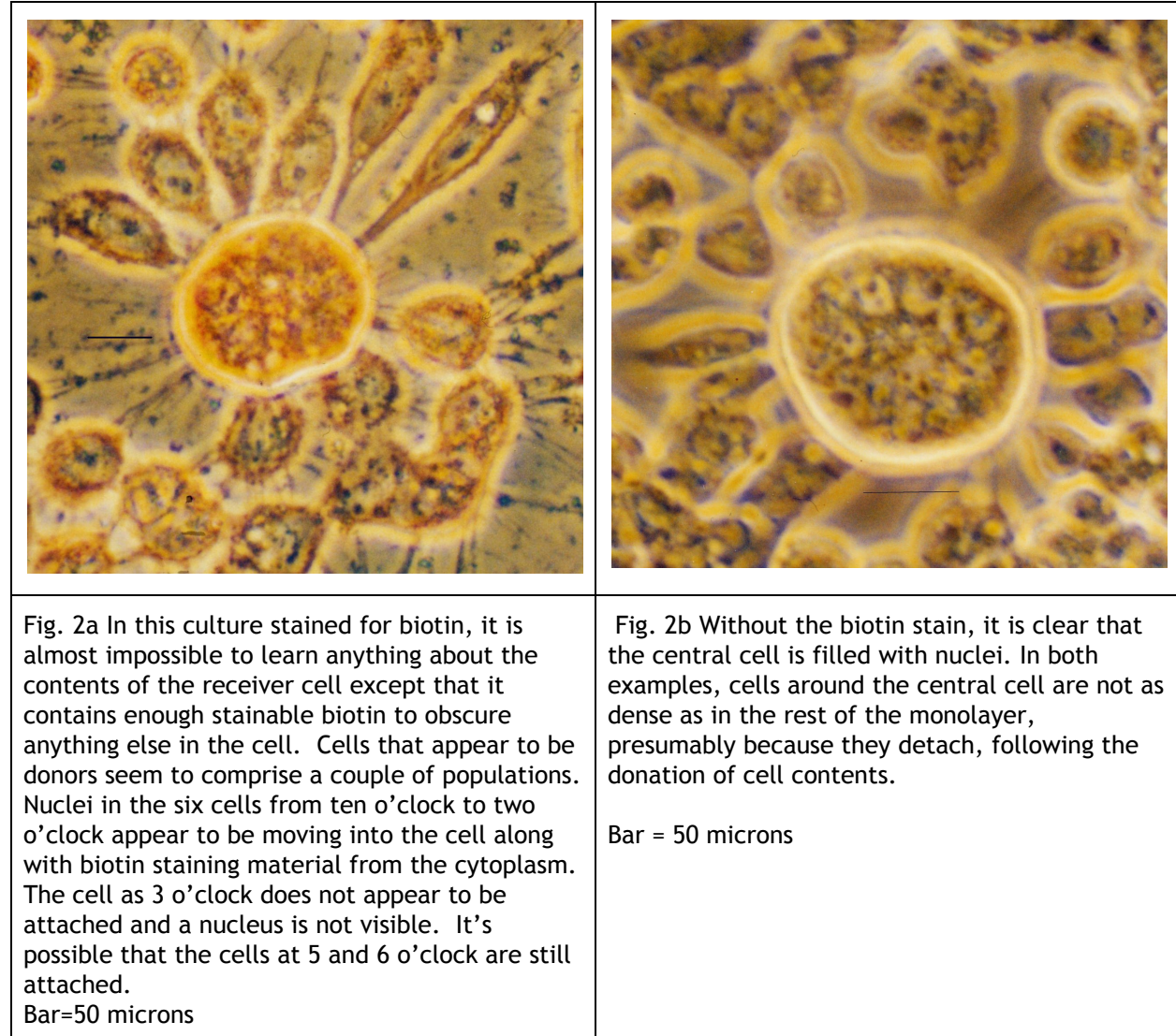
Fig. 1b Enlarged cell and neighboring syncytium. Faint pink stains from biotin are seen in both structures. This polyploid single cell was found in a dish being induced for dome formation. Such cells are also found in cultures that are not being induced. Bar=50 microns

As fig. 1b shows, this enlarged multinucleated cell was found close to a syncytium that is also multinucleated. Fig. 1b demonstrates that two different mechanisms can result in two very different looking polyploid structures in Ishikawa cell cultures. The syncytium in fig. 1b covers an area as large as 50 or more cells following fusion of a similar approximate number of mononucleated cells. Nuclei in the syncytium appear to be fading, possibly signalling a chemical change in chromatin as they begin to fuse into aggregates. The neighboring multinucleated cell is far more compact, but still at least four to five times larger than monolayer cells. There is no visible border around the syncytium other than what is formed by neighboring mononucleated monolayer cells. The multinucleated cell in fig. 1a has a distinct cell membrane and some of the neighboring cells are fusiform with one polar end appearing to be in contact with the enlarged cell.

Staining only for endogenous biotin which we and others have shown is bound to mitochondrial carboxylases (Fleming et.al., 1998; Gamache et. al., 2003), fig 2a reveals two additional characteristics of an enlarged acceptor cell and the surrounding donor cells. So much biotin stain is taken up by the receiving cell in fig. 2a that it is not possible to distinguish much else within the cell suggesting that, in addition to multiple nuclei, large amounts of endogenous biotin in mitochondria are also donated to the receiver cell. In the surrounding cells the endogenous biotin is in the cytosol highlighting the extreme fusiform shape of the cells oriented toward the “accepting” cell. Nuclei with prominent nucleoli in the

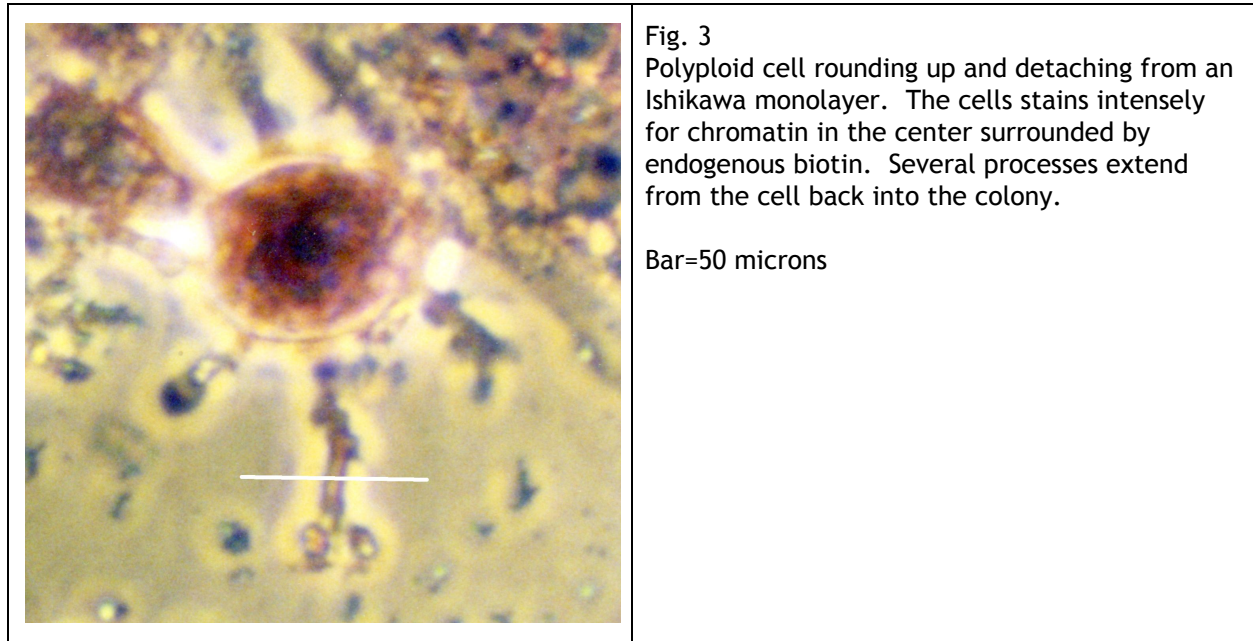
five cells making contact with the receiving cell between 10 o'clock and 2 o'clock appear to have been fixed in the process of moving into the central cell. Nuclei cannot be seen in the cells on the lower right side of the receiving cell and these cells appear to be rounding up, suggesting that they have already donated most of their contents to the receiving cell.

Fig. 2b shows a multinucleated cell in a living culture. The donor cells in this figure are slightly out of focus suggesting that the central cell has not only increased in size in the plane of the dish, but has also begun to swell. One or two fusiform cells can be seen at 9 o'clock. Multiple nuclei are clearly visible within the receiving cell.

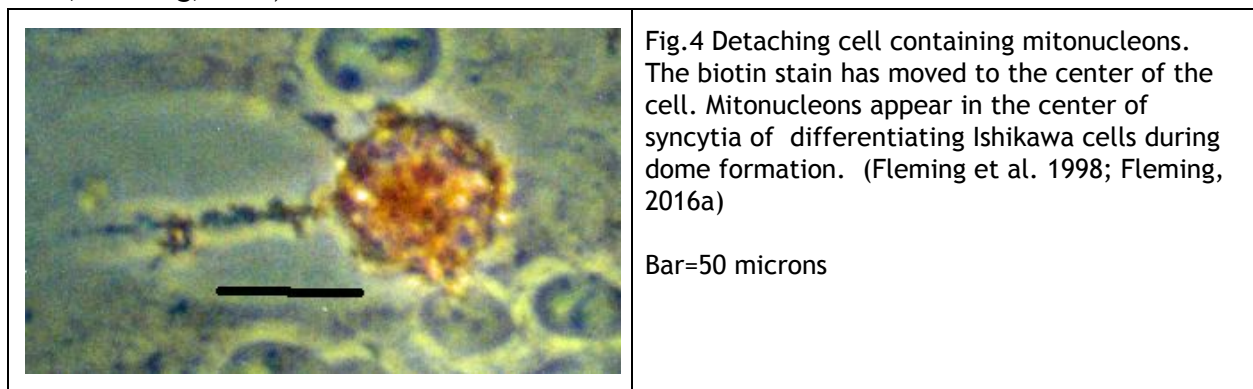


It is particularly obvious in figs. 2 a and b that cells immediately surrounding the enlarged cell are thinning out, a not surprising development if, as the pictures indicate, they have donated nuclei and cytosol into the large central cell. Nurse cells in drosophila oocyte are also known to "die off." (Painter, 1959)

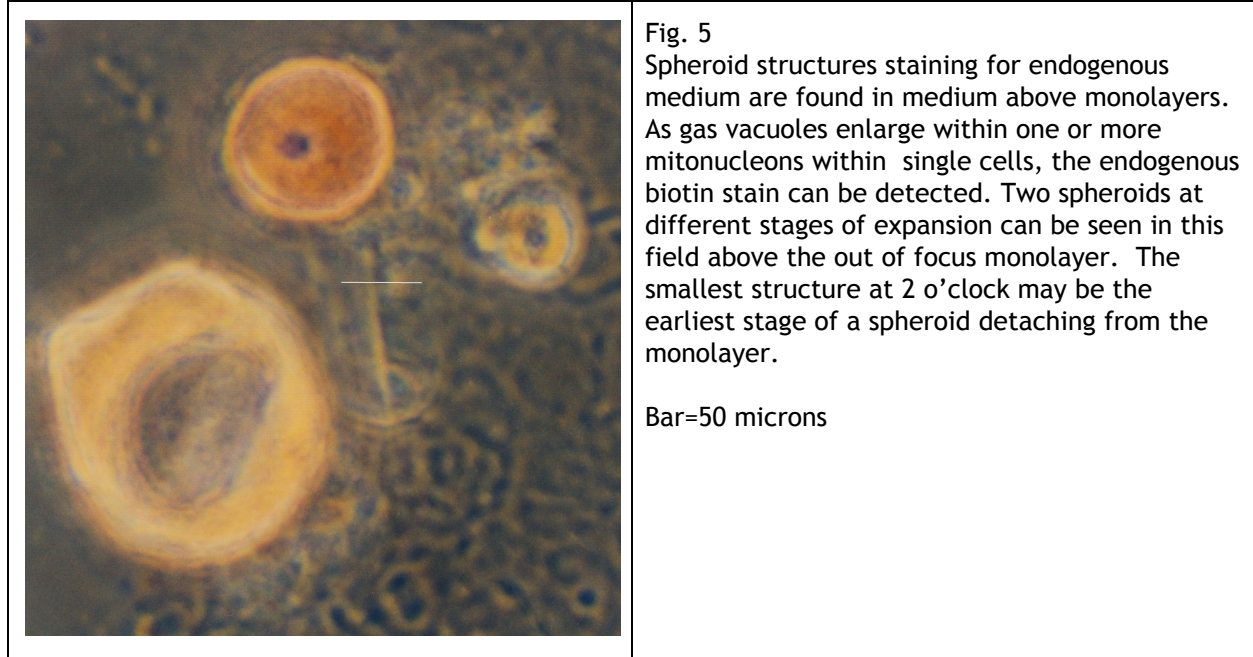
An enlarged cell staining for chromatin in the center with endogenous biotin out to the edges of the cell is shown in fig. 3. Almost all of the surrounding cells have detached. Several processes extend from the multinucleated cell back to the colony.



In fig 4 an enlarged cell above the monolayer, fixed and stained for endogenous biotin, shows biotin-staining structures in the center of the detaching enlarged cell. These structures look like mitonucleons initially seen in Ishikawa syncytia developing into domes. (Fleming et al. 1995; Fleming, 2016)



The mitonucleons are elaborated when multiple giant mitochondria become dense around aggregated nuclei and then round up into spheroidal structures enveloping chromatin. Within these structures, the aggregated nuclei become pycnotic as a result of gas vacuoles forming within the mitonucleon. (Fleming, 2018) Pycnotic chromatin is assumed to be symptomatic of apoptosis. But in dome differentiation, and again in the formation of hollow spheroids, pycnosis does not signal cell death. Retained gas results in a swelling of the cell into a floating spheroid that can be found in the medium above the monolayer (fig. 5).



There appear to be at least 3 structures in this figure that stain for biotin. The smallest, at 2 o'clock is mostly out of focus and may still be attached to the monolayer. The second spheroid at 12 o'clock is clearly detached from the monolayer with a third spheroid nearby. In this spheroid something like a window can be detected in the midst of the biotin stain. Looking at a couple of similarly large spheroids not stained for biotin in Fig. 6, suggests that this "window" reflects some compartmentalization as cellular material is being pressured against the cell wall of the spheroid by a growing gas vacuole.

It is possible to detect two compartments in the unstained enlarging spheroids in fig. 6. One compartment appears to be empty and is, all evidence suggests, filling with gases retained in the mitonucleon(s). Those gases are compressing the cellular material of the other compartment against the wall of the spheroid. Cytoplasm and structures resembling compressed, giant nuclei are being compressed against the cell wall as the mitochondrial superstructure generates and retains gases.



Fig. 6a
Unstained spheroid provides a glimpse of what happens to cellular material as the spheroid vacuole enlarges. Approximately two thirds of the spheroid appears to be empty. The results suggest that this enlarging vacuole flattens cell material as well as nuclei against the membrane of the expanding spheroid.
Bar=25 microns

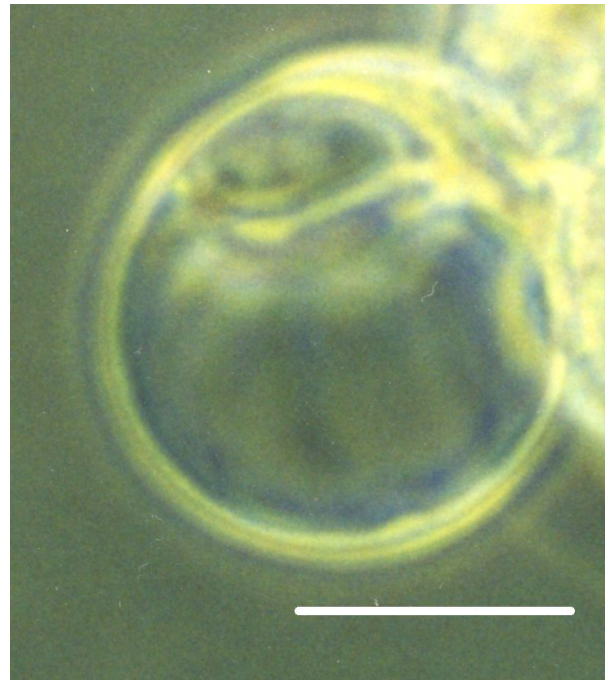


Fig. 6b
Another unstained spheroid in the process of forming a unicellular hollow spheroid.
Bar=50 microns

The pressure exerted by the enlarging gas vacuole eventually results in the structure shown in figs. 7a and 7b, pictures taken of a floating hollow unicellular spheroid focusing first on nuclei and then on one polar end of the spheroid. When the objective of the microscope is focused on two giant nuclei it can be seen that they exist within a rim of cytoplasmic material stretching around the spheroid. Focusing up, the nuclei are no longer visible at one polar edge of the spheroid, although a dark circular structure can be seen, presumably within that thin layer of cytoplasm detectable in fig. 7a.

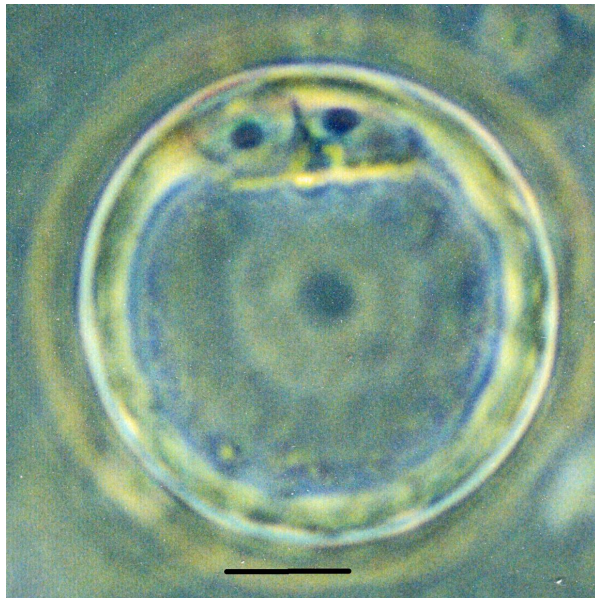


Fig.7a Polyploid unicellular hollow spheroid. Objective focused on giant nuclei. Bar=25 microns

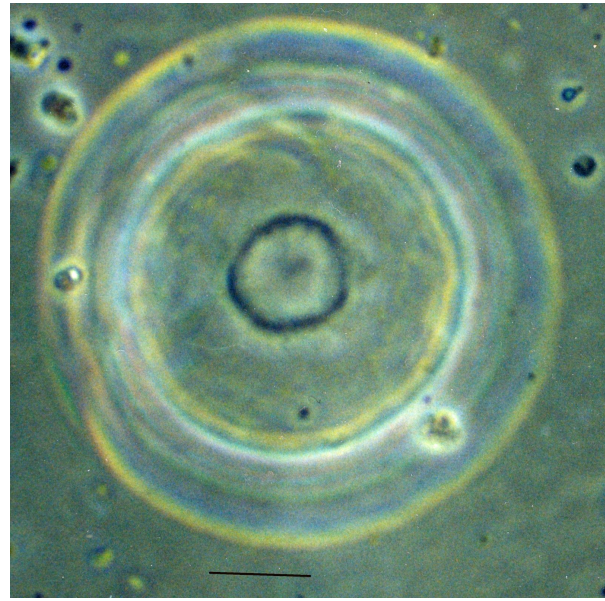


Fig.7b Polyploid unicellular hollow spheroid. Objective focused on polar end of spheroid. Bar=25 microns

Cellular spheroids were carefully collected from the petri dish and transferred into another dish to which serum was added. Structures like that in fig. 7 would be expected to be delicate, but inspection of the dish indicated that multiple spheroids survived the transfer. Under these conditions unicellular hollow spheroids reattach to the surface of the petri dish, releasing some kind of gas or mixture of trapped gases. These bubbles assumed to be trapped in the transient mitonucleon structure dissipate within minutes leaving behind giant cells containing one or more enlarged nuclei.

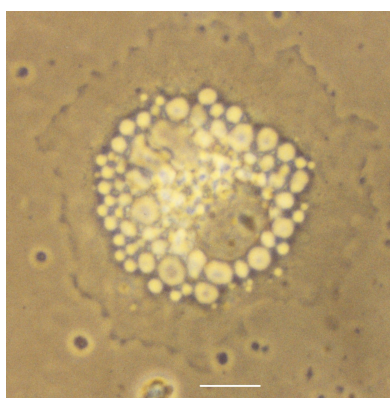


Fig. 8a. Central vacuole of hollow Ishikawa spheroids contain gases. When serum is added to medium containing hollow spheroids, the floating spheroids

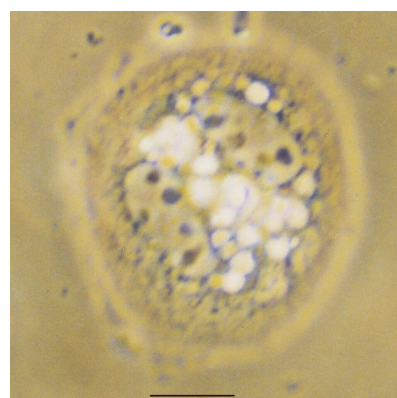


Fig. 8b. Bubbles dissipate rapidly. Bar=25 microns

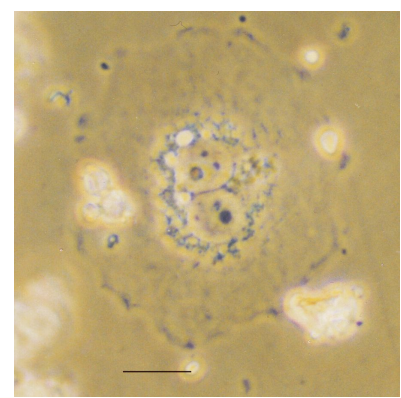


Fig. 8c Multiple giant polyploid cells such as this cell plated down serum-free medium containing hollow spheroids was

“pancake” onto the surface of the petri dish. For a short time after the initiation of reattachment, it is possible to observe gaseous bubbles within the structures.
Bar=25microns

transferred to a culture dish with the addition of serum.
Bar=25 microns

The colonies in figs 8a, b, and c provide additional evidence that the enlargement of multinucleated cells into hollow spheroids occurs because gas is trapped within these structures.

Hollow spheroids are also sometimes observed attached to solid spheroids as shown in Figs. 9a and 9b. In each case, the microscope is focused on the most prominent structure in the hollow spheroid, one or more large nuclei, presumably polyploid, flattened against the outer cell membrane.

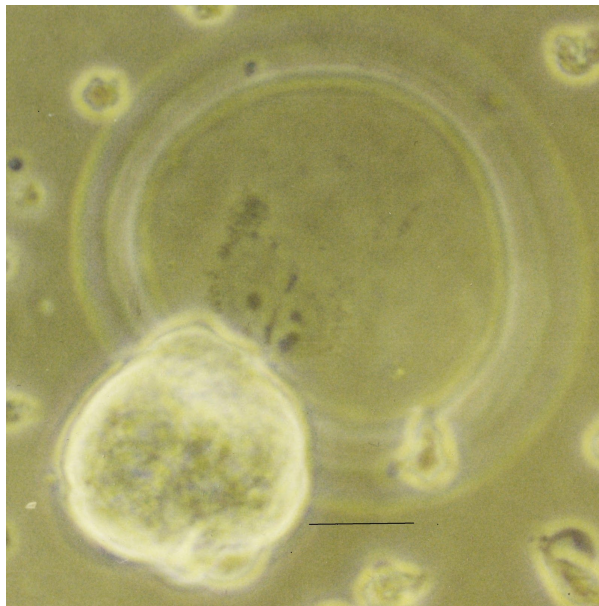


Fig. 9a Hollow spheroid developing apparently attached to a solid spheroid. Based on what is seen in monolayer and shown in this paper, best guess is that a cell associated with the solid spheroid becomes multinucleated, develops mitonucleons that retain gasses.
bar=50 microns

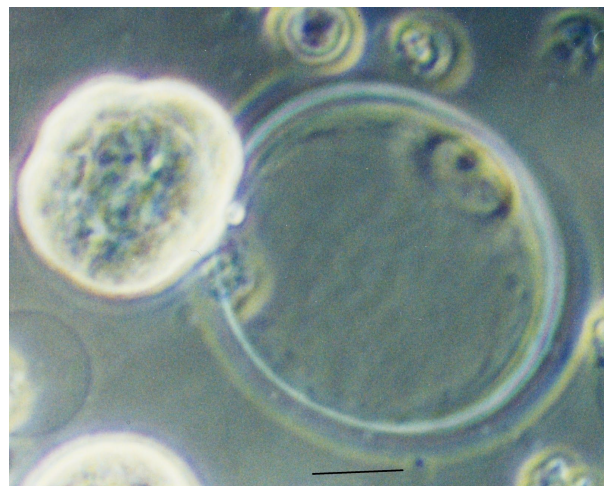


Fig. 9b Another example of a hollow spheroid apparently attached to a solid spheroid.
bar=50microns

When serum is added to medium containing structures like these, spheroid cells revert to monolayers over time. When the solid spheroid also has an attached hollow spheroid, part of the resulting colony presents with the distinctive look of giant nuclei packed together in a common pool of cytoplasm. In fig. 10a, the edges of the extension suggest a splattering of

the outer membrane of the “cell” as the hollow spheroid became attached rather like a “burst” balloon. It is expected that giant nuclei such as those in fig. 10a will bud out, a process demonstrated for giant nuclei in a variety of systems (Zybina & Zybina, 2002; Erenpreisa et al. , Walen, Sundaram et al.) the previously hollow spheroid takes on a distinctive look, different from the rest of the colony with smaller nuclei presumably budding off giant nuclei within an abundance of cytoplasm.

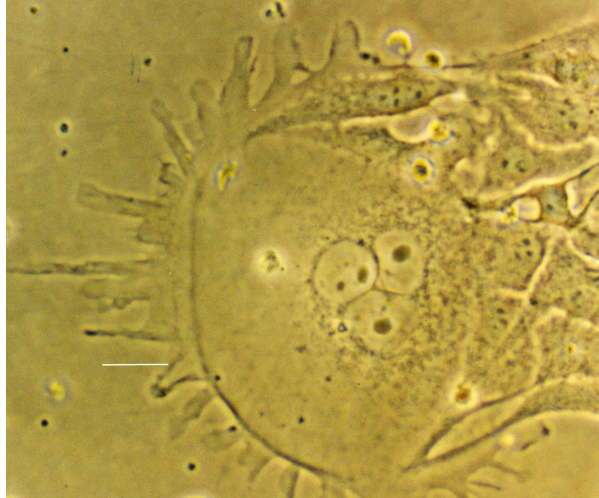


Fig. 10a Just as hollow spheroids can plate down under appropriate conditions an attached hollow spheroid can plate down. The scalloped edge of the structure suggests some internal pressure in what was once a hollow spheroid resulted in a membrane blow-out.

Bar=25 microns

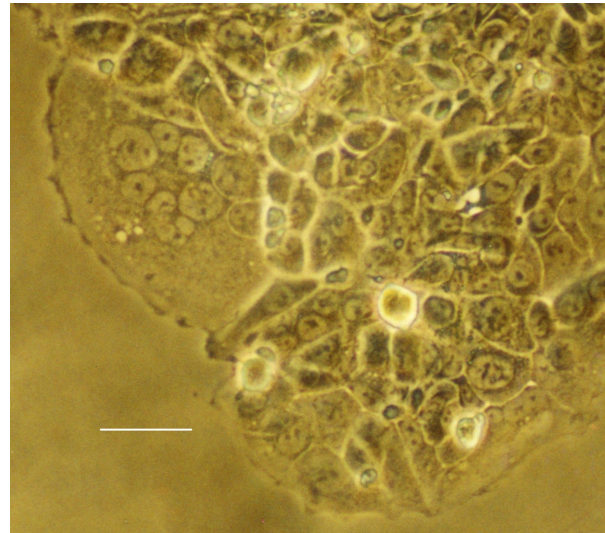


Fig. 10b The absence of distinct cell membranes around the 8 or more nuclei in the extension of this colony suggest that these nuclei budded off from polyloid nuclei associated with a hollow spheroid outgrowth of a solid spheroid. It is worth noting that the nuclei that have budded off are not uniform in size suggesting nuclei with different n values just as has been described in other systems.

Bar=50 microns

Discussion

Polyloid cells, particularly those in which multiple nuclei have fused into one or more giant nuclei such as are seen in figs. 8 and 10, were once thought unable to produce progeny. But in the past couple of decades, polyloid cells have emerged as more than simply diploid cells gone bad. There is growing interest in how they arise and what their functions might be since a number of reports have clearly demonstrated that they can produce progeny. Studying the naturally occurring polyloid trophoblasts for more than two decades, Zybina and Zybina reviewed that work in a 2008 paper discussing the fragmentation of giant nuclei into smaller nuclear fragments and the budding of these into cytoplasm. Erenpreisa and colleagues (2000) demonstrated a similar phenomenon in cells subjected to irradiation that induces mitotic catastrophe. Most cells die, but some cells become polyloid, synthesizing DNA, but not

dividing. Erenpreisa showed that giant polyploid cells surviving this insult are able to give rise to mitotically capable diploid cells in a process both she and Zybina call depolyploidization. Budding is another term that has been used to describe the manner in which polyploid cells give rise to diploid cells. Walen (2002) described the budding off of normal diploid cells from human cells rendered polyploid by viral transformation (2002) as Sundaram et al. (2004) did for polyploid cells that arise when mouse embryo fibroblast lines are exposed to carcinogens,

The consensus seems to be that endocycling, DNA replication without cytokinesis, is the predominant way in which polyploid cells arise, with syncytial formation recognized as an alternative mechanism. It is syncytial formation that gives rise to polyploid predomes in Ishikawa endometrial cells as previously described (Fleming 1998 and Fleming 2016).

In this paper we have shown that monolayer cells can become multinucleated by a third mechanism not previously described in cell monolayers. It appears that fewer than 1% of monolayer cells become polyploid because surrounding cells donate nuclei and cytosol contents into a central enlarging cell. It is not clear what signals within the culture bring about the coordination of cells involved in this process. That it happens is suggested by the presence of fusiform cells surrounding, and in some instances appearing to be attached to, an enlarged polyploid cell. Accessory cells such as these have been called “nurse cells” in other systems. A well-known example involves *Drosophila melanogaster* whose egg chamber contains 16 cells. One cell will become the oocyte, the other 15 cells, the nurse cells, will donate organelles, proteins and nucleic acids to the oocyte, preparing the egg to support the development of an embryo until sustenance from the environment becomes available (Painter, 1959).

Polyploid receiving cells far along in this process are sometimes found with few, if any, surrounding cells presumably because donor cells die following transfer of cell contents. As the polyploid cell itself detaches, multiple cell processes can be detected reaching back into the colony from such cells. Processes back to the cell colony can be detected even as the polyploid cell begins to become elevated, resulting for a time in “tethered” detached cells. Such processes may be similar to cytonemes that are believed to facilitate communication (Kornberg & Sugata R., 2014). Multiple nuclei occupy the center of the cell surrounded by material staining for endogenous biotin.

In the final stages of detachment the biotin stain is found in the center of polyploid cells in structures that resemble the mitochondrial superstructures (mitonucleons) that envelop nuclear aggregates during dome formation (Fleming et al. 1998; Fleming 2016; Fleming 2018). One function of these mitonucleons appears to be the retention of gases (Fleming 2016; Fleming, 2018), and in a single detached cell the retention of gases results in the formation of a unicellular, albeit polyploid, floating spheroid.

Spheroids of various sizes can usually only be seen by focusing above the monolayer. When such spheroids are not stained for biotin the internal structure of hollow spheroids reveals cellular material in the process of being compressed against the cellular membrane.

The results in this paper also show that the gases building up exert pressure resulting in cell contents, including nuclei, becoming marginalized, flattened between the mitonucleon and the outer cell wall (fig. 7) forming unicellular hollow spheroids. The gases at the center of such a structure “become visible” as trapped bubbles in the minutes after hollow spheroids attach to a petri dish in response to the addition of serum. Gas bubbles, initially visible, rapidly disappear.

What cannot be said for certain is the identity of the gases that are building up. Carbon dioxide is a common by-product of aerobic metabolism but it has long been an article of faith that this gas mostly passes freely in and out of cells. In fact however, variability in the rate of diffusion as has been demonstrated in experiments in gastric gland cells (Waisbren et al. 1994). Endevard and colleagues (2013, 2014) have demonstrated a lower rate of diffusion of CO₂ through the apical as compared to the basal membrane of the guinea pig colon. Furthermore, channels have been shown to be involved in CO₂ transport under certain conditions (Nakhoue et al. 1998; Itel et al. 2012). Is the passage of CO₂, the most abundant by-product of mitochondrial metabolism slowed by the mitochondrial superstructure? Interestingly enough Armstrong and his colleagues found giant spheroidal mitochondria in human endometrium at around the time of ovulation, when gland formation begins. This is a finding that suggests what we are studying *in vitro* is relevant *in vivo*. Using an electron microscope, they were able to show that these structures were surrounded a single sac of endoplasmic reticulum. (Armstrong et. al, 1973) Might this unusual presence of an additional membrane explain a build up of gases?

What about other gases known to evolve as a result of metabolism and to be active as gaseous transmitters. Most of these appear to be produced in small amounts and to have a short half life, although half life in a mixture of gases might be different. Perhaps the most intriguing possibility, and purely speculative, is that mitochondrial superstructures bring about changes in the mitochondria that result in a temporary anaerobic environment in which H₂ can be generated. Embley and colleagues (2003) have published a paper speculating that mitochondria and hydrogenosomes are two forms of the same fundamental organelle. And, Boxma et al. (2005) have demonstrated the existence of anaerobic mitochondria that produce hydrogen. Admittedly speculative does a fraction of the total mitochondria in endometrial epithelia forming domes or spheroids “revert” and begin producing hydrogen, a gas that would certainly give spheroids a lift! (Fleming, 2018; Fleming, 2016a)

In this regard, it is important to note that the mitonucleon is transient. Mitonucleons active in Ishikawa differentiation result in the accumulation of gases that unfurl the apical membrane and compress aggregated chromatin against that membrane. The structure becomes distended with gas and subsequently falls apart. The evidence in this paper suggests

that the component parts of the mitonucleon membrane are absorbed back into the giant cell that forms when the spheroid reverts to a monolayer cell. Furthermore, the evidence, particularly for mitonucleons observed during dome formation, is that only a fraction of mitochondria might be generating hydrogen within the mitonucleon, while the remainder of the mitochondria out in the cytoplasm, not confined within the mitochondrial superstructure, are expected to remain capable of aerobic metabolism.

Multicellular Hollow Spheroids

As we have shown, mitonucleons within single cells result in floating spheroids. Spheroids have been of interest as an alternative method for culturing mammalian cells for almost as long as scientists have known how to keep human cells alive in monolayers. Most papers describing cell spheroids are concerned with solid spheroids such as are shown in fig. 9. It is thought that cells interacting with each other in such a three-dimensional structure may more closely mimic the condition of cells *in vivo*. Whereas the terminology for monolayers is relatively simple, the terminology used for spheroids has led to at least 25 different terms for these three dimensional structures, many deriving from tissue origins, with a nod to the methodology used to foster their formation (Weiswald et al.; 2015). Most cell lines such as Ishikawa can be grown as monolayers or as spheroids by manipulating growth conditions. Solid spheroids were elicited in cultures of Ishikawa cells plated with medium containing no serum. As Achilli et al. (2012) recently described, there are at least 7 other culturing conditions that will result in the formation of spheroids by lessening contact between cells and surfaces such as the plastic surface of petri dishes. Furthermore, and relevant to our discussion, there is at least one paper showing that ovarian cancer cells in a monolayer can give rise to multicellular solid spheroids spontaneously and that these are released into the medium following a period of being tethered to the monolayer that produced them (Pease et al. 2012).

A review article in 2008 discusses the biological features unique to spheroids that might make them an important player in dissemination of ovarian epithelial cancer. (Shields et al., 2008) One of the most fascinating differences demonstrated between solid spheroids and monolayers of epithelial ovarian cells appears to be that many fewer cells disaggregated from a spheroid will elicit cancer in a test animal than cells harvested from a monolayer (Odunsi et al., 2014). As few as 2000 cells from a dissociated spheroid of epithelial ovarian cancer cells will, when injected into immune deficient mice, elicit the formation of a tumor. Even a bolus of parent cells from monolayers five times larger than that was not found to be consistently tumorigenic. This observation, and the results from a variety of assays for stem cells, led the researchers to conclude that solid spheroids are enriched in a population of stem cells that are highly tumorigenic.

But there are differences among spheroids. Most of the results listed in the NIH database for cellular spheroids, close to 5000, are about solid spheroids. Fewer than 10% of that number

discuss hollow spheroids most of which are found in malignant ascites fluid. Furthermore, and important for the results in this paper, most of the time the term hollow spheroid has been used to describe multicellular hollow spheroids in which a single layer of 50-200 epithelial cells surrounds the inner lumen. In this paper we have demonstrated a third kind of spheroid which is both unicellular and polyploid.

The earliest paper on multicellular hollow spheroids was published in 1979 by a Japanese research team (Nishida et al.) who called hollow spheroids “mirror balls.” In 1987, Allen’s group (Allen et al.) from Roswell Park published a paper with pictures of hollow spheroids in ascites fluid. They reported on multicellular aggregates found in the peritoneal fluid of patients with ovarian cancer, describing clusters ranging “from compact to loosely adherent groups of cells to spheroids with a central lumen surrounded by a cell monolayer” . Allen alludes to observations of acellular spheres of light density but concludes that these result from a loss of surrounding monolayer cells during processing of the fluid. Maybe, or maybe he was seeing structures like those pictured in fig. 8, which appear acellular if the microscope is not focused on the nuclei (fig. 8b), a phenomenon that would not have been anticipated.

More than a couple of decades passed before many more studies were done. Ishiwata and colleagues (1997) have studied ovarian and endometrial adenocarcinoma cells *in vivo* and *in vitro*. Both cell types formed “hollow cell ball structures” (their term for multicellular hollow spheroids) but the researchers concluded that these arose from solid cell ball structures, hollowed out by necrosis of the internal cells. What they saw may have arisen in that manner. The unicellular hollow spheroids that are the subject of this paper definitely do not start out as solid spheroids, although they are sometimes seen budding out from solid spheroids.

Much of the work of Kato and colleagues (2011) involving hollow spheroids harvested from patients with clear cell carcinoma, attempted to correlate the presence of spheroids with the presence of spherule-like hyaluronan rich stroma in tumor tissue. The researchers also collected spheroids from the abdominal cavity of nude mice and demonstrated that hollow spheroids survive *in vitro* and give rise to daughter hollow spheroids. The evidence in Ishikawa cultures does not contradict those findings but most clearly shows that solid spheroids of Ishikawa endometrial epithelia can give rise to unicellular hollow spheroids.

Interestingly enough hollow spheroids, embedded in normal tissue, have also been found beyond the invasive margin of tumors of the colon and have been identified as an independent risk factor for metastases and shorter survival. (Tamura et al. 2011) It is not obvious how these hollow spheroids develop within tissue but the implication is that they arise from invading cells that are vectors of malignancy. That observation suggests that there may be significant relevance from the point of view of metastasizing solid tumors, that unicellular hollow spheroids can “bud” out from solid spheroids *in vitro*.

The unicellular hollow spheroids being described in this paper arise from single cells that have become multinucleated and enriched in structures containing biotin. When such a cell is

detaching from the colony in which it was created, mitonucleon(s) can be detected in the center of the cell. As recently shown, mitonucleons retain gases (Fleming, 2018). An increasing gas vacuole results in the creation of a structure that looks like the three dimensional realization of "signet cells," with one or more polyploid nuclei and cytosol compressed in the space between two membranes: the outer membrane of the mitonucleon (vacuoles having fused as mitonucleons became distended) and the cell membrane. Gases build up pressing cell contents against the cell membrane. The resulting highly unusual cell structure floats around until conditions are appropriate to once again become attached. Since endometrial cells that detach from the uterus and move out into the peritoneal fluid seem to be the most likely vectors for endometriosis, the mechanisms that such cells use to switch from being attached to being unattached and mobile are of interest. Furthermore the observation of hollow spheroids (Allen et al.) in malignant ascites fluid may be relevant to the metastases of ovarian cancer.

Materials and Methods

It is relevant that the unicellular hollow spheroids described in this report would probably be destroyed by many of the methods used to prepare cell specimens for microscopy. The handling of medium containing unicellular hollow spheroids was kept to a minimum. All of the photomicrographs in this paper are of cultured endometrial epithelia called Ishikawa developed by Nishida and colleagues in 1985. His laboratory established that the line contained receptors for both of the female sex steroids, estradiol and progesterone. The cells were obtained from Erlio Gurpide's laboratory at Mt. Sinai, New York. As described in the first paper on the topic of dome formation in 1995 (Fleming), the cells were cultured in phenol red-free Minimum Essential Medium (MEM) supplemented with 2mM glutamine, 100 U/ml penicillin, 0.1 mg/ml and .25 mg amphotericin B (GIBCO, Grand Island, NY).

Multinucleated cells were found in cultures approaching confluence. Cells seeded at an approximate density of 5×10^5 cells/cm² in MEM containing 5% calf serum (CS) at 37 degrees C and 5% CO₂ were grown for at least one week. Cells were viewed using an Olympus inverted stage microscope at powers of 100X (fig1a), 200X (fig.1b) and 400X (all other figures).

The structures shown in figs. 2b and 6 through 11 were photographed in living cultures.

As first described in a paper published in 1998 (Fleming et al.) endogenous biotin in the structures shown in figs. 1, 2a, 3, 4, and 5 was detected by an assay using extra-avidin conjugated horseradish peroxidase. Dishes of cells were fixed by adding 4% paraformaldehyde in phosphate buffered saline (PBS) to culture dishes at various times after they had been stimulated to form domes. After 10 min, the cells were washed gently four times with 5-10 ml PBS. A solution of 1% Triton X-100 was added to the cells to permeabilize the membrane. Again after 5 min, the culture was washed with successive changes of PBS. After washing, cells were exposed to a 1:200 dilution of Extravidin-conjugated horseradish peroxidase (HRP) (Sigma) for 30 min. After further washing with PBS, a solution of 3-amino-9-ethylcarbazole (20 mg of AEC in 2.5 ml of dimethylformamide, diluted with 47.5 ml of 50 mM potassium acetate adjusted to pH 5.0) was added to the cells together with .25% H₂O₂. This solution was incubated at 37°C for 45 min to allow color to develop. The AEC solution was removed, and the cultures were examined and stored in the presence of PBS at 4°C.

Additionally cells were stained with a solution of hematoxylin and eosin. All of the operations were carried out with care not to expose the monolayer or material above the monolayer to air.

If avidin linked to peroxidase is not added to the cultures, there is no reaction. If avidin without peroxidase is added first to the cultures, followed by avidin-linked to peroxidase, staining is not observed. Staining does not occur if avidin-HRP is not added to the cultures prior to AEC indicating that an endogenous peroxidase is not responsible for the staining. To ensure that avidin was reacting with biotin, we stained domes using streptavidin linked to horseradish peroxidase as well as primary antibody to biotin and secondary antibody-linked to horseradish peroxidase. Staining occurred under all circumstances, indicating that avidin does indeed react with biotin that is endogenously present in cells in significant amounts.

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