Unusual Characteristics of Opaque Ishikawa Endometrial Cells include the Envelopment of Chromosomes with Material containing Endogenous Biotin in the Latter Stages of Cytokinesis

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Abstract

We have identified a small dynamic population of opaque cells in Ishikawa endometrial cultures whose unusual characteristics include the fact that chromosomes become enveloped during the final stages of cytokinesis by material staining for endogenous biotin. Endogenous biotin, ultimately shown to be due to mitochondrial carboxylases, was detected in a membrane that wraps around aggregated nuclei in syncytia that develop as part of the differentiation of domes. (Fleming H et al. 1998). The "wrapped chromosomes" in individual opaque Ishikawa cells stain similarly suggesting a similar origin. We were able to show that opaque cells form from transparent monolayer cells, can be polyploid, and often appear to be detaching from the colony and from the underlying substrate. We were also able to show an opaque cell fissioning asymmetrically, to give rise to a monolayer cell whose nucleus appeared to be wrapped. We believe that the cycle of differentiation of monolayer cells into opaque, polyploid cells and depolyploidization back into monolayer cells is involved in the spatial extension of cells as they develop from discrete colonies into a confluent monolayer. Wrapping of chromosomes may ensure that complete genomes are inherited by daughter cells during depolyploidization.

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Introduction

Differentiation of fluid-filled hemispheres in Ishikawa (Nishida, M. et al.,1985) epithelial monolayers over a 24 hour period is elicited by a factor in fetal calf serum (Fleming, 1995). In seeking to document changes in cellular morphology accompanying what is a surprisingly complex process, it was discovered that transient structures are formed in syncytia when chromatin contained in multiple nuclei is enveloped by a membrane staining for the endogenous biotin linked to enzymes found only in mitochondria (Fleming et al.,1998). In consideration of the apparent "melding" of two otherwise distinct subcellular structures, we call these structures mitonucleons.

Mitonucleons can also form in a minority population of Ishikawa cells characterized by opaque membranes. These cells "grow" to be larger than monolayer cells, sometimes appear to be extruding material out of the cell, and are capable of detaching from neighboring monolayer cells and from the underlying substrate. Mitonucleons formed in syncytia during dome differentiation contain multiple nuclei and are, therefore, polyploid, a characteristic of at least some of the individual opaque cells.

To insure that we were not working with a mixed population, our studies were done using a clone of the Ishikawa cell line

RESULTS

Typically, approximately 5% of the cells in an actively proliferating colony of Ishikawa cells are opaque, as shown in fig. 1.

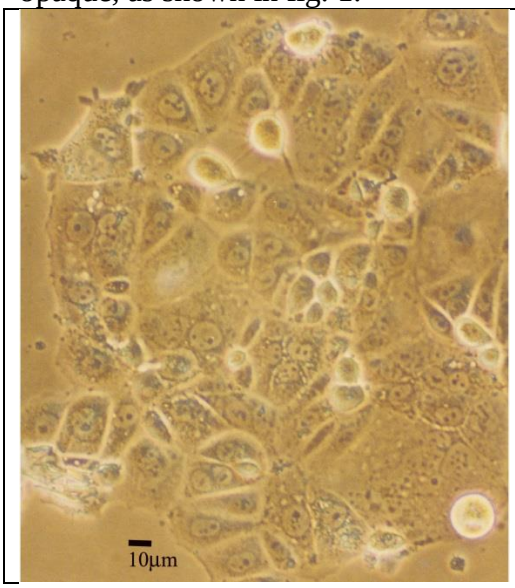


Fig. 1 Typical colony of Ishikawa endometrial epithelial cells in logarithmic growth.

Clearly there is structural heterogeneity among the 200 or so cells in the five day old Ishikawa colony shown on the left. Mononucleated "transparent" cells predominate, but there is also a region in the lower right quadrant that looks like a syncytium. Several binucleated cells can be observed elsewhere in the colony, and ten or so opaque cell structures can be detected throughout the colony, often in pairs.

One of the two opaque cells in the upper half of the colony, with a process extending from it, appears to be detaching from the dish.

In older cultures, the opaque cells can grow to be significantly larger than surrounding monolayer cells, and it is not unusual for such cells to appear to be trailing or extruding material, as can be seen in fig. 2a. We decided to monitor this opaque cell, and the surrounding monolayer, over a period of 20 hours looking for insight into how opaque cells arise and what might be the significance of extensions from those cells.

Figure 2 Observations of formation of an opaque cell in colony over a 20 hour period

Fig. 2a Initial Observations The longer opaque cell process extends through the lamellipodia-like border into the colony, fading near the region to which the arrow points. Discrete vescicles can be seen along most its length	Fig. 2b After two hours Changes are seen in the region to which the arrow points. A region as large as two to three cells begins to take on the quality of opacity and to look as if it is detaching from neighboring cells	Fig. 2c After four hours The developing opaque cell squeezes the monolayer cell that is the origin of the arrow from the left. Vescicles along the length of the process appear diminished.

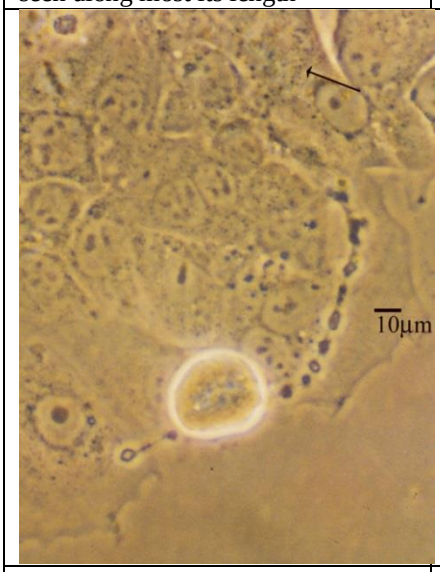
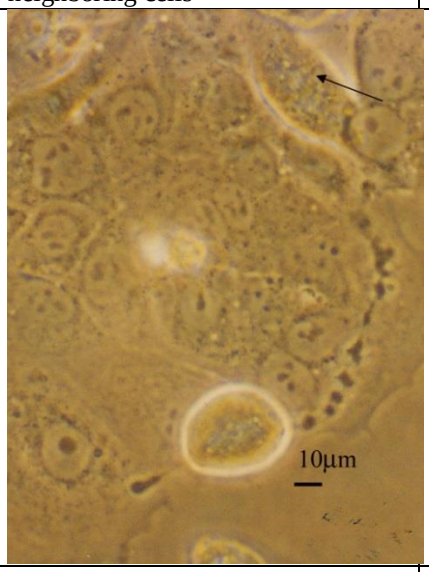
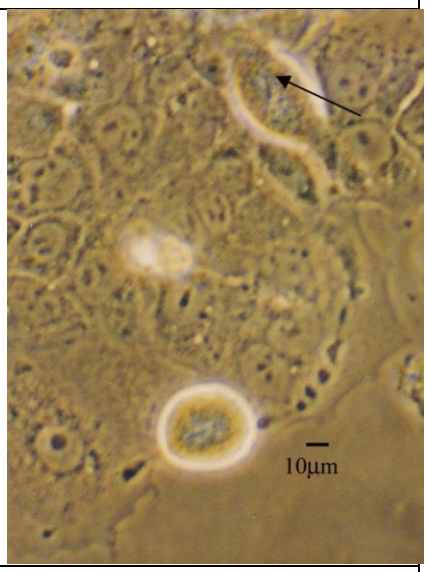
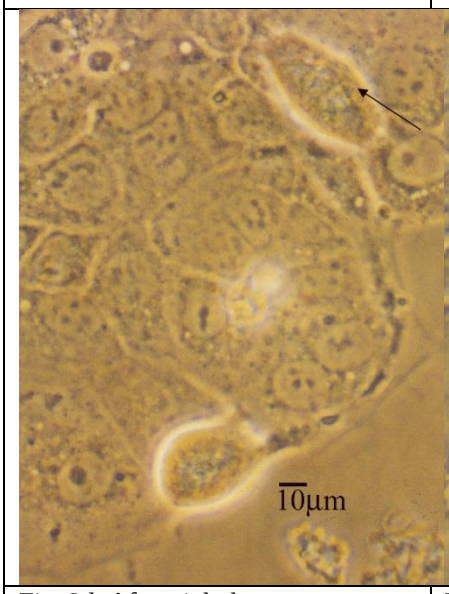
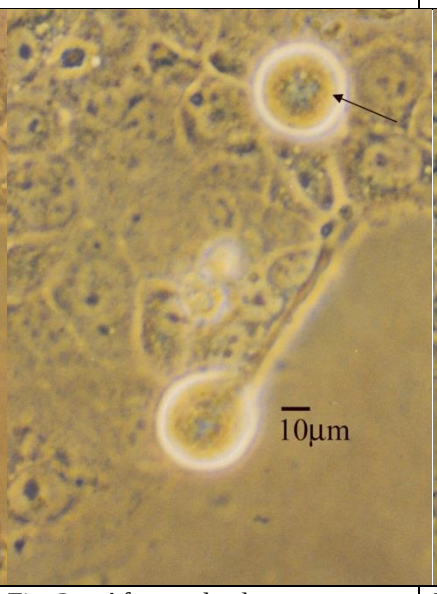
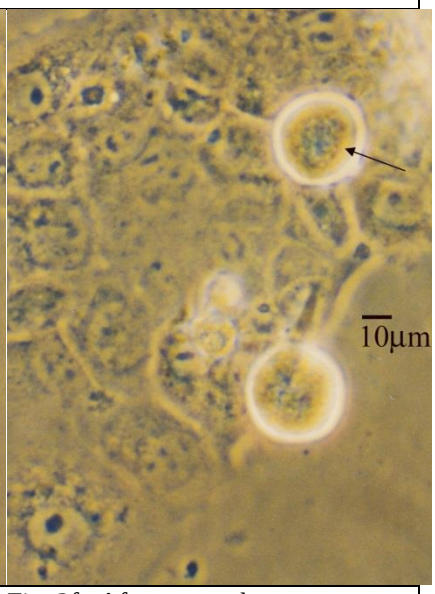

		
		

Fig. 2d After eight hours Lamellipodia-like material through which process travels recedes. The process extending from the right side of the original opaque cell disappears entirely	Fig. 2e. After twelve hours The new opaque cell rounds. The receding edge of colony now appears to be contracting. Original opaque cell is pulled toward new cell, which has rounded up.	Fig. 2f. After twenty hours The original opaque cell continues to move closer to the newly formed opaque cell, and may even be rising above the colony.

In fig. 2a, an opaque cell is "trailing" two extensions, the longer of which stretches from the cell's left side through a lamellipodia-like protrusion along the edge of the colony. Discrete vescicles, relatively clear on the part of the extension closest to the cell, become less distinct as

the extension winds its way into the colony ending almost as a series of "smudges" very near the region where the arrow points. It is in this region that a second opaque cell will start to form over the next 2 hours, raising the possibility that the vescicle-laden extension may release substances that are in some way responsible for this event.

The arrow pointing to the region that will differentiate into an opaque cell is drawn from the uppermost edge of a monolayer cell that does not change throughout the 20 hour observation period except to be squeezed slightly to its left by the opaque cell developing on its right. Pictures were taken at 2 to 4 hour intervals, with the last picture taken after 20 hours. The culture dish was returned to the incubator between observations, and the time when the cultures was outside of the incubator was on the order of 3 to 5 minutes.

It is not possible to detect very much detail in the region that undergoes the differentiation. The size of the developing opaque structure whose outlines are first detected in fig. 2b is approximately that of 2 or 3 monolayer cells, suggesting the possibility that opaque cell formation involves multiple nuclei, a process similar to what is observed in differentiating Ishikawa cells, but on a smaller scale.

The developing opaque cell has contracted by 12 hours into a more typical rounded structure (fig.2e). Over that period, the lamellipodia-like border has receded, the vescicles on the long extension from the original opaque cell have diminished, and the second extension has disappeared completely. By the 12th hour, the longer extension appears to be contracting, pulling the original opaque cell to its left. That movement is even more obvious after 20 hours. There appears to be little keeping the original opaque cell attached to the colony except the now significantly shortened extension. This sequence of changes over a 20 hour period demonstrate that an opaque cell can develop from monolayer cells and that opaque cells can move relative to the monolayer cells within the colony.

In staining such cells, opaqueness is lost, but it is still possible to detect what were opaque cells when they are in the process of detaching from the substrate as is the case for the cell in fig. 3a. An ovoid structure that fills most of this cell stains brightly for endogenous biotin around a core of material that stains for chromatin, looking like the structures we identified in differentiating Ishikawa cells (Fleming et al.,1998)

At the same time as these structures were detected, we found an additional pattern of staining in dividing cells. (fig. 3 b) and wondered how these structures developed.

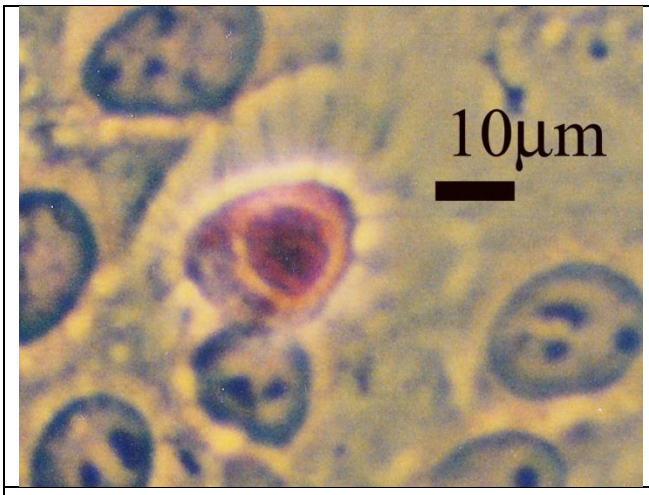


Fig. 3a Mitonucleon in detaching cell
The central structure occupying most of this detaching cell stains for chromatin and for endogenous biotin cell. Surrounding monolayer cells stain only with hematoxylin and eosin.

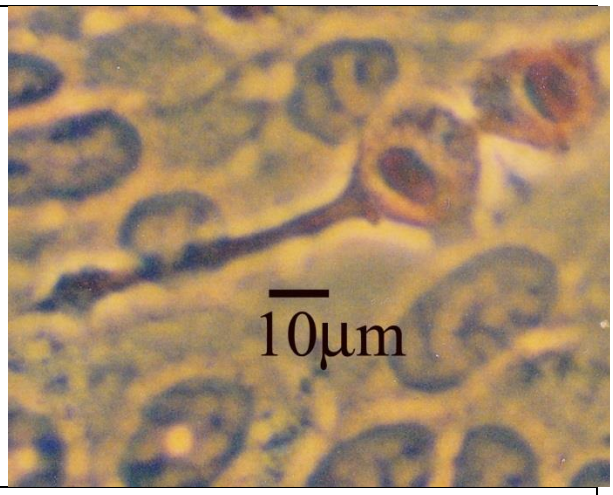


Fig. 3b Mitonucleons in daughter cells
Mitonucleons in dividing cells during the final stages of cytokinesis. One of the daughter cells appears to be discharging significant amounts of material into the colony.

The straight-forward and surprising answer as shown in figures 4 and 5 was that chromosomes become enveloped by material staining for endogenous biotin during cytokinesis. Fig. 4 shows different stages of mitosis and subsequent cytokinesis in Ishikawa cells challenged by a period of four hours when serum was absent from the culture medium, a regimen that increased somewhat the appearance of dividing opaque cells, which may well be related to the observation that polyploidization itself increases in response to stress (Storchova and Pellman, 2004)

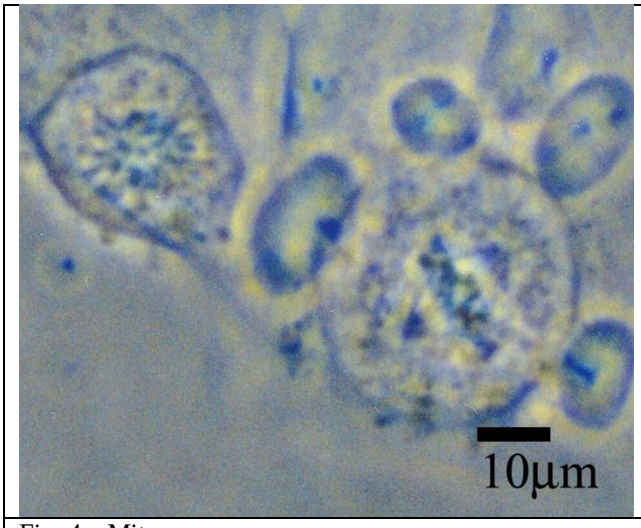


Fig. 4a Mitoses
Cell cultures were fixed only. Cell on the left appears to be in prophase, the cell on the right appears to be in metaphase. The chromosome profiles look normal

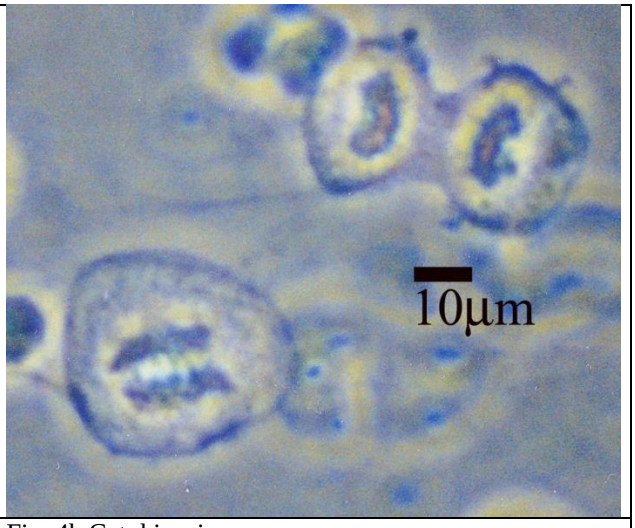


Fig. 4b Cytokinesis
The two mitotic cells in this figure appear to be somewhere between anaphase and telophase. Chromosomes in telophase appear to be enveloped by material that is itself slightly chromatic.

Two large cells in fig. 4a, fixed but not stained, appear to be in prophase and metaphase. The chromatin profile is that of bare chromosomes. The large cells in fig 4b appear to be in anaphase and telophase. The chromatin profile becomes fuzzy during these latter stages of cytokinesis (fig. 4b), particularly during telophase where chromosomal material appears have become "enveloped" by some kind of material that is, itself, slightly chromatic.

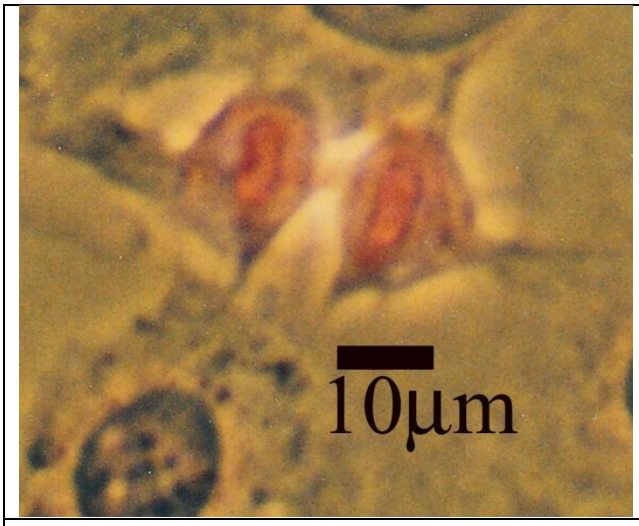


Fig. 5a.Cytokinesis
DNA in daughter cells in what appears to be the final stages of telophase. Barbell shaped chromatin material stains brightly for endogenous biotin. Cell on left appears to be extruding material.

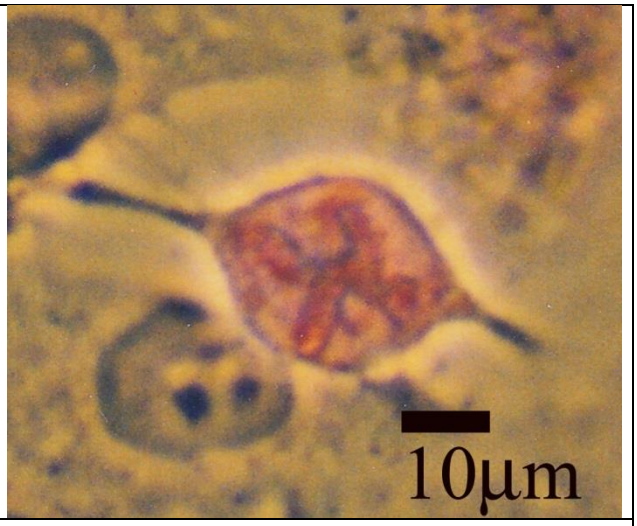
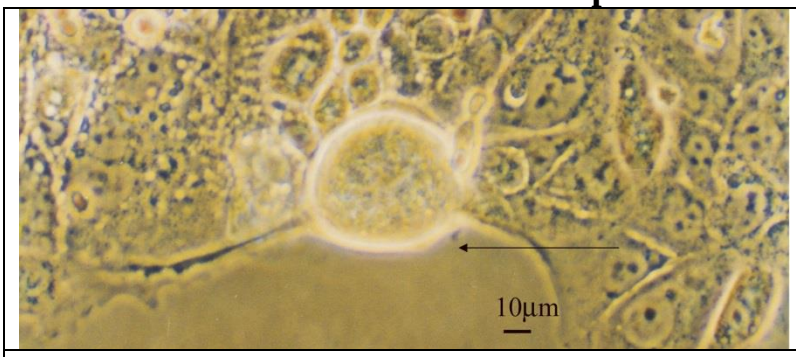
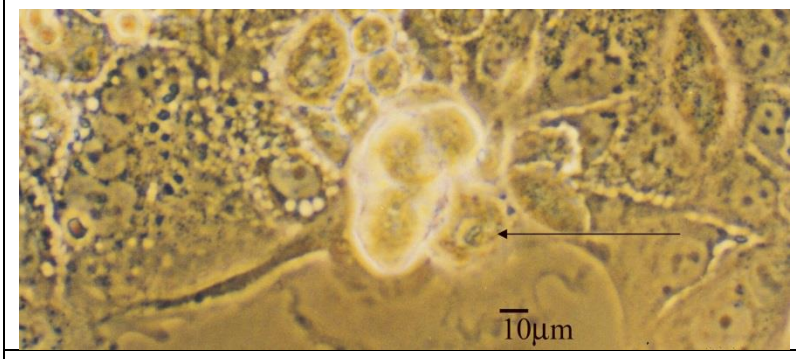
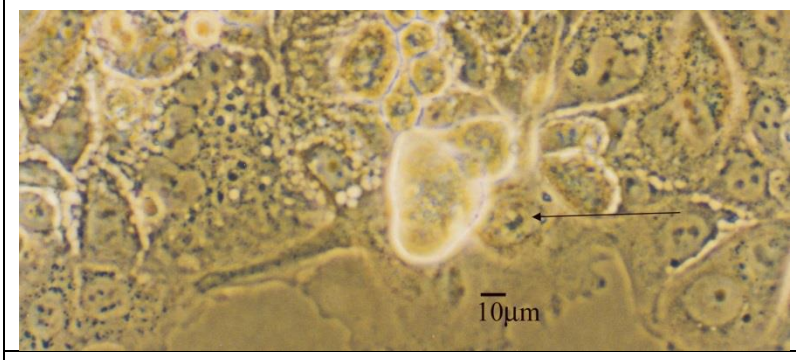
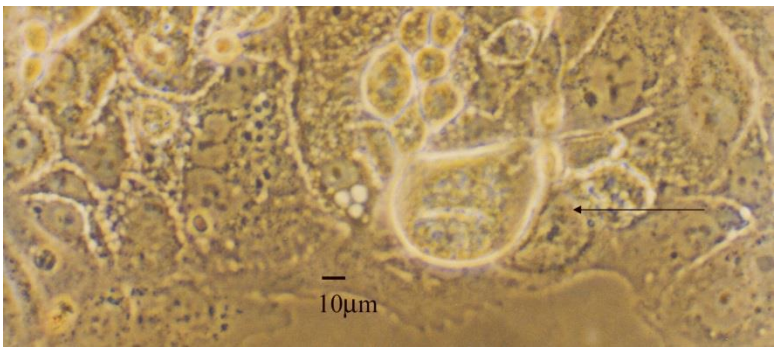


Fig. 5b Detaching cell
What appears to be at least two packets of chromosomes stained for biotin in a giant cell that is detaching.

When we fixed and stained daughter cells in telophase the chromosome packet in each of the cells stained for endogenous biotin (fig 5a), a result expected from what was seen in fig 4 b, although it was surprising that little of the chromatin stain was perceptible. Another surprise was that a neighboring cell contained what appeared to be more than one packet of chromosomes wrapped in material stained for biotin (fig. 5b), prompting the thought that the chromosomes in opaque cells that are "shrink wrapped" toward the end of cytokinesis are fated to become part of a polyploid cell, most likely formed by re-fusing. Whatever else may be the function of wrapped chromatin, such a structure would be one way to ensure that chromosomes are not randomly distributed during asymmetric division or fissioning such as was observed for the very large opaque cell shown in fig. 6.

Figure 6 Observations of fissioning opaque cell in colony over a 12 hour period

	Fig. 6a Giant 0paque cell trailing material from either end. Over 12 hours, this giant cell will undergo substantial changes. The structure will appear to break up into four smaller structures, three of which are opaque and one which might be described as semi-opaque. The arrow is pointing to the region where a new cell will appear.
	Fig. 6b Structures at 4 hours Three opaque and one semi opaque structure arise from the opaque cell in fig. 6a. Colony borders have pushed out and the opaque cell process now appears to flow from underneath the newly fissioned opaque structure. At the center of the semi-opaque cell (arrow), it is possible to see a structure that could be some part of the DNA packet whose formation we have described.
	Fig. 6c Structures at 8 hours Opaque structures are re-fusing. The "coil" in the fourth cell has disappeared and the new cell resembles neighboring monolayer cells.
	Fig. 6d Structures at 12 hours By 12 hours, the newly generated monolayer cell continues to enlarge, perhaps incorporating some of the border material. Refusion of the three remaining opaque structures has progressed and extruded material can no longer be detected. The colony border formed during the previous hours appears to be contracting somewhat even as the newly formed monolayer cell continues to enlarge,

Discussion

Opaque cells, a small but relatively constant population in Ishikawa endometrial cultures, can grow to sizes 2 to 3 times larger than surrounding transparent monolayer cells and are often found to be trailing or exuding material. Examination every several hours (figs. 2 and 6) reveals that opaque cells are dynamic, able to change shape, dissociate from neighboring cells, and even to release from the underlying substrate.

Generation of Opaque Cell

Figure 2 shows the apparently *de novo* formation of an opaque cell in the heart of a colony that already contains one such cell. An arrow points to the region of the colony in which the second opaque cell will develop. There are no obvious distinguishing features for the region except that the long process from the original opaque cell ends nearby. The size of the initial structure that forms (fig. 2b) is two to three times larger than the surrounding monolayer cells. Whether that signifies multiple nuclei cannot be ascertained in the absence of staining although there is evidence in many systems that cell size and degree of ploidy are closely related (Otto, 2007). Multiple nuclei, could become enveloped in the manner documented for syncytial nuclear aggregates by a membrane that stains for endogenous biotin (Fleming. 1998), resulting in a structure such as is seen in fig. 3a, blue at its core from hematoxylin and eosin staining of chromatin surrounded by material staining pink for endogenous biotin.

Intimate Association of Chromatin with Material Staining for Endogenous Biotin

In the differentiating Ishikawa cells, we showed that endogenous biotin increases throughout differentiation, resulting in domes whose cells stain bright pink amidst surrounding unstained monolayer cells. The biotin was ultimately demonstrated to be attached to the mitochondrial carboxylases: proponyl, methylmalonyl, and pyruvate carboxylases (Fleming et al. 1998). Biotin found associated with nuclei in cancer cells, and in nuclei in normal endometrium from women during pregnancy, was also shown to be bound to these mitochondrial carboxylases in a thorough study by Gamachi and his colleagues, involving not only staining of the biotin, but also antibody to the mitochondrial carboxylases (Gamachi et al., 2003). By virtue of the intimate association of chromatin with material containing mitochondrial carboxylases, we have called the structures in Ishikawa cells mitonucleons.

The discovery of mitonucleon formation both in differentiating syncytia and in opaque cells provides one more example, albeit extreme, of the plasticity of mitochondria. The organelles so frequently pictured in cell models as static, ovoid, "stand alone" structures are actually quite dynamic (Bereiter-Hahn, Vöth M.,1994) and can change their shape by elongation, shortening, branching, buckling and swelling. They are capable of fusion and fission, can attach end to end to form threads, and have been shown to interact with cytoskeletal elements (Bereiter-Hahn et al., 2008)

Role for Mitochondria in Differentiation

Proliferation and enlargement of mitochondria has been shown to occur during the differentiation of human stem cells into myocytes (St. John et al., 2006) leading these researchers to suggest that mitochondrial biogenesis may be a key regulatory event. Along with the recognition that the quantity of mitochondria may affect cell fate has come increased interest in the

positioning of mitochondria in cells. In hamster embryos, up through the blastocyst stage, mitochondria were found to be clustered around the nuclei and to delineate the cytocortex subjacent to the plasma membrane (Barnett, Kimura, and Bavister, 1996) . This arrangement persisted through to the formation of the blastocyst cavity, the first of many fluid filled cavities essential in the differentiation of mammalian organisms, and the effective result of Ishikawa dome differentiation(Fleming,1995). On the basis of their studies, Lonergan, Bavister, and Brenner have suggested that proliferation and enlargement of mitochondria and perinuclear location may actually be an indicator of "stemness," _ the ability of cells to differentiate into various specific cell types (Lonergan T, Bavister B, Brenner C, 2007).

Perinuclear mitochondrial clustering may also have a "down side," having been reported to increase the rate of apoptosis (Thomas et al., 2000),to precede cell death in oxidatively stressed human choriocarcinoma cells (Hallman et al., 2004), and to accompany herpes simplex infection of human cells (Murata et al., 2000). All of this suggests that the close association of mitochondria with nuclei, in addition to obvious increased interaction, and possible ready exchange of metabolites including ATP, can have profoundly different long-term consequences by mechanisms mostly still not well understood.

Membrane and Spheroid forming Capacity of Mitochondria

The intimate interaction of mitochondrial membrane and chromatin material seen in Ishikawa cells goes beyond perinuclear clustering. Nuclear aggregates in syncytia are initially surrounded by a transparent membrane staining for endogenous biotin. The resulting structure enlarges into an opaque ovoid structure. Suspecting that nothing as complex as mitochondrial membrane elaboration could be unique to mitonucleon formation, we searched the literature for other examples of such an event. In the past six years, there have been several reports on the "membrane" forming capability of mitochondria. Mitochondrial-derived vescicles were shown by Neuspiel and his colleagues to be synthesized in HeLa cells (Neuspiel et al. 2008). In subsequent papers, quite remarkably, these researchers were able to demonstrate that mitochondria segregate their contents into different "kinds" of vescicles, and speculated that these structures may "communicate" with different intracellular organelles, serving, perhaps, the same purposes as "signaling vescicles" in bacteria. (Andrade-Navarro MA, Sanchez-Pulido L, McBride HM., 2009; Sugiura A, McLelland GL, Fon EA, McBride HM.. 2014)) Although not a focus of the research in this paper, it is the case that we detect vescicles along the process in fig.2a, which could be an example of vescicle-based "communication" within the colony.

In a series of elegant experiments, the mitochondrial outer membrane has been shown to be the source of phagosome membranes in a normal rat kidney cell line starved to elicit phagosome generation (Hailey et al.; 2010) . And, in a recent significant contribution to the field, Ding and his collaborators have found, in an electron microscopic study of murine embryonic fibroblasts treated with a mitochondrial uncoupler, that many of the mitochondria become spheres engulfing various cytosolic components, even including other mitochondria (Ding et al., 2012). Both the inner and outer membranes are involved in these "wrap-arounds".

On the basis of what we observed in Ishikawa syncytia (Fleming et al.,1998), the process that forms mitonucleons appears to be some combination of the elaboration of a translucent, biotin-

containing membrane, around a nuclear aggregate, followed by further development of a spheroid perhaps in the manner described by Ding and his colleagues (Ding et al., 2014

Envelopment of Chromosomes by Material Staining for Endogenous Biotin

In this paper, we report on a second process that can generate mitonucleons in individual cells. As figs 3b, 4 and 5a demonstrate, material staining for endogenous biotin wraps around, and seems to be sufficiently pliant to assume the rod or chevron shape of, chromosomes in daughter cells during the final stages of cytokinesis. Those wrapped chromosomes appear to be the starting point for elaboration of an ovoid structure (fig. 3b), perhaps in a manner similar to what is seen in Ishikawa syncytia (Fleming, et.al. 1998).

At the end of cytokinesis (fig 5a) a “packet” of chromosomes enveloped in material staining for endogenous bioin appears in each daughter cell. But as fig. 5b demonstrates multiple chromosome packets can "turn up" in a single large cell. At least two chromosomal packets lie perpendicular to each other in this cell. The observations of Rengstl and his colleagues investigating the formation of Reed Sternberg giant polyploid cells characteristic of lymphoma may help explain these results. Using time-lapse videography, these researchers were able to show that giant tumor cells can arise when cells undergoing mitosis and cytokinesis, re-fuse after they have reached telophase, sometimes even hours after appearing to completely separate (Rengstl B et al., 2013). This result is a significant contribution to the long standing debate over whether polyploidy results from cell fusion or from a situation in which chromosomes duplicate, in the absence of cytokinesis. The surprising answer, at least in Reed Sternberg cells is that mitosis, cytokinesis, and fusion all occur, but that fusion occurs between daughter cells many of which have just undergone division.

If the structure in fig. 5b similarly results from re-fusion of daughter Ishikawa cells, the chromosome packaging clear from figs 3 and 4, might provide a rationale for why daughter cells undergo most of the cytokinetic process only to re-fuse again, at least in Ishikawa cells. What would otherwise appear to be "wasted energy" if cells were going to re-fuse ensures that complete genomes can be "packaged" in daughter Ishikawa cells before any mixing can occur in polyploid cells, greatly increasing the probability that progeny cells, however they are formed, will inherit a complete set of chromosomes.

Generation of Diploid Cells from Polyploid Cells

At least some of the opaque Ishikawa cells are polyploid (fig. 5b), the chief characteristic of giant cells frequently found in tumors and in cancer cell cultures, as well as in predominantly diploid organisms where they are essential for normal development and physiology (as reviewed by Lee, Davidson, Duronio, 2009). For many years, it was assumed that the polyploid cells in cancer were a "dead end," unable to reproduce. Ignoring that bit of received wisdom, researchers in several laboratories have demonstrated that polyploid cancer cells are capable of generating diploid or paradiploid progeny (Baroja et al., 1998; Illidge et al., 2000; Sundaram et al., 2004; Puig et al., 2008; Ianzini et al.2009; Vitale et al., 2010; Erenpreisa et al., 2011).

Many of the studies of polyploid cells are done in cultures exposed to genotoxic insults designed to kill most of the cells. Polyploid cells that survive and go on to form diploid or paradiploid cells become the "means" to reestablish the cell line. There is significant interest in whether that

ability of a small population of polyploid cells to survive mitotic catastrophe might explain how some cancer cells in vivo survive chemotherapy. Not only are polyploid cells able to survive, there is evidence to suggest that polyploidization may provide "a" or "the" means of cell renewal, and may be associated with the induction of a stemness phenotype (Erenpreisa and Cragg. 2013; Zhang et al.2014b)

Researchers have not always been sure about the exact mechanism whereby polyploid cells generate reproductively viable progeny, although it has been known for more than a century that multipolar mitosis can occur. But there appear to be additional possible ways for polyploid cells to produce diploid or paradiploid cells. Ianzini and his colleagues found evidence of the activation of genes involved in meiosis during depolyploidization (Ianzini et al.2009). Researchers have also, in the past decade and more, begun to demonstrate modes of reproduction formerly thought to be relevant only for lower forms of eukaryotes, such as yeast. Budding of diploid nuclei was, for instance, shown for polyploid cells formed when amniotic cells are transformed by a virus (Walen. 2002)as well as in polyploid cells formed in mouse embryo fibroblast cell lines exposed to carcinogens (Sundaram et al., 2004). Zhang, Wang, and Zhang (2012) describe asymmetric divisions of polyploid giant cells formed by the administration of CoCl2 as including splitting, budding, or burst-like mechanisms.

Production of a monolayer cell in fig. 6 involves fissioning of the giant opaque cell into what appears to be a trio of opaque cells and one semi-opaque cell with an unusual structure at its core. That structure may well be “wrapped” chromosomes such as those whose origins are shown in figs.4 and 5. In succeeding hours, that structure disappears and the semi-opaque cell resolves into a transparent monolayer cell containing a typical nucleus. The opaque progeny which may never have completely dissociated from each other reform one giant opaque and probably polyploid cell over the next 8 hours.

Significance of Opaque Cells

The opaque cells we are studying in Ishikawa cultures did not result from mitotic catastrophe. Opaque cells, as fig. 1 is meant to illustrate, are present in cultures growing under normal culture conditions such as those used for experiments described in figs. 2 and 6. The results in fig. 2 demonstrate that an opaque cell can arise in a region of the colony that is otherwise not remarkable. The study also shows the ways in which an opaque cell can move relative to other cells, and over time, by sliding along and possibly hovering above a colony . Fig. 6 demonstrates dramatic changes in a giant cell as it gives rise to a monlayer cell, as well as lamellipodia that extend the colony.

These results suggest that whatever else is true about opaque cells, they are probably involved in the overall capacity of cells to efficiently populate the surface of a dish or flask. Cell migration, particularly of cancer cells, is an important topic since most cancer related deaths stem from the metastatic spread of cancerous cells. Studies that address this phenomenon are frequently trying to determine how cells move out of the originating organ and into other far-removed organs. Our interest is more local. We are studying confined regions of dish or flask. Nevertheless, it must be assumed that something more than mitosis accounts for the extremely efficient formation of monolayers.

Migration of Epithelial Cells

Cell migration is also essential in normal physiological circumstances such as embryonic development and wound healing. In an elegant study, Bischoff demonstrated that lamellipodia-like structures, cell extensions made mostly of actin common in one-celled organisms, are involved in the migrations of larval epithelial cells required for normal development in Drosophila (Bischoff, 2013)) Lamellipodia had previously been shown to be involved in fibroblast migration (Abercrombie M, Heaysman., Pegrum. 1970). Structures very much like lamellipodia are associated with the two colonies studied in figs. 2 and 6 and undergo changes as the opaque cells evolve. In both of these studies, lamellipodia initially enlarge the colonies in the region of the opaque cells. In fig. 2, the lamellipodia recedes, but in the process of receding, moves the original opaque cell. In fig. 6, the lamellipodia that forms below the giant cell in fig. 6 becomes colonized by the newly forming monolayer cell. In addition to discovering a role for lamellipodia, Bischoff was able to demonstrate movements of whole sheets of epithelial cells . (Bischoff, 2013).. That may well also be happening in culture dishes, but would not be detectable with our less sophisticated monitoring techniques.

What our observations in Ishikawa cells do suggest is that a colony is more than simply the sum of all of its cells. All of the indications are that the colony also contains cell-free regions accessible to, but not part of, individual cells, and possibly secreted by opaque cells. This notion is bolstered by the observation that the opaque cell extension in fig. 2 made up of vesicles moves through what appears to be "common space" up into the center of the colony. And when opaque cells forcefully expel material, most readily seen in fig. 3b and 6a, that material becomes part of the common space, as appears clear from a comparison of fig. 6a, 6b, and 6c. The material can no longer be seen in fig. 6d, perhaps because it has been successfully recycled by monolayer cell uptake. Erenpreisa and her colleagues who have contributed so much to our understanding of the reproductive potential of giant cells established early on in their research that as giant cells give rise to progeny they also extrude degraded chromatin (Erenpreisa et al. 2000; Erenpreisa et al. 2011; Erenpreisa et al. 2012) Perhaps some of what is being expelled from Ishikawa opaque cells is degraded chromatin. For that matter, perhaps some of it is degraded excess mitochondrial material that is being recycled.

We believe that the cycle of differentiation of monolayer cells into opaque, polyploid cells and depolyploidization back into monolayer cells is involved in the spatial extension of cells as they develop from discrete colonies into a confluent monolayer. The question remains about whether, and how, the unusual qualities of migratory behavior and polyploidy observed for opaque cells depends on the intimate association of mitochondrial material with genetic material in the mitonucleon structure. Like so much about the role of mitochondria in processes other than energy production, there is still much to be learned.

Materials and Methods

Ishikawa cells were cultured (Fleming 1995) in phenol red-free, Minimum Essential (MEM) supplemented with 2 mM glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin, and .25 mg amphotericin B (GIBCO, Grand Island, NY). The cells, obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital in New York, were originally detived from an endometrial adenocarcinoma line developed by Nishida et al. (1985), who demonstrated the presence of receptors for both

estradiol and progesterone. Cells seeded at an approximate density of 5 x 10^5 cells/cm2, were grown for 1 -2 weeks in MEM containing 5% calf serum (CS). The experiments shown in figs.2 and 6 were performed while cells were in logarithmic growth phase. Dishes were marked so that we could return to the colony of interest at the desired intervals. Structures were viewed using an Olympus inverted stage microscope at powers of 200X and 400X.

To slightly increase the incidence of opaque cell division for cultures shown in figs. 3, 4, and 5, cultures were incubated in MEM medium without serum for four hours. Fetal calf serum was then added back to the cultures to a final concentration of 10%. This treatment may stress the cells and somewhat synchronize a mitotic response to the stimulus of fresh serum.

Cells were fixed by adding 4% paraformaldehyde in phosphate buffered saline (PBS) to the culture dish. 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 changesof PBS. After washing, cells were exposed to a1:200 dilution of Extravidin-conjugated horse-radish peroxidase (HRP) (Signa) for 30 min. After further washing, a solution of 3-amino-9-ethylcarbazole (AEC), prepared by dissolving 20 mg of AEC in 2.5 ml of dimethylformamide and diluting 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 then stored in the presence of PBS at 4"C. 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 the cell in significant amounts.

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