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Opaque Polyploid Cells in Ishikawa Endometrial Cultures Are Capable of Forming Megamitochondria, Organelles Derived from the Adaptation of Fused Mitochondria Whose Capacity to Develop Gaseous Vacuoles Suggests CO_2 Retention and Hypoxic Metabolism

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Abstract

Opaque polyploid cells capable of forming megamitochondria are a constant feature in colonies of Ishikawa endometrial epithelia, accounting for approximately 5% - 10% of the cells. Opaque cells appear to communicate with other opaque cells via membrane extensions and with other cells in a colony by extracellular vesicles. Opaque cells form first as rectangular structures, somewhat larger than surrounding monolayer cells. The cells eventually round up, remaining in the colony for 20 or more hours before detaching. The most unusual characteristic of Ishikawa opaque cells is their capacity to form mitonucleons, megamitochondria that surround aggregated chromatin. This paper reviews evidence that adaptations resulting in megamitochondria include a loss of the capacity for oxidative phosphorylation leaving the adapted megamitochondria reliant on metabolism such as reductive carboxylation.

Keywords

Mitonucleons, Megamitochondria, Opaque Polyploid Cells, Membrane Extensions, Extracellular Vesicles, Reductive Carboxylation, Endogenous Biotin, Hypoxia

1. Introduction

Despite an assumption that cultured monolayer cells are mostly identical, and

that cells detach from a monolayer only when dying, discoveries have been made by more closely examining the minority, exceptional cells routinely found in and above monolayer cultures. Dr. Jinsong Liu's laboratory [1] has been studying a cell type called polyploid giant cells (PGCC) found in ovarian as well as in other cancer cell lines [2] [3]. Giant cells can proliferate by amitotic means such as asymmetric division. Polyploid cells described as opaque and found in the Ishikawa endometrial cell line [4] are also capable of budding off new "normal" cells.

Dr. Liu and colleagues characterized PGCC's whose formation was stimulated by $CoCl_2$, demonstrating that the giant cells express cancer stem cell markers together with markers characteristic of normal cells. Furthermore, the researchers showed that in long term cultures, with appropriate additions to the media, PGCCs can differentiate into adipose, cartilage and bone [1], a potential for differentiation broadened by results in a subsequent paper [5]. Spheroids derived from single PGCCs can grow into a wide spectrum of human neoplasms, including germ cell tumors, high-grade and low-grade carcinomas and benign tissues leading these researchers to suggest that PGCC's are cancer stem cells and the somatic equivalents of blastomeres.

In 2011, Chaffer and colleagues [6], focusing attention on cells floating above the monolayer as opposed to clinging to the petri dish, presented compelling data demonstrating that this population is enriched for stem cells. They went on to demonstrate that differentiated mammary epithelial cells can convert to a stem-like state in a stochastic manner in culture.

This paper also reports on a small cell population with apparently outsize importance observed in the Ishikawa endometrial epithelial cell line originally isolated in Dr. M. Nishida's laboratory in 1985 [7]. The cell line is capable of forming hemispheres of detached cells arching over reservoirs of fluid in confluent monolayers. Once the routine induction of that process had been harnessed [8], it became possible to study the 20-to-24-hour differentiation for structural changes including the role played by increasing levels of biotin as syncytia of Ishikawa cells formed hemispheres [9] [10] [11]. Elevated levels of endogenous biotin usually observed as a "failed" control have engendered interest in the significance of this carboxylase co-factor and/or warnings that its presence can cause confusion in assays using biotinylated antibodies to detect specific proteins [12]. Endogenous biotin became an important factor in studies of Ishikawa differentiation as the explanation for large spheroidal megamitochondria in syncytia staining bright red following incubation with avidin peroxidase and 3-amino-9-ethylcarbazole (AEC) as a substrate. In Ishikawa cells, mitochondria are observed to fuse around aggregated chromatin forming the mitonucleon, a giant transient organelle that stains darkly for biotin. Subsequently, endogenous biotin was also detected in opaque cells that can be observed detaching from a colony and forming hollow spheroids [13]. As was demonstrated early on in our studies [14], the endogenous biotin is bound to mitochondrial carboxylases whose increasing concentrations accompany mitochondrial biogenesis during dome dif-

ferentiation.

As will be discussed, membrane extensions carrying extracellular vesicles appear to be part of the process resulting in the formation of giant opaque cells. Ishikawa cells like many other cell lines are able to form membrane extensions under a variety of circumstances including in cells adjusting to a change in medium: monolayers transferred into serum-free medium or clustered spheroids formed in serum-free medium transferred into serum-containing medium [15]. The monolayer cultures studied in this paper were not similarly stressed but rather were in logarithmic growth. The observation that the extracellular vesicles extend up into the colony to the region where a second giant cell forms is intriguing circumstantial evidence that giant cells may induce formation of additional giant cells by the materials that are exported. Eventually giant opaque cells detach [13] and can migrate to other regions of the petri dish and start new colonies. This may be one part of the process involved in the proliferation occurring when cell aliquots achieve confluence, a routine event in successful cell culture.

To investigate the origins of opaque cells in typical Ishikawa cultures, we began following cultures hours at-a-time hoping to catch the process of formation of an opaque cell from a typical monolayer cell. Finding a culture in which an opaque cell was forming, photomicrographs were taken at intervals throughout the process over a period of 20 hours (**Figures 3-7**). As the results demonstrate, structural changes could be detected through most of that period. The opaque rounded cell initially present at the start of the observation undergoes the process of detaching from the colony during the observation. Giant opaque cells in Ishikawa cultures are exceptional, particularly in their capacity to develop one or more mitonucleons. Mitonucleons will be discussed in light of that exceptionality and characteristics shared with other megamitochondria.

2. Results

Ishikawa endometrial monolayers are mostly made up of typical epithelial cells, usually dominated by one or two visible nuclei and fitted together with neighboring cells like tiles in a mosaic. But opaque and clearly polyploid cells are always present in cultures, sometimes in groups of 2 or 3. Opaque cells make up approximately 5% - 10% of the monolayer population and are distinguished by size, shape (rounded up as opposed to flat), the presence of substantial cell membranes, and often, but not always, trailing processes such as can be seen in **Figure 1**. Mitonucleons form at the center of such cells, staining brightly for endogenous biotin as seen in the light micrographs in **Figure 1** and **Figure 2**. These giant cells can detach and form hollow spheroids [13] capable of migrating and reattaching elsewhere on the petri dish if fresh serum is added, or of remaining as hollow spheroids and becoming multicellular [16].

Figure 2 shows that enlarged cells containing mitonucleons are capable of communicating quite specifically with each other by an extension passing through

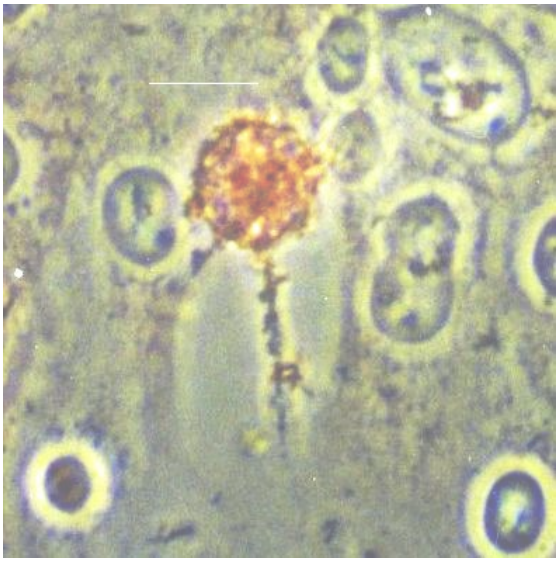

Figure 1. Giant polyploid cell containing mitonucleons can detach from a confluent monolayer. Prior to detachment such cells are characterised by extensions made up of extracellular processes that stretch back into the colony. There appear to be three mitonucleons at the center of the cell featured in this photomicrograph and multiple extracellular vesicles trailing from two processes. Bar = 50 microns.

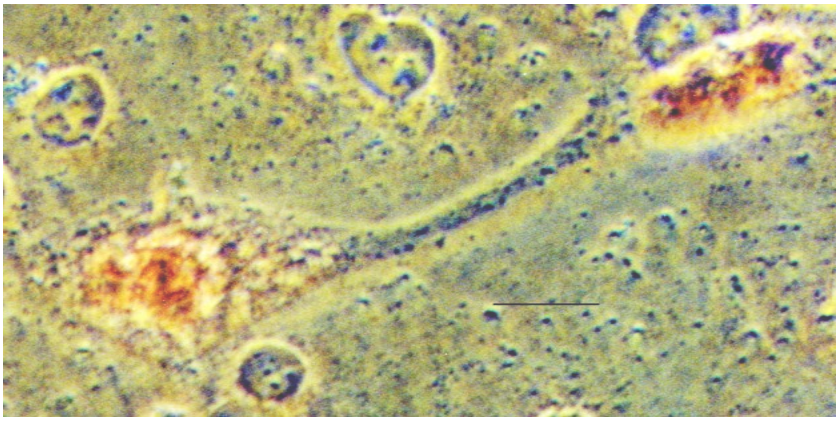

Figure 2. Two cells containing multiple mitonucleons appear to be exchanging material by a cellular process extending between them. Bar = 50 micron.

the colony. The enlarged structures communicating appear to contain multiple mitonucleons.

Figures 3-7 are photomicrographs of a typical colony of Ishikawa cells proliferating as a monolayer in a culture dish. The colony was followed over a period of 20 hours to characterize the behavior of opaque cells as compared to surrounding monolayer cells. Cells have been numbered in the photomicrographs to facilitate comparison of the colony over time. Opaque cell #3 dominates the colony by virtue of size and shape, "glowing" with evidence of cell membranes more substantial than the barely detectable membranes of neighboring monolayer cells. Two exosome-decorated processes extend from this opaque cell, the longer process reaching deep into the colony as it passes through a cytoplasmic

ruffle. A smudge of material seems to define the furthest reach of the process although it might be expected that materials from the vesicles diffuse beyond that point. Changes will be detected at positions 7 and 10 over the next 18 hours.

If a typical monolayer cell was the starting point for differentiation of the second giant cell, the cell could not be detected at the beginning of our observation (Figure 3). Perhaps the process of converting a monolayer cell into an enlarged opaque cell begins with sufficient deconstruction to render the contours of the originating monolayer cell undetectable at some point. A discernable structure does however emerge quickly in the region labeled "10" after only 2 hours. It is dominated by the halo of a substantial membrane forming and by something akin to striations in the center of the differentiating structure. De novo formation such as this, together with asymmetric division [5], may explain how, despite their propensity to wander, enlarged opaque single cells capable of forming mitonucleons persist as a relatively constant population in cultured Ishikawa epithelial cells.

In Figure 3 at the start of the observation, a cell undergoing mitosis is evident in the top left part of the colony. That there are not more mitotic figures observed throughout the 20-hour observation in a culture in logarithmic growth is worth mentioning, although after decades of examining cell cultures, I will admit to generally being underwhelmed by the representation of mitotic figures even

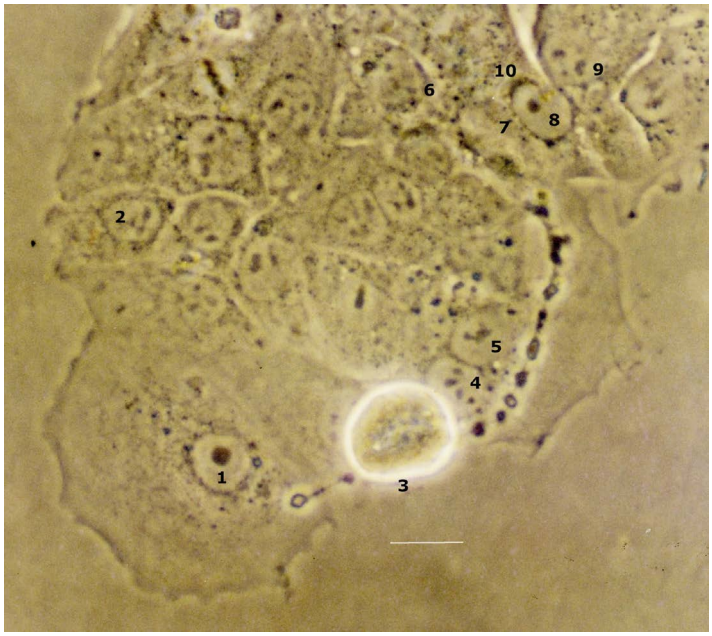

Figure 3. Single opaque cell on the lower edge of the colony. Extensions made up of vesicles stream from either end of giant cell #3. The longer of the two, carrying more than 10 vesicles, passes close by cells #4 and #5 through a cytoplasmic ruffle, ending close to cell #8 to the left of a gap in the culture and cell #9 to the right of that gap. A second much shorter extension emerges from the right side of the opaque cell ending in the cytoplasmic ruffle of cell #1. Cell #2 is approximately 350 microns up and to the left of cell #3. Region 10 defined by cells #6, #8, and #9 is where the new giant cell will develop. Over time a monolayer cell will become more clearly defined at position 7. Bar = 50 microns.

during log growth. Some of the reason for this may lie in the evidence for other modes of proliferation described in the past 20 or so years. Papers have been published describing proliferation of trophoblasts and human cancer cells by processes other than mitosis. Some of the names given to these processes include: budding [17] [18], bursting [1], depolyploidization [19] [20] and neosis [21]. Examples of what is sometimes called asymmetric division have also been observed in Ishikawa cells and indeed there appears to be an example of a cell budding out of giant cell #3 (**Figure 5**) not long before the opaque cell detaches.

Most of what is known about amitotic proliferation is derived from experiments that disrupt the mitotic cycle. Just as some cancers come back after chemotherapy, treated cancer cells do not necessarily all succumb to chemotherapeutic agents in vitro. Using paclitaxel, an effective chemotherapeutic agent, Liu and his colleagues poisoned ovarian cancer cells [5]. Most of the cells died, but a few surviving cells became polyploid giant cells by endoduplication of DNA. These PGCCs were capable of amitotic production of cells by "nuclear budding, nuclear fragmentation or nuclear fission followed by cytofission" [5]. Taking the experiment one step further, the researchers karyotyped the surviving cells and found multiple chromosomal rearrangements including deletions and translocations as well as lower chromosome numbers than parental cells. The authors present evidence that daughter cells acquire new karyotypes with numerous genomic alterations following a single giant cell cycle, and that the karyotypes are not static. They change over time suggesting that an ongoing "editing" process is addressing at least some of the variability arising from amitotic proliferation [5]. On the basis of this study, Dr. Liu and his colleagues have proposed a giant cell cycle that includes the amitotic reproductive behavior of polyploid giant cells and the observation that, as a result of the editing process and asymmetric division, some progeny cells can spiral back into the typical mitotic cycle [22] [23].

After two hours, in addition to the completion of the mitosis observed in **Figure 3**, the photomicrograph in **Figure 4** shows the outline of a large structure forming at position 10. A couple of changes worth noticing include the demarcation between the developing structure and surrounding monolayer cells that have been numbered 6, 8, and 9, and a structure like a monolayer cell emerging at position 7. Something that might be similar to crystalline structures observed in developing megamitochondria [25] can be observed in the interior of developing cell #10.

The extracellular vesicles are somewhat smeared (**Figure 4**), suggesting that packaged materials are seeping out into the colony. Interest in extracellular vesicles has taken off in the past decade, perhaps because of evidence that they might be involved in cancer [26]-[34]. From just a sampling of the literature, the dozen or so vesicular packets decorating the extension stretching from cell #3 into the middle of the colony may contain proteins, RNAs, DNAs, lipids or all of the above. Work from one laboratory suggests that extracellular vesicles are involved in the transfer of cancer pathogenic components, particularly micro RNAs [35]. It is obviously an intriguing possibility, although entirely speculative,

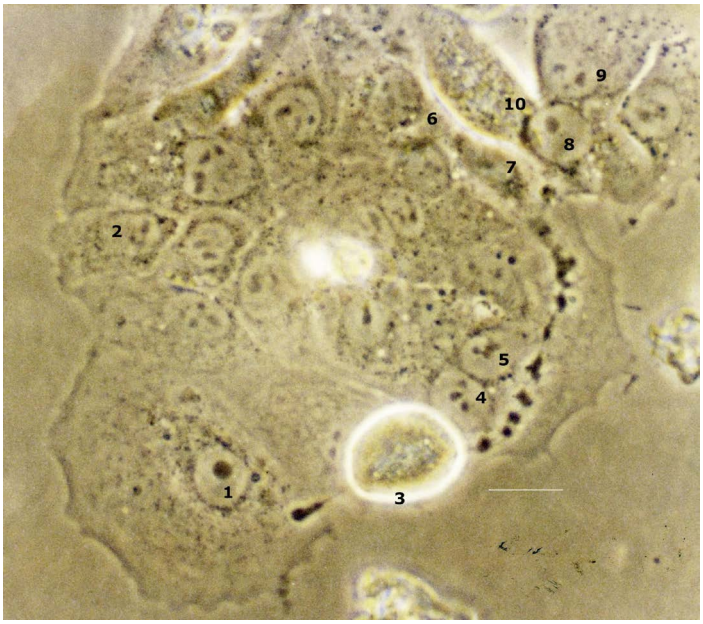

Figure 4. Two hours after the start of the observation, extracellular vesicles can still be detected on the processes extending from cell #3. Some of the vesicles appear to be spreading. The most dramatic change is taking place in the region designated #10. The region is approximately twice as large as surrounding cells, and mostly defined after 2 hours by a border not unlike the outline around opaque cell #3, a characteristic of the phase contrast photomicrograph. Bar = 50 microns.

that the horizontal transfer of material(s) from the vesicles exported by cell #3 might be linked to the differentiation of the second opaque spheroidal cell at position 10. As discussed in the Introduction to this paper, the very significant quality of "stemness" seems to be communicated from one cell to another [6], and the same may be true with regard to the capacity to form polyploid opaque cells and mitonucleons.

Enlarged opaque cells usually undergo detachment and form floating spheroids [13]. They can then reattach at some distance from the original colony, analogous to the EMT process involved in cancer metastases. As a result of his experiments with extracellular vesicles, [36] Gopal and colleagues believe that cells undergoing the EMT process, as is the case for cell #3 (**Figures 3-7**), are reprogrammed with regard to protein and RNA content of extracellular vesicles. The obvious question would be whether that material can result in the induction of a second cell (*i.e.* cell "10") that becomes capable of forming mitonucleons and detaching.

After an additional four hours (**Figure 5**), the smaller extension observed in **Figure 3** and **Figure 4** has been lost, a bud has appeared in the center of the giant cell #3, the cytoplasmic ruffle is shrinking, and cell #10 continues to fill out.

The photomicrograph in **Figure 6** shows the colony after 12 hours. Cell #10 has rounded up. It is no longer possible to observe discrete vesicles between cells #3 and #10 and the cytoplasmic ruffle through which the extension passed has

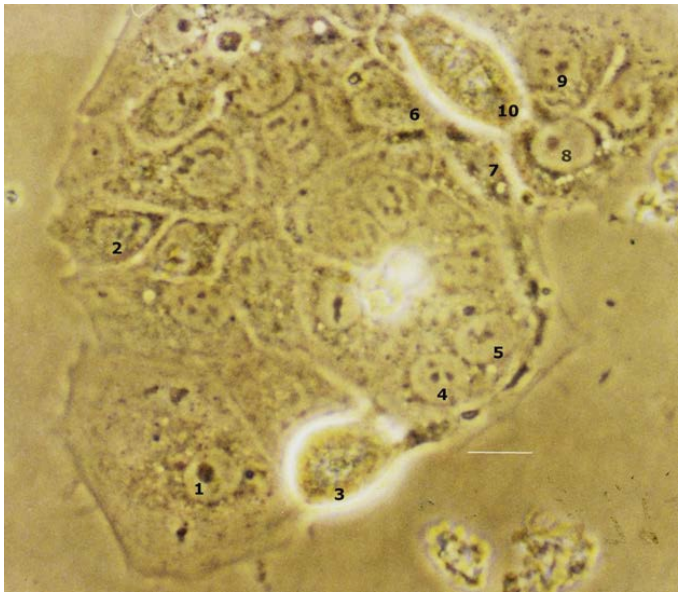

Figure 5. Eight hours after the start of the observations, the extracellular vesicles extending out from cell #3 toward cell #1 have mostly disappeared along with the shorter extension. The vesicles on the longer extension have continued to spread out or diffuse. The rectangular region above cell #8 has become further defined. Concurrent with these changes the gap to the right of the rectangular structure has disappeared. Bar = 50 microns.

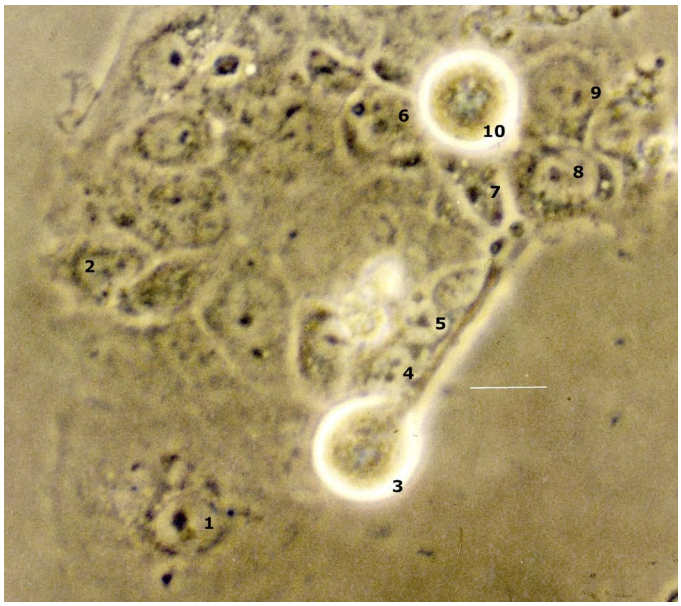

Figure 6. Twelve hours after the start of the observation. Giant cell #10 is fully formed. The cytoplasmic ruffle extending to the right from cell # 3 has disappeared. The nature of the conduit from cell #3 to cell #7 now looks more like a "pipeline" of sorts than the extracellular vesicle-bearing extension in Figures 3-5. Bar = 50 microns.

further contracted. Something like a stream of materials appears to be exiting cell #3.

In the final observation at t = 20 hours (Figure 7), it is obvious that opaque rounded structure #3 has lifted off the plate with a single visible extension

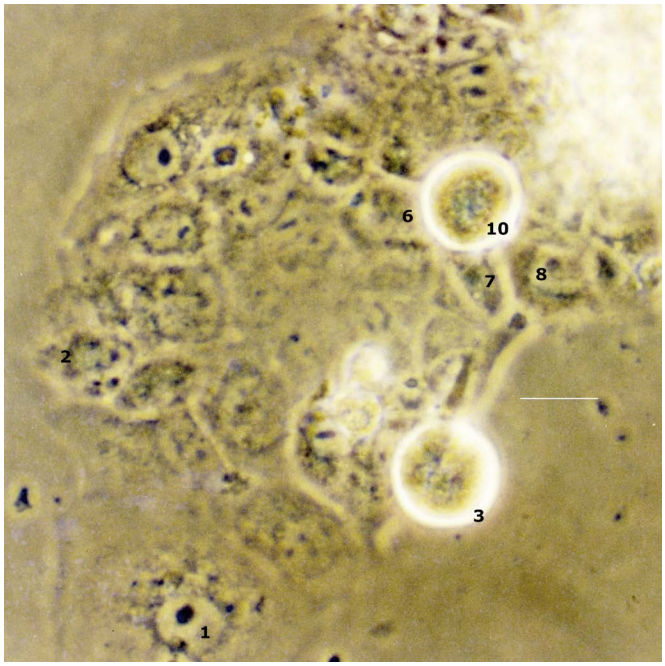

Figure 7. Twenty hours after the start of the observations. Cell #3 has released from the dish although it continues to be tethered to the colony by at least one extension. Evidence for detachment includes the foreshortened appearance of the single visible extension. Cell #3 appears to be floating above cells #4 and 5 that were clearly visible up until 20 hours, as can be seen in **Figure 6**. Finally the apparent distance between cells 1 and 3 has "lengthened" and the apparent distance between cells 3 and 10 has "shortened." Bar = 50 microns.

reaching back into the colony. With the focus of the microscope on the monolayer, it is possible to detect that opaque cell #3 is hovering above cells #4 and #5. The extension still attaching it to the monolayer appears foreshortened and the relationships between cell 1 and cell 3 and between cell 3 and cell 10 have changed in ways that can only be explained by upward movement of cell #3. As already discussed in a previous paper [13], this "behavior" is consonant with the development of a central gaseous vacuole within mitonucleons that develop in giant cells.

What started out as observations about rounded opaque cells in Ishikawa cultures ended up providing information about vesicle-decorated membrane extensions. Extracellular vesicles are on the edge of what is readily detectable by light microscopy. While analyses of the content of extracellular vesicles have become numerous in the past several years, extended observations of the fate of extracellular vesicles in cell cultures such as this are far less common. A short and short-lived extension, 50 microns long with three or four extracellular vesicles, ends in a large expanse of cytoplasm on the edge of the colony surrounding cell #1. The second process is 4 to 5 times longer, passing through a ruffle of cytoplasm on the edge of the colony, "carrying" approximately a dozen extracellular vesicles. By 8 hours the smaller extension has faded completely, seemingly absorbed by the cytoplasm surrounding cell #1, without having any obvious effect.

The second extension is also fading. The cytosol ruffle it is passing through has contracted substantially, leaving what looks more like a "pipeline" similar to the structure connecting two giant cells with mitonucleons in **Figure 2**. The optics have changed from discrete vesicular packets into something that looks like a flow of material out of giant cell # 3 to the region of the colony near cells #7 and # 10. By 20 hours, it is clear that cell #3 has detached and a new opaque cell has formed at position 10. As was described [13], the potential of a detached cell is to form a hollow spheroid that can float to a new location to reattach. If reattachment does not occur, chromatin streaming from giant nuclei in the cytoplasmic rim of the hollow spheroid results in a multicellular spheroid [16]. The clearest evidence of detachment can be observed by a comparison of **Figure 6** and **Figure 7**. The apparent distance from cell #3 to cell #10 has decreased from 260 microns to 192 microns, while the apparent distance from cell #1 to cell #3 has increased from 138 microns to 195 microns.

3. Discussion

3.1. Mitonucleons

Mitonucleons were first observed in Ishikawa cells at regular intervals throughout an epithelial monolayer within hours of imposing conditions that stimulate the formation of domes (fluid-filled hemispheres) [8]. Hybrid and transient, mitonucleons are formed by the fusion of multiple mitochondria around aggregated nuclei in syncytia [9]. Three or four mitonucleons form within a syncytium, fill with gas, and elevate separately so that, for a short period of time, they create multiple protuberances in an "expanding" syncytial apical membrane that most likely includes endoplasmic reticulum membranes associated with the giant mitochondria, an association observed in tissues such as endometrium [37], in the adrenal cortex [38] and in human hepatocytes [25].

Small gas bubbles forms within the chromatin of aggregated nuclei inside the mitonucleon. At least one of the gases in that bubble could be nitric oxide, a gaseous neurotransmitter, that has been shown to form a lethal bubble in nuclei of cells that are extremely stressed at low temperatures [39]. Within the mitonucleon, the bubble does not cause cell death but creates an image that has been called an "optically clear" nucleus, a structure observed in cancerous tissue or in endometrium during pregnancy [9] [40]. Gaseous neurotransmitters can activate enzymes that might be responsible for later fragmentation of DNA. The chromatin aggregate is gradually compressed in a separate compartment up against the unfurling apical membrane of the syncytium as a larger vacuole forms within structures created by fused mitochondria [9]. Each mitonucleon vacuole, together with the bolus of chromatin elevating against the apical membrane, forms a structure that looks like a "signet ring," another structure frequently associated with sectioned biopsied cancer tissue. Our results show that these cells represent two different stages brought about by the activity of mitonucleons.

Some combination of pressure and perhaps of activated enzymes and/or a

change in pH explosively fragments the chromatin and the DNA filaments slip out of membrane protuberances back into the common structure forming an array throughout the syncytium as the breached mitonucleons fade [10]. Such an array opens fragmented DNA to the kinds of epigenetic alterations believed to be involved in differentiation [41]. For a period of time the fundamental distinction between cytosol and nucleus is lost. Chromatin fragments reassociate relatively rapidly forming an irregular mass out of which the nuclei of dome cells emerge, arranging as a layer in what is now the envelope of apical and basal syncytial membranes elevating over accumulating fluid.

Such a process of nuclear proliferation is even more controversial than the amitotic processes already discussed in the Results section. In dome formation, nuclei are "produced" out of a mass of reassociated chromatin [11]. Similarly deconstructed chromatin can stream through hollow spheroids [17] and membrane extensions [15]. Streams of chromatin fragments passing through the envelope of a hollow spheroid look like the seams of a basketball. It is difficult to predict how such streams might look in fixed sectioned tissue. Certainly gas filled spheroids would be collapsed. Since the earliest observations of "exploded" chromatin we have speculated that proteins and DNA associating and dispersing may have something in common with "nuclear envelope limited chromatin" mysterious structures observed in all classes of tissue in mammals [42] [43] for almost 50 years.

Chromatin streaming has all of the problems associated with the amitotic processes of budding and bursting as described for giant cells together with the "complexity" introduced by the necessity of stitching fragmented chromosomes back together again, once the material associates into nuclei. What exactly guarantees that progeny cells contain complements of genetic material and chromosomal structure identical to the parental genome? The answer may be that they do not, but that there is an "editing" process that can correct enough of the problems that arise so that cells can function. Research has been accumulating for some time demonstrating that cells from tumors and cancer cell lines frequently contain more or fewer chromosomes than a typical non-cancer cell, a condition that has been called aneuploidy [44]. The additional problem when chromosomes are fragmented, as has been shown in cell lines treated with chemotherapeutic agents, is that karyotypes of surviving cells display what Heng and associates have called "genome chaos" [45]. Not predictably for cell biologists schooled in the belief that mitotically inherited chromosome complements are an absolute "must" for progeny success, these researchers show that some cells survive even with a chaotic genome. When the genomes are karyotyped every day for more than a week, as was done in this paper by Liu and colleagues [45], significant changes are observed. The peak of fragmentation is measured on day 1. Genome chaos peaks between days 2 and 3. "Normal" genomes represent 50% of what is measured on day 5, peaking on day 8. Approximately 0.1% of the cells in a typical experiment survive and, as described by the authors, some stable clonal populations will be produced from dynamic reorganized ge-

nomes after a few more weeks. All of this suggests that a persistent editing process exists that is capable of bringing enough order to a chaotic genome so that it once again becomes capable of mitosis, but probably is still not identical to the genome of the progenitor cell (evolutionary biologists may find all of this rather exciting, cell biologists less so). Ongoing and effective genome editing and the ultimate survival of a small population of cells makes amitosis by chromatin streaming more acceptable in certain circumstances and there are advantages. It is a process that can produce dozens of "new" cells in a very short period of time so that chromatin streaming might represent an energy and time-efficient mode for proliferation of multiple terminally differentiated cells [15].

In a second example of mitonucleon-dependent differentiation, one or more mitonucleons form at the center of a single multinucleated monolayer epithelial cell. The cell containing mitonucleons detaches from neighboring cells in the colony and then from the substrate, floating up into the medium above the monolayer where it forms a hollow spheroid [13]. The giant gas vacuole forms within the inner mitonucleon membrane, pushing cell contents including polyploid nuclei into a rim of cytoplasm between the outer mitochondrial membrane and the cell membrane [13]. The hollow spheroid is mobile and can reattach elsewhere on the petri dish, releasing gas bubbles and becoming a locus of monolayer growth, a process that evokes the cyclical nature of the epithelial to mesenchymal transition (EMT) involved in cancer metastases. In the dish, it may be one of the mechanisms whereby cells proliferate to form a confluent monolayer. If the spheroid does not reattach, a function of adding serum to the culture medium, the polyploid unicellular spheroid can become multicellular [16] and it does so by chromatin streaming through the cytoplasmic shell. In its entirety, this process results in cell "mobility," a capacity with important functions such as wound healing but one that can be catastrophic as in highly metastatic cancers.

This paper is designed to shed light on the earliest stages of differentiation of opaque cells capable of forming mitonucleons and to advance a theory on the nature of metabolism in mitonucleons that can form gas vacuoles of various sizes [9]; some that quickly fall apart as in dome formation and some that have the capacity for an independent existence as is true for the beehive of multiple spheroids recently described [16].

3.2. Megamitochondria

Mitonucleons appear to be a subset of organelles called megamitochondria by Tadeo Wakabayashi in an extensive review [24]. He summarized work from his and other laboratories, referencing more than 50 years of experimental literature on super-sized mitochondria and cataloging more than 100 observations of megamitochondria in diseased or normal tissue, experimentally induced by exercise and hormones or brought on by drug and even toxin administration. The sheer volume of observations, including examples of megamitochondria in healthy as opposed to dying cells, indicates that megamitochondria are not simply dam-

aged organelles, as has sometimes been assumed. Electron micrographs such as those published by Wakabayashi [24] demonstrate that the internal structure of megamitochondria is substantially different from the "typical" mitochondria engaged in oxidative phosphorylation in aerobic organisms. While the functions of megamitochondria appear to be diverse, Wakabayashi stresses a role for these adapted organelles in reestablishing homeostasis when there is a break-down in the mitochondrial electron transport system, suggesting that the organelles are adapting to neutralize reactive oxygen species and/or free electrons resulting from the break-down. Such a mechanism may go a long way toward explaining the presence of megamitochondria in so many different kinds of diseased tissues: they may develop as a result of the diseased state as opposed to being a cause of it.

Knowledge of giant mitochondria stretches at least back to 1883, as referenced by Charles Bowen [46] in a paper on what were then called nebenkern (giant spherical mitochondria). Furthermore, early researchers following nebenkern formation in living spermatids recognized that the megamitochondria were transient organelles involved in tail elongation. The giant mitochondria in spermatids, together with microtubules, organize the cytoskeletal dynamics responsible for the elongation of spermatid tails from 7 microns to almost 2 centimeters [47] [48]. It is probable that endoplasmic reticulum is involved in this process given that the volume of mitochondria is preserved while its surface area is enlarged.

Electron microscopy provides a window into megamitochondrial structure, although, because of their size, it is often essential to employ serial sectioning and model building to obtain the complete picture [49]. Of particular interest for researchers of endometrial epithelial cells, megamitochondria make an appearance in the epithelial lining of the uterus as a part of the reproductive cycle. Following up on a report by Gompel [50] describing giant mitochondria, Armstrong and colleagues [37] investigated giant mitochondria in uterine tissue as a function of that cycle, employing serially sectioned tissue. By this method, megamitochondria were found in 12 of 14 endometrial specimens collected around the time of ovulation. The report demonstrated that the giant mitochondria were invested with endoplasmic reticulum studded with ribosomes. Armstrong recorded that the appearance of giant mitochondria occurs in the middle of the cycle, declining rapidly after day 18. On the basis of mitonucleon involvement in gland-like differentiation in vitro [9] [10] [11], it seems reasonable to suggest that the megamitochondria observed in tissue are also involved in gland formation for the secretory phase.

Addressing both the size and the 3-dimensional complexity of giant mitochondria, space-filling models were derived from three-dimensional transmission electron microscopy (TEM) [51]. An informative sequence of changes in the formation of megamitochondria was established by Sun and colleagues [52], who treated HeLa cells with etoposide, a disruptor of the electron transport sys-

tem, and recorded changes over a period of 16 hours. They established the leakage of cytochrome C from mitochondria into cytoplasm as an early measurable change in cells treated with this toxin, a phenomenon also observed as an early signal of impending apoptosis. As already described, structures such as optically clear nuclei and signet ring cells, as well as chromatin fragmentation, accompany mitonucleon formation, as they are reported to accompany apoptosis, but that they result not in cell death but in cell differentiation [9] [10] [11]. In Sun's sequence, the leakage of cytochrome C out of mitochondria into the cytoplasm is followed by the uncoupling of oxidative phosphorylation as measured by the loss of mitochondrial membrane potential, essential for oxidative phosphorylation. Fifteen hours after administration of the toxin, researchers observed changes in the internal structures of mitochondria, specifically the transformation of internal membranes from the "typical" christae structure into separate vesicular matrix compartments that can fill with gases and merge. A "snapshot" of the cells after the administration of etoposide revealed four categories of giant mitochondria, different from normal and presumably forming sequentially over a period of 16 hours: in one of those variations, normal christae membranes coexist with vesicular membranes; some mitochondria are observed to be fully vesicular; some of these vesicular mitochondria are also swollen; and finally some vesicular mitochondria are so swollen that almost all internal membranes have been pushed to the edges of the expanding organelle [52].

Recently tomography enabled a complete survey of megamitochondria and normal mitochondria in the livers of deceased patients with non-alcoholic fatty liver disease [25]. Using 4 livers in the study, researchers examined every mitochondrion and concluded that the internal configuration of what they called giant mitochondria "is both multifold and dramatically distinct from their normal-sized counterparts." They observed elongated giant mitochondria, intramitochondrial crystalline structures, and spheroidal giant mitochondrial structures. An important consideration is that in cells that form megamitochondria, typical mitochondria are also found. In one cell, researchers counted almost 2400 mitochondria. Of that number, 55% were giant, the rest were normal, suggesting that both adapted and normal mitochondria present in a single cell may also be active in that cell. In the research into dome formation in Ishikawa cells, while it was certainly the case that all of the syncytial nuclei were subsumed into mitonucleons, it was also the case that additional mitochondria staining for carboxylases did not fuse, remaining small particulate organelles arrayed throughout the syncytium [9]. Variations in metabolism between these structures may be complementary.

A flurry of research during the golden age of the electron microscope in the middle of the last century established that giant mitochondria were also found in organs specializing in anabolic activity such as the adrenal gland that synthesizes most of the steroid hormones [53]. In their studies of the rat adrenal gland, Canick and Purvis also found a mix of giant and typical mitochondria [54]. Synthesis and release of steroid hormones such as cortisol is stimulated by trophic

hormones from the pituitary gland. Time-dependent changes in relative amounts of the two kinds of mitochondria could be elicited by removing the pituitary gland. Under those circumstances, researchers detected an increase in size of lipid droplets and in the volume of giant mitochondria, possibly a build-up of raw materials. Megamitochondria comprise 8% of the total mitochondrial volume in control animals, a value that increased to 65% with the swelling that accompanies hypophysectomy. It has been found that administration of ACTH causes the gradual reversal of these effects of hypophysectomy over a period of 9 days as it restores synthesis of steroid hormones. As described for endometrium, there have been numerous observations in the adrenal gland of the importance of endoplasmic reticulum membranes extending from giant mitochondria.

The contents of vacuoles in megamitochondria, some of which are so large as to be called cavitations [38], has largely remained an open question. Our results with mitonucleons leads to the suggestion that these vacuoles are formed by the retention of CO_2 and perhaps other gases within megamitochondria. Such a possibility was suggested in the systems described in Ishikawa cells by observing the force of an enlarging vacuole that unfurls the apical membrane of a syncytium undergoing differentiation [9] or the "hollowing out" of mitonucleons at the center of a detached cell [13]. Additional clues that megamitochondria with attached endoplasmic reticulum must be retaining gas include the rapidity with which membranes unfurl [9]; the observation that vacuoles rapidly formed can be readily dispelled as gaseous bubbles when hollow spheroids adhere to a petri dish [13], and the observation that vacuoles can become enormous to the point of "bursting" when synthesis of steroid hormones is stopped by the administration of an enzymatic inhibitor [38]. Additionally, it is probable that vacuolar pressure against the chromatin mass in mitonucleons results in pyknotic chromatin. Nothing seems to explain these various observations as well as the formation and expansion of a gaseous vacuole or its dissolution.

The kind of structural change brought about by nebenkern, or even mitonucleons during dome formation, does not appear to explain the presence of megamitochondria in the adrenal gland. Perhaps ready access to CO_2 makes "stored" CO_2 in megamitochondria valuable in anabolic tissue such as the adrenal gland. The gland uses cholesterol as the starting point for synthesis and secretion of various steroid hormones. All 27 carbon atoms of cholesterol are derived from acetyl and there are indications that cells of the adrenal glands responsible for synthesizing steroids are enriched for the same CO_2-fixing enzymes detected in dome formation as demonstrated by the work of Paul and Laufer [55], who correlated the presence of high levels of endogenous biotin with the ability to synthesize steroids in specific cells in the adrenal gland.

3.3. Composition of Gas Vacuoles

The theory then to explain the formation of megamitochondria such as mitonucleons is that the fusion of multiple typical mitochondria into a giant mitochondrion and the association with endoplasmic reticulum may in some in-

stances fundamentally alter the structure so that CO_2 diffuses out from the adapted structure slowly, if at all, and gas fills up vesicular matrix compartments that begin to coalesce into larger vacuoles within the organelle itself. Vacuoles can enlarge and create force and vacuoles can store CO_2. Is the formation of megamitochondria about achieving one or both of these desired ends?

The chief metabolic gas produced by any cell is CO_2, however the widespread assumption that CO_2 diffuses passively through biological membranes, as it does through lipid bilayers in the laboratory, has consistently worked against any suspicion that it can be retained. That assumption was successfully challenged when Boron and colleagues [56] demonstrated differences between apical and basal membrane permeabilities for CO_2 using isolated gastric glands. The research methods of Endeward and Gros [57] demonstrated just how much variability (two orders of magnitude) there can be in biological membranes with regard to CO_2 permeability. These researchers and their colleagues proved that some membranes offer substantially more resistance to CO_2 diffusion than others [58] [59]. Furthermore the laboratory has demonstrated an amazing reciprocal relationship between increasing pressure required for CO_2 to pass through a membrane and declining levels of O_2 consumption [60].

The diffusion of CO_2 through the membranes of typical mitochondria is more rapid than diffusion through most other membranes, an observation from Gros's laboratory that makes sense for the organelle that will produce CO_2 rapidly by oxidative phosphorylation of glucose. The question becomes what happens when normal mitochondria together with endoplasmic reticulum fuse into megamitochondria. Researchers such as Tandler and Hoppel [61] and Wakabayashi [24] were early champions of the notion that megamitochondria form by the fusion of normal mitochondria, a process evident in our studies of dome formation [62]. Wakabayashi did extensive studies on the identity of megamitochondrial lipids to try to understand the underlying biochemical changes that would result in fusible organelles. Wakabayashi [24] compared lipids of megamitochondria with normal mitochondria and found increases in phosphatidylethanolamine and acidic phospholipids as well as in the ratio of unsaturated to saturated fatty acids, changes that might increase fusibility as shown by recent work on phosphatidylethanolamine, phosphatidic acid, and cardiolipin, a negatively charged lipid unique to mitochondria [63] [64]. But in light of results demonstrating that cholesterol is an important variable [58] in determining diffusibility of CO_2 through a membrane, the increases in cholesterol that Wakabayashi found in his studies [16] might be most relevant to the possibility of CO_2 retention by megamitochondria. Such lipid changes together with the close investment of enlarging mitochondrion by a cistern of endoplasmic reticulum [37] could affect passage of gases, particularly since ER itself is believed to be the site of cholesterol synthesis. Although absolute lipid content has not been measured for Ishikawa cells forming mitonucleons, other data suggest that lipids are involved in mitonucleon formation. Fatty acids such as butyrate stimulate the formation of domes

and therefore of mitonucleons more than three-fold [65]. Furthermore the increase in endogenous biotin accompanying mitonucleon formation was ultimately shown to be linked to increases in mitochondrial CoA carboxylases [14], biotin-containing enzymes involved in fatty acid synthesis such as proprionyl CoA carboxylase.

For it to be true that megamitochondria are adapted so that CO_2 retention is the source of vacuoles, it is essential that the formation of megamitochondrial membranes significantly slows the exit of CO_2 and also that the CO_2 be produced by a metabolic pathway other than oxidative phosphorylation, a process reliant on O_2 and the typical christae structure of the internal membranes of normal mitochondria. Together with the loss of mitochondrial membrane potential, the nature of the changes in the inner membrane structure of typical mitochondria that fuse into megamitochondria suggest a point beyond which the chemiosmosis essential to oxidative phosphorylation [66] would no longer be possible. Beyond that point, increasing gas levels suggest an alternative kind of metabolism. Otto Warburg maintained that cancer cells must use alternative metabolic pathways because of an observation of decreased uptake of O_2 by cancer cells. DeBerardinis and his associates [67] extended Warburg's observation by demonstrating that although cancer cells utilize glucose, most of the molecule ends up being excreted as lactate rather than being metabolized by oxidative phosphorylation. In that paper, the researchers presented evidence for the potential of glutamine metabolism as an alternate pathway.

Much has been learned about glutamine metabolism since then. An important piece to the puzzle is experiments demonstrating that glutamine can be processed by reductive carboxylation, bypassing oxidative phosphorylation. Experiments with a melanoma cell line able to proliferate under extreme hypoxia established that reductive carboxylation of alpha ketoglutarate generated from metabolism of glutamine can become much more important than oxidative metabolism as a pathway for production of citrate [68]. The reaction uses an NADP+/NADPH-dependent isocitrate dehydrogenase (IDH2) located in mitochondria. Mullen and colleagues [69] showed that tumor cells with defective mitochondria are able to use glutamine-dependent reductive carboxylation as the major pathway of citrate metabolism. Reductive glutamine metabolism also occurs in cytosol and depends on a second isocitrate dehydrogenase (IDH1) [70].

In fully formed megamitochondria, internal membranes are converted from christae into separate vesicular matrix compartments that can fill with gases [62]. The compartments merge, creating a vacuole that is so large it pushes all membranes to the edge of the megamitochondria [62], as has been shown in electron micrographs. At some point, and it is probably early in the process, megamitochondrial membranes will be unable to carry on oxidative phosphorylation, suggesting that adapted mitochondria may only be capable of reductive carboxylation. Shim and colleagues [71] [72] established a significant connection between glutamine and megamitochondria with the discovery that glutamine, the substrate for reductive carboxylation, is essential for the induction of megami-

tochondria. In fact, megamitochondria may be the organelle best suited for reductive carboxylation, especially if its adaptations make it more or less impervious to oxygen. Depending on the extent of reductive carboxylation, such a possibility suggests that "pools" of CO_2 might increase or decrease depending on whether catabolism or anabolism is the dominant form of metabolism. And it seems reasonable to suggest that the pools of retained CO_2 would be supportive of reductive carboxylation.

It would be hard to overstate the advantages of such a possibility, especially for mitochondria whose principal function is anabolism since, as Zimorski *et al.* [73] recently wrote, "the synthesis of biomass costs thirteen times more energy per cell in the presence of oxygen than in anoxic conditions." Because of the ready diffusion of CO_2 into and out of typical mitochondria, there has not been too much emphasis on supply, but demands for CO_2 would appear to be highly variable. The fact that the adrenal gland needs to excrete steroid hormones on demand and then cease excretion makes the idea of an adapted mitochondria that can store CO_2 seem even more reasonable. Some kind of release of CO_2 from vacuoles in a dissolved state must be imagined since dissolved CO_2 has been shown to be the primary substrate for IDH [74]. The same paper concluded that experiments on the reductive carboxylation of pyruvate catalysed by what the authors call the "malic" enzyme also uses dissolved CO_2 as the primary substrate.

There probably is much more to be learned about reductive carboxylation. Du and colleagues [75] have demonstrated it to be the major metabolic pathway in retinal pigment epithelium Additionally reductive carboxylation is enhanced in detached epithelial cells [76]. And it is noteworthy that, as reported in a study by Labuschagne *et al.* [77], the increase in reductive carboxylation is accompanied by morphological changes in mitochondria. As already mentioned, glutamine is essential for the formation of megamitochondria in Drosophila [71] [72]. One very recent paper even describes an effect of glutamine on the origin and function of cancer cell extracellular vesicles [78].

In the history of the planet, the current levels of oxygen have only been around for the past 500 million years [73]. Elevated oxygen levels resulting in the evolution of oxidative phosphorylation threatened processes that had evolved in the absence of O_2 such as the conversion of gaseous nitrogen into ammonia. I began my professional life studying the enabling adaptations that allow some species of cyanobacteria to convert N_2 to NH_3 even in the presence of O_2. The process involves a 20 to 24 hour differentiation [79] of approximately 10% of vegetative cells into heterocysts, a cell type that maintains a "relatively anoxic microenvironment in a filament that is predominantly oxic." [80] And this is not the only example of cellular adaptation for enzymes whose activity is lost in the presence of O_2. Researchers have shown that anaerobic localities called microoxic niches exist in chloroplasts of Chlamydomonas reinhardtii to protect the activity of [FeFe] hydrogenase from O_2 inactivation, a gas that the organelle itself generates

[81]. One more example of structural changes resulting in an anoxic environment is actually to be found in megamitochondria in a single-celled green protist.

A mutant of Euglena gracilis without chloroplasts, seemingly wholly dependent on oxidative phosphorylation, is nevertheless able to function if the electron transport system is poisoned or if the organism is placed under anaerobic conditions. In these adverse conditions, growth immediately ceases, but without any further changes in medium, Euglena adapt and begin proliferating again after approximately 20 hours. Sharpless and Butow [82] established the induction of a unique NADP+ dependent pyruvate dehydrogenase, insensitive to antimycin or cyanide poisoning and markedly stimulated by AMP. However, the induced enzyme is inactivated by O_2, as researchers found when they tried to purify the enzyme. Its activity was lost as soon as adapted mitochondria were broken open [83].

3.4. Conclusions

The inescapable conclusion is that the adapted structure of Euglena megamitochondria protects the newly induced enzyme from inactivation by O_2. Can we assume similar functions for megamitochondria in higher organisms? Do megamitochondria within opaque giant cells achieve localized hypoxia by virtue of an adapted membrane structure fortified with cholesterol? The observation that megamitochondria retain gases even as evidence of their capacity for oxidative phosphorylation dramatically decreases [52], together with the observations of a reciprocal relationship between membrane permeability to CO_2 and O_2 uptake [61], suggests that adapted mitochondria may be mostly hypoxic.

Finally, this paper demonstrates how opaque multinuclear cells arise in an Ishikawa culture in a process that appears to be an exception to what Virchow posited about all cells arising from other cells. It cannot be proven that a monolayer cell did not exist in region 10 before the start of the observation, but formation of the opaque cell does not appear to be dependent on any prior structure that can be detected. What is observed is a single opaque cell capable of forming a mitonucleon being built up over a period of hours with circumstantial evidence that it develops under the influence of extracellular vesicles arising from another opaque multinuclear cell. Less conclusively but worth mentioning, it appears that a monolayer cell forms at the same time.

If the mitonucleons that form in these extraordinary cells are structures that protect O_2-sensitive enzymes, it may even be possible that the biochemical enzymes essential for H_2 formation may be inducible and active under some circumstances in megamitochondria. It would be hard to overestimate how useful it would be to adapt some of a cell's population of mitochondria so that they are hypoxic or even anoxic, capable of more efficient anabolism, capable of storing CO_2 and perhaps even capable of generating H_2 with the capacity to restore NADP+ to a reduced state.

4. Materials and Methods

Ishikawa endometrial epithelial cells were grown in phenol-red free MEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg amphotericin B (GIBCO, Grand Island, NY). The cell line established by Nishida and colleagues [7] from an endometrial adenocarcinoma was obtained from Dr. Erlio Gurpide's laboratory at Mt. Sinai Hospital in New York. Cells seeded at an approximate density of 5×10^5 cells/cm^2, were grown for 1 - 2 weeks in MEM containing 5% calf serum (CS). The experiments 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 200× and 400×.

The presence of stainable amounts of biotin was used to detect the presence of mitonucleons as in **Figure 1** and **Figure 2**, cultures 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 changes of PBS. After washing, cells were exposed to a 1:200 dilution of Extravidin conjugated horseradish 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 0.25% H_2O_2. 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.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

[1] Zhang, S., Mercado-Uribe, I., Xing, Z., Sun, B., Kuang, J. and Liu, J. (2014) Generation of Cancer Stem-Like Cells through the Formation of Polyploid Giant Cancer Cells. *Oncogene*, **33**, 116-128. https://doi.org/10.1038/onc.2013.96

[2] Mirzayans, R., Andrais, B. and Murray, D. (2018) Roles of Polyploid/Multinucleated Giant Cancer Cells in Metastasis and Disease Relapse Following Anticancer Treatment. *Cancers*, **10**, 118. https://doi.org/10.3390/cancers10040118

[3] Chen, J., Niu, N., Zhang, J., Qi, L., Shen, W., Donkena, K.V., Feng, Z. and Liu, J. (2019) Polyploid Giant Cancer Cells (PGCCs): The Evil Roots of Cancer. *Current Cancer Drug Targets*, **19**, 360-367. https://doi.org/10.2174/1568009618666180703154233

[4] Fleming, H. (2014) Unusual Characteristics of Opaque Ishikawa Endometrial Cells Include the Envelopment of Chromosomes with Material Containing Endogenous Biotin in the Latter Stages of Cytokinesis. *PeerJ PrePrints*, **2**, e772v1. https://doi.org/10.7287/peerj.preprints.772

[5] Niu, N., Zhang, J., Zhang, N., Mercado-Uribe, I., Tao, F., Han, Z., Pathak, S., Multani, A.S., Kuang, J., Yao, J., Bast, R.C., Sood, A.K., Hung, M.C. and Liu, J. (2016) Linking Genomic Reorganization to Tumor Initiation via the Giant Cell Cycle. *Oncogenesis*, **5**, e281. https://doi.org/10.1038/oncsis.2016.75

[6] Chaffer, C.L., Brueckmann, I., Scheel, C., Kaestli, A.J., Wiggins, P.A., Rodrigues, L.O., Brooks, M., Reinhardt, F., Su, Y., Polyak, K., Arendt, L.M., Kuperwasser, C., Bierie, B. and Weinberg, R.A. (2011) Normal and Neoplastic Nonstem Cells can Spontaneously Convert to a Stem-Like State. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 7950-7955. https://doi.org/10.1073/pnas.1102454108

[7] Nishida, M., Kasahara, K., Kaneko, M. and Iwasaki, H. (1985) Establishment of a New Human Endometrial Adenocarcinoma Cell Line, Ishikawa Cells, Containing Estrogen and Progesterone Receptors. *Acta Obstetrica et Gynaecologica Japonica*, **37**, 1103-1111. (In Japanese)

[8] Fleming, H. (1995) Differentiation in Human Endometrial Cells in Monolayer Culture: Dependence on a Factor in Fetal Bovine Serum. *Journal of Cellular Biochemistry*, **57**, 262-270. https://doi.org/10.1002/jcb.240570210

[9] Fleming, H. (2016) Mitonucleons Formed during Differentiation of Ishikawa Endometrial Cells Generate Vacuoles That Elevate Monolayer Syncytia: Differentiation of Ishikawa Domes, Part 1. *PeerJ PrePrints*, **4**, e1728v1. https://doi.org/10.7287/peerj.preprints.1728

[10] Fleming, H. (2016) Pyknotic Chromatin in Mitonucleons Elevating in Syncytia Undergo Karyorrhexis and Karyolysis before Coalescing into an Irregular Chromatin Mass: Differentiation of Ishikawa Domes, Part 2. *PeerJ PrePrints*, **4**, e1729v1. https://doi.org/10.7287/peerj.preprints.1729v1

[11] Fleming, H. (2016) Chromatin Mass from Previously Aggregated, Pyknotic, and Fragmented Monolayer Nuclei Is a Source for Dome Cell Nuclei Generated by Amitosis: Differentiation of Ishikawa Domes, Part 3. *PeerJ PrePrints*, **4**, e1730v1. https://doi.org/10.7287/peerj.preprints.1730v1

[12] Hollinshead, M., Sanderson, J. and Vaux, D.J. (1997) Anti-Biotin Antibodies Offer Superior Organelle-Specific Labeling of Mitochondria over Avidin or Streptavidin. *The Journal of Histochemistry and Cytochemistry*: *Official Journal of the Histochemistry Society*, **45**, 1053-1057. https://doi.org/10.1177/002215549704500803

[13] Fleming, H. (2018) Polyploid Monolayer Ishikawa Endometrial Cells form Unicellular Hollow Spheroids Capable of Migration. *PeerJ PrePrints*, **6**, e26793v1. https://doi.org/10.7287/peerj.preprints.26793v1

[14] Fleming, H., Condon, R., Peterson, G., Guck, I., Prescott, E., Chatfield, K. and Duff, M. (1998) Role of Biotin-Containing Membranes and Nuclear Distribution in Dif-

ferentiating Human Endometrial Cells. *Journal of Cellular Biochemistry*, **71**, 400-415. https://doi.org/10.1002/(SICI)1097-4644(19981201)71:3<400::AID-JCB9>3.0.CO;2-W

[15] Fleming, H. (2019) Tubular Membranes Extended between Monolayer Cells, from Solid Spheroids, and from Clustered Hollow Spheroids in Ishikawa Endometrial Cell Cultures Can Carry Chromatin Granules and Mitonucleons. *PeerJ PrePrints*, **7**, e27895v1. https://doi.org/10.7287/peerj.preprints.27895

[16] Fleming, H. (2019) Chromatin Streaming from Giant Polyploid Nuclei in Ishikawa Endometrial Hollow Spheroids Results in the Amitotic Proliferation of Nuclei That Fill the Spheroid Envelope. *PeerJ PrePrints*, **7**, e27463v1. https://doi.org/10.7287/peerj.preprints.27463

[17] Isakova, G.K. and Mead, R.A. (2004) Occurrence of Amitotic Division of Trophoblast Cell Nuclei in Blastocysts of the Western Spotted Skunk (*Spilogale putorius latifrons*). *Hereditas*, **140**, 177-184. https://doi.org/10.1111/j.1601-5223.2004.01749.x

[18] Walen, K.H. (2008) Genetic Stability of Senescence Reverted Cells: Genome Reduction Division of Polyploidy Cells, Aneuploidy and Neoplasia. *Cell Cycle*, **7**, 1623-1629. https://doi.org/10.4161/cc.7.11.5964

[19] Zybina, T.G. and Zybina, E.V. (2020) Role of Cell Cycling and Polyploidy in Placental Trophoblast of Different Mammalian Species. *Reproduction in Domestic Animals = Zuchthygiene*, **55**, 895-904. https://doi.org/10.1111/rda.13732

[20] Erenpreisa, J., Kalejs, M., Ianzini, F., Kosmacek, E.A., Mackey, M.A. and Emzinsh, D. (2005) Segregation of Genomes in Polyploid Tumour Cells Following Mitotic Catastrophe. *Cell Biology International*, **29**, 1005-1011. https://doi.org/10.1016/j.cellbi.2005.10.008

[21] Sundaram, M., Guernsey, D.L., Rajaraman, M.M. and Rajaraman, R. (2004) Neosis: A Novel Type of Cell Division in Cancer. *Cancer Biology & Therapy*, **3**, 207-218. https://doi.org/10.4161/cbt.3.2.663

[22] Niu, N., Mercado-Uribe, I. and Liu, J. (2017) Dedifferentiation into Blastomere-Like Cancer Stem Cells via Formation of Polyploid Giant Cancer Cells. *Oncogene*, **36**, 4887-4900. https://doi.org/10.1038/onc.2017.72

[23] Liu, J. (2018) The Dualistic Origin of Human Tumors. *Seminars in Cancer Biology*, **53**, 1-16. https://doi.org/10.1016/j.semcancer.2018.07.004

[24] Wakabayashi, T. (2002) Megamitochondria Formation—Physiology and Pathology. *Journal of Cellular and Molecular Medicine, 6,* 497-538. https://doi.org/10.1111/j.1582-4934.2002.tb00452.x

[25] Shami, G.J., Cheng, D., Verhaegh, P., Koek, G., Wisse, E. and Braet, F. (2021) Three-Dimensional Ultrastructure of Giant Mitochondria in Human Non-Alcoholic Fatty Liver Disease. *Scientific Reports*, **11**, Article No. 3319. https://doi.org/10.1038/s41598-021-82884-z

[26] Minciacchi, V.R., Freeman, M.R. and Di Vizio, D. (2015) Extracellular Vesicles in Cancer: Exosomes, Microvesicles and the Emerging Role of Large Oncosomes. *Seminars in Cell & Developmental Biology*, **40**, 41-51. https://doi.org/10.1016/j.semcdb.2015.02.010

[27] Becker, A., Thakur, B.K., Weiss, J.M., Kim, H.S., Peinado, H. and Lyden, D. (2016) Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell*, **30**, 836-848. https://doi.org/10.1016/j.ccell.2016.10.009

[28] Fujita, Y., Yoshioka, Y. and Ochiya, T. (2016) Extracellular Vesicle Transfer of Cancer Pathogenic Components. *Cancer Science*, **107**, 385-390. https://doi.org/10.1111/cas.12896

[29] Ciardiello, C., Cavallini, L., Spinelli, C., Yang, J., Reis-Sobreiro, M., de Candia, P., Minciacchi, V.R. and Di Vizio, D. (2016) Focus on Extracellular Vesicles: New Frontiers of Cell-to-Cell Communication in Cancer. *International Journal of Molecular Sciences*, **17**, 175. https://doi.org/10.3390/ijms17020175

[30] Tkach, M. and Thery, C. (2016) Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell*, **164**, 1226-1232. https://doi.org/10.1016/j.cell.2016.01.043

[31] Nogués, L., Benito-Martin, A., Hergueta-Redondo, M. and Peinado, H. (2018) The Influence of Tumour-Derived Extracellular Vesicles on Local and Distal Metastatic Dissemination. *Molecular Aspects of Medicine*, **60**, 15-26. https://doi.org/10.1016/j.mam.2017.11.012

[32] Han, L., Lam, E.W.F. and Sun, Y. (2019) Extracellular Vesicles in the Tumor Microenvironment: Old Stories, but New Tales. *Molecular Cancer*, **18**, 59. https://doi.org/10.1186/s12943-019-0980-8

[33] Tao, S.C. and Guo, S.C. (2020) Role of Extracellular Vesicles in Tumour Microenvironment. *Cell Communication and Signaling*, **18**, 163. https://doi.org/10.1186/s12943-019-0980-8

[34] Yokoi, A. and Ochiya, T. (2021) Exosomes and Extracellular Vesicles: Rethinking the Essential Values in Cancer Biology. *Seminars in Cancer Biology*. (Advance Online Publication) https://doi.org/10.1016/j.semcancer.2021.03.032

[35] Hu, W., Liu, C., Bi, Z.Y., Zhou, Q., Zhang, H., Li, L.L., Zhang, J., Zhu, W., Song, Y.Y., Zhang, F., Yang, H.M., Bi, Y.Y., He, Q.Q., Tan, G.J., Sun, C.C. and Li, D.J. (2020) Comprehensive Landscape of Extracellular Vesicle-Derived RNAs in Cancer Initiation, Progression, Metastasis and Cancer Immunology. *Molecular Cancer*, **19**, 102. https://doi.org/10.1186/s12943-020-01199-1

[36] Gopal, S.K., Greening, D.W., Rai, A., Chen, M., Xu, R., Shafiq, A., Mathias, R.A., Zhu, H.J. and Simpson, R.J. (2017) Extracellular Vesicles: Their Role in Cancer Biology and Epithelial-Mesenchymal Transition. *The Biochemical Journal*, **474**, 21-45. https://doi.org/10.1042/BCJ20160006

[37] Armstrong, E.M., More, I.A., McSeveney, D. and Carty, M. (1973) The Giant Mitochondrion-Endoplasmic Reticulum Unit of the Human Endometrial Glandular Cell. *Journal of Anatomy*, **116**, 375-383.

[38] Kadioglu, D. and Harrison, R.G. (1971) The Functional Relationships of Mitochondria in the Rat Adrenal Cortex. *Journal of Anatomy*, **110**, 283-296.

[39] Chang, N.S. (2016) Bubbling Cell Death: A Hot Air Balloon Released from the Nucleus in the Cold. *Experimental Biology and Medicine* (*Maywood*, *N.J.*), **241**, 1306-1315. https://doi.org/10.1177/1535370216644531

[40] Gamachi, A., Kashima, K., Daa, T., Nakatani, Y., Tsujimoto, M. and Yokoyama, S. (2003) Aberrant Intranuclear Localization of Biotin, Biotin-Binding Enzymes, and Beta-Catenin in Pregnancy Related Endometrium and Morule-Associated Neoplastic Lesions. *Modern Pathology*, **16**, 1124-1131. https://doi.org/10.1097/01.MP.0000092953.20717.48

[41] Handy, D.E., Castro, R. and Loscalzo, J. (2011) Epigenetic Modifications: Basic Mechanisms and Role in Cardiovascular Disease. *Circulation*, **123**, 2145-2156. https://doi.org/10.1161/CIRCULATIONAHA.110.956839

[42] Davies, H.G. and Haynes, M.E. (1975) Light- and Electron-Microscope Observations on Certain Leukocytes in a Teleost Fish and a Comparison of the Envelope-Limited Monolayers of Chromatin Structural Units in Different Species. *Journal of Cell Science*, **17**, 263-285. https://doi.org/10.1242/jcs.17.3.263

[43] Olins, A.L., Buendia, B., Herrmann, H., Lichter, P. and Olins, D.E. (1998) Retinoic Acid Induction of Nuclear Envelope-Limited Chromatin Sheets in HL-60. *Experimental Cell Research*, **245**, 91-104. https://doi.org/10.1006/excr.1998.4210

[44] Duesberg, P., Li, R., Fabarius, A. and Hehlmann, R. (2006) Aneuploidy and Cancer: from Correlation to Causation. *Contributions to Microbiology*, **13**, 16-44. https://doi.org/10.1159/000092963

[45] Liu, G., Stevens, J.B., Horne, S.D., Abdallah, B.Y., Ye, K.J., Bremer, S.W., Ye, C.J., Chen, D.J. and Heng, H.H. (2014) Genome Chaos: Survival Strategy during Crisis. *Cell Cycle* (*Georgetown*, *Tex.*), **13**, 528-537. https://doi.org/10.4161/cc.27378

[46] Bowen, R.H. (1927) On the Structure of the Nebenkern in the Insect Spermatid and the Origin of Nebenkern Patterns. *Biological Bulletin*, **42**, 53-84. https://doi.org/10.2307/1536580

[47] Devine, R.L., Powers, E.L. and Tahmisian, T.N. (1956) Light and Electron Microscope Studies of Morphological Changes of Mitochondria during Spermatogenesis in the Grasshopper. *The Journal of Biophysical and Biochemical Cytology*, **2**, 325-330. https://doi.org/10.2307/1536580

[48] Noguchi, T., Koizumi, M. and Hayashi, S. (2011) Sustained Elongation of Sperm Tail Promoted by Local Remodeling of Giant Mitochondria in Drosophila. *Current Biology*, **21**, 805-814. https://doi.org/10.1016/j.cub.2011.04.016

[49] Coaker, T., Downie, T. and More, I.A. (1982) Complex Giant Mitochondria in the Human Endometrial Glandular Cell: Serial Sectioning, High-Voltage Electron Microscopic, and Three-Dimensional Reconstruction Studies. *Journal of Ultrastructure Research*, **78**, u283-u291. https://doi.org/10.1016/j.cub.2011.04.016

[50] Gompel, C. (1964) Structure fine des mitochondries de la cellule glandulaire endometriale humaine au cours du cycle menstruel. *Journal de Microscopie*, **3**, 427-436.

[51] Mannella, C.A., Marko, M., Penczek, P., Barnard, D. and Frank, J. (1994) The Internal Compartmentation of Rat-Liver Mitochondria: Tomographic Study Using the High-Voltage Transmission Electron Microscope. *Microscopy Research and Technique*, **27**, 278-283. https://doi.org/10.1002/jemt.1070270403

[52] Sun, M.G., Williams, J., Munoz-Pinedo, C., Perkins, G.A., Brown, J.M., Ellisman, M.H., Green, D.R. and Frey, T.G. (2007) Correlated Three-Dimensional Light and Electron Microscopy Reveals Transformation of Mitochondria during Apoptosis. *Nature Cell Biology*, **9**, 1057-1065. https://doi.org/10.1038/ncb1630

[53] Sabatini, D.D. and DeRobertis, E.D. (1961) Ultrastructural Zonation of Adrenocortex in the Rat. *The Journal of Biophysical and Biochemical Cytology*, **9**, 105-119. https://doi.org/10.1083/jcb.9.1.105

[54] Canick, J.A. and Purvis, J.L. (1972) The Maintenance of Mitochondrial Size in the Rat Adrenal Cortex Zona Fasciculata by ACTH. *Experimental and Molecular Pathology*, **16**, 79-93. https://doi.org/10.1016/0014-4800(72)90022-6

[55] Paul, A. and Laufer, E. (2011) Endogenous Biotin as a Marker of Adrenocortical Cells with Steroidogenic Potential. *Molecular and Cellular Endocrinology*, **336**, 133-140. https://doi.org/10.1016/j.mce.2011.01.015

[56] Boron, W.F., Waisbren, S.J., Modlin, I.M. and Geibel, J.P. (1994) Unique Permeability Barrier of the Apical Surface of Parietal and Chief Cells in Isolated Perfused Gastric Glands. *The Journal of Experimental Biology*, **196**, 347-360. https://doi.org/10.1242/jeb.196.1.347

[57] Endeward, V. and Gros, G. (2005) Low Carbon Dioxide Permeability of the Apical Epithelial Membrane of Guinea-Pig Colon. *The Journal of Physiology*, **567**, 253-265. https://doi.org/10.1113/jphysiol.2005.085761

[58] Itel, F., Al-Samir, S., Öberg, F., Chami, M., Kumar, M., Supuran, C.T., Deen, P.M., Meier, W., Hedfalk, K., Gros, G. and Endeward, V. (2012) CO_2 Permeability of Cell Membranes Is Regulated by Membrane Cholesterol and Protein Gas Channels. *FASEB Journal*, **26**, 5182-5191. https://doi.org/10.1096/fj.12-209916

[59] Arias-Hidalgo, M., Hegermann, J., Tsiavaliaris, G., Carta, F., Supuran, C.T., Gros, G. and Endeward, V. (2016) CO_2 and HCO_3-Permeability of the Rat Liver Mitochondrial Membrane. *Cellular Physiology and Biochemistry*, **39**, 2014-2024. https://doi.org/10.1159/000447897

[60] Arias-Hidalgo, M., Yuan, Q., Carta, F., Supuran, C.T., Gros, G. and Endeward, V. (2018) CO_2 Permeability of Rat Hepatocytes and Relation of CO_2 Permeability to CO_2 Production. *Cellular Physiology and Biochemistry*: *International Journal of Experimental Cellular Physiology*, *Biochemistry*, *and Pharmacology*, **46**, 1198-1208. https://doi.org/10.1159/000489070

[61] Tandler, B. and Hoppel, C.L. (1986) Studies on Giant Mitochondria. *Annals of the New York Academy of Sciences*, **488**, 65-81. https://doi.org/10.1111/j.1749-6632.1986.tb46548.x

[62] Fleming, H. (2018) Mitochondrial/Nuclear Superstructures Drive Morphological Changes in Endometrial Epithelia by Pressure Exerted When Gas Vacuoles Form and Coalesce Within Superstructures. *Advances in Bioscience and Biotechnology*, **9**, 224-242. https://doi.org/10.4236/abb.2018.95016

[63] Joshi, A.S., Thompson, M.N., Fei, N., Huttemann, M. and Greenberg, M.L. (2012) Cardiolipin and Mitochondrial Phosphatidylethanolamine Have Overlapping Functions in Mitochondrial Fusion in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **287**, 17589-17597. https://doi.org/10.1074/jbc.M111.330167

[64] Ha, E.E. and Frohman, M.A. (2014) Regulation of Mitochondrial Morphology by Lipids. *Biofactors*, **40**, 419-424. https://doi.org/10.1002/biof.1169

[65] Fleming, H., Begley, M., Campi, T., Condon, R., Dobyns, K., McDonagh, J. and Wallace, S. (1995) Induction of Heat Labile Alkaline Phosphatase by Butyrate in Differentiating Endometrial Cells. *Journal of Cellular Biochemistry*, **58**, 509-516. https://doi.org/10.1002/jcb.240580414

[66] Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation. *Biological Reviews*, **41**, 445-502. https://doi.org/10.1111/j.1469-185X.1966.tb01501.x

[67] DeBerardinis, R.J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S. and Thompson, C.B. (2007) Beyond Aerobic Glycolysis: Transformed Cells Can Engage in Glutamine Metabolism That Exceeds the Requirement for Protein and Nucleotide Synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 19345-19350. https://doi.org/10.1073/pnas.0709747104

[68] Wise, D.R., Ward, P.S., Shay, J.E., Cross, J.R., Gruber, J.J., Sachdeva, U.M., Platt, J.M., DeMatteo, R.G., Simon, M.C. and Thompson, C.B. (2011) Hypoxia Promotes Isocitrate Dehydrogenase-Dependent Carboxylation of α-Ketoglutarate to Citrate to Support Cell Growth and Viability. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 19611-19616. https://doi.org/10.1073/pnas.1117773108

[69] Mullen, A.R., Wheaton, W.W., Jin, E.S., Chen, P.H., Sullivan, L.B., Cheng, T., Yang, Y., Linehan, W.M., Chandel, N.S. and DeBerardinis, R.J. (2011) Reductive Carboxylation Supports Growth in Tumour Cells with Defective Mitochondria. *Nature*, **481**, 385-388. https://doi.org/10.1038/nature10642

[70] Metallo, C.M., Gameiro, P.A., Bell, E.L., Mattaini, K.R., Yang, J., Hiller, K., Jewell,

C.M., Johnson, Z.R., Irvine, D.J., Guarente, L., Kelleher, J.K., Vander Heiden, M.G., Iliopoulos, O. and Stephanopoulos, G. (2011) Reductive Glutamine Metabolism by IDH1 Mediates Lipogenesis under Hypoxia. *Nature*, **481**, 380-384. https://doi.org/10.1038/nature10602

[71] Shim, M.S., Kim, J.Y., Jung, H.K., Lee, K.H., Xu, X.M., Carlson, B.A., Kim, K.W., Kim, I.Y., Hatfield, D.L. and Lee, B.J. (2009) Elevation of Glutamine Level by Selenophosphate Synthetase 1 Knockdown Induces Mega Mitochondrial Formation in Drosophila Cells. *Journal of Biological Chemistry*, **284**, 32881-32894. https://doi.org/10.1074/jbc.M109.026492

[72] Shim, M.S., Kim, J.Y., Lee, K.H., Jung, H.K., Carlson, B.A., Xu, X.M., Hatfield, D.L. and Lee, B.J. (2011) L(2)01810 Is a Novel Type of Glutamate Transporter That Is Responsible for Mega Mitochondrial Formation. *The Biochemical Journal*, **439**, 277-286. https://doi.org/10.1042/BJ20110582

[73] Zimorski, V., Mentel, M., Tielens, A.G.M. and Martin, W.F. (2019) Energy Metabolism in Anaerobic Eukaryotes and Earth's Late Oxygenation. *Free Radical Biology and Medicine*, **140**, 279-294. https://doi.org/10.1016/j.freeradbiomed.2019.03.030

[74] Dalziel, K. and Londesborough, J.C. (1968) The Mechanisms of Reductive Carboxylation Reactions. Carbon Dioxide or Bicarbonate as Substrate of Nicotinamide-Adenine Dinucleotide Phosphate-Linked Isocitrate Dehydrogenase and Malic Enzyme. *The Biochemical Journal*, **110**, 223-230. https://doi.org/10.1042/bj1100223

[75] Du, J., Yanagida, A., Knight, K., Engel, A.L., Vo, A.H., Jankowski, C., Sadilek, M., Tran, V.T., Manson, M.A., Ramakrishnan, A., Hurley, J.B. and Chao, J.R. (2016) Reductive Carboxylation Is a Major Metabolic Pathway in the Retinal Pigment Epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, **113**, 14710-14715. https://doi.org/10.1073/pnas.1604572113

[76] Jiang, L., Shestov, A.A., Swain, P., Yang, C., Parker, S.J., Wang, Q.A., Terada, L.S., Adams, N.D., McCabe, M.T., Pietrak, B., Schmidt, S., Metallo, C.M., Dranka, B.P., Schwartz, B. and DeBerardinis, R.J. (2016) Reductive Carboxylation Supports Redox Homeostasis during Anchorage-Independent Growth. *Nature*, **532**, 255-258. https://doi.org/10.1038/nature17393

[77] Labuschagne, C.F., Cheung, E.C., Blagih, J., Domart, M.C. and Vousden, K.H. (2019) Cell Clustering Promotes a Metabolic Switch That Supports Metastatic Colonization. *Cell Metabolism*, **30**, 720-734.e5. https://doi.org/10.1016/j.cmet.2019.07.014

[78] Fan, S.J., Kroeger, B., Marie, P.P., Bridges, E.M., Mason, J.D., McCormick, K., Zois, C.E., Sheldon, H., Khalid Alham, N., Johnson, E., Ellis, M., Stefana, M.I., Mendes, C.C., Wainwright, S.M., Cunningham, C., Hamdy, F.C., Morris, J.F., Harris, A.L., Wilson, C. and Goberdhan, D.C. (2020) Glutamine Deprivation Alters the Origin and Function of Cancer Cell Exosomes. *The EMBO Journal*, **39**, e103009. https://doi.org/10.15252/embj.2019103009

[79] Fleming, H. and Haselkorn, R. (1973) Differentiation in Nostoc Muscorum: Nitrogenase Is Synthesized in Heterocysts. *Proceedings of the National Academy of Sciences of the United States of America*, **70**, 2727-2731. https://doi.org/10.1073/pnas.70.10.2727

[80] Thiel, T. and Pratte, B. (2001) Effect on Heterocyst Differentiation of Nitrogen Fixation in Vegetative Cells of the Cyanobacterium *Anabaena variabilis* ATCC 29413. *Journal of Bacteriology*, **183**, 280-286. https://doi.org/10.1128/JB.183.1.280-286.2001

[81] Liran, O., Semyatich, R., Milrad, Y., Eilenberg, H., Weiner, I. and Yacoby, I. (2016) Microoxic Niches within the Thylakoid Stroma of Air-Grown *Chlamydomonas*

reinhardtii Protect [FeFe]-Hydrogenase and Support Hydrogen Production under Fully Aerobic Environment. *Plant Physiology*, **172**, 264-271. https://doi.org/10.1104/pp.16.01063

[82] Sharpless, T. and Butow, R. (1970) An Inducible Alternate Terminal Oxidase in *Euglena gracilis* Mitochondria. *Journal of Biological Chemistry*, **245**, 58-70. https://doi.org/10.1016/S0021-9258(18)63422-2

[83] Inui, H., Miyatake, K., Nakano, Y. and Kitaoka, S. (1984) Occurrence of Oxygen-Sensitive, NADP+-Dependent Pyruvate Dehydrogenase in Mitochondria of *Euglena gracilis*. *Journal of Biochemistry*, **96**, 931-934. https://doi.org/10.1093/oxfordjournals.jbchem.a134913