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Megamitochondria Initiate Differentiation of Monolayer Cells into Detached Dome Cells That Proliferate by a Schizogony-Like Amitotic Process

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Abstract

Mitonucleon-initiated dome formation involves structural changes occurring over a 20 to 24 hour period in monolayer cells induced by a serum factor. The earliest observable change is the fusion of monolayer cells into a syncytium in which nuclei aggregate and become surrounded by a membrane that stains for endogenous biotin. Each of these structures is further surrounded by a fraction of the mitochondria that arise in the syncytium following initiation of dome formation. The mitochondria fuse around the chromatin aggregate in a structure we have called a mitonucleon. Within mitonucleons, a gaseous vacuole is generated that can be seen in protrusions of the apical membrane pressuring chromatin into a pyknotic state. Eventually that pressure, together with whatever enzymatic changes have occurred in the bolus of chromatin, results in DNA fragmentation. The fragments drawn out through the syncytium by a unipolar spindle are arrayed in a configuration that appears open both to epigenetic changes and to DNA repair and replication by polyteny. The fragmented DNA stretched across the syncytial space, hardly detectable by light microscopy, becomes visible approximately half way through the differentiation as the filaments thicken in what looks like replication by polyteny. This "recycling" of attached monolayer cells into detached dome cells must include DNA replication since the number of cells in the resulting domes is greater than the number of monolayer cells by 30% or more. The resulting DNA associates into a mass of chromatin which will "segment" into polyploid structures and then into what appear to be diploid nuclei over a period of 2 to 4 hours. When the layer of nuclei has filled the syncytium, the nuclei are cellularized, forming dome cells rising up from the monolayer and arching over a fluid cavity. Dome cells can extend into gland-like structures

by the same mitonucleon dependent amitotic process observed in dome formation. Some of the characteristics of this process resemble the amitotic process of schizogony among single-celled eukaryotic parasites of the apicomplexan phylum. Mitonucleon initiated amitotic proliferation results in synthesis of dozens of dome cell nuclei in a period of 20 to 24 hours, so it is much more efficient than mitosis. Cells generated by this process and their progeny would also not be sensitive to agents that inhibit mitosis suggesting that the process, as an alternative to mitosis, might be activated in cancers that become resistant to some cytotoxic drugs.

Keywords

Mitochondria, Megamitochondria, Mitonucleons, Polyteny, Schizogony, Polyploid Reduction Divisions, Cellularization, Pyknotic Nuclear Aggregates, Molded Nuclei, Karryorhexis and Karyolysis

1. Introduction

A key event in the induction of domes in monolayers of human endometrial cells is the formation of transient structures called mitonucleons in syncytia essential to the formation of domes or fluid-filled hemispheres within 24 hours [1]. Induction of this differentiation event in a cell line isolated by Nishida and colleagues [2] is achieved by the addition of at least 10% fetal calf serum to resting, confluent monolayers, and is enhanced by the addition of progesterone. The megamitochondrial structures called mitonucleons form within 5 to 7 hours in syncytia created by cell fusions throughout the monolayer [3]. Apical and basal syncytial membranes containing mitonucleons detach from the petri dish, remaining in contact with the monolayer by cuboidal cells around their circumference and elevating as fluid accumulates under the basal membrane forming a hemicyst [4] [5]. In some clones of Ishikawa endometrial cells, hemispheres grow out into gland-like structures within two to three weeks after initial induction of domes [6].

Mitonucleons are detected as large (often 15 to 30 microns) structures in syncytia stained by a streptavidin assay for endogenous biotin. An overall increase in endogenous biotin throughout the syncytium results from increased biogenesis of mitochondria [4] [5] [6], and within that increasing population, a subset of mitochondria fuses around aggregates of multiple nuclei (8 or more nuclei in each aggregate; 3 to 4 aggregates within each syncytium). Since observing the role of mitonucleons in dome formation, we have also documented the apparently spontaneous formation of mitonucleons in single multinucleated Ishikawa monolayer cells that detach from the monolayer to become hollow spheroids [7].

Mitonucleons belong to a category of megamitochondria that has been studied for more than 5 decades, most extensively reviewed by Dr. Wakabayashi [8], who concurred with research suggesting that megamitochondria are formed

by the fusion of typical, at least with regard to size, mitochondria. One truth about at least some megamitochondria is that mitochondrial christae are lost or rearranged [9] resulting in the shut-down of measurable electronic potential, essential for oxidative phosphorylation. The authors of that study assumed that that this was a terminal condition, but anaerobic metabolism must continue in mitonucleons since they are characterized by the formation and expansion of gas vacuoles [4], assumed to contain CO_2, the constant abundant gaseous product of catabolism by anaerobic, and well as aerobic, cycles. As we have shown, mitonucleons are explicitly formed around aggregated chromatin that is then compressed by the expanding gas vacuole into a pyknotic state [3]. That pressure together with whatever enzymes are generated within the nuclear aggregate ultimately results in chromatin fragmentation similar to the well-known processes of karyorrhexis and karyolysis [5]. The resulting fragmented chromatin leaking from breached mitonucleons associates in the apical-basal envelope of elevating syncytial membranes. The mass of reassociated chromatin resolves into individual nuclei that populate the apical-basal envelope eventually forming a sheet of amitotically generated nuclei around which cell membranes form [6].

Numerous examples of amitosis appearing in the past two decades [6] have described processes whereby one or a few cells arise from polyploid cells, frequently budding out through the cell membrane. The amitotic process for Ishikawa cells stimulated to form domes is fundamentally different in that it involves the generation of many nuclei in a common cytoplasm over a period of 2 to 4 hours that only become cellularized after arranging themselves as a two-dimensional array in the syncytial envelope. As will be discussed, groundbreaking work on parasites such as those causing malaria has demonstrated a similar amitotic process called schizogony in which eukaryotic single-celled organisms of the Apicomplexan phylum replicate nuclei in host cells. In this paper, the stages in proliferation of dome cells are described in greater detail than in the preprint [6], explicitly comparing a process initiated by mitonucleon formation in human endometrial cells with the process of schizogony, and with the consideration that such a process might be one source of replicating cells in cancers resistant to drugs that inhibit mitosis.

2. Results

An early distinguishing characteristic of dome-forming Ishikawa syncytia is a dramatic increase in the number of mitochondria as detected by streptavidin peroxidase binding to the endogenous biotin of mitochondrial enzymes. **Figure 1(a)** shows a syncytium within the first 4 hours after initiation of dome formation as previously described [4]. Most of the biotin stain is associated with particulate structures, but it is also possible to detect a transparent membrane staining for biotin wrapped around a nuclear aggregate. Nuclei thus segregated, are further enveloped in a structure made up of fused mitochondria (**Figure 1(b)**), creating the subcellular structure we have called a mitonucleon. As **Figure 1(c)** shows, after one or more hours, all of the aggregated nuclei become

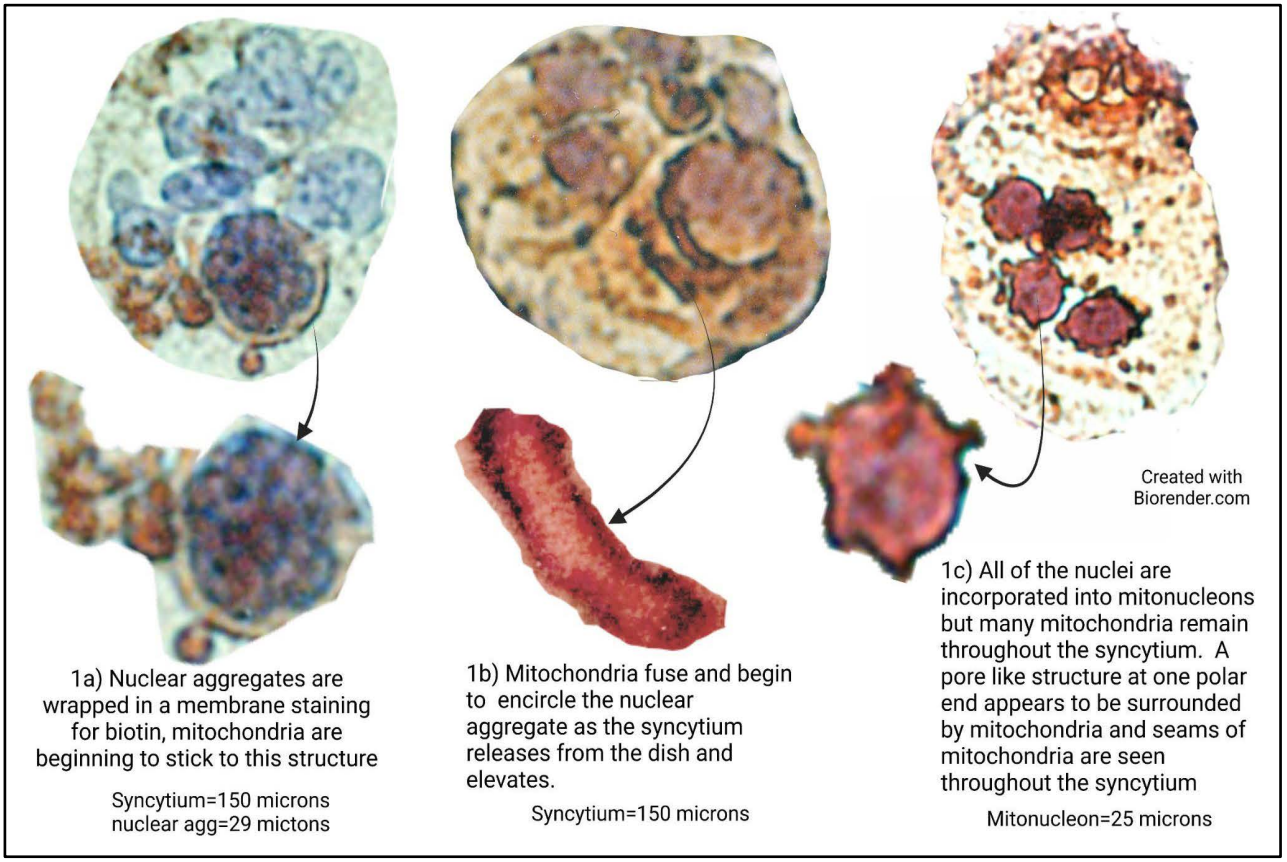

Figure 1. Syncytia open up in monolayers stimulated to form domes. Nuclei aggregate and mitochondria proliferate (a). Mitochondria are observed to fuse contiguous with nuclear aggregates (b). As syncytia elevate mitochondrial fusion around chromatin aggregates is completed forming mitonucleons (c).

surrounded by fused mitochondria. The resulting multiple mitonucleons "round up" as the envelope of apical and basal syncytial membranes elevates over accumulating fluid. While all of the chromatin becomes sheathed in fused mitochondria, not all of the mitochondria are found in those structures as **Figure 1(c)** clearly shows. Numerous mitochondria remain free in the syncytium, some amassed in what looks like a pore of unknown function at one end of the syncytium, some linearly arrayed along with many free mitochondria presumably involved in the production of energy and essential metabolites including nucleotides and fatty acids for the process of differentiation. Structural "differentiation" among mitochondria opens the question of whether different metabolic cycles might dominate in structurally different mitochondria.

At least 3 mitonucleons staining for biotin and elevating with the syncytial membranes can be detected in **Figure 2(a)** as demonstrated by focusing first on monolayer cells surrounding the syncytium (left panel) and then on the top of the syncytium that has elevated into a predome (right panel). **Figure 2(b)** shows an unfixed, and importantly, unstained syncytium observed at approximately the same time. When the biotin stain of the mitonucleons is not present, it becomes possible to detect enlarging vacuoles pressuring chromatin, forming a structure

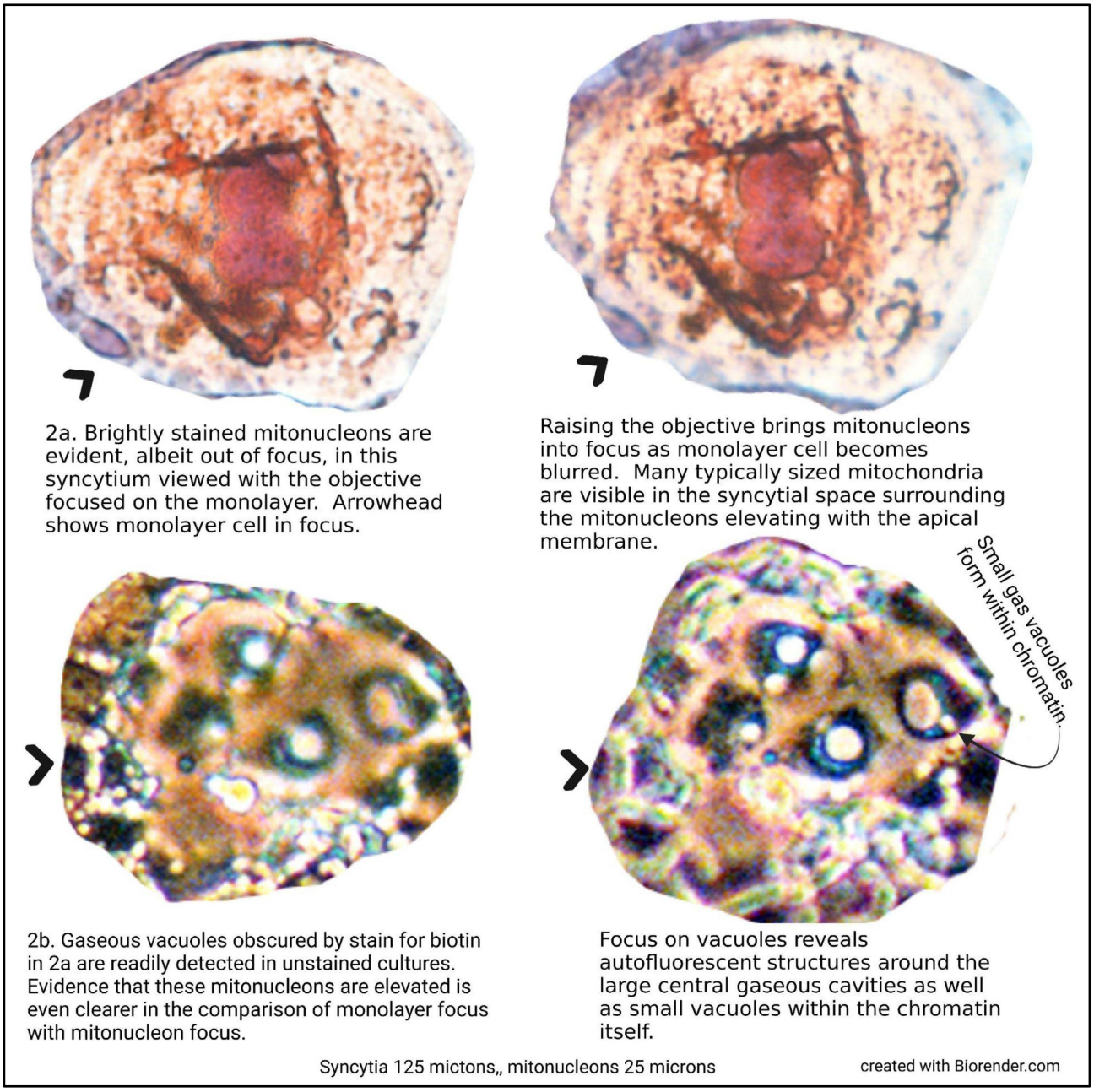

Figure 2. Syncytial membranes elevating to form predomes.

that is frequently found associated with cross sections of cancer cells and sometimes called "optically clear" nuclei. Gamachi and colleagues [10] called the structures "intranuclear inclusions" in a paper clearly and comprehensively demonstrating that the biotin associated with these inclusions was bound to carboxylase enzymes usually found only in mitochondria. The biotin stain in domes has also been shown to be associated with mitochondrial enzymes containing biotin [3], demonstrating that the intimate association of fused mitochondria with chromatin early in differentiation likely explains the existence of "intranuclear inclusions" in endometrium and in cancer [10].

A close look at the mitonucleon in **Figure 2(b)** shows autofluorescent structures that could arise from activity of electron transport elements [11] engaged perhaps in anaerobic metabolism in the fused mitochondria creating the gas vacuoles that pressure a chromatin bolus [4]. In a previous paper, it was shown that the formation of the large central gaseous vacuole begins with the merging of multiple gas vacuoles within the fused mitochondria early in the process [12]. The comparative photomicrographs in **Figure 2(a)** and **Figure 2(b)** attest to the three-dimensionality of predomes since when monolayer cells (arrows) are in focus, mitonucleons are blurred. Upon raising the objective, the monolayer cell becomes blurred and the mitonucleons come into focus.

Single photomicrographs contain information on predome elevations as well as the effects of increasing pressure on the bolus of chromatin within mitonucleons. The microscope objective for the photomicrograph shown in **Figure 3(a)** was focused on a polyploid nucleus pressured against the inner membrane of a mitonucleon within a relatively transparent protrusion of the apical membrane expanding upward. The membrane protrusion itself is apparent even though it is mostly out of focus. In **Figure 3(b)** two protrusions are detectable in a predome. A tight nuclear aggregate sits atop the protrusion on the right. Material in the protrusion on the left is filamentous and appears to be moving downward out of the protrusion. The relatively short-lived, but seemingly extensive protrusions appear to be flattening in the predome in **Figure 3(c)**. Chromatin in the left-most protrusion can also be detected moving out of the protrusion and "pits" can be seen in the membrane, related perhaps to the passage of medium through the syncytial envelope into the cyst forming below the membrane envelope.

Vacuoles such as those shown in **Figure 2** continue to expand during the final

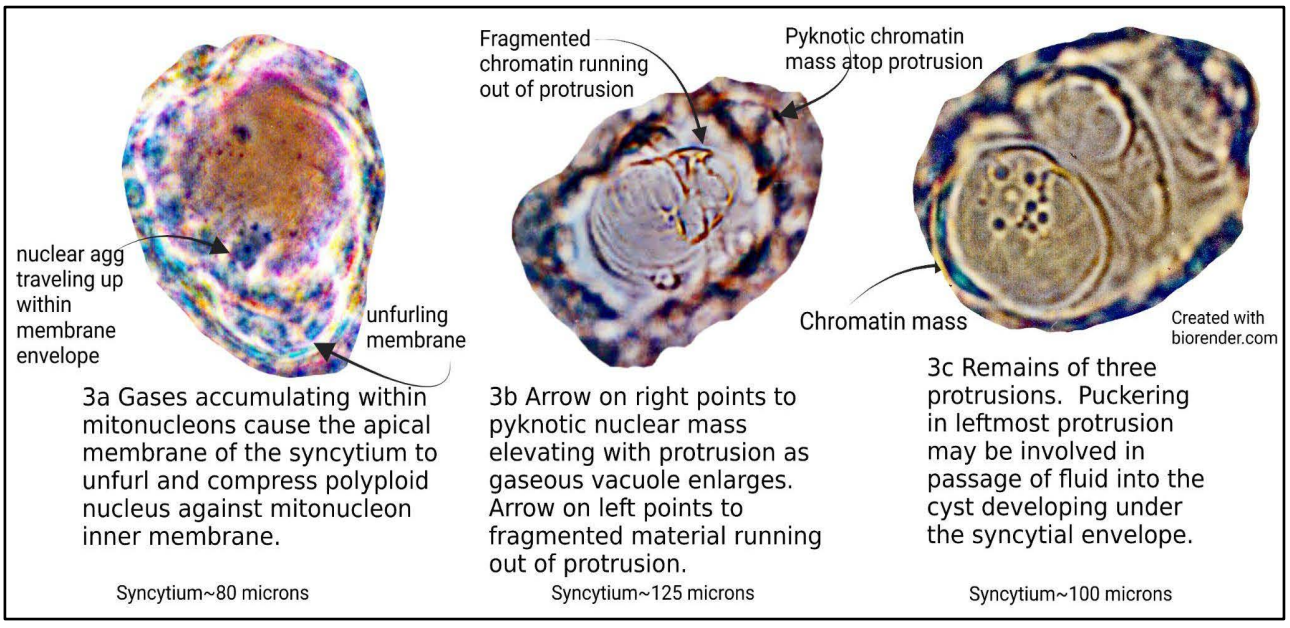

Figure 3. Membrane protrusions arise as chromatin in mitonucleons is pressured against apical membranes of syncytia.

stages of predome formation as shown in Figure 4, with the pressure eventually breaching mitonucleon membranes. Chromatin aggregates from monolayer nuclei within mitonucleons become marginalized and compressed to the point of appearing pyknotic. The mitonucleons in Figure 4(a) are at slightly different stages with regard to the vacuolar enlargement. Prominent among the changes during the final hour of the predome stage, black structures appear associated with mitonucleon membranes. The black structures are approximately the size of centrosomes and appear to be involved in chromatin fragmentation, as will be described. It is intriguing that the relaxation of the pyknotic chromatin mass in the upper mitonucleon of the syncytium in Figure 4(b) results in the reappearance of aggregated nuclei as opposed to a single polyploid nucleus or a bolus of chromatin. In the lower mitonucleon in Figure 4(b), assumed to be further along because it has been breached, the level of hematoxylin stain in a nuclear mass is diminished. Pressure on the chromatin together with whatever enzymatic activity has occurred within the chromatin bolus appears to be responsible for deconstruction of the nuclear structure. The process of "chromatin fading", essentially because it ceases to stain with hematoxylin, and subsequently fragmenting has been observed frequently enough in tissue to have earned the Greek names of karyolysis (fading) and karyorrhexis (fragmenting), terms referring to evidence that a nucleus is deconstructing. The breached mitonucleons in Figure 4(c) open up into the common cytosol of the syncytium. The remains of nucleons are observable at 1 o'clock, 3 o'clock, 5 o'clock, 9 o'clock. "Empty", breached

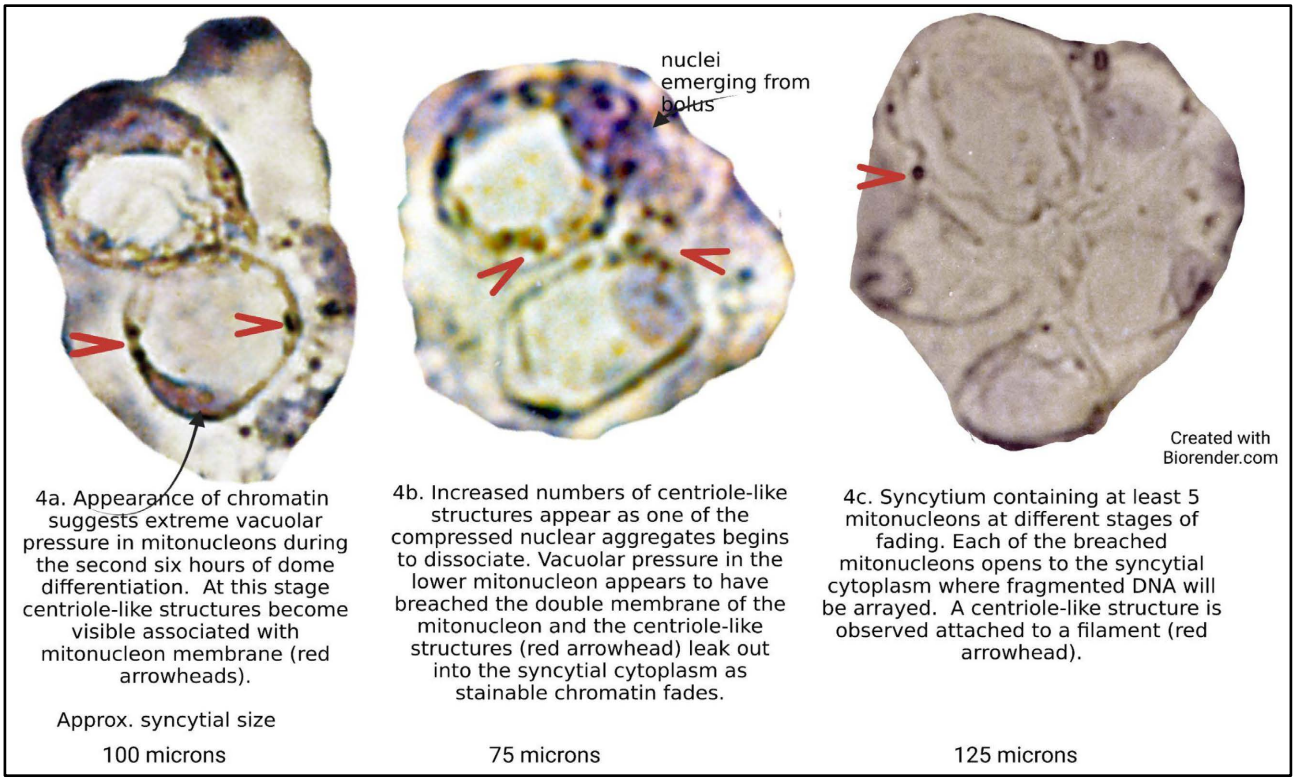

Figure 4. Mitonucleons in predomes 8 to 10 hours after the start of dome differentiation.

mitonucleons are found at approximately 6 o'clock and 11 o'clock. The latter mitonucleon contains two of the black centrosome-sized structures attached to a tubule, favoring the proposition that these products, appearing as mitonucleons begin to fade, may indeed function in organizing chromatin fragments. DNA fragmentation is not an uncommon observation although it is usually assumed to signal cell death by apoptosis. The fragmented DNA in predomes will reassociate, having been arrayed and opened up to epigenetic changes and presumably open to repair and replication by polytenization, modifying and ultimately replicating DNA of monolayer cells that will become the chromatin of dome cells. Fragmentation in Ishikawa predomes exemplifies, as others have shown, that enzymes responsible for DNA fragmentation are observable during cellular differentiations. In a review intriguingly entitled "New roles for old enzymes: killer caspases as the engine of cell behavior changes", Connolly, Jager, and Fearnhead [13] described how different caspases, enzymes involved in DNA fragmentation, exhibit functions aside from programmed cell death, including differentiation programs for at least 13 different cell types. Research has indicated that the ligation of DNA strand breaks formed during myoblast differentiation or ligation of breaks in lymphocytes following mitogenic activation are repaired by the activity of nuclear adenosine diphosphoribosyltrasferase [14].

Additional information about mitonucleon-generated apical membrane protrusions can be gleaned from damaged syncytial predomes, such as the image shown in **Figure 5**. The predome in this photomicrograph was stained for biotin without fixation. The process appears to have resulted in a blow-out of one of the protrusions. Debris can be observed adjacent to the blow-out, with a plume of "stuff" releasing up into the medium that more than likely contains the chromatin

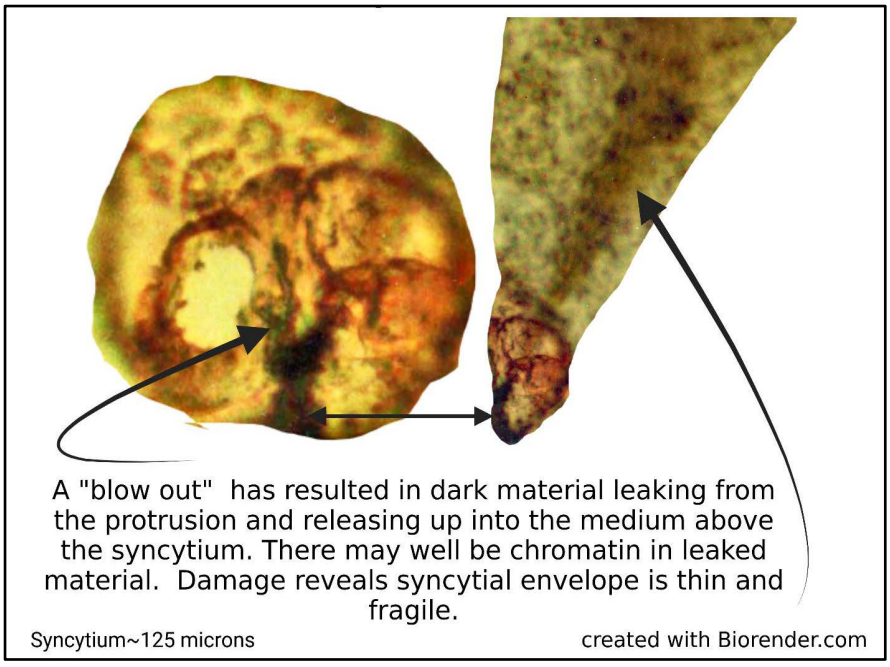

Figure 5. Damaged protrusion in predome stained for the presence of biotin.

pressured up into the projection. As can be seen from what remains, membranes of the syncytial envelope are thin and apparently delicate. The blow-out itself is indirect evidence of the gas vacuole in protrusions and may be a hint of the power of vacuolar propulsion that could be involved with a process like netosis, albeit that phenomenon has been seen in an entirely different cell type.

The syncytium in **Figure 6** demonstrates deconstruction of nuclei previously contained in mitonucleons. Focusing on the syncytium at two different levels, DNA fragments can be observed being drawn down from deconstructing nuclei by a structure resembling a microtubular spindle apparently associated with numerous centrosome-like structures that first appear just before the mitonucleon is breached (**Figure 4**). Fragments can be observed below and above the disintegrating nuclei. A syncytium stained with hematoxylin when the chromatin fragments are completely drawn out as in **Figure 6(c)** appears "empty" except for some heterochromatin at one polar end and an arc of barely detectable filamentous material extending from the heterochromatin down to the other polar end of the syncytium. Chromatin arrays similar to this were observed 40 years ago by Mazur and colleagues [15] in uterine endometrial tissue from first trimester abortions. Mazur used the term "nuclear clearing" to indicate that by light microscopy and hematoxylin staining, typical nuclei could no longer be detected, karyolysis and karyorrhexis had taken place. Using electron microscopy, the researchers showed that nuclear material had not been destroyed, rather it had been deconstructed into granules and "stretched" out into an array of

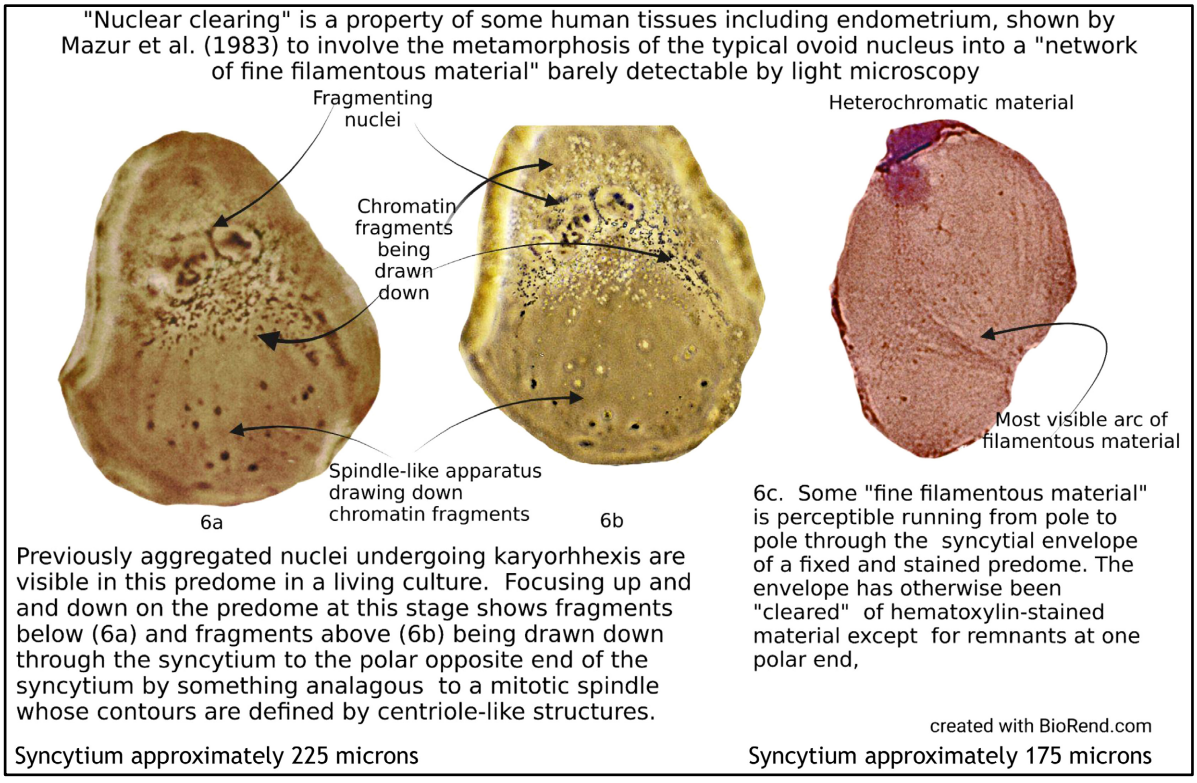

Figure 6. Chromatin fragmentation 9 to 12 hours after the start of differentiation.

chromatin filaments. Arrays of chromatin fibers detectable by electron micrography have also been observed in cancer tissue, for example in thyroid cancers [16] and in pulmonary blastoma [17]. These reports contain electron micrographs of quasi-parallel chromatin fibers following one axis of a structure that is somewhat larger than the typical cell, obviously without the boundary of the typical nuclear membrane.

Phenomena described in this paper as occurring during differentiation of domes have been found associated with various cancers. Optically clear nuclei, cells with pyknotic nuclei called "signet ring" cells and "nuclear fading" are examples. This paper establishes those structural anomalies as relevant to a time-line of differentiation. During the first half of dome differentiation as shown in **Figures 1-6**, the images can be seen to represent sequential structural changes that will result in arrayed DNA presumably open to epigenetic changes, to repair of fragmented DNA, and to replication by polyteny. **Figure 7(a)** depicts chromatin coming back into focus for light microscopy approximately 12 hours after the start of differentiation as an array of parallel filaments containing thickened regions suggestive of polyteny. Zybina and colleagues [18] [19] described non-classical polyteny in trophoblast polyploid cells as did Isakova and Mead [20] who reported that some nuclei are characterized by chromatin threads of different thickness which lie parallel to one another and frequently look "beaded," approximating the vision of chromatin fibers shown in **Figure 7(a)**.

An even rarer sight can be seen in the damaged predome in **Figure 7(b)**.

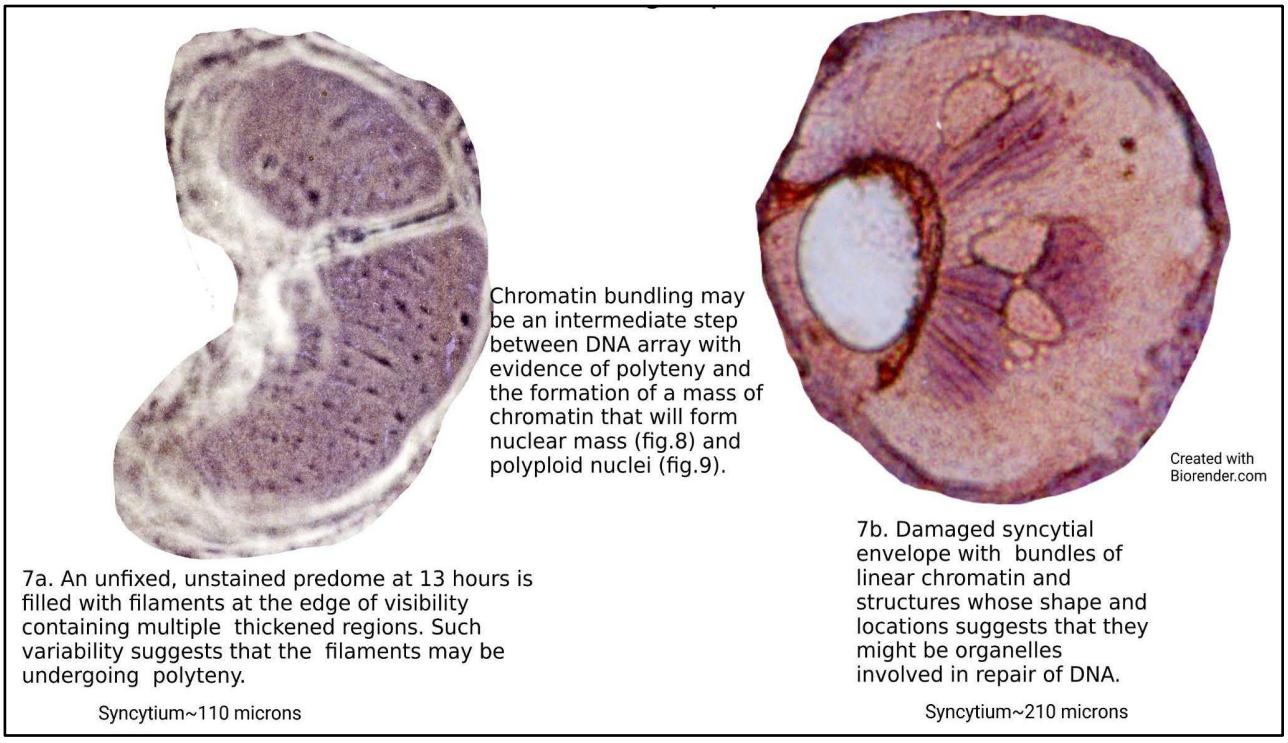

Figure 7. Arrayed DNA that may be undergoing polyteny and bundled DNA in a damaged predome.

Chromatin bundles that take up hematoxylin are apparent at 12 to 14 hours. In an extensive study of polyteny in mosquito development, Dr. Mary Grell [21] describes bundles of DNA to be a product of polyteny as sister chromatids associate with each other.

The outlines of organelles that may be involved with repair of DNA in the bundle are visible lying between the chromatin and the outer membrane. In their descriptions of polyteny in trophoblasts, the Zybinas also describe bundles of DNA [19]. Other information from staining such as the spread of the streptavidin peroxidase stain may reflect damage caused by the blowout of the protrusion.

As reassociation proceeds, a featureless mass of chromatin becomes visible 13 to 14 hours after the start of dome formation, staining with hematoxylin as in Figure 8(a). Linear stretches, as well as punctile forms, that stain for biotin occupy

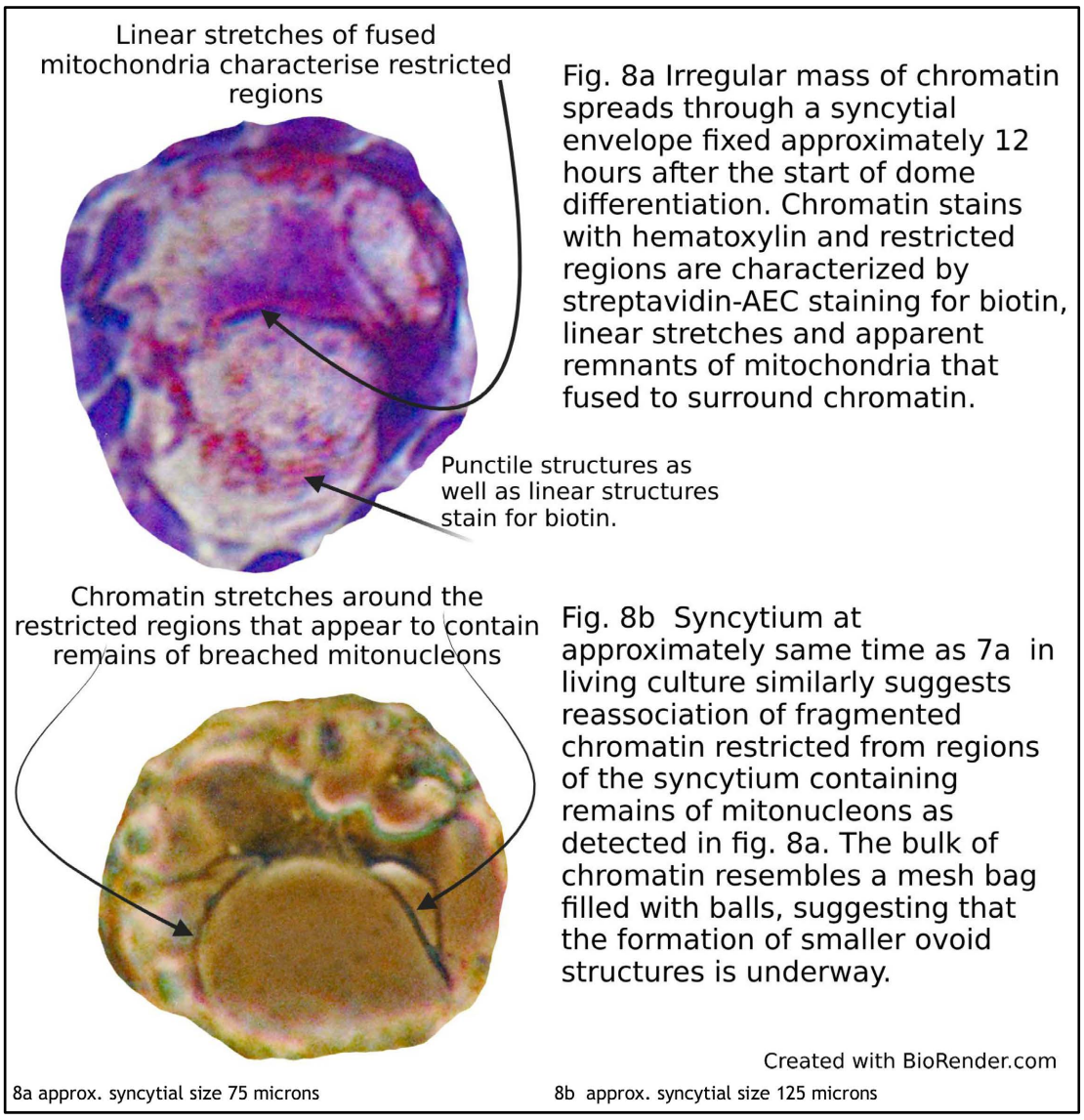

Figure 8. Arrayed chromatin reassociates into a mass stretched around collapsed mitonucleon structures.

the regions around which chromatin stretches. A similar mass of chromatin in the unstained syncytium at approximately 13 hours (**Figure 8(b)**) looks a little like a mesh bag filled with ovoid structures. Chromatin emanates from the mass, streaming around restricted regions. Over the next two to three hours these "restricted regions", that include remains of mitonucleons, will disappear presumably as previously fused mitochondria fission into individual mitochondria and spread through the syncytial envelope. In the same time period, the massed chromatin will give rise to nuclei within the common cytoplasm of the syncytial envelope by somatic cell polyploid reduction as described by Grell [21] and before her by Dr. Charles Berger [22] for epithelial cells of the ilium of Culex pipiens.

The mass of chromatin begins to take shape from 13 to 15 hours as seen in **Figure 9(a)**. The bulk of chromatin appears to be segmenting into ovoid structures whose size suggests polyploidy. The clearest evidence of chromatin segmenting into nuclei can be seen on the leading edge of a chromatin mass (**Figure 9(b)**) that may be slightly further along than **Figure 9(a)**. Five or six ovoid structures highlighted by what appears to be nucleoli are observed moving out from the

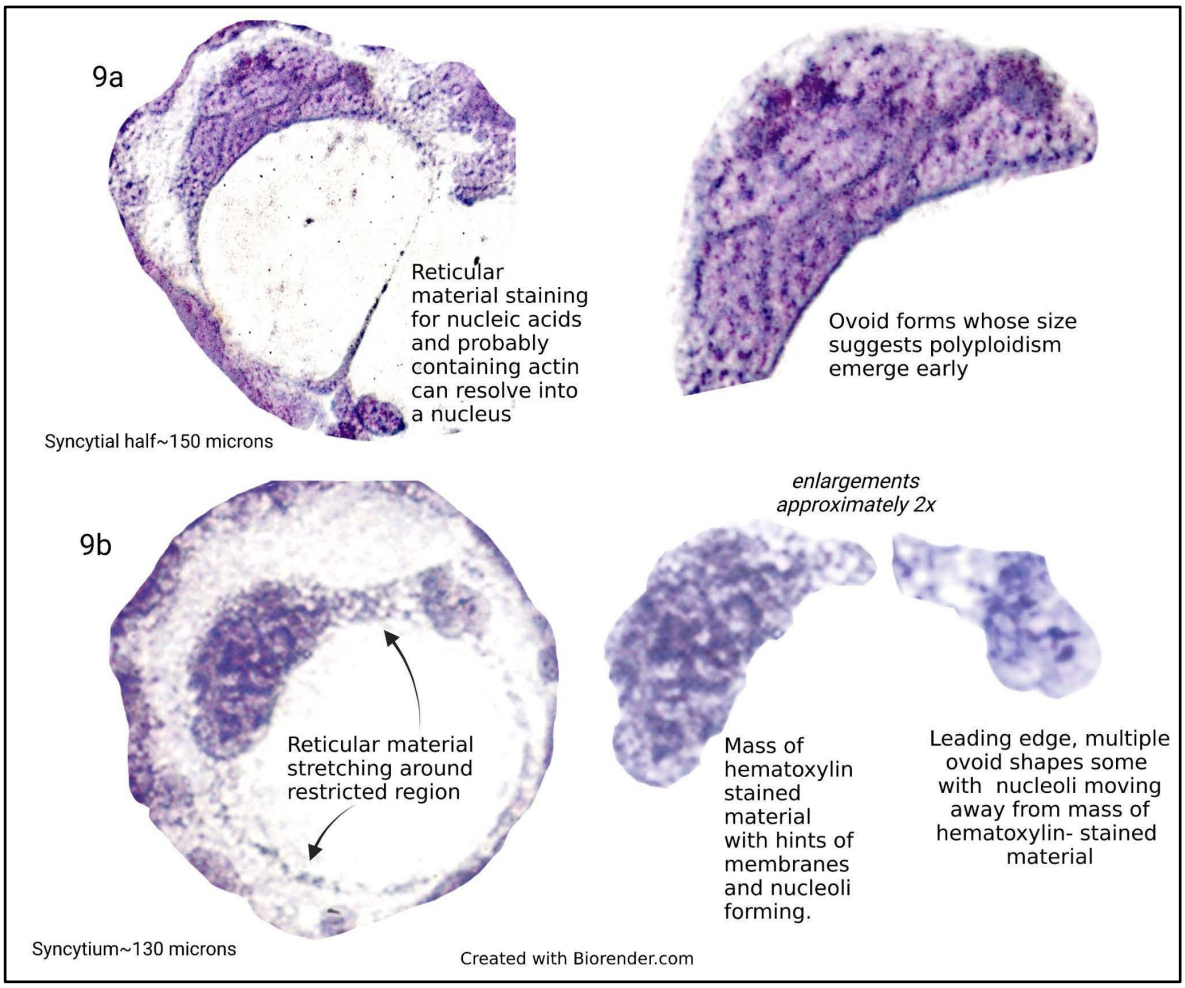

Figure 9. Ovoid forms emerging from chromatin mass adjacent to a restricted region outlined by streaming chromatin.

large mass of chromatin. In both syncytia, reticular material is stretched around restricted regions similar to those described for **Figure 8**. Clusters of nuclei such as these when observed in lung cancers have been called molded nuclei [23], and are also reported to be characteristic of Merkel cell carcinoma [24]. Molded nuclei can be added to optically clear nuclei, signet ring cells, and nuclear fading as one more structural change generated by mitonucleon-initiated production of nuclei within the syncytial space.

Over a period of 4 to 6 or so hours, monolayer nuclei pressured in mitonucleons have experienced transient strand breaks opening DNA to epigenetic changes, repair and polyteny. The fragments pulled apart by spindles may remain physically associated with these microtubule structures. When chromatin "reappears", it is as a mass resolving into polyploid structures and then into single nuclei that will populate the syncytial membrane envelope. The evidence in this paper suggests that mitonucleon-initiated differentiation and proliferation may be an example of a polytene cycle suggested by MJ Pearson [25] as an "alternative" to the mitotic cycle. During early stages of chromatin dispersal most of the nuclei appear in polyploid structures highlighted in **Figure 10** by 2- to 4-fold enlargements (double arrows). A sleeve of nuclei moving from the left edge

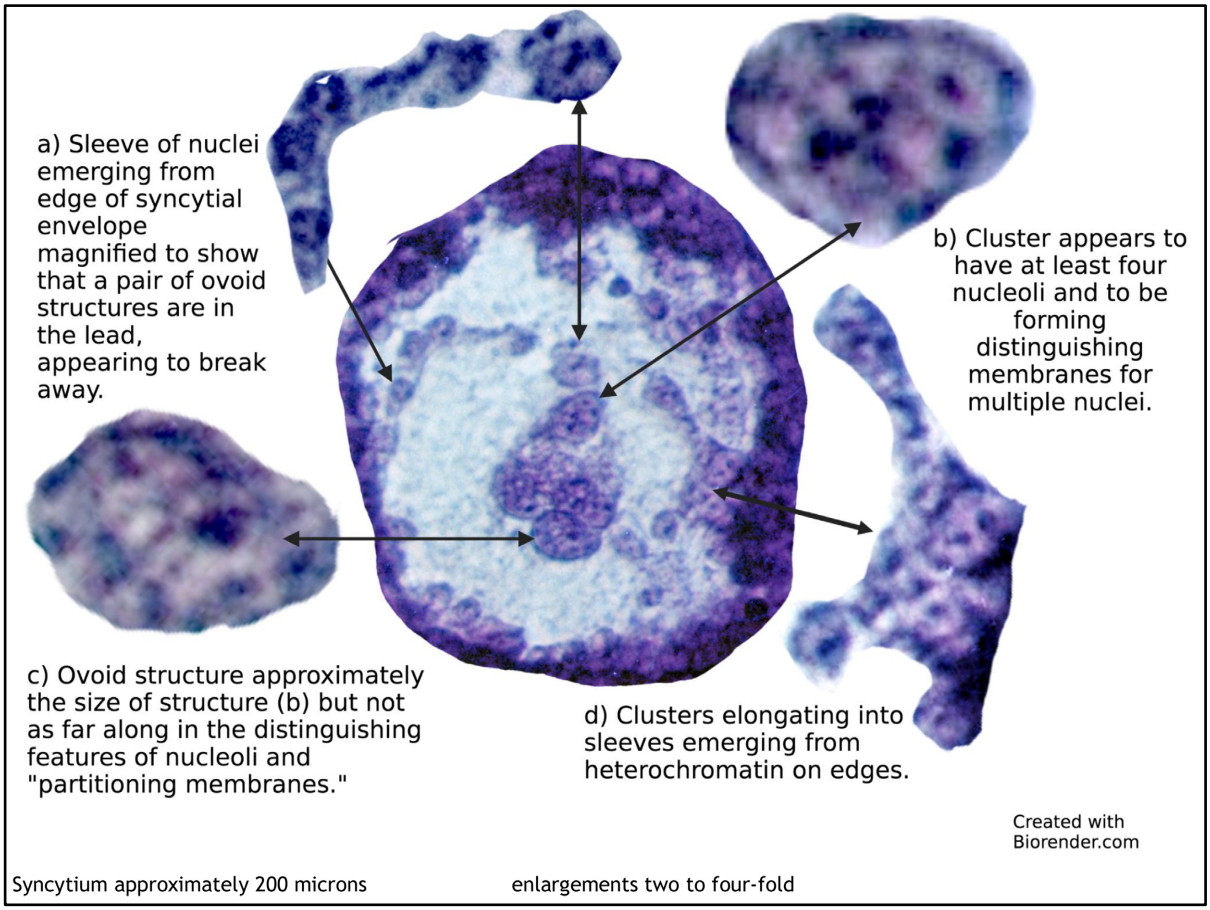

Figure 10. Distribution of polyploid structures with clusters and sleeves along with single nuclei beginning to fill the apical-basal syncytial envelope.

of the syncytium arches over clustered nuclei in the center of the envelope with a pair of nuclei moving out from the "leading" edge. Linear arrays of nuclei seen in cancer are sometimes called Indian files and have been observed in serous effusions from a variety of carcinomas including breast, gastric and ovarian carcinomas [26].

Enlarged images of two similarly-sized clusters from the center of the syncytial envelope (**Figure 10(b)** and **Figure 10(c)**) suggest that "pieces" of the chromatin mass containing 6 or more molded, and probably still assembling, nuclei break apart from a mass of chromatin such as is shown in **Figure 9**. Judging by the size of nucleoli in the approximately four-fold enlargements of the clusters, nuclei in **Figure 10(b)** are further along in assembly than nuclei in cluster **Figure 10(c)**.

The structure enlarged as **Figure 10(d)** appears to be a mix of sleeves and clusters emerging from heterochromatin along the edge of the syncytium. Nuclei in polyploid structures such as those shown in **Figure 10** dissociate from sleeve or cluster and become distributed throughout the syncytial envelope as observed in **Figures 11(a)-(d)**. In light of these observations, an obvious question is how a

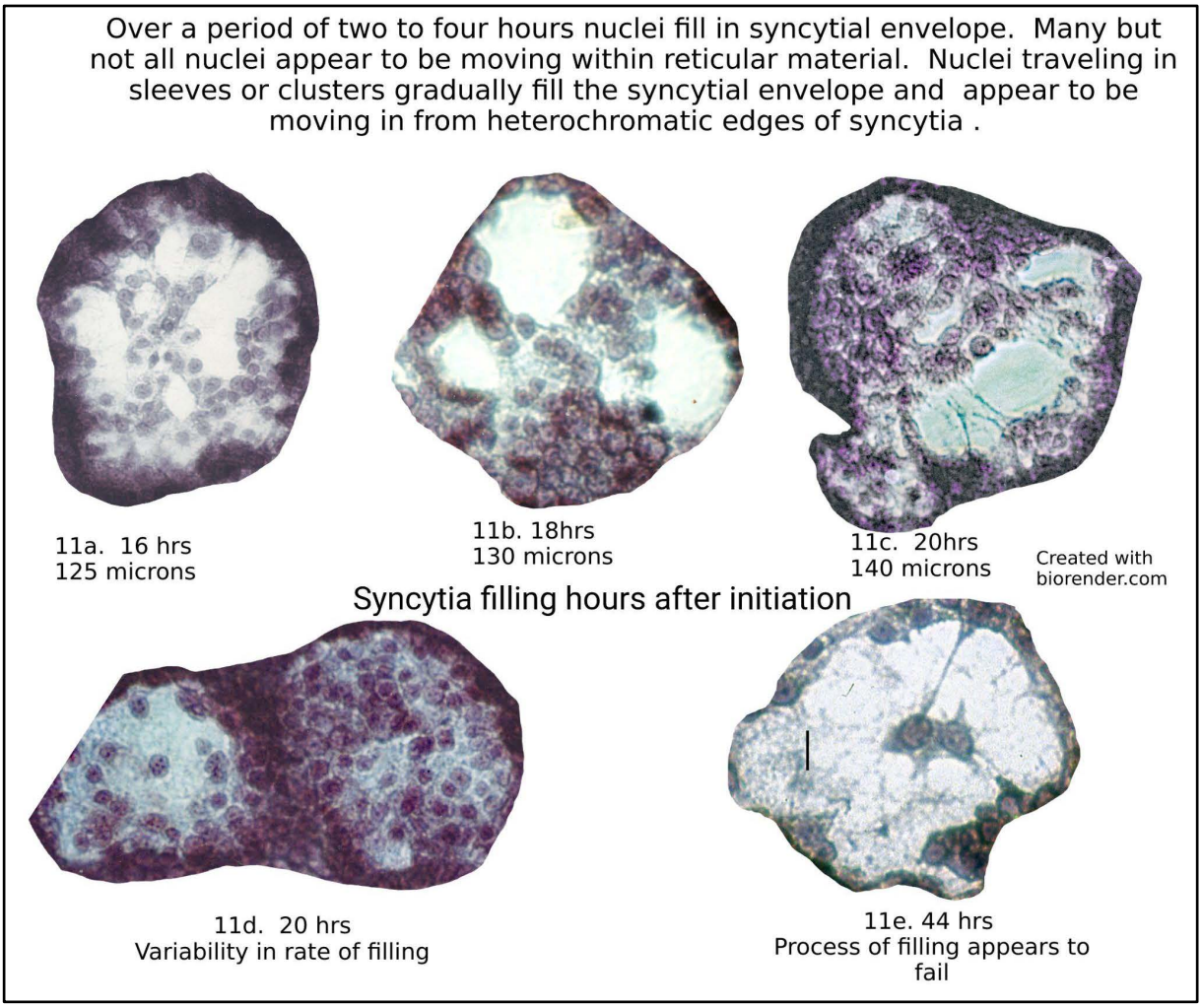

Figure 11. Nuclei fill syncytia in final stages of dome differentiation.

mass of chromatin containing multiple copies of each chromosome sorts itself out into functional nuclei with something like the correct number and complement of chromosome territories. This is a question that has been addressed by scientists studying single-celled eukaryotic parasites that also form multiple nuclei within the common cytoplasm of host cells. The organisms belong to the apicomplexan phylum and they replicate in host cells by a process called schizogony whose central characteristics include the production of multiple nuclei within a specific cell in a host organism. Variability [27] [28] [29] characterizes schizogony within the commonality that cell proliferation does not occur by mitosis but amitotically. Some representatives of the phylum such as Toxoplasmosis (infecting cats and other mammals) produce just two progeny [30], the same number of "daughter" nuclei as formed in a typical mitosis. Other members of the phylum such as various species of Plasmodium, parasites that cause malaria, produce 16 to 30 progeny over a period of 44 to 48 hours after a single merozoite infection of a red blood cell in a human host. Characteristics of DNA synthesis during schizogony differ from the typical S phase of mitosis. Multiple rounds of DNA synthesis frequently characterize schizogony as opposed to the single round of DNA synthesis for each mitotic cycle. Sarcocystis [31], as well as Plasmodium falciparum [32] [33] are capable of 4 to 5 rounds of DNA synthesis. Ganter and colleagues have identified a *Plasmodium*-specific kinase *Pf*CRK4 as the key cell-cycle regulator that orchestrates multiple rounds of DNA "observed" during the last half of a schizogony cycle in *Plasmodium falciparum* [34].

Reassuring images of mitotic reproduction with identical condensed chromosomes being pulled apart and physically segregated by cell membranes forming around the "identical cargoes" are not seen either in schizogony nor in dome nuclei formation. In their review of division of nuclei for apicomplexans, Francia and Striepen [28] ask the question raised by observing multiple nuclei forming within common cytosol, "how do parasites keep track of their chromosomes in polyploid stages?" In schizogony, centrosomes [35] or centrosome plaques [36] [37] along with microtubules have been shown to be essential for the process. Affirming a role for centrosomes, it has been proposed [28] that these structures, together with microtubules, physically tether cellular components providing, "spatial and temporal control of apicomplexan" nuclear production. The authors speculate that "persistent kinetochore attachment of chromosomes to spindle microtubules' provides a mechanism to maintain the integrity of chromosomal sets through polyploid stages". The schizogony-like process of dome formation suggests that the centrosome-sized structures appearing in mitonucleons, not long before they are breached, may be similarly involved with organization of reconstructed chromatin fragments.

Common to the schizogonic process and to dome-cell nuclear proliferation is the fact that synthesis and assembly of nuclei occurs within the cytoplasm of the generative space: host cell for apicomplexans, syncytial envelope for dome forming Ishikawa cells. In apicomplexans the process of replicating nuclei culminates in cellularization and "weaponization" of progeny that are then released

from the destroyed host cell ready to infect another cell and create more progeny. Rudlaff and colleagues [38] have constructed models representing this process derived from electron microscopy specific for measuring volumes. Analysis of sequential sections through plasmodium infected red blood cells over the period of the infection reveals some details of the process analogous to what is seen in dome nuclei formation, including an early segmentation stage when some of the nuclei are polyploid and appear jagged, followed by a mid-segmentation stage with more typical nuclei.

During Ishikawa dome formation, many nuclei form in syncytial cytoplasm initially en masse, rapidly downsizing to polyploid structures and continuing ploidy reduction until single nuclei fill the syncytial space, in a process that takes 2 to 4 hours. Varying stages of dome differentiation during the critical hours when syncytia are filling with nuclei are shown in **Figure 11**. As filling proceeds, and restricted regions diminish in size, there is variability in the rate of filling even in syncytia that are side by side as illustrated in **Figure 11(d)**. The final stage of the process called cellularization converts nuclei into cells that become the epithelial monolayer overlaying the dome hemicyst. Whether condensed chromosomes are pulled apart and deposited into new cells during mitosis where they organize as chromosome territories [39] [40] or whether uncondensed chromosome territories are organized by centrosomes or centrosome plaques, chromosomes must be put together appropriately to form stable, yet dynamic nuclei. It has been proposed by Dundr and Mistelli that nuclear self-assembly is a dynamic process [41] that, in addition to chromatin and the nucleolus, involves nuclear bodies such as speckles, stress bodies, Cajal bodies, paraspeckles and other structures essential to nuclear activity, as well as the nuclear membrane.

With so many moving parts, it may not be surprising that from time to time the process fails. Perhaps that explains why, a full 20 hours after at least one of the syncytia in **Figure 11(d)** is approaching completion, the syncytium in **Figure 11(e)** appears to be stuck at a very early phase. Reticular material is wrapped around the two nuclei in the center of the syncytium suggesting that self assembly has been significantly stalled perhaps by the scarcity of some essential element.

A recent preprint [42] contained details of nuclei forming in an Ishikawa spheroid that reattached when serum was added to the serum-free medium in which it had formed. The cytosol was filled with granules surrounding nuclei in the process of self-assembly. The nucleolus appears to be "in touch" with the developing nuclear membrane by a spur of chromatin, a visual supporting the theory that the nuclear envelope is involved in chromatin organization [43], and that the nucleolus and ribosomal DNA are central factors in the spatial organization of the genome [44]. In the variety of patterns observable as nuclei fill syncytial membrane envelopes, there are hints that nuclear self-assembly can occur beyond the segregation of nuclei from the initial chromatin mass (**Figure**

9). Figure 12(a) shows nuclei forming within streaming chromatin stretched around restricted regions occupied by breached mitonucleons. Additionally, some syncytial nuclei appear to be moving within reticulum as in Figure 12(b) perhaps having been formed in the heterochromatic regions along the edges of syncytia.

These images together with those in Figure 10 and Figure 11 suggest that nuclei move and are moved. Actin and actin filaments have been shown to be in nuclei [45]. Gunasekaran and colleagues [46] suggest that the dynamic nature of actin is relevant to key nuclear events and is essential in developmental programs. There is a fascinating complexity to what must be concluded from figures 10 through 12 about the purposefulness of nuclei arranging themselves into a "sheet" that will be converted into a "tissue." A cell biologist once counseled me that only video evidence will bring certainty about fine points such as these. The comings and goings of "purposeful" nuclei as well as nuclei "along for the ride" will certainly be fascinating.

Nuclei forming outside of polyploid structures looking like "blobs" attached to the reticulum stretching around restricted areas reinforces the suggestion that the

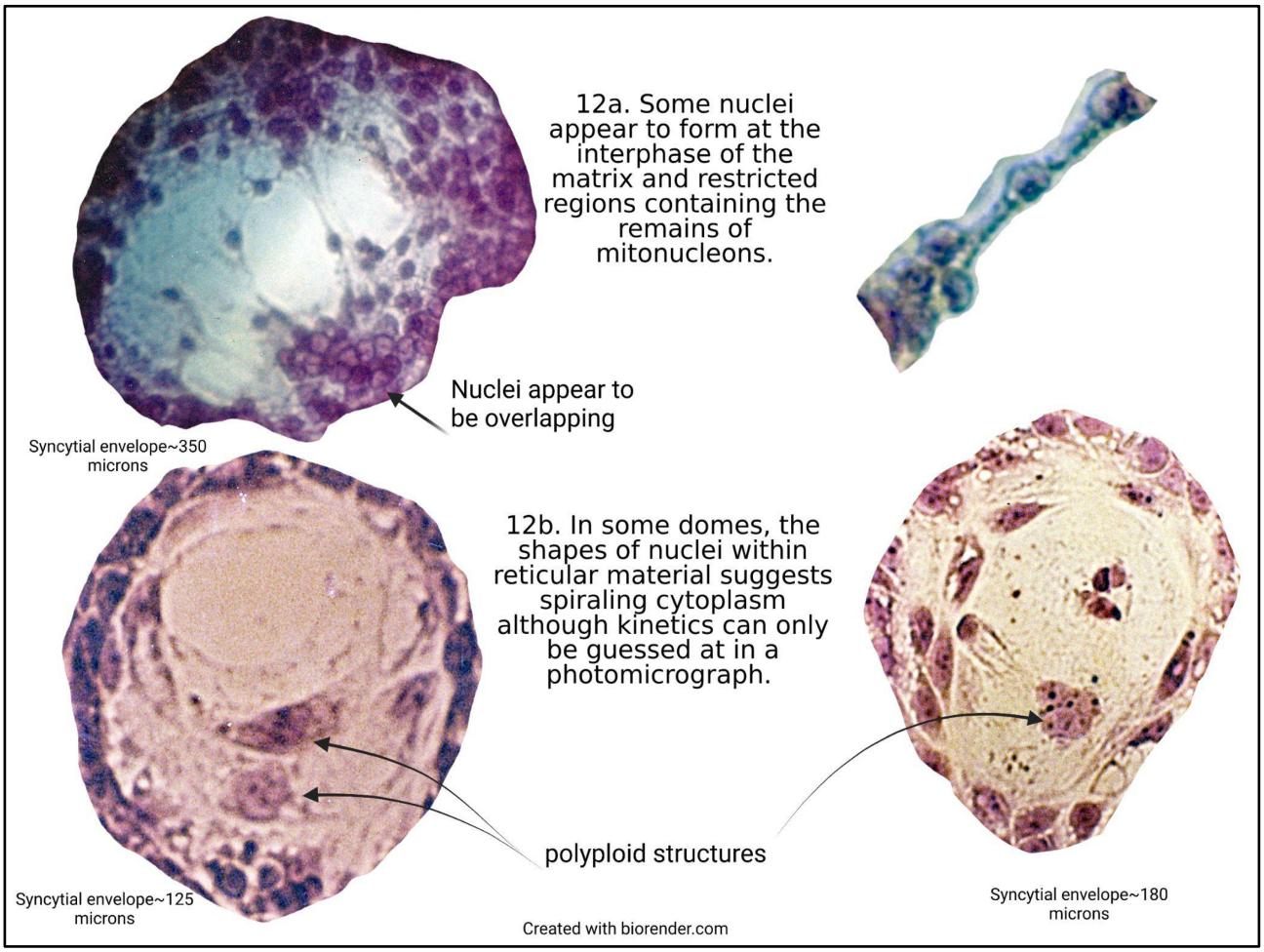

Figure 12. Variety of polyploid nuclear forms moving into place to form dome cell membrane.

reticular material contains "streaming chromatin", a phenomenon we described in a recent publication [42]. Streaming chromatin may be related to the 50+ year-old mysterious structure sometimes called chromatin sheets whose function is still not well understood. Found to be present in many different organisms [47] the chromatin fragments appear to be bounded by nuclear envelope proteins such as laminin B. "Envelope limited chromatin sheets" or ELCS, the name proposed by Olins and Olins [48], is mostly made up of approximately 10 micron chromatin fibers, bounded by nuclear envelope proteins. Chromatin sheets can be adorned with blebs, projections, appendages and pockets as has been described by Ghadially [49]. It is possible that during the final phase of dome differentiation, the syncytium becomes an envelope of apical and basal membranes that might appear in cross sections as linear chromatin together with occasional blebs and pockets.

The final phase of dome formation is illustrated by the unstained domes in **Figure 13**. A dome half-filled with irregularly shaped nuclei is shown in **Figure 13(a)**,

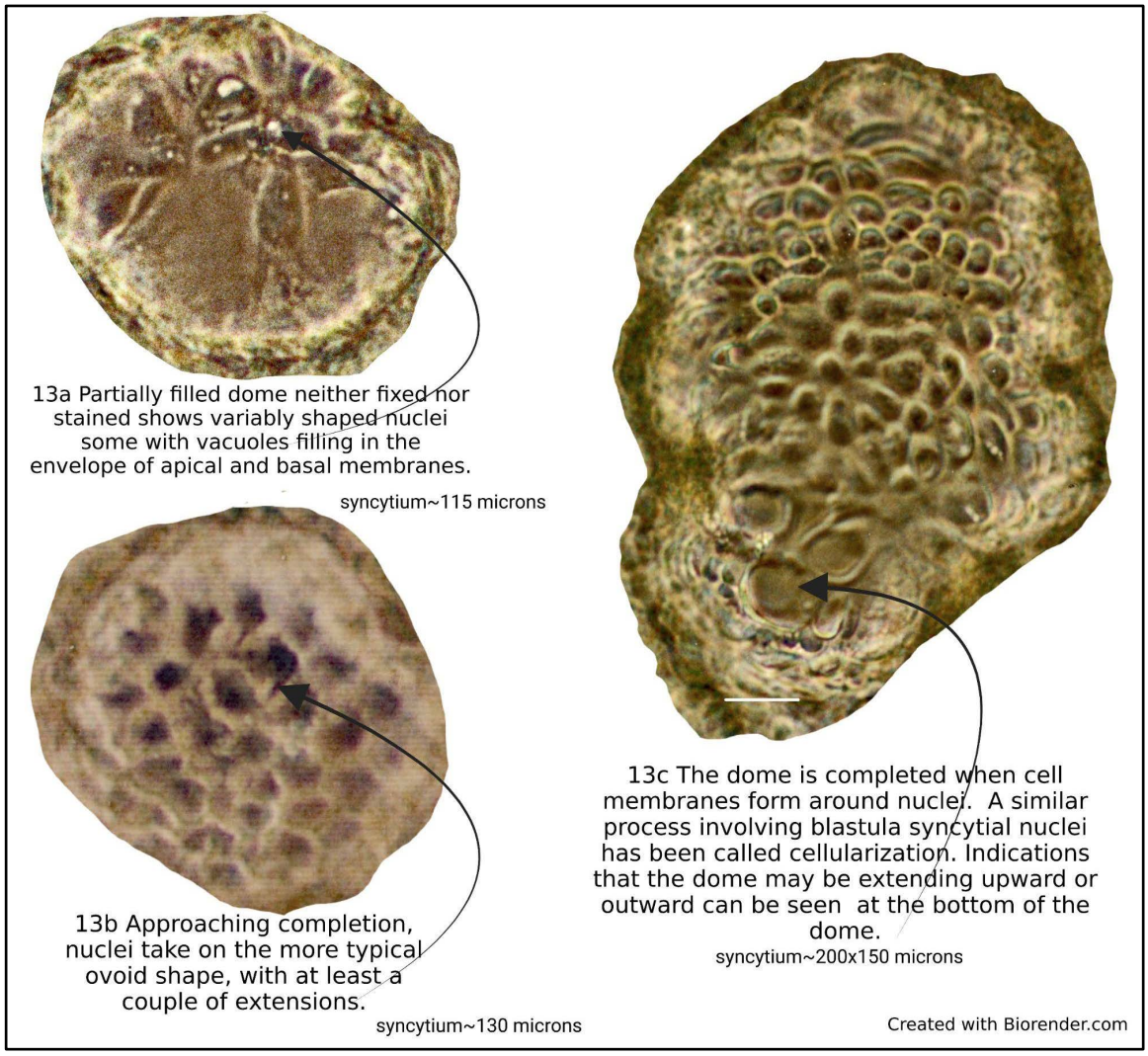

Figure 13. Nearly complete domes inform on the final stages of dome formation.

some nuclei contain small gas vacuoles, most do not conform to the more typical ovoid shapes generally observed for epithelium, but they do appear to be aligned so that nuclear membranes are contiguous. Clearly the syncytium is not yet filled, but as it approaches being filled, nuclei take on more typical shapes and "distance" themselves from each other as demonstrated in **Figure 13(b)**. Such observations suggest that the mechanism for schizogonic-like amitosis by polyteny may be somewhat open-ended until sufficient numbers of nuclei have been formed to fill the syncytial envelope.

The final step is shown in **Figure 13(c)**. From one polar end to the other, the surface of the dome takes on the more familiar appearance of cells suggesting that membranes are forming around the nuclei in a process that has been called cellularization. Longcar and Singer [50] who investigated cellularization in drosophila described three phases. Initially membranes form between nuclei, followed by membranes forming on the apical surface and finally forming in the basal periplasmic space. Cellularization has also been investigated in the ichthyosporean *Sphaeroforma artica*, a close unicellular relative of animals. The process has been shown to involve coordinated inward plasma membrane invaginations dependent on an actomyosin network resulting in the formation of a polarized layer of cells resembling a layer of epithelia [51]. In the bottom third of the maturing dome in **Figure 13(c)**, it appears that even as it is in the process of finalizing cellularization, this dome is beginning to form an extension.

What may be unique about cellularization in dome formation is that it happens within a membrane envelope of opposed apical and basal syncytial membranes that may well be involved in the process. Aside from the mechanical aspects of cellularization, it's important to remember that essential organelles, particularly mitochondria, must be enclosed together with nuclei. Cellularization in most of the apicomplexan organisms is even more complex than what we have described for dome cells since the progeny must be equipped to live outside of the host cell and to "infect again".

Domes enlarge by a process that recapitulates their creation. An early sign that a dome is extending itself is the appearance of marginalized chromatin and vacuoles as shown in **Figure 14(a)** (compare with **Figure 2(b)**), usually at one end or the other of a dome. Fragmented DNA extending through a syncytial gap in the dome in panel **Figure 14(b)** (compare with **Figure 7(a)**) demonstrates that the formation of extensions involve the complete cycle of DNA fragmentation even when dome cells, and not monolayer cells, are the starting point. Reassociated massed chromatin can be observed in panel **Figure 14(c)** (compare with **Figure 8(a)**).

An example of extension completion is shown in **Figure 15**. Mitochondria are already spread throughout the syncytial envelope of the extension. Wide distribution of the mitochondria should guarantee their incorporation into "cells" when nuclei are appropriately arrayed and cellularization can proceed. Polyploid

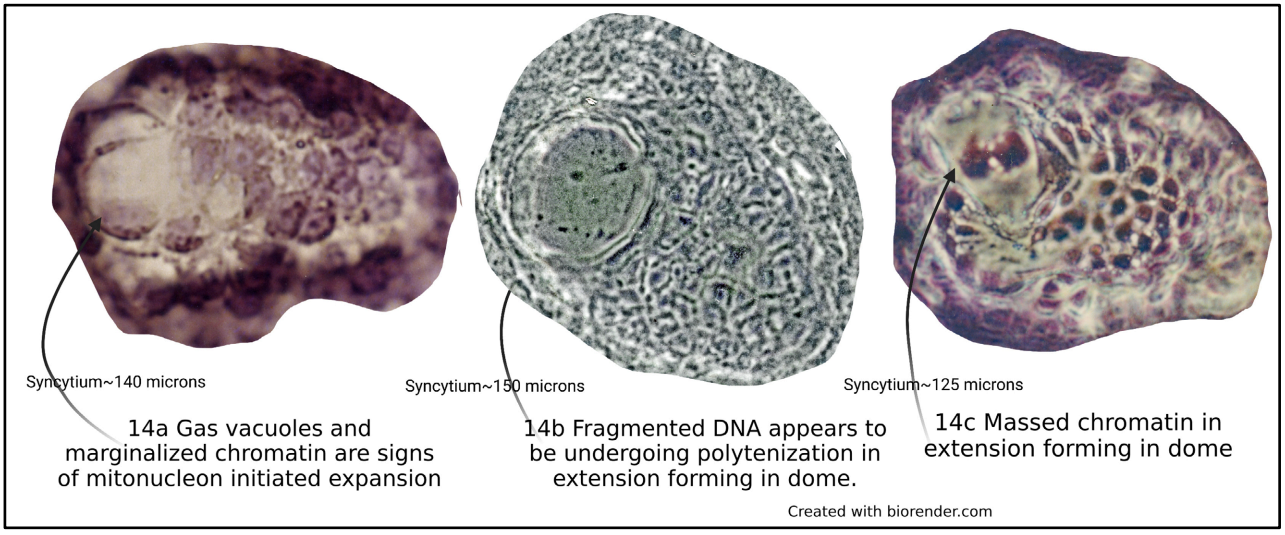

Figure 14. Stages of formation of dome extensions.

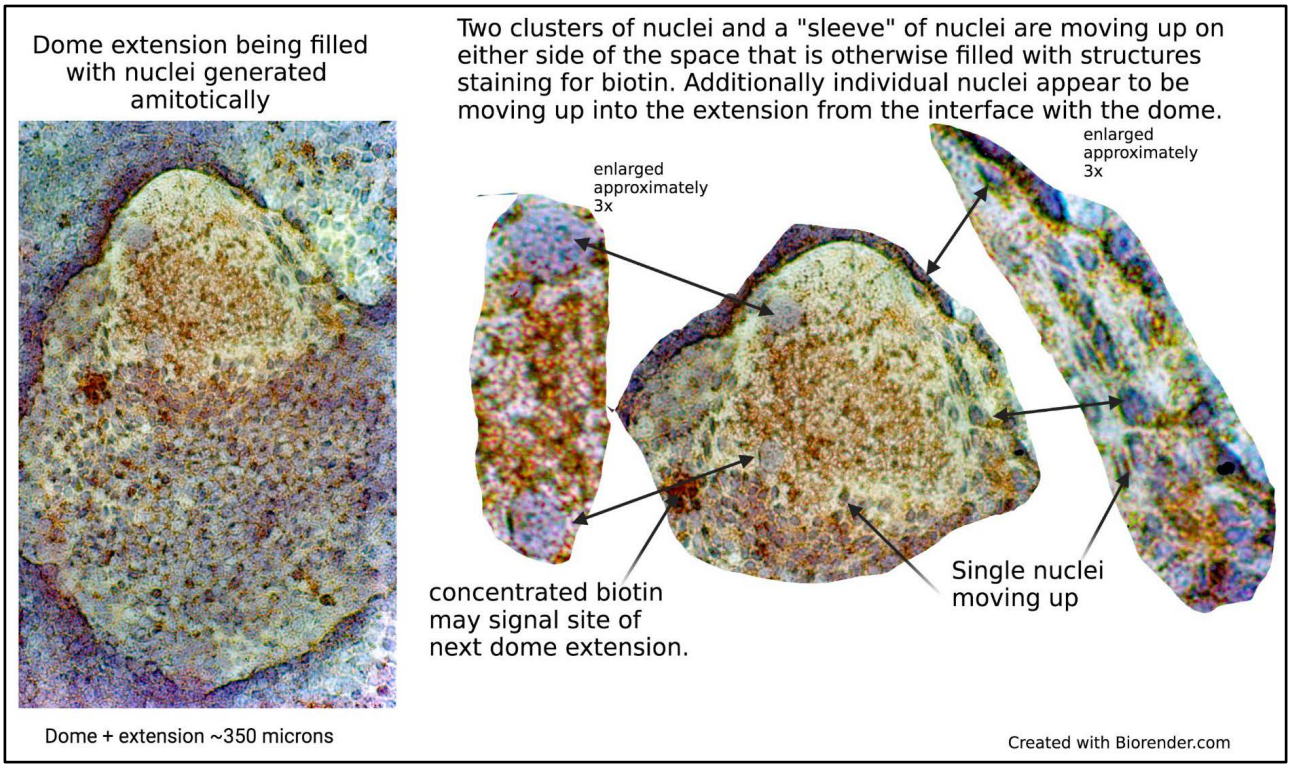

Figure 15. Domes extend by amitotic nuclear proliferation.

nuclear structures are beginning to move into the envelope extension. On the right side of the extension, nuclei are "moving" up in something like an "Indian file" similar to what was seen in the sleeves of nuclei shown in **Figure 10**. Two separate similarly sized polyploid nuclear clusters can be identified on the left side of the extension. Single nuclei are beginning to move up from the interface between the dome and the extension. This image, like those in **Figure 10** and **Figure 11** is one more example that nuclei formed as a result of mitonucleon in-

itiated differentiation, distribute throughout a syncytium in polyploid structures that eventually give rise to single nuclei. It is not clear why amitotic expansion sometimes results in a broadening of the dome as in Figure 15 or in a gland-like extension as in Figure 16 although the shape of domes was found to vary among different clones of dome forming Ishikawa cells (unpublished observation). When a petri dish full of domes is "nurtured" over time, it becomes possible to observe structures like those in Figure 16. The tell-tale signs of dome extension are recognized in Figure 16(a) by gaps in the cells that define the circumference of the dome. Cells proliferate outward as "tubes" and sometimes tubes extending out from one dome connect with neighboring domes as in Figure 16(b). Figure 16 shows that mitonucleon-initiated amitotic division that creates cells capable of thriving detached from the surface of the petri dish is well suited to form gland-like three-dimensional structures.

Mitonucleon dependent amitosis described in this paper produces dozens of nuclei in the period of time (20 or so hours) required for mitosis to generate one or two pairs of nuclei. Figure 17 illustrates structures for each of the stages of this process as described in the results.

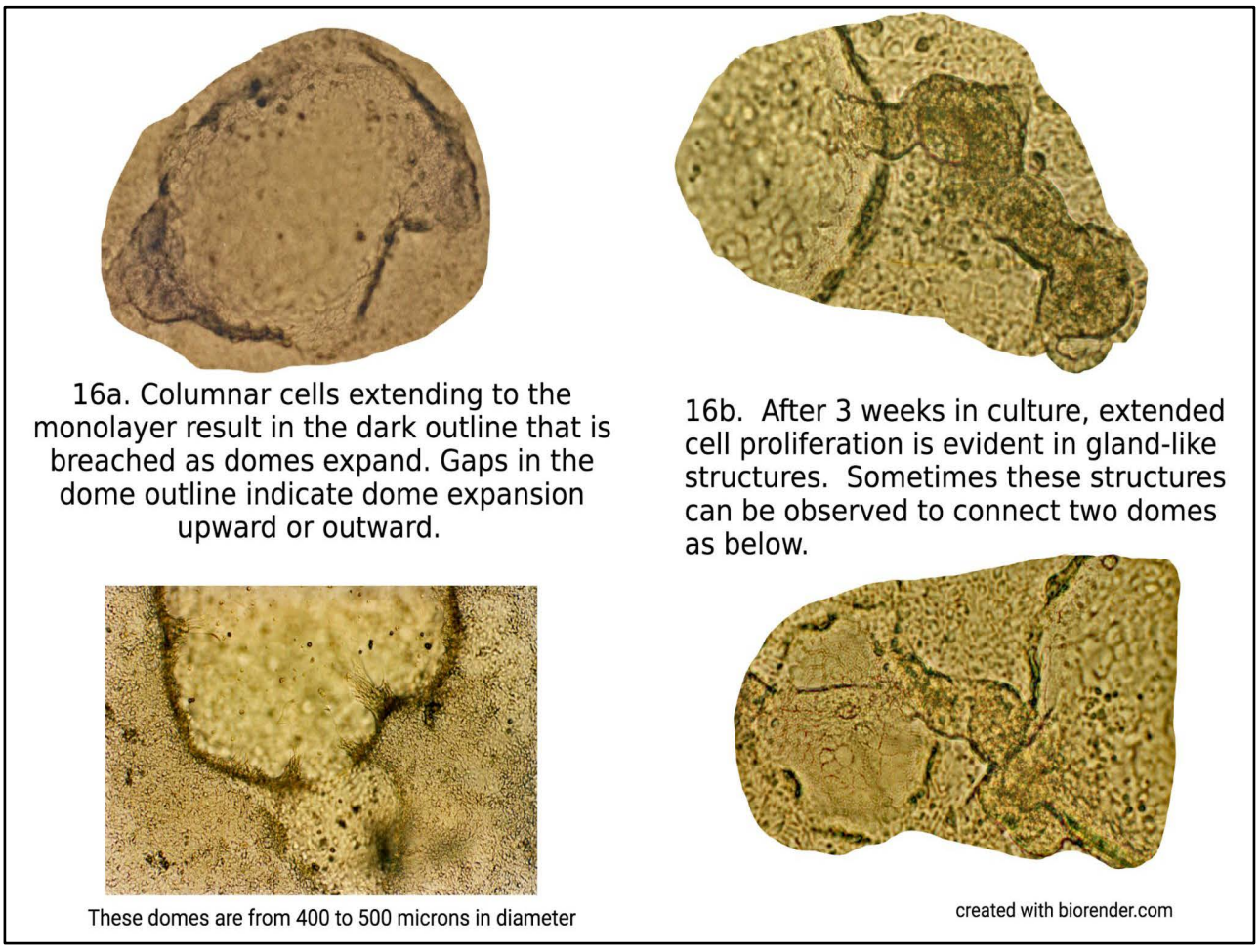

Figure 16. Domes extend upward and outward eventually forming gland-like structures.

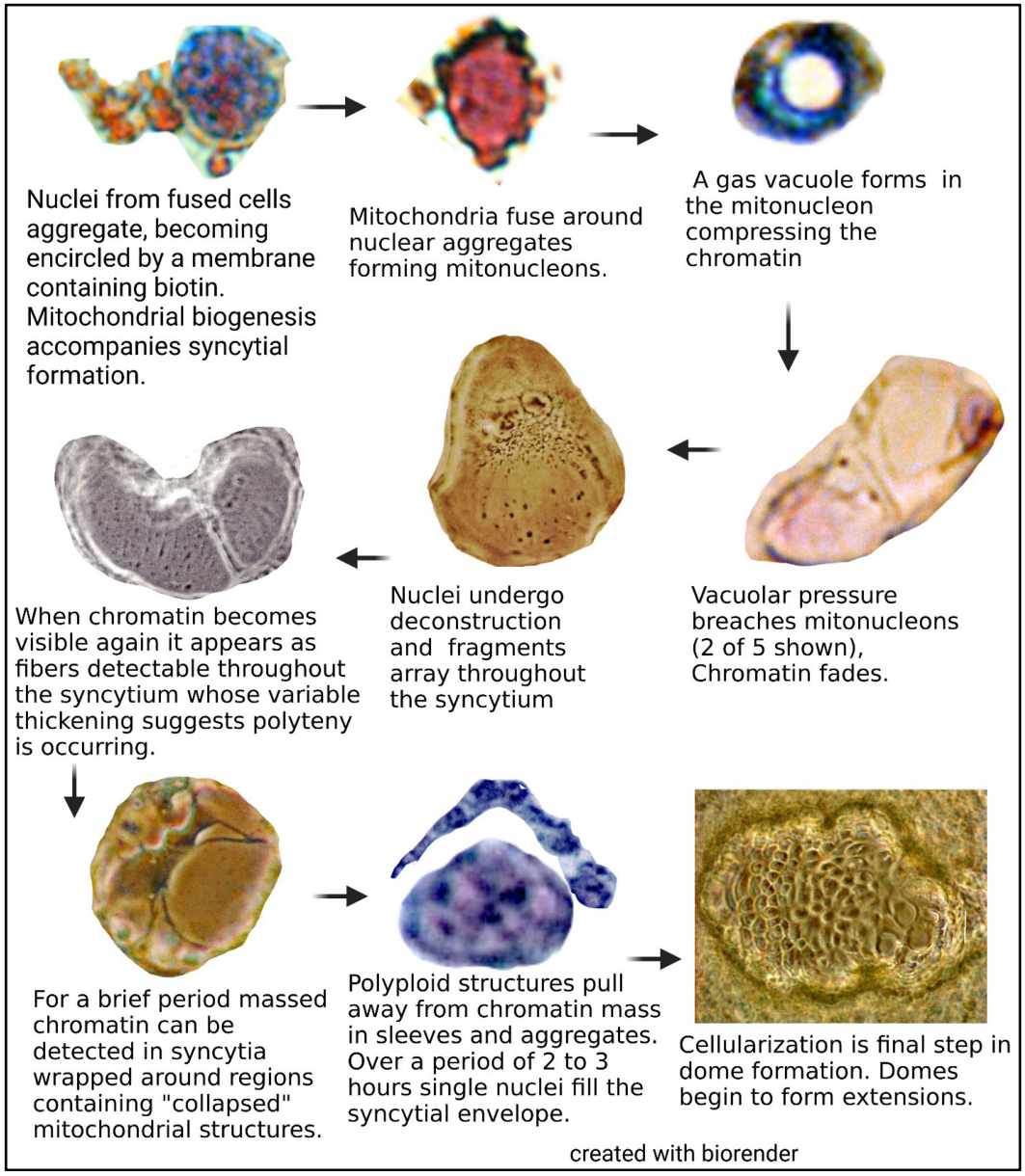

Figure 17. Stages of dome cell proliferation elicited by formation of mitonucleons.

3. Discussion

Differentiation and proliferation, the special province of fetuses and embryos and, of course, the newly fertilized egg, also occur in adult organisms and quite elegantly in the reproductive cycle. Gland formation in the female uterus during the reproductive years is a window into processes of development and differentiation that cannot otherwise be easily researched. This paper documents the structural changes over a 20 to 24 hour period when monolayers of human endometrial cells are stimulated to form domes that can elongate into gland-like structures. Perhaps the most unexpected finding is the intimate association of fused mitochondria around nuclear aggregates in a structure called a mitonucleon. Perhaps the most consequential finding is that the production of cells

occurs by a process distinct from mitosis that could be operative in the presence of agents that prevent mitosis-dependent proliferation. Since cell proliferation resistant to cytostatic agents is the devastating explanation for most cancer deaths, mitonucleon-initiated proliferation deserves attention both as a system instructive of differentiation processes and as a mechanism that may become activated in cancer.

Mitonucleons, physical evidence of mitochondrial interaction with nuclei, are temporary organelles with consequential effects. I recently presented evidence [52] that their structure must cause a change in mitochondrial metabolism from aerobic to anaerobic with the possibility that the resulting anoxic organelle may generate energy and metabolites using the alternative pathway for generation of citrate that includes CO_2 fixation. In that paper, I also suggested that CO_2 does not as readily pass out of mitonucleons as it does out of mitochondria that have not fused. Evidence for that includes the formation of a gaseous vacuole that pressures the chromatin aggregates within the mitonucleon into a pyknotic state. Eventually that pressure, together with whatever enzymatic changes occur in the bolus of compressed chromatin, results in DNA fragmentation.

The fragments are drawn out through the syncytial envelope by what appears to be a spindle and arrayed in a configuration open both to epigenetic changes and to DNA replication by polyteny. The subsequent "recycling" of attached monolayer cell DNA into detached dome cells must include additional DNA synthesis since the number of cells in the product domes is greater than the number of monolayer cells that formed the syncytium by more than 30%. Beginning at approximately 12 hours after initiation of dome formation, DNA fibers can be observed stretched across the syncytial space in filaments with varying thickenings suggestive of polyteny. The resulting DNA then reassociates into a mass of chromatin that once again stains with hematoxylin and can be seen together with the remains of the mitonucleons (**Figure 8**). The chromatin mass morphs into a mass of nuclei (**Figure 9**) that will undergo ploidy reduction into smaller polyploid structures and ultimately into what appears to be typical diploid nuclei that arrange themselves in a layer throughout the syncytial space. During this process nuclei and mitochondria function within the syncytium but outside the confines of cellular structures until very late stages of the differentiation when cellularization occurs.

Evidence of polyteny without any evidence of mitosis and the prevalence of polyploid structures "reducing" to single nuclei over time, suggests an alternate proliferative cycle that, as described in MJ Pearson's 1974 review [25] "departs from a normal mitotic cycle" and "is the property of a nucleus in permanent interphase." Pearson discusses studies by Dr. Charles Berger [22] of large epithelial cells in the ileum of Culex pipiens as a possible example of such an alternate proliferative cycle. Berger discovered that nuclei containing up to 96 chromosomes were reduced in a stepwise manner to 48, 24, 12 and finally to 6N nuclei, the diploid number. Berger asserted that the high ploidy of the original larval cells

was not achieved by endomitosis but by DNA replication outside of the mitotic cycle in nuclei that are in interphase.

Endocycling, a variant of the mitotic cycle that occurs in the absence of cytokinesis, is also reported to be responsible for polyteny and polyploidy and has been extensively investigated in drosophila [53] [54]. Apparently most polyploid cells do not revert but there are exceptions. Exceptions include hepatocytes in mice and men [55] as well as intestinal papillar cells in drosophila [56], that are reported to revert by multipolar mitosis.

Berger was aware of endocycling or endoreplication as it is sometimes called but was convinced that his observations indicated somatic polyploid reduction that was a function of an alternative cell cycle. He encouraged a graduate student to repeat his experiments. Both Berger, and the graduate student Mary Grell [21], were quite explicit that the process of metamorphosis of Culex pipiens is outside of the mitotic cycle. In painstaking analysis of chromosomes during the process, Grell described the role of sister chromatids in the formation of bundles of chromatin. Presumably it is the bundles of chromatin organized perhaps by microtubules that form polyploid nuclei when the sister chromatids finally separate. Grell described a regular arrangement of "sister chromosomes in pairs, in pairs of pairs, and in pairs of such pairs resulting in bundles of DNA".

The process during dome differentiation is not measured by counting chromosomes but by following a decrease in multiple polyploid structures and an increase in individual nuclei during the final several hours of dome formation. A mass of chromatin is "whittled" down over time by "somatic ploidy divisions" to nuclei that will subsequently form a single-celled epithelial membrane. Data relevant to such a process in giant trophoblasts published more than 50 years ago and in more recent papers from the Zybina laboratory [18] [19] recognizing that polyteny was part of the life cycle of trophoblasts, described the process in this way, "The final step of the trophoblast giant cell differentiation is characterized by a transition from polyteny to polyploidy, with subsequent fragmentation of the highly polyploid nucleus into fragments of low ploidy." In essence, this is what we observe in the last 3 to 4 hours of dome formation. Varmuza and colleagues studying mouse trophoblast giant cells similarly concluded "that the mechanism of giant cell DNA amplification involves multiple rounds of DNA replication in the absence of both karyokinesis and cytokinesis, and that sister chromatids, but not homologous chromosomes, remain closely associated during this process [57]."

Dome cell nuclei are formed in the common cytoplasm of the syncytial envelope. Given that the DNA from monolayer cells involved in the initiation of dome formation is recycled, it is altogether possible that one or two rounds of polyteny provide enough chromatin for domes or for dome extensions. More may be needed for the formation of multicellular spheroids. Mitonucleon-initiated dome and spheroid formation amounts to an assembly-line production of "tissue" by amitosis whose efficiency would certainly be useful during

fetal and embryological development. As previously noted, multiple rounds of DNA synthesis characterize the latter half of the schizogony cycle in apicomplexans.

Other examples of amitosis in metazoans include nuclei budding through plasma membranes of amniotic cells transformed by a virus [58] as well as in mouse embryo fibroblast lines exposed to carcinogens [59], Liu and his colleagues [60] established methods using $CoCl_2$ to stimulate generation of polyploid giant cells and showed that these cells reduce to 2n cells through processes that have been called budding, splitting and bursting, "growth patterns very different from traditional mitotic growth of eukaryotic diploid cells", leading the authors to suggest that PGCC's are using an evolutionarily conserved mechanism to achieve fast growth by feeding into the mitotic growth cycle [61]. Some of the most exotic examples of amitosis have been reported by Gostjeva and colleagues [62] who examined cells in the fetal gut (5 - 7 weeks), as well as neoplastic cells of colonic adenomas and adenocarcinomas. The nuclei in the fetal gut are gauzy and bell shaped, capable of dividing symmetrically by an amitotic nuclear fission process that resembles the "separation of two paper cups." Furthermore, they report that seven other nuclear morphotypes emerge from the bell-shaped nuclei within the syncytia by asymmetrical amitotic nuclear fission. Phenomena like this only rarely seen in adult tissues reinforce the suspicion that amitosis is an essential feature of early growth and differentiation in metazoans, particularly important in the fetus and embryo when 37 trillion cells plus or minus must be created.

But the results in this paper suggest that amitotic production of nuclei and ultimately of cells can persist in adult organisms. All indications are that the process of mitonucleon-initiated differentiation and proliferation is a normal and not a pathological process in a tissue that is routinely renewed. Tolerance for some variability may be higher in cells that will secrete products into a common space as is the case for glands. And all indications are that the process of schizogony-like production of cells is more efficient than mitosis.

Nevertheless the possibility that amitosis is involved in any aspect of cell proliferation in metazoans is unsettling for some cell biologists, reflecting concerns that the process may be more prone to result in variable genomes with catastrophic consequences. In fact, research over the past two decades suggests that there is a much higher tolerance for genome variability than was generally believed in the twentieth century. Using 21st century sequencing methods, scientists have documented surprising variations in DNA within organisms. In 2004, two laboratories reported widespread variations in the number of copies of genes among different normal individuals [63] [64]. Copy number variations (CNV's) were also demonstrated for mouse embryonic stem cells leading Liang *et al.* [65] to suggest for mice and humans that "all somatic tissues in individuals will be mosaics composed of variants of the zygotic genome". Researchers have reported CNV's within differentiated human tissues from a single individual [66],

CNV's among cells within a single organism and, possibly most surprising, even differences among cells within a single organ [67]. It is relevant to note that genome diversity seems to have an upside in certain organs. It is estimated that polyploid hepatocytes comprise approximately 50% of adult human hepatocytes and that their depolyploidization is a source of diploid cells and genome variability when liver is damaged. The multipolar spindles and chromosome segregation defects observed in dividing human hepatocytes result in aneuploidy suggested to be advantageous [55] for the organ that is in the line of defense against toxins.

Of course the reality that malignant tumors are characterized by aneuploidy has been known for more than a century. Clearly and unfortunately, the genomic variability in tumors is among the most tolerated of genome variations, a reflection perhaps of the fact that tumors have no essential function other than proliferating and they can do this even with variable genomes. As was noted in a recent review, "aneuploidy is a context-dependent, cancer-type-specific oncogenic event that may have clinical relevance as a prognostic marker and as a potential therapeutic target [68]." The proliferative process discussed in this paper is one that is intimately involved with mitochondria so in considering whether mitonucleon-initiated amitotic proliferation may be involved in tumor formation a hint might come from research on the role of mitochondria in cancer and in tumor formation.

Some of the most compelling work on a role for mitochondria in cancer has come from the laboratory of Dr. Michael Lisanti. He and his colleagues have shown that some cells from breast cancer cell lines and from metastatic breast cancer can be separated from the general population because they contain measurably higher levels of mitochondria. Furthermore these cells are resistant to paclitaxel, a toxin that inhibits mitosis [69]. The overall increase in mitochondrial mass could have something to do with the biogenesis of mitochondria characteristic of mitonucleon formation as discussed in this paper. In another paper, Dr. Lisanti reports "a strict dependence on mitochondrial biogenesis for the clonal expansion and survival of cancer stem cells [70]."

Results from Dr. David Diaz-Carballo's laboratory suggest that a structure like the mitonucleon can be observed in cancer cells found to be resistant to cytotoxic agents [71]. He and his colleagues, studying such a population of cells, found that "a portion of resistant tumor cells displayed nuclear encapsulation via mitochondrial aggregation in the nuclear perimeter", noting that although such structures could be found in untreated tumor cells their numbers were more pronounced in resistant entities [72]. Given that detectable mitonucleons are present for a fraction of the polytene cycle as described in this paper, it is relevant that multinucleated oncogenerative cells were also found among tumor cell lines of different embryonic origins that were etoposide-inducible [72].

The evidence that an amitotic process can reliably generate nuclei with the potential for cellularization raises the possibility that the inappropriate activa-

tion of mitonucleon-generated proliferation may be one way cancer cells can escape the effects of cytostatic agents targeting mitosis. Furthermore it is relevant, as has been reported in this paper and more fully described in a preprint [7], that single cells containing multiple nuclei can detach from a monolayer after forming mitonucleons and can form spheroids capable of survival, migration, and even of reverting to monolayer growth if serum is added back to the medium. Furthermore, it has been shown that mitonucleons can be transferred out of a cell in membranous tubules, a possible mode of invasion into normal tissue as suggested in a recent preprint [73].

4. Materials and Methods

Ishikawa cells were cultured in phenol red-free, Minimum Essential (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 cells, obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital in New York, were originally derived from an endometrial adenocarcinoma line developed by Nishida *et al.* [2] who demonstrated the presence of receptors for both estradiol and progesterone. 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), and then transferred to medium containing 1% calf serum. Cultures left in MEM with 1% CS could survive for an additional 3 - 5 days with little proliferation. Assays for dome formation were done in confluent cultures, although differentiation has been observed to occur, to a limited extent, in non-confluent cultures. Differentiation was initiated with the addition of 10% - 15% fetal bovine serum (FBS). Multiple dishes were fixed and stained for biotin and/or for chromatin at different times during differentiation. Structures were viewed using an Olympus inverted stage microscope at powers of 100×, 200× and 400×. For purposes of comparison, organelles of interest were edited out from the original photos and put into figures that could be labeled, captioned, and where the sequence of changes could be clearly established. The transformation was achieved using Adobe photoshop and Biorender software. The approximate size of the organelles is indicated under each of these figures. In order to provide more information on some of the smaller polyploid structures, enlargements were made as indicated.

Unfixed and unstained pictures of differentiating structures were taken in living cultures. Photomicrographs of fixed and stained cells were taken following fixation 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. After another 5 min, the culture was washed with successive changes and then incubated with a 1:200 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% 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 reacts with biotin that is endogenously present in the cell in significant amounts.

The methods used in these experiments have appeared in a number of published papers. The studies that uncovered mitonucleon-initiated differentiation and proliferation required relatively synchronized dome formation [1] rather than the sporadic formation that initially sparked my interest. Domes could be induced in resting confluent cultures over a period of 20 to 24 hours in a process that was enhanced as reported both by progesterone and by DMSO [1] [3]. These methods allowed us to monitor and record morphological changes throughout the transparent intact syncytial space as well as the space above the differentiating dome structure. Examining morphology in situ, so to speak, obviates any distortions that might result from even gentle centrifugation, not to mention the routine methods for fixing, staining, and sectioning tissue.

For each stage observed in the process of mitonucleon-initiated dome formation, I searched the massive literature on cell structure, most of which is on fixed, stained, and sectioned tissue. For instance, there are many studies in which it has been shown that pyknotic nuclei precede apoptosis. The results with Ishikawa endometrial cells presented in this paper indicate that pyknotic nuclei can also be evidence that cellular differentiation is occurring. In particular, in the case of endometrial epithelia, cells that usually prosper by proliferating as attached cells undergo a 20 to 24-hour process of differentiation resulting in their ability to fare even better as detached cells capable of migration.

Conflicts of Interest

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

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