Chromatin streaming from giant polyploid nuclei in Ishikawa endometrial hollow spheroids results in the amitotic proliferation of nuclei that fill the spheroid envelope

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Abstract

This paper describes the amitotic proliferation of nuclei that fill the envelope of Ishikawa hollow spheroids. The presence of hollow spheroids in malignant ascites fluid has intrigued cancer researchers because of their potential as vectors that spread cancer. Little is understood about how they form. Observations in Ishikawa endometrial cell cultures demonstrate that nuclei filling the spheroid envelope are generated amitotically from giant nuclei by the same mechanism responsible for cell formation in domes. Transient structures of aggregated chromatin surrounded by fused giant mitochondria, the initiating structure for dome formation, are also the starting point for the differentiation of unicellular polyploid hollow spheroids. Nuclei from monolayer cells are transferred from neighboring cells into a single enlarged cell where they aggregate and become surrounding by giant fused mitochondria. A gaseous vacuole forms inside the resulting mitonucleon, expanding the cell and pressuring all of the cell material, including polyploid nuclei, between the outer membrane of the mitonucleon and the inner membrane of the cell. The resulting unicellular hollow spheroid detaches from the colony, capable of migration from the site of its formation. Ultimately, pressure on the aggregated chromatin, along with possible enzyme activation, results in the release of streams of chromatin granules that initially travel as if guided by microtubules through the shell of the hollow spheroid. Granules dissolve into filaments and, as initially described in dome formation, this material self-assembles into clusters of nuclei. Nuclei move out of these clusters into a regular array within the spheroid envelope, with formation of cell membranes as the final step in the creation of hollow spheroids with a central vacuole surrounded by dozens of cells. The spiral arrangement of cells around a central vacuole characteristic of the membranes of domes and spheroids, as well as colonies of nuclei similar to those produced by amitosis in differentiating Ishikawa cells, have been identified in tumor tissue that survives chemotherapy, suggesting that amitotic cell proliferation may at least partially explain the population of cancer tumor cells in humans that persist even when mitotically produced cells succumb to chemotherapy.

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Introduction

This paper describes a second example of amitosis in Ishikawa endometrial epithelia as chromatin from attached monolayer cells is recycled into the detached cells of a multicellular spheroid.

The first example of such processing was observed in the differentiation of adherent monolayer cells into domes (a layer of secretory cells elevated as a hemi-cyst surrounding fluid) in a confluent monolayer (Fleming, 1995). With the appropriate stimulation (Fleming et al. 1998), nuclei in cells previously committed to grow in a monolayer undergo a process that reprograms chromatin. Upon being recycled and reprogrammed, nuclei are reformed by amitosis and develop into cells that populate a detached envelope capable of secreting proteins into a lumen (Fleming, 1999).

The dramatic changes in cell structure accompanying hemi-cyst or dome formation starts with the formation of syncytia throughout an Ishikawa endometrial monolayer as dozens of monolayer cells fuse. The syncytial nuclei aggregate, and extensive mitochondrial biogenesis can be detected within two to three hours. Some of the mitochondria also begin to fuse adjacent to nuclear aggregates in syncytia, ultimately surrounding the chromatin aggregates (Fleming 2016 a). These transient subcellular structures called mitonucleons are similar to giant spheroidal or cup-shaped mitochondria such as nebenkern, a structure characteristically formed in insects during spermatogenesis (Fleming 2018b). Giant spheroidal mitochondria have also been observed in human endometrial tissue at the time of ovulation when glands begin to form in tissue entering the secretory phase of the menstrual cycle (Armstrong et. al., 1973).

By 9 to 12 hours after the start of differentiation, 3 to 4 mitonucleons can be detected in each syncytium. The aggregated chromatin, (Fleming et al., 1998) engulfed by multiple fused mitochondria, develops small gas vacuoles soon after becoming enclosed. Such vacuoles are also observed in cancerous endometrial tissue where they are reported to form in morules and have been shown to be associated with mitochondrial carboxylases, presumably due to their close association with surrounding mitochondria (Gamachi et al., 2003). The content of the small gaseous vacuoles in chromatin is still unknown, however nitric oxide NO is a gas that can be generated in nuclei. Among other physiological effects, it has been implicated in regulation of apoptosis (Chung et. al., 2001) and shown to stimulate mitochondrial biogenesis (Nisoli and Carruba, 2006). A paper recently described a bubble containing nitric oxide as being released from the nuclei of temperature-stressed HeLa cells under certain conditions (Chang, 2016).

Independently, the extensive membranes of the surrounding mitochondrial spheroid also form numerous gaseous vacuoles that eventually merge into a large vacuole elevating the apical membrane and compressing the chromatin aggregate at the center of the cup-shaped mitochondrion, to approximately one tenth of its size. When found in vivo, such nuclear aggregates are sometimes called pyknotic and assumed to be a signal of impending apoptosis. In Ishikawa differentiation, the pyknotic nuclei are actually a signal for impending

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reprogramming of chromatin previously contained in monolayer cells. Eventually, perhaps due to accumulating gases, mitonucleon membranes break down and the enveloped chromatin "explodes" into particulate and filamentous chromatin, a reaction that has been shown for isolated nuclei treated with a protease (Székvölgyi et. al 2007) under alkaline conditions.

Fragmented chromatin arrays have been observed in human endometrial cells, as Mazur and colleagues showed in electron micrographs (1983). Further investigation of these chromatin arrays is scanty, perhaps because of the obvious challenges of working with healthy human endometrium. Perhaps even more problematic is the fact that in the past two decades the breakdown of DNA into fragments is usually assumed to signal the onset of apoptosis. That may be true some of the time, but it is not true with regard to the DNA fragmentation observed in Ishikawa cell in the two processes described in this paper. In a salient review of many more examples of DNA fragmentation not associated with apoptosis, Sjaste & Sjaste (2007) hypothesize that DNA fragmentation is an epigenetic tool for regulation of the differentiation process.

In differentiating Ishikawa cells, chromatin is initially elevated as giant spherical structures into protrusions that appear to consist of membranes unfurling above each of the mitonucleons. The nuclear structure "disappears" as chromatin fragments and the material flows back into the cytosol initiating the formation of a chromatin array (Fleming, 2016a,b). DNA in that array is "relaxed" and fragmented, suggesting a state ideal for reprogramming by enzymatic changes and/or by new associations with histones and other proteins. The fragmented DNA coalesces and nuclei form out of that mass of chromatin as a colony (Fleming, 2016c). Nuclei then move apart into a regular array, with the probable involvement of microtubules.

The final step in the differentiation process is that membranes form around the redistributed nuclei and, after only 24 to 30 hours, dozens of cells can be detected in the newly formed hemi-cyst. These cells have been reprogrammed to form the hemi-cyst, attached (except at the base) only to each other and capable of secreting proteins into a fluid-filled lumen that has been forming under the dome. Over a period of weeks, the hemi-cysts can further develop into gland-like structures such as those found in many human organs, including the lining of the uterus (Fleming 1999) where they are renewed every 28 days in premenopausal women. The endometrial, epithelial cell line capable of this differentiation is called Ishikawa and was isolated by Nishida and his colleagues (1985).

This paper explores a second example of chromatin recycling and reprogramming, similar to the process of dome formation and also involving the formation of mitonucleons. In this instance the development of mitonucleons occurs within a single cell and leads to its detachment and conversion from a multinucleated cell to a hollow unicellular polyploid spheroid (Fleming 2018a). This buoyant structure detaches, is able to migrate, and can re-attach to form a new colony of cells during a process that results in the release of gas bubbles. It is also the case that unicellular hollow spheroids can give rise to multicellular spheroids in which multiple nuclei surround the "hollow" center of the spheroid as this paper will show. The nuclei of those cells are formed by an amitotic process that involves streaming chromatin granules and appears to be the same process previously shown to occur in domes.

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Results

Figure 1 shows an example of a unicellular polyploid spheroid formed from a multinucleated monolayer cell as previously described (Fleming, 2018a). The mitonucleon fills with gases and the contents of the cell including polyploid nuclei and cytosol are pressured into a shell on the outer edge of the developing hollow spheroid. Polyploid spheroids can readily reattach if serum is added back to the medium. Dissipating gas bubbles are visible for a short period of time following reattachment.(Fleming, 2018a) and the giant cells with giant nuclei left behind resemble trophoblasts (Zybina 1979; Zybina & Zybina,2007) or the cultured human cells that survive mitotic death (Erenpreisa et al.2011). Such giant cells have been demonstrated capable of forming more nearly normal (2n) nuclei in a process called de-polyploidization by these two pioneering scientists. Recently Zhang et al.(2014) have been able to elicit formation of giant cells with giant polyploid nuclei by the addition of $CoCl_2$ to cultures. They present evidence that these giant cells can behave like stem cells.

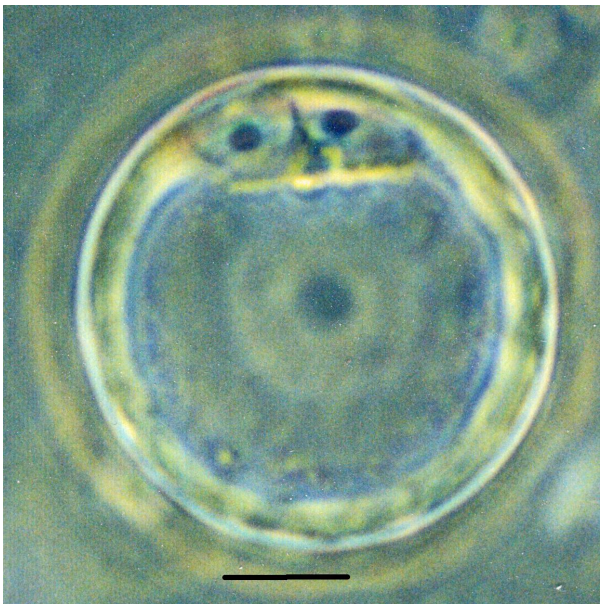

Fig. 1. Polyploid unicellular spheroid. A mitonucleon forms in a multinucleated monolayer cell as described (Fleming, 2018). The giant polyploid nuclei are several times larger than monolayer nuclei, approximating the size of polyploid nuclei in trophoblasts, the giant cells in placenta. An enlarging gas vacuole has compressed the nuclei and cytoplasm into what amounts to a spherical shell. Under culturing conditions favoring attachment, this structure can initiate a colony.
bar=25 microns

The other possible fate for this unusual unicellular spheroid is that the giant polyploid nuclei can give rise to multiple nuclei by an amitotic process similar to that summarised in the introduction to this paper (Fleming; 2016 a,b,c). The chromatin from giant nuclei can be reprogrammed and recycled into nuclei that eventually populate the membrane envelope of the spheroid. The earliest sign of such changes in the unicellular polyploid spheroid is the formation of small gas vacuoles in the region of the giant nuclei within the envelope created by the outer membrane of the distended mitonucleon and the inner membrane of the cell in which it formed (fig. 2a). There are many more gas vacuoles around the nuclei in picture 2b and an event in the polyploid nucleus, possibly activation of an enzyme, results in granular chromatin beginning to flow (arrow).

Appearance of gas vacuoles in chromatin was also an early event in dome formation (Fleming, 2016b). In fact, as table 1 at the end of the Results section shows, there are many similarities in the process of forming an envelope of apical and basal membranes that will be

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filled by multiple dome nuclei and the process of forming the envelope of a hollow spheroid similarly capable of being filled by multiple spheroid nuclei.

Ultimately multiple streams of granular chromatin are observed to flow from the spheroid nuclei in all directions (fig.3), like spokes in a wheel, traveling from polyploid nuclei at one end of the spheroid through the membrane envelope to the other end of the spheroid with a linearity that suggests microtubule involvement. The streaming chromatin appears to be granular.

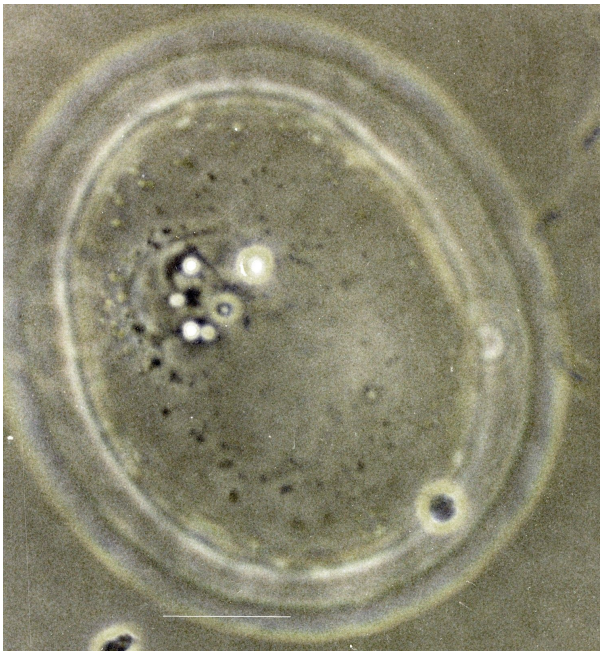

Fig. 2a. A polyploid unicellular spheroid. This structure results when a mitonucleon forms in a multinucleated monolayer cell as described (Fleming, 2018). Discontinuities are observed in the hollow spheroid in the vicinity of the giant nucleus. These may be similar to gas vacuoles observed within chromatin material during the early hours of dome formation.
Bar=25 microns

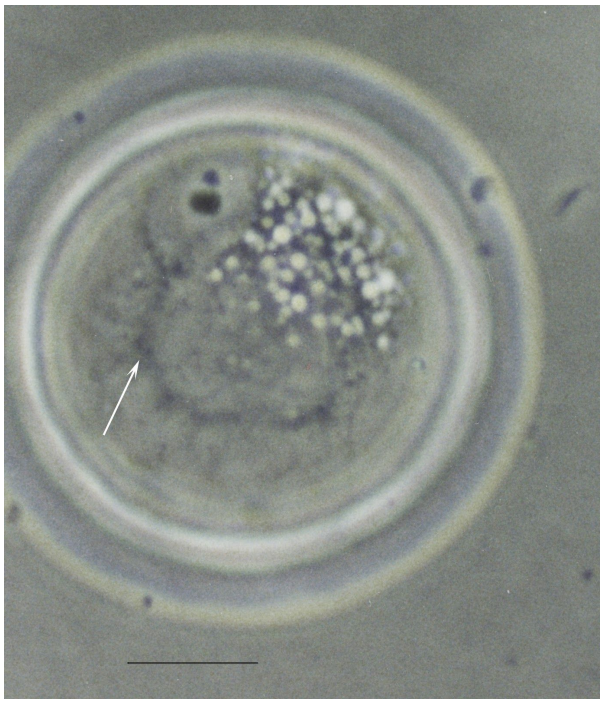

Fig. 2b. A polyploid unicellular spheroid further along in the process of forming a multicellular spheroid. Gas vacuoles in the vicinity of giant nuclei have increased. The arrow points to material beginning to stream from one or more of the giant nuclei (Fleming, 2016b).

As discussed in the paper describing formation of unicellular spheroids, vacuolated partial spheroids are also observed to form on the edges of solid spheroids (Fleming, 2018a). Such a structure made up of 2, possibly 3, contiguous vacuolated structures is shown in fig. 4. The hollow attached spheroids allow the observation of chromatin moving through a spheroid envelope. The focus shows the profile of one of the streams with a couple of additional streams alongside, out of focus. Measuring in profile, the size of the granules averages 9 to 10 microns, similar to the size of a nucleosome (Olins et. al., 1977; Olins & Olins, 2003).

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Chromatin streaming from giant nuclei was originally described as a process in formation of domes (Fleming, 2016b), although its visualization in spheroids is more dramatic.

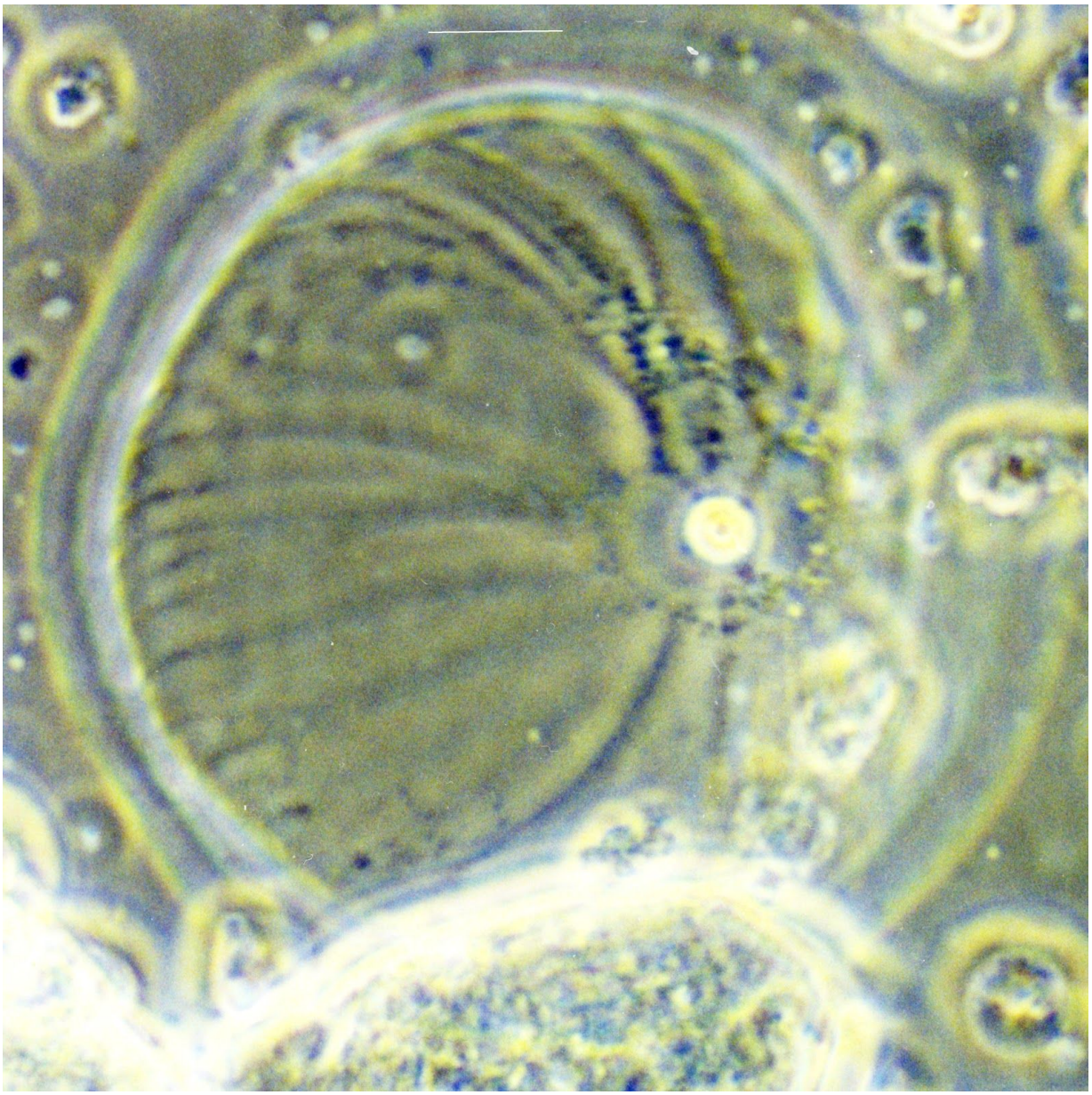

Fig. 3. represents an advanced stage of chromatin decompression with granules streaming from nuclei through the spheroid envelope. Multiple streams of chromatin material can be seen flowing through the spheroid envelope toward the polar end opposite from the nuclei. The straight lines of the flow suggests that the material has become attached to microtubules, as appeared to be the case during dome formation. The granularity of the chromatin can be discerned most clearly in the material flowing from the nucleus between 11 and 12 o'clock.Bar=25 microns

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At some point, presumably when the distribution of chromatin is complete, the neat rows of chromatin granules reconfigure into an array of wavy lines (fig. 5a) perhaps signaling the conversion of granules into filaments.

Chromatin arrays such as these have been found in endometrial tissue taken at the time of ovulation as well as in tissue treated with medroxyprogesterone (Mazur et. al., 1983). In a paper filled with fascinating electron micrographs, Mazur and his associates describe a chromatin array that almost fills the cell as "a network of uniform, fine filaments that measured 7 to 8 microns in diameter." They go on to say that the filaments appear like

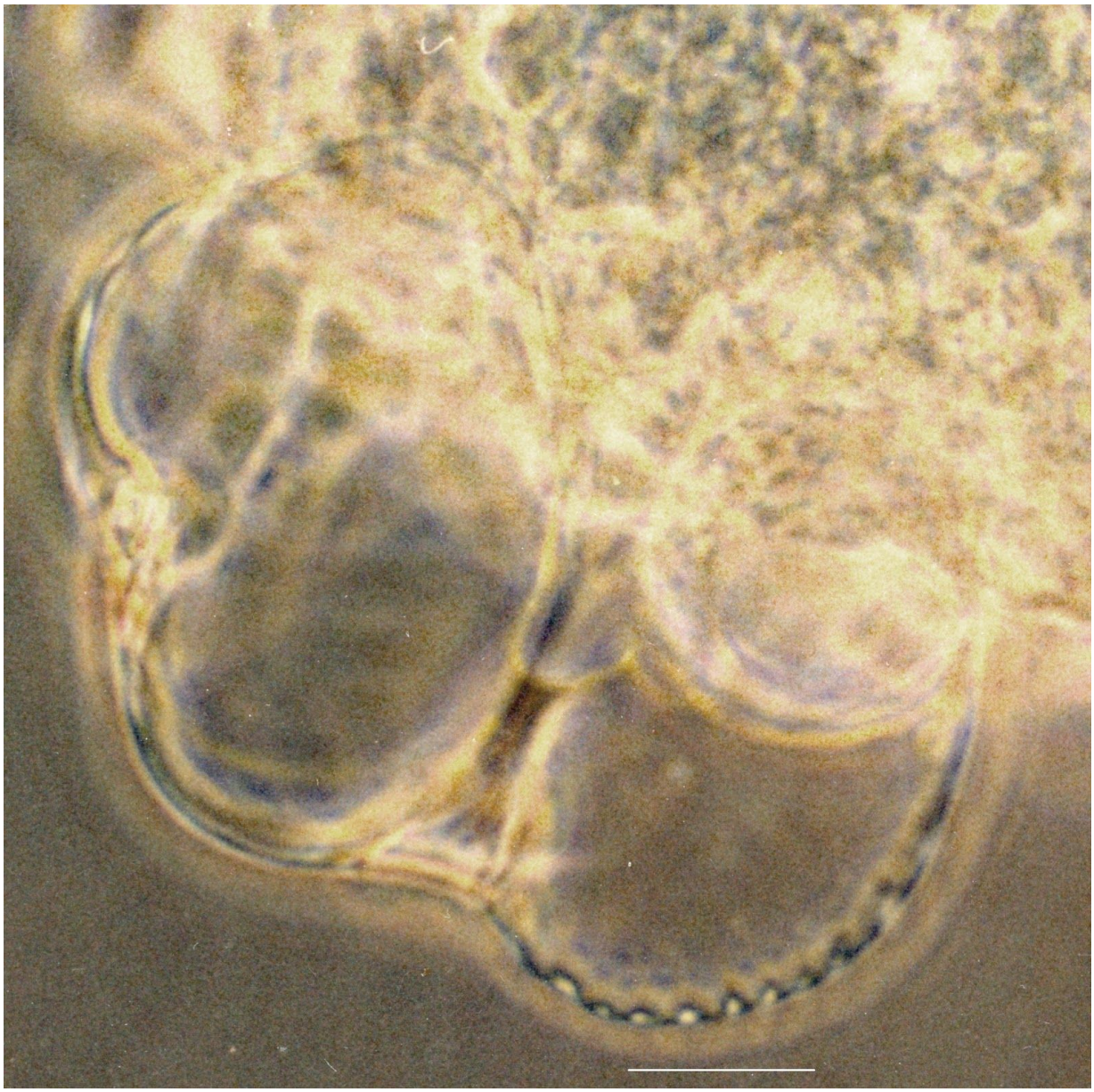

Fig. 4. This structure is of particular interest because it provides a profile of chromatin granules moving, likely being moved, through the spheroid envelope. Two additional lines of granules appear out of focus parallel to the granules that are in focus, as would be expected on the basis of figure 3. bar=50 microns

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"wavy strands arranged along the long axis of the cell." DNA fragmentation is usually taken as a signal that apoptosis is occuring in a cell. Our results show that chromatin fragmentation such as observed in domes (Fleming, 2016b) and in hollow spheroids is part of a process that recycles and presumably allows reprogramming of DNA. Such an array of DNA filaments has been observed elsewhere such as in cancer cells (Yang et.al.1995).

The chromatin array is not a stable state and, in what may be the most surprising part of the process, like humpty dumpty, the chromatin pulls itself together again and does so rapidly. In that regard, there is evidence that eukaryotic DNA is interrupted by linkers (Székvölgyi, 2007) whose destruction by a protease or by a dramatic change in pH can lead to the formation of free 50-100 Kbp chromatin fragments. Whatever the process, the effects can be readily reversed in the systems under discussion. Chromatin rapidly coalesces into an irregular mass that quickly forms "colonies" of nuclei often in the midst of reticular material that also stains for nucleic acids (Fleming, 2016c)

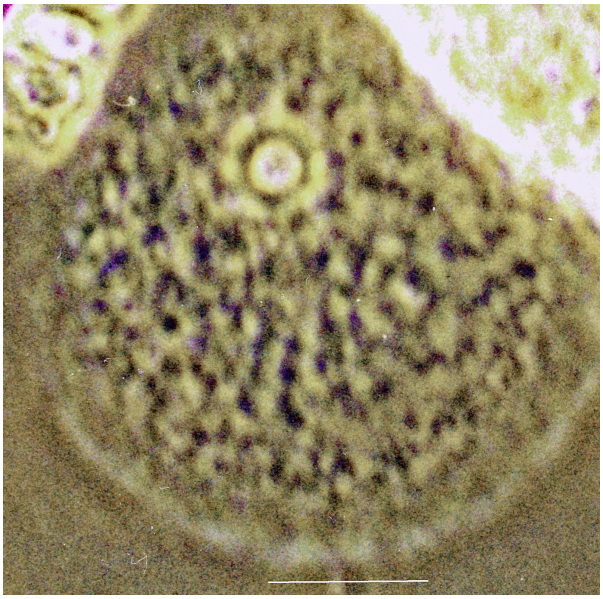

Fig. 5a. Chromatin array in hollow spheroid.
At some point, presumably when the distribution of chromatin is complete, the neat rows of chromatin granules are replaced by an array of wavy lines that appear to be interacting chromatin filaments.
bar=25 microns

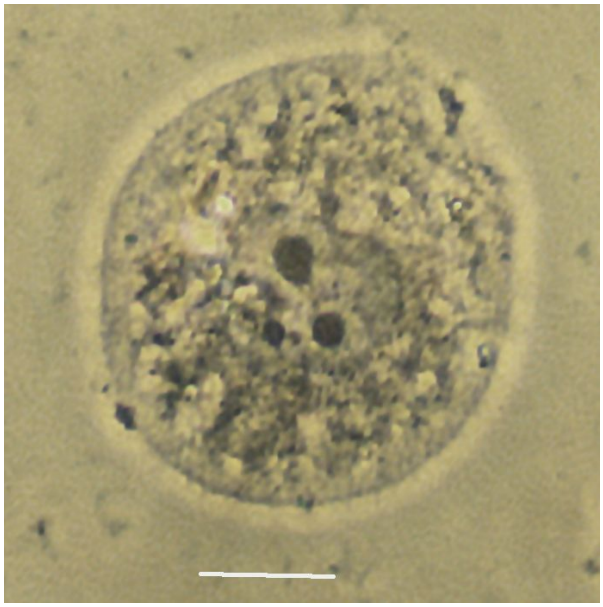

Fig. 5b. The two large dark bodies and one smaller body at the center of the spheroid look like heterochromatin, These structures could represent the initiation of nucleoli formation. Nucleoli are known to be centers of ribosomal RNA and their early appearance may be essential for protein synthesis. Furthermore the nucleoli appear to serve as an organizing structure for nuclei that will arise in the spheroid envelope. bar =25 microns

A chromatin array (as in fig. 5a) would be open to a variety of changes to the DNA molecule itself and/or to changes in proteins associated with the molecule, such as histones. For example, butyrate, which enhances dome differentiation (Fleming 1995) is a known inhibitor of histone deacetylation (Sealy L and Chalkley R. 1978). Histone acetylation is an example of a change that could occur when chromatin is reprogrammed. Obviously any number of other enzymatic changes could be effected in such a chromatin array.

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Generalizing about a process from the outcome, the chromatin array must lend itself to reprogramming chromatin from formerly adherent monolayer cells into chromatin that supports anchorless secretory cells in gland-like structures or in hollow spheroids. Out of the "apparent chaos" in figure 5a, the earliest glimpse of familiar structures appears to be the formation of heterochromatin, possibly multiple nucleoli, emerging from the wavy strands in the background as in fig. 5b.

Finally in figs. 6a and 6b, the familiar structure of nucleus plus nucleolus swims into focus in the spheroid envelope. In Fig. 6a at least 3 structures that look like "typical" nuclei are emerging. The nucleoli are substantial, suggestive perhaps of some intense ribosomal RNA synthesis. The focus in Fig. 6 b is on the spheroid envelope that is yet to be filled with nuclei, although other material is clearly filling up the spheroid envelope, leaving a transparent window through which "ghosts" of nuclei are fuzzily apparent on the out-of-focus side of the spheroid. Once again, interpretation of the process is informed by observations in domes (Fleming, 2016c) where even as the chromatin fibers are rapidly coalescing, typical nuclei are forming. Regions of the spheroid envelope contiguous with the nuclei that are forming contain material while other regions still appear to be empty.

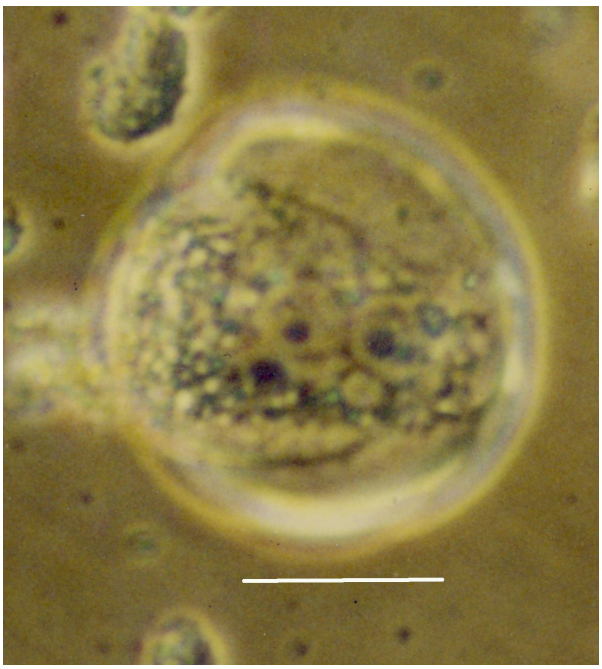

Fig. 6a. Observation of nuclei forming in hollow spheroid. Structures shaped like nuclei and containing at least one nucleolus are emerging side by side from amassing chromatin in this hollow spheroid. bar=50 microns

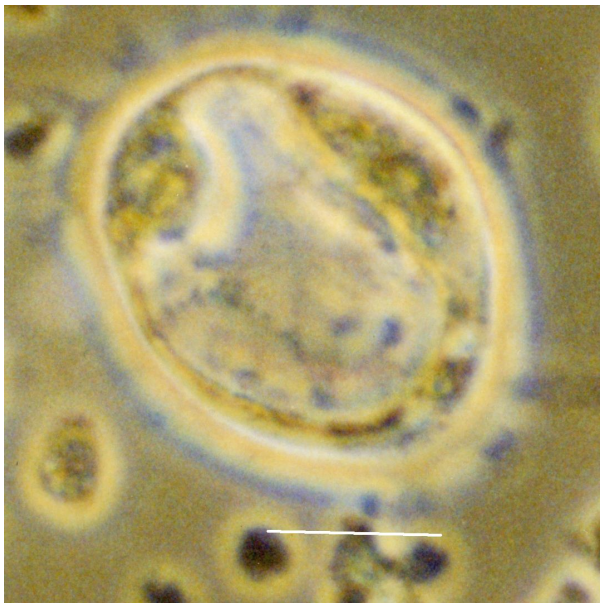

Fig. 6b. Some parts of the membrane of this hollow spheroid are still transparent. Through that transparent region it is possible to discern what looks like nucleoli in faintly outlined nuclei on the other side of the spheroid. bar=50 micron

Fig. 7 is an image of a spheroid containing multiple nuclei, but still under-populated. The nuclei formed by amitotic chromatin streaming initially appear as colonies with nuclei sometimes overlapping. Finally the spheroid shell in fig. 8 looks to be a complete shell of

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cells, an entirely different look from the semi-transparent hollow spheroid pushing out from that shell. After nuclei have spread throughout the spheroid, cell structure is completed with the addition of membranes around each nucleus. A better representation of this process was found in dome formation. It was possible to detect "cells" in three different states: with membranes, without membranes, and forming vacuoles characteristic of mitonucleons indicating that the dome was enlarging by amitosis (Fleming, 2016c, fig.5).

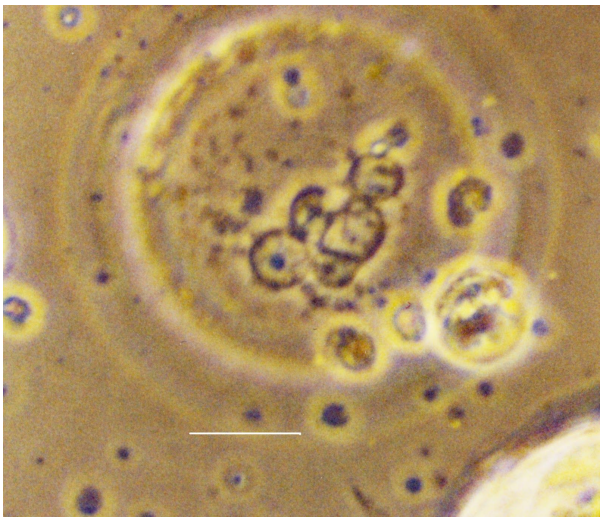

Fig. 7. Colony of nuclei detected in one part of a hollow spheroid
As is characteristic of nuclei formed by chromatin streaming, some of the nuclei appear to be overlapping.

bar=25 microns

Hollow spheroids can be free floating, as in figs. 1, 2a, and 2b; or they can be attached to multicellular hollow spheroids as in figure 8 or to solid spheroids. The composite structure

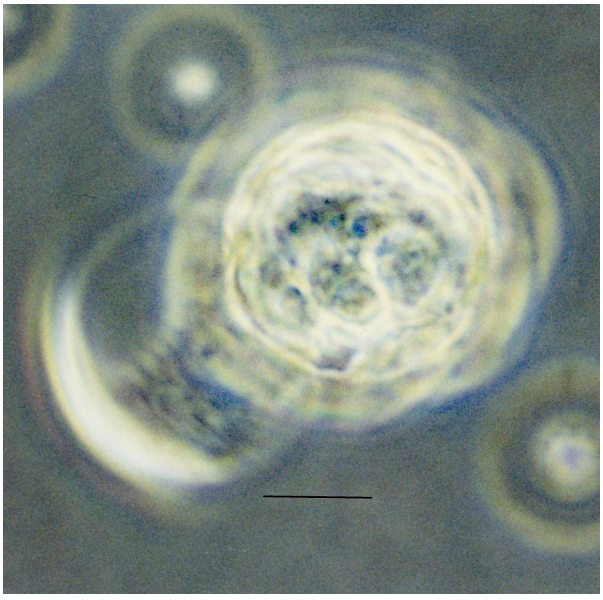

Fig. 8a. Focusing on what appears to be a complete shell of nuclei in what we must now presume to be a multicellular hollow spheroid. bar=50 microns

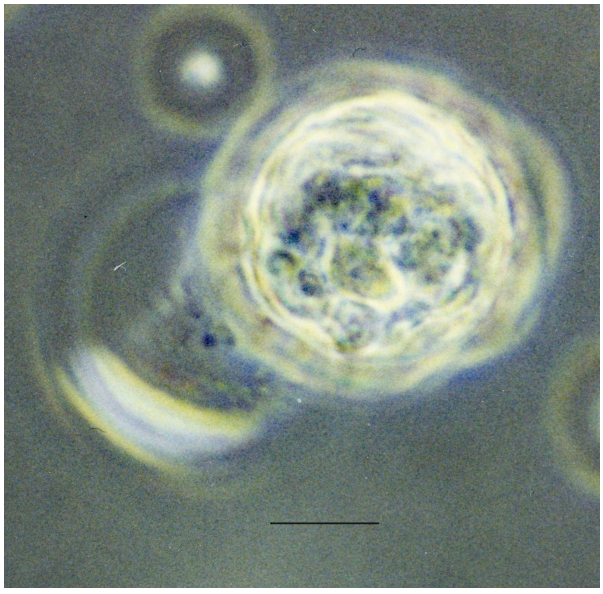

Fig. 8b. "Daughter" hollow spheroid arising from the back of the newly formed multicellular hollow spheroid. bar=50 microns

can reattach to the dish when fresh serum is added to the medium, a process that has allowed us to observe a few more details about amitotic production of nuclei.

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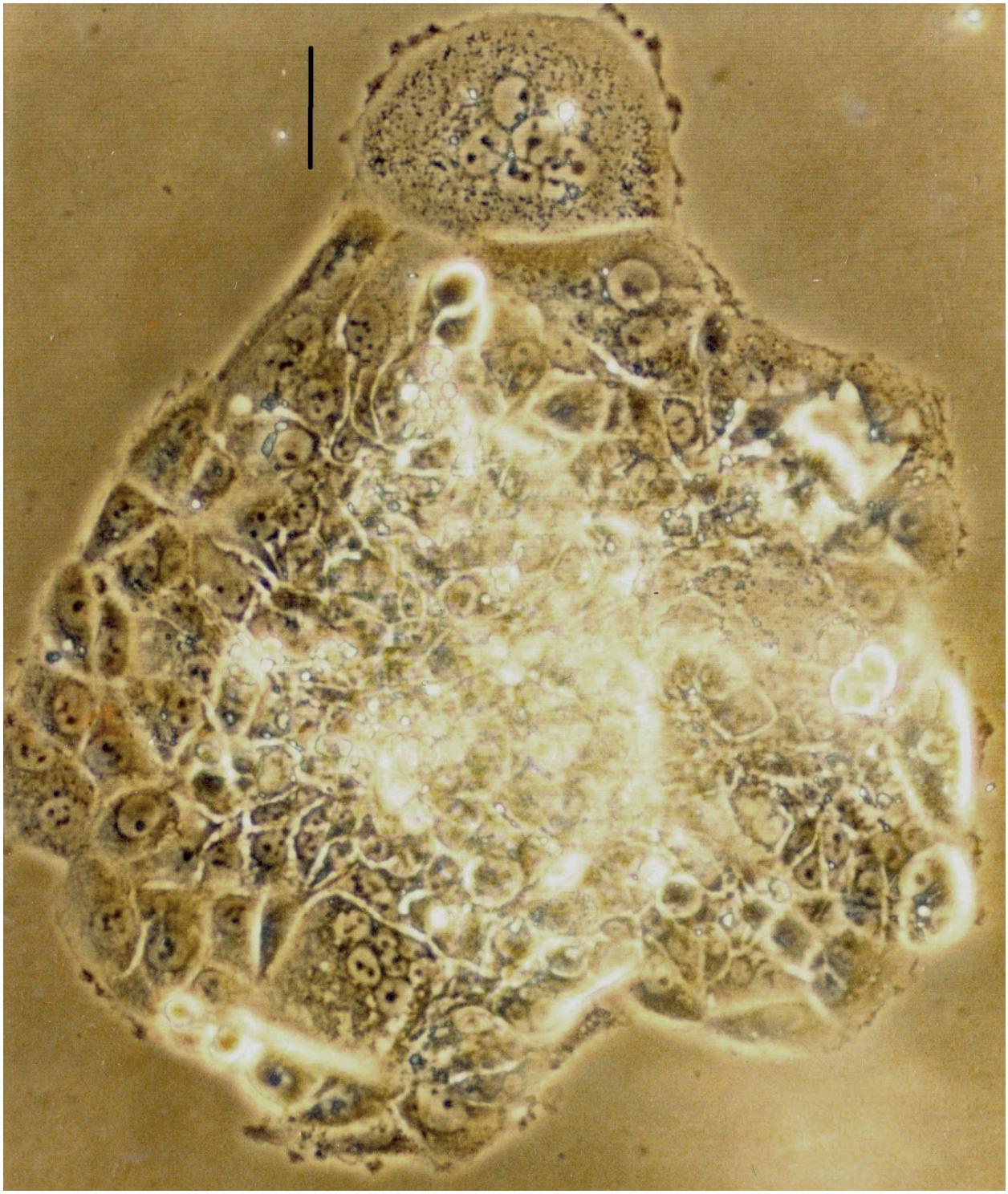

Fig. 9. As this solid spheroid attaches to a petri dish a variety of cell types can be seen. There are single cells, cells with two nuclei, and opaque cells. The fuzzy center of the structure suggests that not all of the cells originally in the spheroid have moved out into the monolayer. It is the two multinucleated structures, one at 7 o'clock and one at 12 o'clock that are of interest for this paper. Both of these structures are enlarged in fig.10.
Bar = 100 microns

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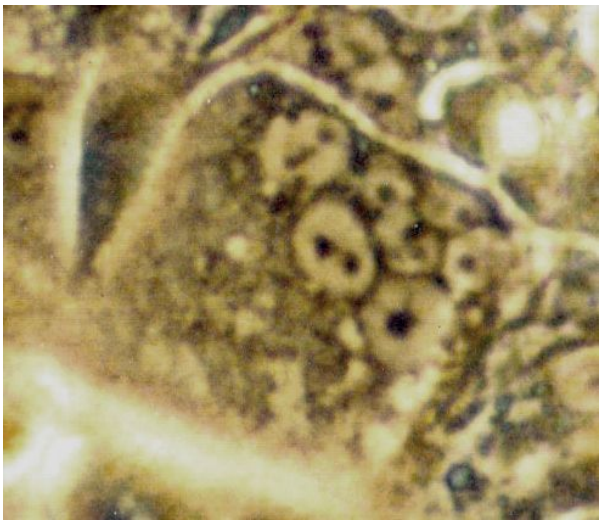

Fig. 10a. At least 6 nuclei can be detected in this structure. Such a structure is usually assumed to arise from the formation of a syncytium. But a colony such as this could could also arise from a hollow spheroid on the edge of a solid spheroid. That is the obvious conclusion for the second multinucleated structure enlarged in fig. 10b.

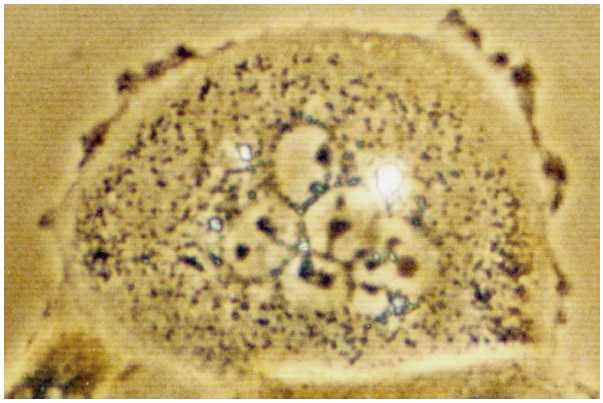

Fig. 10b. Attached hollow spheroid in the process of forming nuclei from chromatin granules. Nucleoli appear to be an organizing force with one or two nucleoli appearing in the midst of each of the five or 6 nuclei that are forming. Most of the nucleoli appear to be connected to nuclear membranes that are forming. Heterochromatin that may well become organizing nucleoli can be detected in the outer reaches of what is assumed to have been a hollow spheroid, although it is missing in some places perhaps because it had already become the center of a nucleus being formed.

As fig. 9 illustrates, when Ishikawa solid spheroids become attached to dishes upon the addition of serum, spheroid cells migrate out and form a monolayer. There is always some heterogeneity among the cells (Fleming, 2014). The largest population are typical cells with a single nucleus, but it is also possible to see cells with two nuclei. Opaque cells, often in pairs like the “yin and yang” cells in the top half of the colony are frequently observed. Specifically of interest to this paper, are the two multinucleated structures further enlarged in figs. 10a and 10b that are characterized by “colonies” of nuclei.

The structure in fig. 10a shows a small colony of 6 or 7 nuclei. Such a figure would normally be assumed to arise as a result of syncytial formation. Our results suggest the possibility that the structure arose as a result of amitotic streaming. Fig. 10b shows a structure that, from the observations of chromatin streaming shared in this paper (Fleming 2016 a,b,c) and from the work of Dundr and Misteli (Dundr & Mistelli, 2001, Mistelli, 2001 and Dundr & Mistelli, 2010) looks like self assembly of nuclei from granular chromatin material, the process assumed to be taking place in the spheroid images in figs. 5b and 6.

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Multiple nucleoli appear to be in contact with granules approximating ovoid nuclear membranes surrounding one or more nucleoli. The nuclei are forming so close together in a colony that, at this stage, they almost appear to be "sharing" half formed nuclear membranes, perhaps facilitating interaction. The surrounding cytosol is filled with additional granules. Clumps of heterochromatic material are apparent on the scalloped edges of the collapsed hollow spheroid.

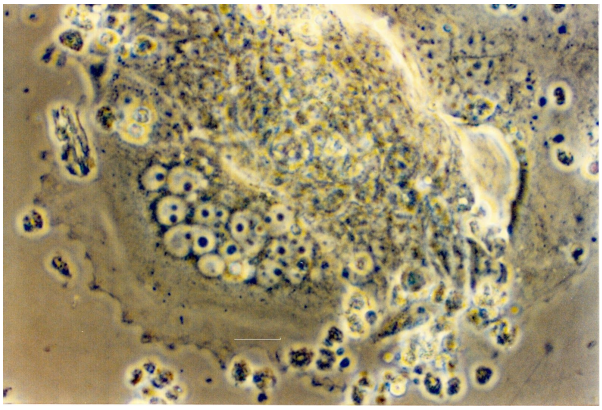

Fig. 11a. Approximately 15 to 20 nuclei were formed amitotically in this hollow spheroid. Nucleoli are apparent in most of the nuclei. Two boundaries can be detected moving out from the nuclei. The first is slightly darker and may be cytosol. The second has a scalloped edge as would be expected for the frayed edges of a collapsed hollow spheroid. bar=50 micron

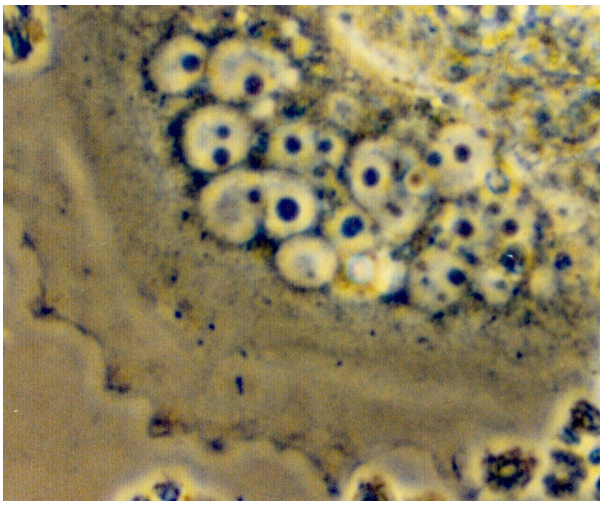

Fig. 11b. This amitotically produced colony of nuclei is further along in the process of self assembly than the colony in fig.10b. Far fewer granules can be detected outside the perimeter of the colony. It does appear that the process of self-assembly was further along, although granules although granules crowding in along the edges of the outermost nuclei suggest that self assembly was not complete.

Another photomicrograph of a monolayer originating from a solid spheroid and an attached hollow spheroid is shown in figs. 11 a and b. Even fewer cells appear to have moved out of the center of the solid spheroid, but a colony of more than a dozen nuclei can be detected attached to the developing monolayer in another structure that appears to have been formed by a collapsing hollow spheroid. The comparison of the structures in 10b and 11b suggest that the detached hollow spheroid in 11b was further along in the process of nuclear self assembly before collapsing and adhering to the dish. More nuclei have formed and far fewer granules are visible in the surrounding cytosol. Diminished amounts of heterochromatin are associated with the scalloped edges of what was a hollow spheroid.

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Amitotic nuclear proliferation in membrane envelopes of domes and hollow spheroids

Reprogramming chromatin from monolayer cells that form syncytia, into fluid-filled hemicysts(Fleming 2016 a,b.c)	Reprogramming chromatin from monolayer cells into form multicellular hollow spheroids (Fleming 2018a)
Multiple nuclei result from formation of a syncytium when as many as 50 monolayer cells fuse.	Multiple nuclei result from nurse-cell like dumping of cellular contents into a receiving cell. Donor cells and the acceptor cell are attached monolayer cells at the start of the process.
Particulate structures staining for the endogenous biotin of mitochondrial carboxylases increases dramatically.	Numbers of mitochondrial structures in the receiving cell increases as a result of nurse-cell like activity.
Some of these mitochondria aggregate and fuse around 3 to 4 chromatin aggregates forming giant spheroidal mitochondria, more than 10 times larger than typical mitochondria.	Formation of one or more mitonucleons in the enlarged multinucleated cell can be seen within a cell detaching from a colony of cells.
Formation of gaseous vacuoles within multiple mitonucleons elevates the apical membrane of syncytium and pressures chomatin aggregates into pyknotic structures characteristic of “signet cells.” Evidence suggests that inner membranes of the giant mitochondria can accumulate gases because they have become vesicular.	The gaseous vacuole growing within a single giant spheroidal mitonucleon exerts pressure on the mitonucleon outer membrane pressing it against the cell membrane. Nuclei and cell contents exist in the envelope formed by the outer membrane of the mitonucleon and inner membrane of the cell.
Pyknotic giant nuclei explode into granules that travel from the nuclear origin to the opposite end of the predome.	Pyknotic giant nuclei explode into granules that travel through the envelope of the developing hollow spheroid.
Chromatin breaks down and forms an array (consisting of DNA filaments) throughout the envelope created by the apical and basal membranes of the predomes.	Chromatin array (consisting of DNA filaments) fills the shell of the hollow spheroid.
Chromatin re associates and rapidly forms a colony of nuclei.	Chromatin re associates, nuclei appear in aggregates in various regions of the hollow spheroid.
Nuclei move out and become redistributed within the envelope of the apical and basal membrane. Throughout the process, fluid has been accumulating under the basal membrane.	Nuclei distribute throughout the shell of the hollow spheroid.
Membranes form around each of the redistributed nuclei completing the process of dome formation. At this point in time (24 to 30 hours after the start of differentiation), dome cells look very much like surrounding monolayer cells.	Membranes form converting what is essentially a unicellular hollow spheroid with giant nuclei into a multicellular hollow spheroid.

Table I: Comparing structural changes during dome formation in Ishikawa monolayers and in detaching multinucleated single cells that become converted into hollow spheroid cells.

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Discussion

The photomicrographs in this paper demonstrate amitotic proliferation by chromatin streaming in floating hollow spheroids and in collapsed hollow spheroids. Amitosis was previously identified as responsible for dome formation in Ishikawa endometrial epithelial cells (Fleming, 2016 a,b,c). What can be said about both processes is that chromatin from multiple monolayer nuclei is recycled, reprogrammed, and assembled into multiple nuclei that ultimately populate a membrane envelope. Some aspects of this process can be observed more readily in hollow spheroids than in domes but as table 1 indicates, similar processes result in the creation of multiple nuclei. Mitonucleon initiated reprogramming of cells appears to be something like a "biological assembly line," efficiently producing multiple reprogrammed nuclei in a 24 to 30 hour time period, not much longer than the time it takes for a single cell to undergo mitosis.

Recycling and Reprogramming multiple monolayer nuclei in spheroids

As shown in a previous paper describing formation of single cell hollow spheroids (Fleming 2018a), nuclei from multiple surrounding cells are donated into a single central monolayer cell where they aggregate and become enveloped by fusing mitochondria. A single giant vacuole forms in the resulting mitonucleon, compressing cell contents and nuclear chromatin between the outer membrane of the mitonucleon and the inner membrane of the cell. The resulting polyploid cell detaches from the colony as it begins to enlarge.

The earliest sign that such a unicellular polyploid hollow spheroid is going to become multicellular is the formation of small gas bubbles in the region of the polyploid nuclei. The pressure exerted on the polyploid nuclei appears to have "flattened" them between outer mitonucleon and inner cellular membranes (fig. 2). Many more gas vacuoles are apparent in the unicellular hollow spheroid in fig. 2b with an arrow indicating chromatin beginning to stream from polyploid nuclei, a process that is at an advanced stage in fig. 3. The hollow spheroid shell is filled with multiple streams of 9 to 10 micron granules, approximately the size of nucleosomes (Olins and Olins,2002), although such a measurement, is being made at the limits of resolution of the light microscope.

At any rate, granule streaming may result from pressure on the nucleus together with activation of one or more proteases/nucleases responsible for the "reduction" or "relaxation" of chromatin. Fascinating experiments have shown that physical stress on nuclei (Roy et al. 2018), may be a part of a reprogramming process, and a recent paper has presented evidence that the nucleosomes of giant cells contain unusual histones that impart increased flexibility to the chromatin (Hayakawa, K.,et.al.,2018). The granular streams resolve into an array of filaments (fig.5a) Inter-fiber nucleosome interaction (Maeshima et al.,2010) appears to result in interdigitating fibers in a state that Maeshima has likened to a polymer melt or self-oligomer state. In our systems that array is temporary: the fibers coalesce into a mass that rather quickly reassembles into a colony of oval nuclei that eventually, with rearrangements that probably involve microtubules (Mazia 1993), fill the spheroid envelope.

Reassembling chromatin into nuclei in spheroids

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Two, possibly three, heterochromatic regions in fig. 5b are among the earliest signs of organization emerging from a chromatin array such as is shown in fig. 5a. It becomes possible to detect nuclear membrane forming around heterochromatic regions in figs. 6a and 6b. Hollow spheroids partially filled or completely filled by newly formed nuclei are shown in figs. 7 and 8. Fig. 8 also shows that a hollow attached spheroid, a structure previously described (Fleming, 2018a), can be seen attached to the multicellular spheroid. Once the process of amitosis by chromatin streaming begins, structures formed by this process appear to extend themselves by the formation of more hollow spheroids on the perimeter of spheroids or extending from domes.

Figs. 9 and 10 demonstrate the distinguishing features of nuclei formed by amitotic streaming. Fig. 9 is a photomicrograph of a structure formed upon addition of fresh serum to a petri dish containing spheroids. A multicellular spheroid has attached to the surface of the petri dish and most of its cells have spread out into a monolayer. Fig. 10a is an enlargement of a region in the colony containing multiple nuclei that might be due to syncytial formation or to amitosis by chromatin streaming. But the structure at the top of the colony enlarged in fig. 9b seems to have attached to the dish while still in the process of forming nuclei, a rare sighting and one that seems to bear out the theory that nuclei can be formed by self-assembly. (Dundr & Mistelli, 2001, Mistelli, 2001 and Dundr & Mistelli, 2010) The central observation in this picture is that the self-assembly appears to involve interaction of granules.

Heterochromatin resembling nucleoli is both at the center of the formation and in contact with granules that have begun to arrange themselves in the typical ovoid shape of nuclear membranes. Furthermore, those granules arranged around one or more nucleoli approximate nuclear membranes that almost appear to be shared because the nuclei are so close one to another. It seems reasonable to assume that the membrane-forming granules contain nuclear membrane proteins such as laminin as well as nucleic acids attached to those proteins. Other granules, containing chromatin and, perhaps, nuclear bodies must be assumed to be assimilated into the interior of the nucleus. This supposition is based partly on the concept of the “functionally compartmentalized nucleus” with specific chromosomal material attached to proteins on the outer edges of nuclear membranes and other chromosomal material entirely in the nucleus within chromosomal territories (Cremer et al., 1993) (Croft et al., 1999) (Cremer & Cremer 2010). Heterochromatin also appears on the scalloped edges of the colony and granules are abundant in what appears to be cytosol surrounding the self-assembling nuclei.

Interpretation of this uncommon sight suggests that an attached hollow spheroid plated down onto the surface of the petri dish along with the spheroid to which it was attached while still in the midst of amitotic production of nuclei as illustrated in figs. 2 through 8. This supposition is strengthened by comparing this figure to fig. 11 b showing a similar extension with some important differences. Granules are not abundant in the cytosol in fig. 11b, perhaps because many more nuclei have already formed. There is a demarcation in the material surrounding the nuclear colony that may signal the difference between “cytosol” present in the spheroid and “cytosol” being synthesized after the spheroid has reattached. Finally, somewhat less heterochromatic material is visible on the scalloped edges of the structure. The formation of nuclei within a colony in what was previously a hollow spheroid appears to be almost complete in the extension in fig. 11b.

Self-Assembling Nuclei

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Studies of self-assembly of smaller entities such as nuclear bodies led Dundr and Mistelli to theorize that the entire nucleus could form by self-assembly(Dundr & Mistelli, 2001, Mistelli, 2001 and Dundr & Mistelli, 2010). The researchers emphasized that the process is dynamic and, 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 (Dundr & Mistelli, 2010) although not necessarily identifiable in our photomicrograph. One estimate is that the nucleolus alone contains more than 700 proteins (Andersen et al., 2005) moving in and out of association with each other and with nuclear bodies.

Fig. 9b suggests that not only is the nucleolus the center of activity, in the early stages of nuclear assembly, it appears to be "in touch" with the developing nuclear membrane, a visual that supports the theories that the nuclear envelope is involved in chromatin organization (Zuleger et al., 2011), and that the nucleolus and ribosomal DNA are central factors in the spatial organization of the genome (O'Sullivan et. al., 2015). Self-assembly, as well as the consistent formation of chromosome territories (Cremer & Cremer,2010) in interphase nuclei have been assumed to reflect thousands of weak and strong chemical bonds intrinsic to the dynamic interaction of chromatin, nuclear bodies, and nuclear membranes. Rippe (2007) suggests that the thermodynamic underpinnings of self-assembly of the nucleus resides in the high concentration of macromolecules favoring the formation of nuclear-subcompartments in a reversible self-organizing manner manner, emphasizing that hydrophobic interactions make a significant contribution to complex formations within the nucleus.

Quality control of proliferation by amitotic chromatin streaming

One final and potentially important observation from fig. 11b is that there seems to be some variation in the size of the nuclei formed by amitotic streaming and nuclear self-assembly. This could be true because self-assembly is proceeding at a variable rate in neighboring nuclei. Even so, such variability brings up the fundamental question of quality control. How does amitotic proliferation by chromatin streaming ensure that self-assembling nuclei contain two complete copies of each of the essential chromosomes-no more, no less?

There is a ready answer for that question with regard to mitosis. Newly created cells resulting from mitosis can be followed visually as matching chromosomes line up in the middle of a parent cell and are then pulled apart, so that a full complement of chromosomes is delivered to each polar end of the dividing cell. Extensive experimental work since Walther Flemming first described the process in 1883 has detailed much of the molecular biology of this exquisite process, including the checks and balances ensuring that each of the daughter cells receives the appropriate array of chromatic material (Mitchison & Salmon, 2001).

But it is not nearly so obvious how chromatin streaming accomplishes what has always been considered essential to proliferation: the delivery of a complete genome to each of the progeny nuclei. The answer may rest in the thermodynamics of chromosome interaction with RNA, nuclear bodies, and nuclear membrane protein, as the complex system that is the nucleus seeks steady state equilibrium. Nevertheless, the optics suggest that the amitotic process, particularly by streaming, is almost certainly less reliable than the familiar "push/pull" of mitosis. And by extension, it might be expected that more of the nuclei formed by chromatin streaming may not contain the right number of chromosomes, a condition called aneuploidy. Thus if amitotic production of cells by chromatin streaming, an appropriate form of proliferation under some circumstances, begins to occur when it is not appropriate, the result could be enhanced production of aneuploid cells such as is observed in

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cancer where as it turns out, the number of aneuploid cells is reported to be significant, sometimes greater than 50%.

Short of the absence of a chromosome or the addition of an extra chromosome, multiple subtler changes are known to occur when the genomes of cells even in the same organ are compared. This phenomenon is called somatic mosaicism and the chromosomal alterations include deletions, amplifications, and translocations, most of which are assumed to occur during proliferation. Somatic mosaicism has proven to be more prevalent than previously suspected (O'Huallachain et al., 2012) and it is reasonable to assume that such deviations might also be more frequent in cells produced by chromatin streaming.

While aneuploidy in germinal cells is usually fatal, the consequences of somatic cellular aneuploidy are variable. Surprisingly, cells can survive and even function in the aneuploid state as is certainly the case with regard to somatic mosaicism. Weaver and Cleveland(2007) described observations suggesting that aneuploidy could be both an instigator and an inhibitor of tumorigenesis, a paradox that Sheltzer and Amon (2012) have reviewed pointing out that there even appear to be advantages in some examples of aneuploidy and mosaicism. But the possibility of genome variability in progeny cells, even now that it is understood to be more common than previously suspected, may be what most troubles biologists who are asked to consider evidence that amitosis exists alongside mitosis in metazoans.

Common forms of amitosis

Nevertheless, and even in a scientific climate of skepticism about the role of amitosis in the proliferation of cells in metazoans, researchers continue to discover examples of multiple amitotic processes.

Fissioning is an amitotic process whereby a nucleus presumed to contain two complements of DNA, splits with both halves moving apart as a cell membrane pinches off between the newly separated 2n nuclei. The process has been reported in placental tissue as well as in cells grown from that tissue in rats (Ferguson and Palm, 1976), in human trophoblasts (Cotte et al., 1980), and in mouse trophoblasts (Kuhn, Therman and Susman, 1991). Amitosis by fissioning has also been reported in mammalian liver cells (David and Uerlings, 1992) and human adrenal cells (Magalhaes, Pignatelli, and Magalhaes, 1991). Chen and Wan (1986) not only reported amitosis by fissioning in rat liver, but also presented a mechanism for a four-stage amitotic process whereby chromatin threads are reproduced and equally distributed to daughter cells as the nucleus splits in two.

There are other variations on amitosis. A couple of decades of research has shown that polyploid cells once assumed to be reproductive dead ends can be "reduced" to diploid or nearly diploid cells without the appearance of mitotic chromosomes. The nuclear envelope of the trophoblast has been shown to be involved in the subdivision of a highly polyploid nucleus into low-ploidy nuclei (Zybina, 1979; Zybina and Zybina, 2008). Polyploid cells are also of interest in determining how some cells survive chemotherapy. Erenpreisa and colleagues have shown that following treatment of cultured cells with mitosis-inhibiting chemicals (similar to what is used in chemotherapy), a small population of induced polyploid cells survives, greatly increasing its ploidy level through DNA synthesis without cytokinesis (endomitosis). Eventually this population can give rise to "normal" diploid cells by formation of polyploid chromatin bouquets that return to an interphase state, and separate into several secondary nuclei (Erenpreisa et. al., 2011). An intriguing system recently has been described in which polyploid cells formed in the Drosophila intestine can undergo de-polyploidization to form

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intestinal stem cells to replace stem cells lost when drosophila are starved for a period of two days (Lucchetta, E. M., & Ohlstein, B., 2017).

Additionally, there are multiple reports of amitosis occurring when nuclei bud out through the plasma membrane of a polyploid cell. Such a process has been shown to occur in amniotic cells transformed by a virus (Walen, 2002) as well as in mouse embryo fibroblast lines exposed to carcinogens (Sundaram et al., 2004). A similar process called extrusion has been described for mink trophoblasts, a tissue in which fissioning is also observed (Isakova and Shilaova, 2002).

A previously unreported kind of amitosis has been described by Thilly and his colleagues (2014) as occurring in the early weeks of embryo development in metazoans, when a more efficient approach to proliferation might be most needed to fulfill the production quota of trillions of new cells in 40 weeks. Cells from this stage have been called metakaryotic as opposed to eukaryotic and present a wonderland of gauzy and fragile bell shaped nuclei that proliferate by amitosis. In addition to being one more example suggesting that amitosis is relevant to at least some of the proliferation in metazoans, researchers offer the insight that DNA synthesis may involve a double stranded RNA-DNA intermediate in the alternative S phase of what has to be an alternative cell cycle. (Thilly et.al., 2014).

For the amitotic proliferation by chromatin streaming of cells within a dome, it is not known how much DNA synthesis and repair is carried out in addition to recycling of DNA from monolayer cells. The fact that domes can extend out into gland-like structures with the additional proliferation proceeding by the same method that created the dome suggests that DNA is being synthesized.

Niches for proliferation by amitotic chromatin streaming

What the various modes of amitosis have in common is that they are mechanisms for cell proliferation without the formation of chromosomes and without cytokinesis: two processes that obviously require time and energy. There may be circumstances when efficiency is most critical such as in the earliest stages of proliferation of some cells in a complete organism. Is cell proliferation by amitotic streaming most relevant for at least some of the 37.2 trillion human cells (according to a google search and “wonderopolis.org”) that need to be produced in a period of 40 weeks?

The two examples of chromatin streaming described in table 1 suggest additional situations that might favor amitosis by chromatin streaming. Possible deleterious effects of aneuploidy or mosaicism may be offset when progeny cells will be active within a colony and need to be produced rapidly. Perhaps it is less essential for every cell in a gland to contain every gene or even every chromosome if, in the aggregate, all of the genes have been passed on to one or more cells capable of synthesizing and secreting products into a common lumen.

An important aspect of the two examples of amitosis by chromatin streaming described in this paper is their anchorage independence. Is chromatin streaming the only proliferative process suitable for proliferation of anchorage independent cells? This is an intriguing possibility in light of the fact that metastases are believed to result from migration of anchorage-independent cells. But it is not hard to imagine that anchorage-independent cells play a role in normal fetal development and that the reappearance of such cells in the adult organism may be unwelcome anomaly.

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Finally, amitosis by chromatin streaming may be the metazoan's answer to the efficient proliferation of "disposable cells," cells whose future does not require the capacity for mitosis. Furthermore, there is some evidence that Ishikawa cells differ in their ability to effect amitosis by chromatin streaming. When Ishikawa endometrial cells were cloned, a range of dome-forming capacity was observed in the initial clones, including some clones in which syncytia formed but in which differeration did not proceed. Clones also differed with regard to the size of the domes that formed.

Amitosis by chromatin streaming outside the petri dish

In vivo results suggest that cells other than Ishikawa endometrial epithelial cells are capable of proliferation by chromatin streaming. Most of the examples are in cancers where much of what is "known" about cell structure for human cells is, of necessity, based on two dimensional microscopic data of processed tissue. Amitotic differentiation for Ishikawa cells discussed in this paper was studied in living cultured human endometrial cells by focusing on and above monolayers throughout the period of differentiation of a dome or spheroid, a perspective simply not available for excised cancer tissue. As this paper shows, the amitotic formation of cells in an arched membrane envelope deviates dramatically from mitotic formation of an attached layer of cells. Nevertheless, the resulting regular arrays of cells may not be readily distinguished in single cross-sections through excised tissue.

In an extremely valuable research project, Diaz-Carballo and associates (2014) studied sequential paraffin sections of chemotherapy-resistant tumors, looking for cell types able to survive an onslaught of cytotoxic drugs. Reconstructing the data from these sequences, the researchers found that the most strikingly amplified cell types were "spiral cells" marked by a number of nuclei arranged in a helical pattern around an empty central space, details that simply could not be revealed in "a stand-alone"cross section. This three dimensional structure of spiral cells enclosing a lumen is just what would be expected for the appearance of cells formed by amitotic chromatin streaming in a membrane envelope as described for dome formation (Fleming et al. 1998; Fleming 2016 a,b,c). Furthermore, the fact that such nuclei are formed amitotically is a possible explanation for why the cells are resistant to therapies that target mitosis. In the same paper, the researchers describe finding isolated nuclei in colonies and speculated that these nuclei had come together because cells had lost their cytoplasm. But colonies of nuclei such as those observed in figs. 9 and 10 are exactly what would be expected if the cytostatics had stimulated amitotic proliferation by chromatin streaming.

Hollow spheroids and even envelopes filled with cells are delicate structures that would almost certainly look different when fixed and stained as part of tumor tissue. Structures such as the spheroids in figs. 1 through 7 might deflate as a result of fixing, staining and sectioning, or as a result of even modest centrifugation forces. And at least some images of "hobnailed cells" frequently observed in cancers (Fadare et al, 2015) look like a multicellular spheroid that has been deflated. The aptly named envelope limited chromatin, observed in many different cell types, became an object of interest more than 50 years ago as reviewed by Davies and Haynes in 1975 and more recently by Olins and Olins (2009). The structure did not appear to fit in with the imagery of mitosis. In addition to its unusual geometry, there is frequently evidence that one edge of the envelope is in contact with cytosol, a fact that may spring from the lack of compartmentalization of chromatin within nuclei for the brief period

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of chromatin streaming. “Envelope limited chromatin” might result from the displacement of envelopes that were previously part of structures such as seen in figs. 3 through 6.

Furthermore, it has been possible to find descriptions of photomicrographs suggestive of proliferation by chromatin streaming in two dimensional sections through cancer tissue. In a thorough review of papillary thyroid cancer LiVolsi (2011) reports observations that could be explained if amitotic chromatin streaming is a relevant process in this kind of thyroid cancer. She describes “layers of cells with crowded oval nuclei,” so crowded that nuclei sometimes are overlapping. On the one hand, such overcrowding would be expected to result from active proliferation, on the other hand Dr. LiVolsi notes that mitoses are exceptional. Perhaps these nuclei result from amitotic proliferation by chromatin streaming.

For the histology of pancreatobiliary adenocarcinoma, Dursun et al (2010) describe “infiltrating nests of tumor cells with large vacuoles and “signet-ring” like appearance imparting a cribriform growth pattern. As has been described elsewhere (Fleming 2016a), signet ring-like appearance characterizes mitonucleons at the stage when the enlarging gas vacuole is pressuring chromatin against the mitonucleon membrane. Darsun goes on to say that the “vacuoles range from one to five cells in size, often merging to form multilocular spaces, separated by a thin rim of cell membrane.” Additionally the researchers observed that the vacuoles “compress the cytoplasmic organelles to the periphery of the cell, forming a thin bridging membrane.” This is a description that would probably characterize a cross section through multiple attached hollow spheroids, such as those in fig.4, if formed in the midst of tissue and not destroyed by fixing and staining.

In another study, a microcystic histological pattern with signet ring cells such as are frequently found in neoplasms of the pancreas and the brain was described for ovarian mixed epithelial carcinomas (Che et al. 2001) Vacuolated, cribiform, and microcystic, are terms frequently used to describe cross sections through cancer tissue. It is possible that gaseous vacuoles such as those formed in mitonucleons are the cause of at least some of these structures and suggests that amitotic proliferation may be ongoing.

Finally it is interesting to note that hyperplasia, uncontrolled proliferation that is not cancer- can give rise to cancer in some tissues, including endometrium (reviewed by Sanderson et al., 2016). Of particular interest to those studying hyperplasia in endometrium is the fact that squamous morules are a common component of premalignant glandular lesions. Squamous morules may be how spiral cells (or dome cells) present in a single section through excised tissue. And, as one important study noted, proliferation rates by mitoses were undetectable or extremely low for all cells in squamous morules in the endometrium of 66 patients (Lin et al.2009). The spiral cells as described by Diaz-Carballo and associates in chemoresistant tumor tissue might look like morules in single tissue sections.

If amitotic streaming is one source of hyperplasia, and if the resulting cells are more prone to aneuploidy, as might be expected without the checks and balances of mitosis, there could be multiple “karyotypic-phenotypic variations” in every morule detected in hyperplasia. Duesberg and his colleagues propose (Hirpara et.al.,2018), that while many karyotypic-phenotypic variations are not malignant, some might lead to malignancy. Many of the aneuploid cells will not even be able to undergo mitosis, although they may still generate new cells by amitosis. But among these karyotypic-phenotypic variations the “perfect storm” of variation might result in a cell capable of being cloned, and in the worst case scenario, capable of becoming migratory and invasive! At any rate, it would not be difficult to

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determine just how much aneuploidy is introduced in monolayers engaged in dome formation to test whether chromatin streaming does indeed result in aneuploidy.

Relevance of amitosis

The debate over a role for amitosis in metazoan cell proliferation is ongoing. In the first decade of the last century, Theodor Boveri made pronouncements on the matter that cell biologists continue to take very seriously. Boveri believed that cells produced by amitosis must, subsequent to being produced, be able to divide by mitosis to form cells with the correct number of chromosomes. This stipulation may not conform to the reality that some cells in the body are "terminally differentiated." The epithelial cells that form glands in the uterine endometrium are among the most accessible of terminally differentiated cells in adult organisms. Most of the cells generated in such glands are flagrantly "terminal" if/when implantation does not occur as well as post partum. Our studies with Ishikawa endometrial cells *in vitro* demonstrate that the process of gland formation can be initiated and extended by mitonucleon-dependent amitosis. There does not appear to be a need to prove that these cells can revert to mitosis, although it is altogether probable that stem cells are left behind following menses that do still divide mitotically.

It was Boveri who recognized that large populations of aneuploid cells could be found in tumors (Holland & Cleveland, 2009). This observation has been taken as an indication that some event(s) has shifted the mitotic process from mostly reliable as would seem to be the case in normal tissue (Knouse et.al., 2009) to distressingly unreliable in cancer (Bakker et al., 2016). A simpler explanation would be that aneuploidy becomes more evident when an amitotic form of proliferation such as chromatin streaming is producing a larger proportion of progeny cells. Chromatin streaming and the resulting amitotic proliferation could contribute to the tumor cell population and generate "karyotypic-phenotypic variations" capable of becoming malignant.

Chromatin Streaming and Cell Dogma

Boveri also said that amitosis must give rise to separate nuclei within a cytoplasmic domain, granting his imprimatur to the theory positing the cell (nucleus plus cytoplasm) as the most basic unit of a biological organism (Boveri, 1907). But as Baluska et al.(2004, 2012) have reminded us in at least two reviews, cell theory (now cell dogma) is flawed. Unifying as it may be to assume that all cells must come from cells and that the cell is the smallest indivisible unit of biological life, Baluska, a researcher knowledgeable about the plant as well as the animal kingdom described ways in which cell dogma falls short of explaining cell organization in the plant kingdom (Baluska et al., 2004).

Furthermore the process being described in this paper demonstrates the self assembly of nuclei from chromatin granules as the starting point for the formation of cells that fill envelopes, even if some of those cells are not "normal." Nuclei form in a colony and then move out of the colony by a process that most likely involves "cell bodies," nuclei plus microtubules, a concept originating from Daniel Mazia (1993) and expanded on in Baluska's review (2004).

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Images of this process were among a number of surprises in the final phase of formation of dome cells (Fleming, 2016c). In some of these pictures, multiple nuclei are observed side by side within the envelope created by apical and basal membranes. In other pictures, single nuclei appear to have spiraled out of the tight association characteristic of their formation. By the end of the differentiation process, nuclei have moved to equidistant positions that render them a "sheet", albeit a "curved sheet". The formation of cell membranes around equidistant nuclei in cytosol seem to be the final essential structural event in dome formation and in multicellular spheroids. Although membrane repair has been very much in evidence as daughter cells complete cytokinesis, it was shown only recently it was shown that plasma membranes can form *de novo* in animal cells (Shimoda, 2004), and this appears to be what happens.

In these details, amitosis by chromatin streaming appears to embody an exception to cell theory. A piece of the puzzle will be to determine how DNA synthesis and/or repair is controlled during the amitotic cycle. The amount of DNA in the initial output of dome cells may match the amount of DNA from the recycled monolayer cells that form syncytia. However further outgrowth of domes into gland-like structures suggests that DNA is being synthesized (Fleming, 1999).

Gaseous vacuole formation within giant spheroidal mitochondria

Approximately halfway through dome formation, one must focus above the Ishikawa monolayer in order to detect structural changes. As pre-dome structures move cell material up into the third dimension, the most dramatic change is also the most difficult to detect and record. Gossamer membranes unfurl from each of the mitonucleons. In profile, it is possible to detect that a chromatin aggregate in the familiar shape of an ovoid nucleus is initially at the top of the developing protrusion. The nuclear structure rapidly falls apart and spreads out of the protrusions down into the cytosol at the base of the protrusion beginning the process of chromatin array formation.

Much of what happens in the formation of hollow spheroids also occurs above the monolayer . At some point, the detaching cell/spheroid begins to float up from the monolayer, tethered to the colony for a period of time by cell processes that may be like cytonemes, thin processes extending from cells and believed to be involved in cell communication (Sugata and Kornberg, 2014). Eventually the spheroid becomes free-floating (Fleming, 2018a). The obvious question is what creates the pressure that unfurls membranes and the buoyancy that elevates detached cells/spheroids (Fleming, 2018b). The likely answer, given how quickly the pressure builds up (and dissipates) seems to be that a transient gas vacuole forms. And, in fact, dissipating gas bubbles are fleetingly visible when a hollow spheroid reattaches (Fleming, 2018a). Much less obvious is the identity of the gas or gases.

The fact that giant mitochondria were involved in the buildup of vacuoles initially suggested that CO_2 might somehow be retained since it is an abundant by-product of glucose metabolism by oxidative phosphorylation in mitochondria. The decades-old research that CO_2 passes freely out of cells has been challenged by studies that look at changes in the rate of CO_2 passage through membranes of differing composition (Weisbren et.al.,1994; Nakhoul et al., 1998, Itel et. al. 2002, Endevard & Gros 2012).

However, there is evidence that these mitochondria undergo structural changes seemingly essential for their ability to form transient gas vacuoles but antithetical to the process of oxidative phosphorylation. In the context of research done over the past two decades it would

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seem that mitochondria are capable of a variety of activities in addition to oxidative phosphorylation (Roger et.al.,2017). Research into mitochondrial membrane structure has revealed that there are at least two different profiles of inner mitochondrial membranes. Christae, a membrane structure essential for the electron transport characteristic of oxidative phosphorylation is what is usually represented in cell models. But more recently tubular membranes have been described, and the morphology of these membranes suggests that they would be better able retain gas (Frey and Manella). In fact such vesicles are observable in giant mitochondria in Ishikawa epithelial cells (Fleming, 2018b).

Sun and colleagues showed such vesicular membranes forming in some mitochondria soon after HeLa cells were exposed to etoposide. It was further shown that morphologically altered mitochondria lose the membrane potential essential for oxidative phosphorylation (Sun et. al. 2007). The resulting vesicular mitochondria ultimately become swollen as the potential of mitochondrial membranes drops to zero. Mitochondria whose inner membranes have been remodeled into vesicles are no longer able to generate CO_2 by oxidative phosphorylation, nevertheless they do swell. If that swelling is due to production of a gas, might it be hydrogen?

Discoveries in the past 20 years have suggested that there may be a continuum among organelles that generate only hydrogen, the hydrogenosomes, and mitochondria. Müller (1993) and his colleagues pioneering research in hydrogenosomes has explored a dizzying variety of metabolic schemes involved in the production of H_2 (Muller et al., 2012), during anaerobic energy metabolism in eukaryotes. With a particular interest in the evolutionary relationship between hydrogenosomes and mitochondria Embly et al. (2003) have concluded that the "facility by which ciliates make hydrogenosomes must result from modification of pre-existing mitochondria." Stechmann and colleagues (2008) have demonstrated that the unicellular anaerobe Blastocystis has organelles that have metabolic properties of aerobic and anaerobic mitochondria as well as of hydrogenosomes. And, finally, genes have been found bearing the hallmark signatures of [Fe]-hydrogenases on the human genome and in the genomes of other aerobic eukaryotes (Horner et al. 2018). It may be possible that while a permanent hydrogenosome cannot be found in metazoans, the organism can convert some parts of a giant mitochondria into an organelle that can generate H2, a gas eminently suitable for conferring buoyancy on detached biological structures.

In addition to synthesizing the enzymes necessary for H_2 generation, it is essential that the process of mitochondrial christae conversion into vesicles create a transiently anaerobic environment in which H_2 can be generated. The informed reader might well ask: how likely is it that the mitochondria of an aerobe, even if fusion events result in a giant mitochondrion, would protect an anaerobic enzymatic reaction. Amazingly enough, Inui and his colleagues demonstrated exactly this phenomenon in Euglena. They characterized a pyruvate:NADP+ oxidoreductase that is highly sensitive to O_2 inactivation if extracted from the euglena mitochondria but is stable in isolated, intact giant mitochondria. (Inui et al. 1990)

These observations are being made at the same time as studies suggest that mitochondria are much more adaptable than previously understood with regard to metabolism. A long standing observation by Otto Warburg (1953) demonstrated that aerobic glycolysis and not oxidative phosphorylation was the preferred form of metabolism in cancer cells. Warburg concluded that mitochondria had been damaged. Recent studies are focusing on observations that some of the resulting glycolysis actually occurs in mitochondria. This fact is some of the evidence that mitochondrial energy pathways can be reprogrammed to meet the challenges of high

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energy demand, better utilization of available fuels and macromolecular synthesis for rapid cell division and migration. (Jia et al. 2018). It is this apparently still unfolding story of how much more there is to know about mitochondria (Tielens et al., 2002), together with the suspicion that the physical characteristics of H_2 might best fulfill the functions described in this paper, that lead to my suggestion that cup-shaped or giant spheroidal mitochondria might be capable of a transient generation of H_2 in this and other examples of the functioning fused mitochondria. Nebenkern, involved in spermatid tail elongation in insects, is a structure recently discussed in this regard (Fleming, 2018b).

Gas vacuoles in chromatin

In addition to a large gaseous vacuole, small bubbles are observed in aggregated chromatin in mitonucleons in differentiating Ishikawa cells. The nuclear "clearing" resulting from these bubbles is a phenomenon apparently underlying so-called optically clear nuclei (Hapke & Dehner, 1979) that have been observed in cancer tissues over the past 50 years. (reviewed in Fleming 2016a). These bubbles do not appear to have any continuity with the large vacuoles in giant mitochondria that we have been discussing.

Gaseous neurotransmitters such as nitric oxide (N0), carbon monoxide (C0) and hydrogen sulfide (H2S) are involved in physiological functions (Li & Moore, 2007), although they are generally assumed to be present in small quantities and to have short half lives. NO has been implicated in apoptosis (Chung et al., 2001) and has been shown to stimulate mitochondrial biogenesis (Nisoli E. & Carruba MO, 2006). One example of fatal NO production was shown in the release of a bubble of NO from nuclei of cells subjected to cold shock (Chang et al., 2002). Perhaps the profound changes that occur in nuclei during differentiation of Ishikawa monolayer cells into domes or into spheroids are the result of the accumulation of gaseous neurotransmitters.

Spheroids, Mitochondria and Metastasis

Spontaneous formation of a polyploid unicellular hollow spheroid described for Ishikawa monolayers is one of the two mitonucleon dependent amitotic processes described in table 1. The giant mitochondrial structure that surrounds the chromatin of aggregated nuclei is therefore central to the formation of unicellular and multicellular hollow spheroids. From a number of clever experimental approaches, additional evidence is accumulating that implicates mitochondria in metastases. Noting that an inactivating mutation in mitochondrial respiratory chain complex I enhanced the ability of Lewis cells (lung carcinoma cell line) to metastasize (Ishikawa et. al. 2002), Porporata and colleagues (2014) pursued the existence of a metabolic phenotype associated with tumor metastases in their ovarian carcinoma cell line. In light of studies with mitonucleons, it seems relevant that their selection process resulted in the creation of a cell line that was significantly more invasive than the parental cell line and contained giant mitochondria not observed in the parent cell. Farnie and his associates (2015), studying breast cancer cell lines used a flourescent tag to separate out cell populations with higher than average levels of mitochondrial material (mito high) and those with lower than average mitochondrial material (mito low). Their mito high breast cells formed mammospheres more efficiently and showed a 2.4 fold enrichment in tumor-initiating cell activity over the mito-low cells.

These studies taken together link giant mitochondria with spheroids and with metastases. In endometrial epithelia, the formation of giant mitochondrial structures that envelop aggregated chromatin has been shown to be the starting point for the spontaneous formation

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of hollow spheroids. This paper shows that by amitotic chromatin streaming, unicellular hollow spheroids can be converted into multicellular hollow spheroids. By reason of their mobility either one of these structures are possible vectors in endometriosis and perhaps even metastatic cancer, a possibility supported by the history of observations (Allen et al.,1987; Shield et al. 2009) of hollow spheroids in malignant ascites fluid (reviewed in Fleming, 2018a).

The phenomenon of amitosis by streaming chromatin, along with evidence that such a process may occur in cancer tissue, is an intriguing possibility. For one thing, it might explain why some cancers become resistant to chemotherapy. If proliferation in some cancer tissue occurs by a combination of mitosis and amitosis, it would suggest that tumors could become chemoresistant when/if the process of amitosis becomes responsible for most of the proliferation. If this form of cell proliferation is most characteristic of developing embryos, its reprise in tumors might not be surprising in the context of research that has uncovered similarities between early embryo development and tumorigenesis (Ma et al.,2010).

As already noted once the process of amitosis begins,the resulting structures are extended by amitosis, suggesting that many different karyotypes could be formed. According to a timeline in a recent review (Ye et al.,2018), aneuploidy is the first step in the development of a clinically detectable tumor. The authors suggest that if elevated chromosome instability follows, along with macro-and micro cellular evolution, aneuploidy can result (at least some of the time) in malignant cancer. It is possible that the production of cells by amitotic chromatin streaming is responsible for the hypertrophy that sometimes, but not always, becomes malignant.

As researchers continue to study therapeutic agents capable of slowing down or stopping mitotic proliferation in cancer, it may be possible to find agents capable of slowing down amitotic cell proliferation. A proliferative process that is altogether different from mitosis, but could be a factor in the progression of cancer, seems to be worth further investigation.

Materials and Methods

All of the photomicrographs in this paper are of cultured endometrial epithelia called Ishikawa developed by Nishida and colleagues in 1985. His laboratory established that the line contained receptors for both of the female sex steroids, estradiol and progesterone. The cells were obtained from Erlio Gurpide's laboratory at Mt. Sinai, New York. It is relevant that the unicellular hollow spheroids described in this report would probably be destroyed by many of the methods commonly used to prepare cell specimens for microscopy. The handling of medium containing unicellular hollow spheroids was kept to a minimum.

As described in the first paper on the topic of dome formation in 1995 (Fleming), the cells were cultured in phenol red-free Minimum Essential Medium (MEM) supplemented with 2mM glutamine, 100U/ml penicillin, 0.1 mg/ml and .25 mg amphotericin B (GIBCO, Grand Island, NY). Cells were seeded at an approximate density of 5×10^5 cells/cm^2 in MEM containing 5% calf serum (CS) at 37 degrees C and 5% CO_2 were grown for at least one week. Cells were viewed using an Olympus inverted stage microscope at powers of 100X (fig1a), 200X (fig.1b) and 400X (all other figures). All of the structures in figs. 1 through 11 were photographed in living cultures.

Spontaneous hollow spheroids were observed to form from monolayer cells when medium without serum was added to cultures. Hollow spheroids were transferred into petri dishes using techniques least likely to disrupt their delicate three dimensional structures. In point of fact medium bathing the monolayer was simply decanted, carefully in a laminar flow hood. Serum was added to some of the dishes containing hollow spheroids. Some of the hollow spheroids maintained without serum spontaneously initiated amitotic cell proliferation as described in this paper. Solid spheroids were formed by plating cells in medium without serum. Some of the solid spheroids

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developed attached hollow spheroids as previously described (Fleming 2018) Solid spheroids were decanted fro dishes and serum was added to the medium. The cultures in figs. 9 and 10 were observed to form, with extensions characteristic of amitotic proliferation.

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