Tubular membranes extended between monolayer cells, from solid spheroids, and from clustered hollow spheroids in Ishikawa endometrial cell cultures can carry chromatin granules and mitonucleons

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Abstract

Membrane tubule extensions, recently recognized an important communication element in mammalian cells are demonstrated to form in Ishikawa endometrial epithelial cells growing in monolayers, and to extend from solid spheroids and from clustered hollow spheroids. Two kinds of chromatin cargoes travel through these tubules. Chromatin granules can pass through an endometrial tubule bridge extending from one monolayer fragment to another. The passage of granules over time from one of the fragments appears to support the self-assembly of nuclei in the other colony fragment. Similarly, in a process detected by observing an open-ended membrane tubule extending from a solid cell spheroid a nucleus formed over a period of 3 hours. Indications are that chromatin granules such as those observed in the amitotic processes of epithelial dome cell formation and of hollow spheroid cell formation are contributing to the build up of nuclei. Mitonucleons, a transient subcellular organelle consisting of fused mitochondria intimately associated with aggregated chromatin are also observed to pass through tubular membrane extensions. Multiple membrane extensions can be shown to extend from clusters of unicellular polyploid hollow spheroids whose formation is described for the first time in this paper.

Introduction

The existence of tubular extensions between cells has emerged as a particularly exciting area of research relatively recently in the century-plus history of cell biology. Throughout that same century, biologists, especially those studying development, understood the importance of communication between and among cells in multicellular organisms, particularly the role of directed morphogen diffusion (Müller et al., 2013). The advantages of even more direct communication between cells were obvious, but typical methods of investigation, such as those involved in fixing and sectioning tissue were not ideal for detection of intercellular conduits, delicate structures (frequently less than a micron wide) that stretch over distances of hundreds of microns. Even in living cultures, it’s essential to look sharp to detect membrane tubular extensions.

Researchers studying sea urchins and cultured insect cells are credited with some of the earliest observations of “communication” by membrane tubules, frequently called filopodia. Karp and Solursh (1985) culturing primary mesenchymel cells from sea urchins demonstrated thin, elongated, filopodia between cells in culture. Locke (1987) studied filopodial bridges in cultured insect cells, forming between cells “losing” contact as they moved away from each other and suggested that such
membrane tubules re-established or maintained cell-to-cell continuity. Tubules were also observed in the normal course of tissue morphogenesis. Ramirez-Weber and Kornberg (1999) identified cellular processes that they called cytonemes projecting from wing disc cells to the signaling center in Drosophila. Relevant to the discussion in this paper, the researchers also described the formation of similar cell-to-cell extensions in co-cultures of wing disc and signaling center cells. Much of what was discovered for sea urchins and insects was “rediscovered” in mammalian cell cultures starting with Ramirez and Kornberg’s observation that cultured mouse limb buds also formed cytonemes.

Despite that foreshadowing, it took some time for this new research area to gain acceptance. To get a sense of the trauma that can accompany the appearance of an exciting but challenging “new discovery” (or even sometimes its rediscovery [Platner 1886 and Flemming 1891]), read Monya Baker’s news feature “How the Internet of cells has biologists buzzing” (2017). Not only does Ms. Baker clearly and concisely describe seminal research; she evokes the conflicts and self-serving skepticism that often accompany a challenge to orthodoxy. Scientists and their enterprising postdocs “soft-peddle” exciting discoveries until at least some of the establishment catches up with them. Publication of a relevant research paper from the laboratory of a prominent scientist can be an ice-breaker, but it takes time for the word to spread. Along the way grant applications with resources on the line, sometimes bring out the worst of “peer judgement” (is the principal investigator concerned that what appears to be a tubule might actually be a scratch in the petri dish?). And when all else fails, there is the hard-to-answer (and equally hard to credit) suggestion that perhaps cells have learned tricks in petri dishes that have nothing to do with what happens in vivo (because we know exactly what that is with our mostly two-dimensional view of “pickled” tissue). Initial reactions to the reality of membrane extensions, as described by Ms. Baker, illustrate the paradox of a field of study dedicated to discovery but predisposed to reject what are perceived to be challenges to beloved orthodoxies rather than relevant refinements. Inevitably, as has happened many times before, skepticism was mostly overcome, hopefully without any damage to hapless graduate students or postdocs whose work on membrane tubules appeared too early in the cycle.

Amin Rustom and his colleagues in Dr. Hans-Hermann Gerdes laboratory showed elegant pictures of tubules stretching from cell to cell in a rat pheochromocytoma cell line (2004). The research team demonstrated that these tubules carry endosomes, calling the structures “tunneling nanotubes” to convey something about size and function. In that same year, Onfelt and colleagues (2004) presented evidence that “nanotubular highways” (their preferred descriptive name for tubules) represent a novel mechanism for intercellular communication in immunology. Additional discoveries were made in the Gerdes laboratory where, approximately 10 years later, Gerdes and his colleagues began the important process of comparing and contrasting the data accumulating about membrane tubules in a review that he unfortunately was unable to see to completion (Austefjord et al. 2014).

During the decade following the introduction of tunneling nanotubes, research scientists produced evidence that tubular extensions were not only relevant for in vivo communication but also carried various and important cargos from one cell to another. Lou and his team (2014) published pictures of membrane tubules in cultured cells from human mesothelioma lung cancer and studied cultured fragments of tumor tissue from that same disease. By maintaining the three dimensionality of tissue, the latter technique promises a close approach to observation in vivo and tunneling nanotubes were found to be present. In an experimental tour de force, Dr. Frank Winkler and his 39 co-researchers published a paper in 2015 that “looked” into the brains of mice injected with cancer cells derived from human brain tumors. Not only did they observe tubular membranes galore developing among the cells, they pinpointed the growth of tubules (8 to be exact) that could be shown to deliver a new nucleus to
an area in the living mouse’s brain that had suffered the targeted death of a single cell from a laser assault, a remarkable observation (Osswald et al., 2015).

Research into tubular structures continues to flourish with studies on structures known by a variety of names including cytonemes, filopodial bridges, tunneling nanotubes, cellular bridges, nanotubular highways, tubular bridges, and others (Sisakhtneshad and Khosravi 2016; Vignais et al., 2017; Yamashita et al., 2018). Yamashita and colleagues suggest that while honoring each of these names, the entire category of structures could be called “thin membrane protrusions” a term sufficiently inclusive to call up more than 8000 references in the NIH “Pub Med” data base. The existence of membrane tubules actualizes the staggeringly important, and no longer underappreciated, fact that there is significant continuity of cytoplasm and membranes in complex multicellular animal systems (Rustom, 2016). As will be discussed, researchers have made many relevant observations about tubules and there is clearly more to be learned. The question of names is being sorted out as additional examples of tubules are described. Frankly the process of finding names that provide some indication of function is ongoing even for an organelle that has been the object of study for much longer. Mitochondria whose distinction is enlargement by fusion of smaller mitochondria have variously been called: nebenkern, spheroidal mitochondria, and cup-shaped mitochondria. In endometrium researchers, by serial cross sections, demonstrated that what was once thought to be multiple average sized mitochondria were actually part of a giant mitochondrion-endoplasmic reticulum unit made up of fused mitochondria (Armstrong et al., 1983). These structures are similar to those observed in studies of Ishikawa endometrial epithelia where the term mitonucleon is used to signal the intimate association of fused mitochondria with chromatin (Fleming, 2018b) essential for differentiation of domes that elongate into tube-like structures and hollow spheroids (Fleming, 2018a).

This paper demonstrates for the first time that mitonucleons can be carried by membrane tubules that form in cultures of Ishikawa endometrial cells. Calibers of the tubules observed in Ishikawa epithelial cells extend from hundreds of nanometers (almost the limit of what can be seen by light microscopy) to approximately 1-3 micron, somewhat larger than cytonemes and tunneling nanotubes but close to what has been observed for bronchial epithelial bridges that have been shown to carry whole cells (Zani et al. 2010). Additionally the transport of chromatin granules between Ishikawa endometrial epithelial cells, from spheroids and from clustered hollow spheroids formed by these cells, will be described in this paper.
Results

A photomicrograph of an endometrial epithelial bridge (approximately 2.5 microns wide) extending from a monolayer Ishikawa endometrial cell to a neighboring Ishikawa colony over a distance of around 225 microns is shown in fig. 1. The tube emerges from a nucleolus in one cell and fuses with a cytoplasmic ruffle on the edge of a neighboring colony. Serum-free medium had been added to this dish of cells two days previously. A low level of serum has been recognized as one of the conditions that elicit the formation of tubular extensions (Lou et al. 2012). The tubule itself between a cell and a thriving colony some distance away might be the prototype for many of the tubular extensions observed in other cell cultures although the tubules do not always originate from nucleoli.

The second not so typical membrane tubule, shown at two different times in figs. 2 and 3, links two colony fragments in a monolayer stressed by the absence of serum from medium for five days. Many monolayer cells detach under those conditions. The two colony fragments in fig. 2 persisted and established a tubular connection that is a little longer (approximately 280
microns) and a little thinner (1 to 2 microns) than the process observed in fig. 1. The fragment on the right in fig. 2 contains a pair of unusual structures within a dramatic flourish of membranes. What appears to be the donating colony fragment contains granules concentrated along one of the membranes leading to the opening into the tubule.

Fig. 2 Two membrane fragments in a stressed monolayer linked by a thin membrane tubular bridge. Neither fragment is typical of what is usually observed for colonies of these cells, perhaps because the monolayer was stressed by the absence of serum for 5 days. Bar = 50 microns

Fig. 3 shows these structures after 3 hours. An amazing transformation has occurred in the colony fragment on the right. It has doubled in size, two apparently typical nuclei fill out most of the membrane flourish. The concentration of granules in the colony fragment on the left has diminished and at least three small vacuoles have appeared. Fig. 4 shows enlargements of the structures in figs. 2 and 3, with panels a and b contrasting the anucleic fragment seen at the start of the observation and three hours later; panels c and d contrast the colony fragment in which nuclei form over that same time period.
Fig. 3 The appearance of both fragments changes dramatically over the period of three hours. Most of the dark granular material at the mouth of the tubular extension of the structure on the left has disappeared and small vacuoles have appeared. Two nuclei are being built up in the fragment on the right. The granular outlines of the nuclei resemble images of formation of nuclei in hollow spheroids due to chromatin streaming (Fleming, 2019) Bar = 50 microns

Recently published evidence that chromatin granules amitotically form nuclei in hollow spheroids (Fleming, 2019) bolsters a theory that chromatin granules passing through the membrane tubule over a period of three hours contributed to the formation of two nuclei. Granularity is still evident in the nuclear membranes in the enlargement shown in panel 4 d. Similar granularity was observed in nuclei formed in hollow spheroids “caught” in the process of adhering to the surface of a petri dish during amitosis (Fleming, 2019). Dispersed chromatin was initially shown to be involved in the differentiation of monolayer Ishikawa endometrial epithelia into detached dome cells (Fleming 2016 a,b,c) and subsequently as the product that appears when giant polyploid nuclei in “unicellular” hollow spheroids “deconstruct”(Fleming 2019). This paper suggests that chromatin material, specifically chromatin granules, can be added to the list of “cargos” for thin membrane tubules that includes among other things: prions (Gerdes, 2009; Goussset et al. 2009), retroviruses (Xu etal., 2009; Rudnicka and Schwartz, 2009; Panasiuk etal.,) and, as will be discussed in some detail, mitochondria (Vignais et al., 2017).
Fig. 4 Enlargements of structures at the ends of the tubule in fig. 2 and fig. 3. Panels a and c are enlargements of the photomicrograph taken at the start of the observation (fig. 2); panels b and d were enlarged from the photomicrograph taken three hours later. Granules are observed concentrated at the entrance to the tubule in panel a. Some kind of structure, perhaps scaffolding for nuclei can be detected filling about half of the membrane in panel c, along with an elaborate flourishing membrane of about equal size. Panel d shows that most of the membrane scaffolding fills out, doubling the size of the fragment with the formation of two nuclei.
Fig. 5 A flourish of membranes at the end of a tubular process extending from a solid spheroid. When this tubular extension was initially observed in a living culture, there was little structural detail in the membranes extending out from the end of the process flowing from a solid spheroid. Dark material and a bulge of autofluorescent material can be detected in the tubule. These materials are observed to move during the 3 hours between the observations of the structure in figs. 5 and 6. What appears to be a nucleus is forming in the membrane flourish at the end of the tubule. Bar = 50 micron

Fig. 6 Material flowing into the membrane flourish appears to be self assembling into a nucleus. Bar = 50 micron

The tubule in fig. 5 is approximately 250 microns long, with a prominent bulge 75 microns from the spheroid and a flourished membrane at the end of the extension. Over a period of 3 hours, the bulge moves 75 microns toward the membrane flourish (fig. 6). In that same period, the outlines of something resembling a nucleus become visible within the flourish at
the end of the tubular process. The material passing through the tubule is not uniform. Dark granular material and auto-fluorescent material can be detected.

Fixing and staining tubules arising from solid spheroids reveals some of what is being transported from solid spheroids into tubular processes. A large, perhaps polyploid, nucleus stained with hematoxylin is apparent at the end of the process in fig. 7. A thin rim of endogenous biotin stained with avidin-linked peroxidase and AEC surrounds the nucleus. Hematoxylin stained granular material can be observed in the cytoplasm surrounding the nucleus. Approximately half-way down the tubule extending from the spheroid is another structure staining both for chromatin and for endogenous biotin. The other half of that structure can be seen in fig. 8, showing the solid spheroid from which the tubular extension originates with granular material “entering” the tubule and a clearer picture of the structure traveling through the extension. Chromatin is surrounded by endogenous biotin in a structure initially observed in syncytia formed in a monolayer of Ishikawa cells stimulated to form domes (Fleming 2016a) and ultimately shown to result from mitochondria fusing around aggregated chromatin (Fleming 2018a).

Figure 9 is a tubule that contains two mitonucleons that may be further along in formation. The mitonucleon on the right appears to have begun generating and retaining gas, a process that has been discussed in some detail for dome formation (Fleming 2018b), and that occurs in the development of a hollow spheroid from a monolayer cell (Fleming, 2018a).
Figure 8. Process extending from a solid spheroid. Granular material can be detected at the mouth of the tubule. Material halfway down the tubule (also visible in fig. 7) stains for the endogenous biotin of mitochondria and a central core of chromatin characteristic of mitonucleons. Bar = 50 microns

Figure 9. Tubular membrane process extending from another solid spheroid contains two structures that stain like mitonucleons. One of the structure appears to have already started to generate a gaseous vacuole. Bar = 50 microns
Clusters of unicellular hollow spheroids are also capable of forming membrane extensions.

Extensions similar to those emerging from monolayer fragments and from solid spheroids have also been detected extending from clusters of hollow spheroids, shown in this paper for the first time. Looking like a bunch of grapes and staining for endogenous biotin, a cluster of enlarging cells is shown detaching from a monolayer colony in fig. 10. Multiple hollow spheroids appear to form in the same way as a single hollow spheroids (Fleming 2018a), starting with monolayer cells in which spheroidal mitochondrial structures staining for endogenous biotin appear to completely surround aggregated chromatin material. (Fleming, 2016a). The final point of attachment is itself a tubular membrane extending back into the monolayer. The developing hollow spheroid cluster can float above the monolayer, as was observed for single hollow spheroids (Fleming, 2017a), as gas vacuoles continue to expand (fig.11). As vacuoles reach the limits of their expansion, the resulting structure looks like a “beehive” (fig 12). Each of the hollow spheroids is outlined by endogenous biotin that has expanded with the mitonucleon to the point where it is compressing nuclei and other cell structures into a
spheroidal rim against the cell membrane (Fleming 2018a). Fig. 13 shows a beehive structure, not fixed and seemingly slightly deflated but clearly showing nuclei or chromatin granules in spheroid rims. The outermost rims surrounding vacuoles appear to be continuous at some points.

Figure 12. The endogenous biotin previously shown to be bound to mitochondrial carboxylases highlights the membranes of a cluster of hollow spheroids. The focus of the microscope is on the surface of the multiple spheroid structure. The structure may be in the process of becoming anchored to the petri dish by a structure at the top of the beehive. The entire structure is larger than 250 by 200 microns. Bar = 25 microns

Figure 13. A cluster of hollow spheroids whose irregular shapes suggest deflation. In a single hollow spheroid, nuclear and cytosolic material are pressured against the cell wall by developing internal vacuoles. This photomicrograph demonstrates that clustered hollow spheroids are similarly structured with the possibility that the rims of individual hollow spheroids can run together within a cluster. Bar = 25 microns

A structure that looks like a nucleus can be observed in the interstitial space between two of the beehive “chambers” in fig. 13. It may be relevant to emphasize that the cluster is made up not of multicellular hollow spheroids but rather of polyploid hollow spheroids that are in effect “single cells,” albeit extraordinary cells whose monolayer attachments to each other survive through the period of time between the release of a cluster of what were monolayer cells to the appearance of what is a cluster of hollow spheroids.
Figures 10 through 13 illustrate the variability in sizes of hollow spheroids, an obvious function of the increasing size of the gas vacuoles. Furthermore, one especially large vacuole in the structure in fig. 13 suggests that vacuoles themselves may fuse.

A cluster of hollow spheroids can, under appropriate conditions such as the addition of fresh medium with added glucosamine but without serum, “bristle” with multiple membrane extensions from the surface of a structure that is, as already described, mostly vacuoles surrounded by rims of cytoplasm containing nuclei (figs. 14 and 15).

Figure 14 is a photomicrograph of the upper most surface of this three-dimensional cluster of hollow spheroids. Two prominent extensions arise from nucleoli (much like the extension shown in fig. 1). Focusing down from the surface of the cluster, fig. 15 brings the ends of these extensions into focus. One of the extensions is capped by a flourish of membranes much like what was observed in fig 5. The other process ends with two “rectangular” cargoes. The presence of an electron lucent structure in the midst of the membrane flourish, and in one of the rectangular packages, suggesting that the rectangular package may be an as yet unfurled membrane flourish.
Figure 15. Honeycomb of hollow spheroids bristling with tubular membrane extensions. Fillapodia extend out from the edges of some of the hollow spheroids and these also have tubular membrane extensions. The hollow spheroids are approximately 40 microns. An enlargement of the tubule extending from the right side of the honeycomb is shown in fig. 16.

Filipodia extending out from the structure are sprouting additional shorter tubules. Tubular extensions range from barely visible by light microscopy (hundreds of nanometers) to micron size. No details of structure can be detected in the hollow spheroids that cap off the ends of two extensions. Experiences with single hollow spheroids (Fleming, 2018a) suggest that one possible explanation is that nuclei compressed against the rim of the structure are on the other side of the spheroid. Interestingly, there is, what appears to be debris associated with each of the spheroids.

The intriguing tubule extending from the right side of the cluster, enlarged in fig. 16, contains a bolus of material, as well as a membranous flare and is capped by a hollow spheroid. Granular material comes into focus in the membrane flare. The sum total of these various extensions suggest that this intimidatingly complex beehive structure is prepared to “put down roots” using some of the structures that have been the subject of this paper. The clusters of hollow spheroids, in figs. 11 through 16 outlines one way in which a cluster of cells may successfully move from attached monolayer to mobile structure to re-attaching mass, an important “trick” in the playbook of cancer metastases.
Figure 16. Scanning the 35mm photomicrograph of a membrane flourish in fig. 15 at a higher resolution reveals that the contents of the flourish are granular.

Discussion

Research over the past 20 years has put to rest any doubts about the existence of membrane tubules and their importance in cellular physiology. The field is indebted to the authors of comprehensive reviews (Sisakhtnezhad and Khosravi 2016; Vignais et al. 2017; Yamashita et al. 2018) who have documented the appearance of tubular membrane extensions in more than 20 different cell lines as well as in tissue cultures and in a living brain. The evidence in this
paper demonstrates, for the first time, that such structures are also formed by endometrial epithelial cells (Ishikawa cell line from the laboratory of Nishida et al., 1985).

What tubules carry
The initial function of membrane tubules was understood to be the passage of morphogens, and perhaps ions such as calcium, from one cell to another. But intriguing bulges led scientists early on to investigate whether larger cargoes could pass through tubules or bridges. Not only was this discovered to be the case: researchers demonstrated physiological changes accompanying at least some of the cargoes, highlighting the importance of membrane tubules to cell communality. Among multiple examples, it was shown that endothelial progenitor cells took on a cardiomyogenic phenotype as a result of the transport of organelles, including mitochondria, through membrane tubules that form between the two cell types in co-culture (Koyanagi et al. 2005). Spees and colleagues (2006) demonstrated that mitochondrial transfer through membrane tubules from adult stem cells can rescue aerobic respiration in mammalian cells with nonfunctional mitochondria. In a co-culture of bone marrow derived mesenchymal and endothelial cells, it was shown that the transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance of the cancer cells (Pasquier et al., 2013). One more observation is that the transfer of mitochondria via tunneling nanotubes can rescue apoptotic PC12 cells. (Wang & Gerdes, 2015)

Another important fact about membrane tubules, learned early on, is that their numbers increase when cells are stressed. Donghui et al. (2005) demonstrated that hydrogen peroxide increased intercellular connections in astrocytes. Low serum together with high glucose levels resulted in increased numbers of tubules in mesothelioma cells (Lou et al., 2014) and tubule formation was stimulated in ovarian (Desir et al., 2016), as well as in colon cancer cells (Lou et al., 2018), by hypoxia. In vivo Osswald and colleagues (2015) showed that a microtube (another name for the larger membrane extensions containing microtubules) could be observed growing to the region of a laser damaged brain cell within 12 hours of the event; with a “replacement nucleus” traveling through the tubule over the next 24 hours.

The movement of nuclear material has been reported in other systems. Zani et al. (2010) reported migration of whole cells through what the authors called type 2 epithelial bridges whose width was in the range of one or more microns and whose composition included microtubules along with actin. Antanavičiūtė and colleagues (2015) described the movement of DAPI stained vesicles containing nucleic acids through tubules. The results in this paper indicate that chromatin granules and mitonucleons can also pass through tubular membranes.

Origins of tubules in endometrial cells
Although tubular membrane extensions are being reported for the first time in cultured human endometrial cells, it appears that the origins of some tubular membranes were initially observed more than 50 years ago. Researching structures in human endometrium during the reproductive cycle, electron microscopists discovered an actin-based structure in nucleoli that they showed to be a compact whorled arrangement of tubules around an electron-lucent core (Clyman, 1963; Terzakis, 1965). Researchers called this a nucleolar channel system and showed that its appearance was related to the human reproductive cycle with the number of such structures peaking on day 19 of the cycle (More et al. 1974). More recently the
formation of these systems has been shown to be dependent on a threshold level of progesterone (Nejat et al., 2014).

Researchers assumed that the nucleolar channel system acted within the cell of origin, having detected on occasion that a tubule extended from the nucleolus out into the cytoplasm (Wang Tzung & Schneider, 1992). Tubules were not reported to extend any further, perhaps because their fragility, together with their small diameter, made survival during tissue processing unlikely. Some of the tubules shown in this paper clearly emerge from a nucleolus. Nucleolar channel membrane systems may or may not be unique to endometrial cells and to oocytes where they have also been detected (Funaki et al., 1995). But it is the case, even in Ishikawa cell culture, that not all membrane tubules can be traced back to nucleoli.

The membrane tubule in fig. 2 illustrates that fact. What appears to be the originating colony fragment does not contain anything remotely resembling a nucleus. Dark granules, mostly concentrated at the “mouth” of the extension, appear to be the origin of the tubule that is attached to another monolayer fragment containing some kind of internal structure amidst a flourish of membranes. Over a period of three hours, granular material at the mouth of the tubule diminishes, while the structure to which the extension is attached doubles in size with the formation of two “typical” nuclei filling most of the membrane flourish. On the basis of this observation, the best guess is that one fragment of a colony is “donating” nuclear material to a second, perhaps “healthier,” fragment to complete assembly of nuclei. Actin filaments associated with cell membranes are suspected of involvement in membrane tubular extensions by a complex process that includes actin polymerization (Drab et al., 2018).

The movement of chromatin granules through a tubular membranes is also the most reasonable explanation for the changes that occur in the membrane flourish in figs. 5 and 6. The flourish appears mostly featureless in fig. 5 but, after 3 hours (fig. 6), is observed to contain the “granular outlines” of a nucleus similar to what was found for nuclei in reattaching hollow spheroids seemingly stopped in the midst of amitosis (Fleming, 2019). Chromatin streaming in hollow spheroids (Fleming, 2019) produces colonies of nuclei that eventually position themselves equidistantly in dome or spheroid and then form membranes. Despite skepticism about whether amitosis can achieve precise distribution of the parental genome into daughter cells, reports of different forms of amitosis in cultured mammalian cells (reviewed Fleming, 2016c) continue to appear. At the same time, it has become obvious, particularly for cancer cells, that such precise distribution does not actually occur as evidenced by a large population of aneuploid cells that are found (reviewed, Fleming, 2019). So perhaps that rationale for rejecting a proliferative process widely “used” by successful lower forms of life can be put to rest.

Credible reports of what might be called niche amitosis responsible for production of normal progeny cells from giant cells have been observed by many researchers (reviewed in Fleming, 2016c and in Fleming, 2019). Liu and colleagues made an important advance in such studies by isolating polyploid giant cancer cells induced by hypoxia in human ovarian cancer cell lines and demonstrating that the polyploid nuclei give rise to regular sized cancer cells by amitotic processes described as “budding, splitting, and bursting” (Zhang et al., 2014). In a recent paper, Liu and his colleagues proposed the existence of a cell cycle relevant to giant cells that “integrates” amitotic processes such as these with the more familiar process of cell proliferation by mitosis (Niu et al., 2016).
Origins and function of chromatin granules
Amitosis by chromatin streaming discussed in this paper appears to be outside the cell cycle, although it mostly appears to involve polyploid nuclei. The process resulting from the fragmentation of nuclei into chromatin granules has been observed during dome differentiation (Fleming 2016 a,b,c) and during conversion of a single cell hollow spheroid into a multicellular hollow spheroid (Fleming, 2019). Fundamental changes within an aggregate of nuclei, including, most probably, a change in pH, results in an “explosive” deconstruction of chromatin into granules, a process strikingly like the phenomenon that Székvölgyi and colleagues (2008) were able to demonstrate for isolated nuclei. In predomes and in the shell of hollow spheroids, chromatin granules become DNA filaments whose epigenetic alteration is probably essential for differentiation. The dispersed chromatin then coalesces into a mass from which arises a colony of nuclei that is the basis for dome or spheroid cells. Formed as a cluster, nuclei eventually move apart and generate or perhaps sequester cytosol from what resembles a giant multinucleated cell, ultimately forming cell membranes around each nucleus. Not only is nuclear assembly from chromatin granules a kind of amitotic proliferation, it also constitutes a significant exception to cell theory (Fleming 2016 a,b,c; Fleming 2019).

Results to date suggested that chromatin streaming might be another kind of niche amitosis, responsible for “assembly-line” proliferation of multiple nuclei in adult organisms (Fleming, 2019). The process is obviously more efficient than mitosis, producing dozens of cells over approximately the period of time required for completion of a round of mitosis (Fleming, 2016c). The fact that domes can eventually form gland-like structures highlight the possibility (Fleming, 2016c) that streaming chromatin granules may be responsible for production of cells such as those that make up endometrial glands, structures that will flourish for a relatively short period before being replaced. Furthermore such a process might be involved when the demands for proliferation are at their greatest and trillions of cells are being produced during the 40 week gestation period of a fertilized human zygote (Fleming, 2019).

Results in this paper suggest, for the first time, that chromatin granules may also be involved in building up individual nuclei on an “as needed” basis. The monolayer fragments in fig. 2, challenged by the addition of medium without serum, are also with nuclei. Over time, granular material traveling from one fragment to the other appears to have contributed to the assembly of two nuclei in one of the fragments. Similarly in figs 5 and 6, a tubular process appears to be “delivering” materials into a membrane flourish resulting in a structure that looks like a nucleus. As many will surely ask, is there an in vivo analogue to this process? The amazing work of Winkler and colleagues already described in this paper documents a fascinating example of nature “swapping out” a nucleus via a membrane tubule seemingly formed precisely for that process (Osswald et al. 2015). It is possible to wonder whether the nucleus being delivered was formed early on in the tubular process from chromatin granules.

Mitonucleons are also observed in Ishikawa epithelial tubules
In addition to streams of chromatin granules that appear to“build” nuclei (figs.2,3; 5,6), fused mitochondria as detected by staining for endogenous biotin also pass through endometrial epithelial tubules. In the unfixed, unstained process in figs. 5 and 6, a
significant concentration of mitochondria was suggested by an auto-fluorescent bulge moving through the tubule apparently behind, but probably admixed with, dark granular material. When processes were stained for endogenous biotin as well as for chromatin, a familiar maroon structure was observed created by the salmon stained endogenous biotin of mitochondria enveloping hematoxylin stained chromatin. This structure called a mitonucleon was initially observed in dome formation (Fleming, 2016a) where it was shown to be a transient organelle able that results in retention of gases that compress aggregated chromatin into pyknotic-like structures (Fleming, 2016 a,b,c). As has been highlighted in previous papers this nuclear compression resembles the earliest stages of apoptosis except that cell differentiation rather than cell death is the outcome.

By following the formation of mitonucleons in monolayer cells, the process of formation of detached hollow spheroids could be described (Fleming 2018a). This is a structure that has intrigued cancer researchers for more than a couple of decades, without much being understood concerning its origins. As the gas vacuole within the mitonucleon in a single monolayer cell expands, it pressures polyploid nuclei against the cytoplasmic membrane and the developing spheroid detaches from the monolayer, having been converted from an anchored into a mobile “cell”. Researchers studying spheroid formation in pancreatic cancer cell lines (Feng et al. 2017), elicited structures capable of growth as various kinds of spheroids by an alternative method called high-throughput single-cell derived sphere formation. The method is like cloning but uses a sophisticated device to guarantee single cell distribution in conditions favoring non-attachment (Chen et al., 2015). Cells that can proliferate under such conditions interact with each other to form spheroids. In Feng’s laboratory, cultures of “single cell-derived” tumor spheroids, appear to contain unicellular (characterized by a rim of cytoplasm) hollow spheroids and multicellular (golf-ball-like) hollow spheroids, as well as solid spheroids. Such results are consistent with the observation in Ishikawa endometrial cells that a unicellular hollow spheroid is the starting point for differentiation of a multicellular hollow spheroid (Fleming, 2019). Multicellular hollow spheroids may well proliferate inward to form solid spheroids, a process observed for pancreatic spheroids (Feng et al., 2017) but not yet observed in Ishikawa spheroids. What has been observed is that hollow spheroids can proliferate outward by forming additional attached hollow spheroids (Fleming, 2018a).

Spheroids for other epithelial cell lines sometimes named according to tissue origins such as neurospheres and mammospheres frequently arise in cancer cell lines from specific tissues. A common observation with regard to spheroids is that cells from such structures form more tumors in compromised animal models than the same number of the parent cells grown as a monolayer. This observation has led to the suggestion that spheroids are enriched for tumorigenic cells sometimes called tumor stem cells (Dontu et al.2003). Studies linking spheroids to tumorigenicity include research done with ovarian cancer cell lines. As few as 2000 cells from a dissociated spheroid of epithelial ovarian cancer cells, injected into immune deficient mice, elicited the formation of a tumor. Even a bolus of 10,000 parent cells from monolayers (even monolayers resulting from the outgrowth of solid spheroids) was not found to be as tumorigenic (Liao et al., 2014). In research that supports the tumorigenic capacity of spheroid cells, Espina et al. (2014) showed that mammospheres arising spontaneously from human ductal cancer tissue fragments consistently generated mammary xenograft tumors in an immune-compromised mouse.
In Ishikawa endometrial cells, the formation of mitonucleons and the ensuing generation of gas vacuoles converts a monolayer cell into a floating spheroid with giant polyploid nuclei pressured within a rim of cytoplasm between the outer membrane of the mitonucleon and the cell membrane, fairly exotic as cells go. If fetal calf serum is added to medium containing such structures, the spheroid can reattach, releasing bubbles of gas as it does so and forming an attached cell with one or more giant nuclei (Fleming, 2018a). Without that intervention, activity within the cytoplasmic rim of unicellular hollow spheroids, apparently initiated by the generation of small vacuoles in the region of polyploid giant nuclei, converts typical giant nuclei into chromatin granules that can be observed streaming through the cytoplasmic rim (Fleming, 2019). The chromatin granules become an array of filaments, open no doubt to essential epigenetic changes, and then re associates to form colonies of nuclei that separate, become bounded by membranes and ultimately fill the cytoplasmic rim of a hollow spheroid in a structure some have called “golf ball” spheroids. (Fleming, 2019).

**Mitionucleon involvement in metastases**

The observation that the mitonucleon is responsible for hollow spheroid formation in Ishikawa endometrial epithelia (Fleming, 2018a), together with the fact that spheroid cells have been frequently shown to be more tumorigenic than monolayer cells, suggests circumstantially that mitochondria or some form of fused mitochondria, characteristic of mitonucleons, are involved in tumorigenicity. More direct evidence for mitochondrial involvement in cancer metastases has been provided by some very clever experiments. Porporata and colleagues (2014) pursued the existence of a metabolic phenotype associated with tumor metastases in their ovarian carcinoma cell line. Their selection process resulted in the creation of a cell line that was significantly more invasive than the parental cells and that contained giant mitochondria not observed in the parent cell. In another approach, Farnie and associates (2015), studying breast cancer cell lines, used a fluorescent 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. And, in an approach dependent on membrane tubules, Lu and colleagues (2017) demonstrated that the transfer of mitochondria from a highly invasive kidney cancer cell line to a less invasive kidney cell line via tubules increased the invasiveness of the latter.

In an important and possibly underappreciated observation, Tamura and colleagues (2011) studied hollow spheroids just beyond the leading edge of invasive colon cancer. The researchers looked for correlations between the presence of hollow spheroids and the clinicopathological characteristics of 314 patients with colorectal cancer from whom the cancers were removed. By serial cross-sectioning they identified 96 patients having complete hollow spheroids beyond the invasive front. Furthermore, the research team demonstrated that the presence of hollow spheroids was an independent risk factor for metastases and predictive of shorter survival time for patients. The invasion appears to involve lymph nodes Tamura and colleagues suggest that solid spheroids invaded normal tissue and were hollowed out by apoptosis. Results with Ishikawa cells suggest an alternative, possibly more efficient process. An invading mitonucleon could be responsible for generating a hollow spheroid if delivered by a membrane tubular process from a structure that has invaded lymph fluid.
Results in this paper show that chromatin and mitochondria in endometrial tubules can form mitonucleons, and that tubular cellular bridges can accommodate the relatively compact mitonucleon whose width is not much larger than that of a nucleus. Once it has been introduced, the mitonucleon can expand into a single cell hollow spheroid and then into a multicellular hollow spheroid (Fleming 2016 a,b,c and Fleming 2019) making it a little like a “cellular trojan horse.” Mitonucleons that generate hollow spheroids would represent a previously unknown invasive structure, something of a hybrid between the well documented forms of single tumor cell and collective tumor cell invasion (Khalil et al.,2017).

Are invading hollow spheroids precursors to micropapillary cancer? It may be relevant to this discussion that in colon, as well as in other cancers, nests of epithelial cells are found whose growth pattern suggests origins different from surrounding cancer cells, a phenomenon that has been called micropapillary cancer. Interest in micropapillary cancer has been growing because in addition to colon, it has been observed in lung, breast, urinary bladder, stomach and other cancers (Vyas et al.,2019). As reported by Haupt and colleagues (2007), micropapillary carcinoma is an aggressive variant of cancer associated with frequent lymphovascular invasion and poor clinical outcome. Having reviewed approximately 375 cases of colorectal cancer, Verdu and colleagues (2011) detected a micropapillary component in cancers for 60 patients. Noting a greater depth of invasion and more positive lymph nodes in cancers with a micropapillary component the authors concluded that colorectal micropapillary carcinoma is more aggressive than conventional colorectal adenocarcinoma.

In addition to Tamura’s observations of hollow spheroids invading normal tissue, other sightings have suggested that hollow spheroids are involved in human cancers. In 1987, Allen and colleagues reported on multicellular aggregates found in the peritoneal fluid of patients with ovarian cancer, describing clusters that included “spheroids with a central lumen surrounded by a cell monolayer.” Such hollow spheroids were subsequently demonstrated capable of giving rise to monolayers (Burleson et al., 2006). Reviewing research on spheroids found in malignant ascites, Shield et al.(2009) characterized them as capable of tumorgenesis in vivo and as exhibiting a reduced response to chemotherapeutic drugs when compared to monolayers in vitro.

Hollow spheroids have been harder to find in the blood of cancer patients despite extensive studies demonstrating that tumor cells and tumor cell clusters are present in a significant proportion of patients with cancer, both before and after the removal of a primary tumor.(Allard et al., 2004). Denes and colleagues (2017) observing that most available methods to detect cancer tumor cells include steps that destabilize or eliminate spheroids, developed a new light scatter flow cytometry blood test that allowed them to search for and find hollow spheroids in 46.3% of metastatic patients but not in the blood of normal subjects, nor in patients with non-metastatic cancer. Finding evidence of regions of hypoxia in these structures, a condition favoring metastases, the research team suggested that hollow spheroids might afford a premetastatic niche for tumor stem cells. Along these same lines, Hou and colleagues have suggested that tumor stem cells in circulating clusters might have a survival advantage (Hou et al. 2017).
Are clusters of hollow spheroids involved in metastases?
The characteristics of a unique structure of clustered hollow spheroids is described for the first time in this paper. An obvious question is whether anything like it has already been seen in vivo, an inquiry that turns up a surprisingly rich history of research on tumor cell clusters mostly isolated from blood, and stretching back more than 50 years (Hong et al. 2016; Umer et al. 2018). Almost consistently, starting with Watanabe in 1954, researchers have concluded that tumor cell clusters are more likely to contribute to metastases than single tumor cells. Liotta et al. (1973) showed that the injection of single tumor cells and tumor cell clumps (or clusters as they have been called recently) resulted in many metastatic foci, but clumped tumor cells produced a significantly greater number of metastatic foci than did the same number of cells in single-cell form and larger-sized clumps produced still more metastatic foci than smaller-sized clumps. Aceto and colleagues (2014) presented evidence that tumor cell clusters are up to 50-fold more likely to form metastases. Furthermore they demonstrated that the clusters detach as such from tumors, rather than forming from single tumor cells in the bloodstream, suggesting that their cohesion may identify a novel and potentially targetable step in the blood-borne dissemination of cancer. The relevance of tumor cell clusters in metastases was recently reviewed by Cheung & Ewald (2016) with a model of metastatic dissemination that highlights the activities of clusters of tumor cells that retain epithelial properties. Overall, there appears to be recognition that at least some tumor cell clusters may be more than the sum of their parts relating perhaps to discussions about the distinct phenotypic and molecular characteristics of cells in tumor clusters or microemboli in comparison with single cancer tumor cells (Umer et al., 2018).

Single hollow spheroids were difficult to detect in vivo because of their relative “fragility,” and it is probable that spheroid clusters are similarly fragile. In fact, the cluster in fig. 14 appears to be deflating, a fact that provided us with evidence of its intriguing internal structure but might ultimately result in a structure that is no longer recognizable. Additionally, it has long been understood that blood-borne cancer cells and cancer cell clusters can get hung up in capillaries as they pass through the blood system. On the basis of size alone, clusters of hollow spheroids released in vivo would be expected to quickly become sequestered. In one recent study, comparing numbers of cancer tumor cells in portal and arterial blood during a pancreatic tumor resection, a significant uptake (approximately 40%) of putative cancer cells was demonstrated by researchers, who suggested that tumor cell structures might have become caught up in liver or lung compartments (Vilhav et al., 2018).

As was true in the detection of single hollow spheroids, the best place to begin to look for delicate structures such as these may well be in carefully handled malignant ascites fluid. It is relevant that Kashima and associates (2010) examining ascites fluid in a woman with cancer of the large intestine described small papillary clusters with a smooth surface showing peripherally located cytoplasm surrounding a rare central lumen. The researchers dubbed these “inside-out” cell clusters and concluded that the patient had micropapillary cancer. Given a clear, concise description that is so evocative of hollow spheroids, it is reasonable to wonder whether clusters of hollow-spheroids might also be found.

In the long history of looking for tumor cell clusters in the blood of cancer patients (Hou et al., 2017; Umer et al., 2018), it is possible that research groups have found structures whose silhouettes resemble that of a hollow spheroid cluster. Larger clusters are sometimes called...
CTM an acronym for circulating tumor microemboli. As new methods of isolation, reviewed by Umer and colleagues (2018) continue to be tested, the potential destruction of cancer cells and clusters is an ongoing concern (Yu et al., 2011). Glaves and associates (1988) studied dissemination of cells from human renal adenocarcinomas using density gradients, a gentler method than some separation strategies currently used. The pictures published as a result of this study contain at least one example of a structure that could correspond to the hollow spheroid clusters shown in this paper. Staining for endogenous biotin such as was done for the cluster of hollow spheroids in fig.10 might be of some help in making an identification. Molnar and colleagues (2001) similarly found clustered tumor cells in the peripheral blood of colon cancer patients. Furthermore, they identified what they characterized as dendritic-like processes emanating from such clusters, leaving us with a tantalizing “maybe” as to whether what they identified bears any similarity to the structures shown in this paper.

Hollow spheroid clusters have distinct phenotypic characteristics that might support a role for them as metastatic agents. Foremost, upon release from the monolayer they can be transported in fluids. Yet, as is shown in figs. 15 and 16, they possess abundant resources to reattach and send out filopodia, believed by some to “drive cancer cell invasion” (Jacquemet et al., 2015). An additional relevant question would be whether structures like this could become dormant, perhaps because of adaptive metabolism, surviving in metastatic niches for years before “springing” to life? With all the work underway to study tumor cell clusters, researchers might want to keep in mind the potential importance of hollow spheroids and clustered hollow spheroids. If such “inside” out cell structures comprise at least one kind of metastatic structures, their vulnerability can be probed in vitro. It might be possible to identify a toxin that attacks the particular physiology of the “inside” out cell structures, inhibiting their ability to spread cancer.

Materials and Methods

The subject of this paper is the formation of tubular membrane protrusions by Ishikawa endometrial epithelial cells. Tubules have been observed to form in monolayers as well as to extend from solid spheroids. Examples are shown in figs. 1 through 7. The tubules carry granules that contribute to “building” nuclei and they carry mitonucleons, transient organelles essential to the formation of predomes (Fleming, 2016a,b,c) and unicellular hollow spheroids (Fleming, 2018a). In both of those examples, vacuole formation within the mitonucleon compresses chromatin aggregates and initiates the amitotic process of chromatin streaming. The revelation in this paper is that mitonucleons can transit through tubules. Whereas figs. 1 through 5 are photomicrographs of living culture, the mitonucleon was detected by fixing and staining cultures containing membrane tubules.

Fixation was achieved by adding 4% paraformaldehyde in phosphate buffered saline (PBS) to the culture dish. After 10 min, the cells were washed gently four times with 5 · 10 ml PBS. A solution of 1% Triton X 100 was added to the cells to permeabilize the membrane. Again after 5 min, the culture was washed with successive changes of PBS. After washing, cells were exposed to a 1:200 dilution of Extravidin conjugated horse-radish peroxidase (HRP) (Sigma) 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$_2$O$_2$. 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. Controls were carried out to ensure that the stain was reacting specifically with endogenous biotin. If avidin linked to peroxidase is not added to the cultures, there is no reaction. If avidin without peroxidase is added first to the cultures, followed by avidin linked to peroxidase, staining is not observed. Staining does not occur if avidin HRP is not added to the cultures prior to AEC indicating that an endogenous peroxidase is not responsible for the staining. To ensure that avidin was reacting with biotin, we stained domes using streptavidin linked to horseradish peroxidase as well as primary antibody to biotin and secondary antibody linked to horseradish peroxidase. Staining occurred under all circumstances, indicating that avidin does indeed react with biotin that is endogenously present in the cell in significant amounts. The endogenous biotin had previously been shown to be associated with mitochondrial carboxylases. (Fleming etal., 1998)
Cells for all of these experiments were grown in phenol-red free Minimal Essential Medium with 10% calf serum. Additionally, 2 mM glutamine was added. Culture conditions eliciting formation of tubules were similar to those used by Lou and colleagues (2014) except that phenol-red-free Minimum Essential Medium contained no serum, but did contain 2 mM glutamine in addition to fresh glucose. Serum-free medium was added to confluent monolayers, a condition that stresses the cells and result in the formation of tubular membrane extensions. Solid spheroids were formed when trypsinized Ishikawa monolayers were resuspended in serum-free medium in petri dishes. Spheroids formed in that manner were carefully transferred to dishes to which additional fresh serum-free medium was added. Spheroids were monitored for the formation of tubular membrane extensions. Solid and 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. Some of the hollow spheroids maintained without serum spontaneously initiated amitotic cell proliferation as previously described (Fleming, 2019). Cells were viewed using an Olympus inverted stage microscope at powers of 100X, 200X and 400X. Tubule formation was observed in cultures starved for serum. The most dramatic tubule forming activity was observed for clustered Ishikawa hollow spheroids, a structure whose formation is documented in the second half of the paper. The formation of a cluster of hollow spheroids follows almost the identical process for the formation of a single hollow spheroid (Fleming, 2018a). As was true for the single hollow spheroid, its formation proceeds from the formation of mitonucleons except that multiple cells containing mitonucleons detach together, remain together as a cluster, and eventually form a structure with the appearance of a beehive, gaseous vacuoles surrounded by rims containing nuclear material within cytosol, including mitochondria as detected by the presence of endogenous biotin.

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