Amitosis generates differentiated cell nuclei from chomatin mass formed after monolayer nuclei are aggregated, beome pyknotic, and are fragmented: Differentiation of Epithelial Domes, Part 3.

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Abstract

Ishikawa endometrial epithelial cells are capable of differentiation from monolayer cells into fluid-enclosing hemispheres through a surprisingly complex series of structural changes as discussed in this and in two accompanying papers (Fleming, 2016a; Fleming 2016b). The process starts with the dissolution of cell membranes in defined regions throughout a monolayer that has been stimulated to differentiate (Fleming, 1995). Aggregated nuclei become wrapped in membranes containing mitochondrial carboxylases, and apparently generated by contiguous mitochondria. These mitonucleons are involved in vacuole formation that elevates the syncytium into a predome (Fleming, 2015a). The mitonucleons begin to fall apart several hours after formation as the enveloping membranes are breached and the pyknotic chromatin undergoes profound changes (Fleming, 2015b). Chromatin deconstruction, with attendant disappearance of the typical ovoid nuclear structure, results in chromatin fibers that fill the envelope formed by the apical and basal membranes of the syncytium, now stretching over a cavity filling with fluid. In the next several hours, hematoxylin staining, greatly diminished when nuclei were fragmented, reappears in an irregular mass of chromatin out of which nuclei form amitotically and increase in numbers until they fill the envelope. Subsequently cell membranes form around the nuclei. Domes can enlarge and even extend into tubules by becoming vacuolized and undergoing the same amitotic process that created the dome initially.

Introduction

Structural changes in Ishikawa epithelial cells begin with the fusion of foci of monolayer cells 4 to 6 hours after inducing differentiation. Mitochondria proliferate and nuclei aggregate in the center of the resulting syncytium becoming surrounded by a double membrane staining for the endogenous biotin contained in mitochondrial carboxylases (Fleming, 1998). The membranes appear to be elaborated from mitochondria contiguous to the nuclear aggregates. Small vacuoles form in the heterochromatin along with a larger vacuole within the double membrane surrounding the aggregate, in a structure we have called a mitonucleon. The overlying apical membrane of the syncytium, along with the aggregated vacuolated heterochromatin in the mitonucleon, elevates and fluid begins to accumulate between the basal layer of the syncytium and the petri dish to which cells previously adhered. (Fleming 2016a). These changes occur within the first 10 to 12 hours of the differentiation.

As described in the accompanying paper (Fleming 2016b), the mitonucleon and the enclosed heterochromatin are compressed against the rising apical membrane. At what may be the peak of that elevation when the enlargement of the vacuole and the compression of the heterochromatin presents like the structure that has been called a

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"signet ring" cell, the mitonucleon begins to come apart. The earliest signs are a breach in the double membrane as centriole-sized structures, previously contained within the double membrane, leak back into the syncytium at the base of the apical membrane protrusion. At the same time, previously aggregated nuclei begin to disaggregate. The rather dramatic event following on this is the "explosion" of nuclei as the heterochromatin is deconstructed into an array of chromatin granules barely visible under the light microscope. Chromatin granules and fibers appear to bind to microtubule-like structures, stretching along one axis of the predome structure, from pole to pole resulting in a quasiparallel array of filaments that is transient.

The next observation of chromatin material is as an irregular hematoxylin-staining mass, probably resulting from coalescence of chromatin filaments (Fleming 2016b), spreading within the apical-basal membrane envelope around protrusions. Almost as soon as the "sheet" can be seen, it is also possible to see ovoid structures emerging from the mass. This is the beginning of the final phase of the differentiation as dozens of nuclei form out of the mass of chromatin by a process that appears to be amitotic.

Results

Figure 1a shows a swath of material staining with hematoxylin in an Ishikawa predome, approximately 13 hours after the start of differentiation. Shapes seem to be emerging, although it is a little like interpreting an inkblot. In fig. 1a, a clump of 4 to 5 almost ovoid shapes can be seen "pulling away" from a larger clump of similar structures. Hematoxylin staining material connects the emerging nuclei. Additionally a thin "strand" of staining material encircles what appears to be an apical membrane protrusion. In Figure 1b the emerging nuclei are more irregular, with at least two structures at 12 o'clock and 3 o'clock staining more darkly with hematoxylin suggesting that some of the nuclei may initially emerge as polyploid structures. Whether the irregularly shaped nuclei might be similar to the "immature nuclear morphologies" found in induced HL-60 granulocyte differentiation (Olins et. al. 1998; Collins, 1987) is an interesting question, since that is an example of differentiation shown to involve an increase in unusual linear chromatin structures.

Protrusions in the apical membrane, detectable by the exclusion of hematoxylin stain are seen in many but not all of these predomes. The evidence suggests that protrusions visible during the first half of dome formation are due to accumulation of gasses, perhaps CO2, that are, to some extent, exchanged for H20 (Fleming, 2016a). The appearance of protrusions at this stage is highlighted by the manner in which hematoxylin staining material stretches around gaps as in fig. 1a, 2a, and 2b.

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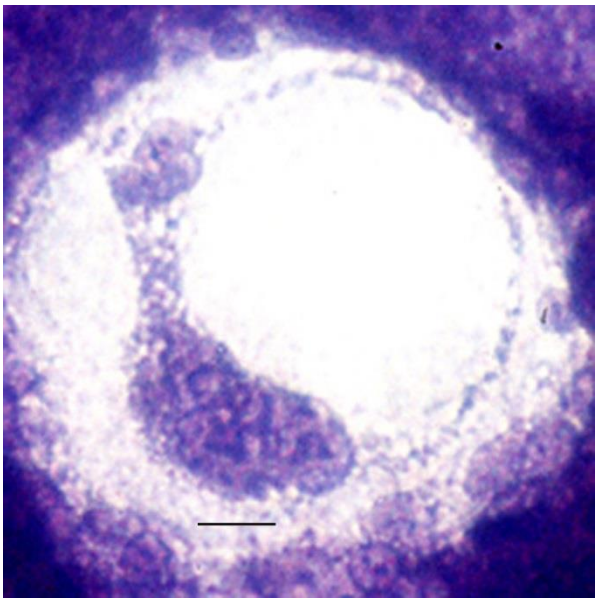

Fig. 1a. Early stages of nuclear production in predomes
Predome was fixed and stained with hematoxylin and eosin 12 hours after the start of differentiation.
Bar=25 microns

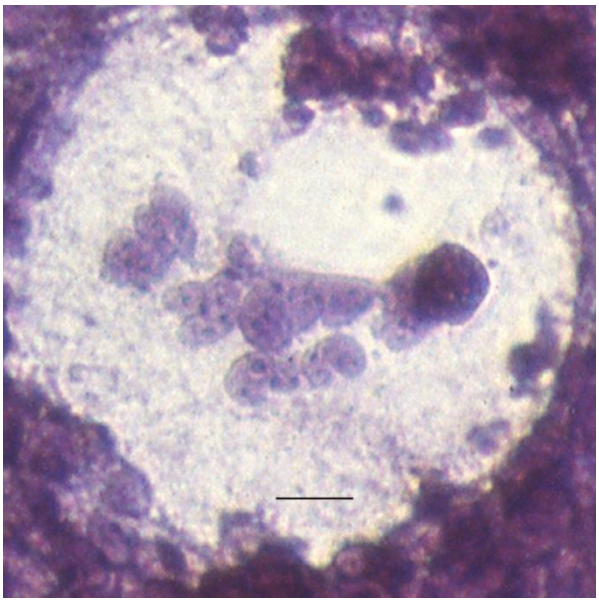

Fig. 1b. Early stages of nuclear production in predomes
Predome was fixed and stained with hematoxylin and eosin 13 hours after the start of differentiation.
Bar=50 microns

Figs 2a and 2b show predomes fixed and stained at 14, and at 16, hours after the start of differentiation. Almost everywhere in the predome envelope it is possible to observe either ovoid structures or reticular material, variably staining with hematoxylin, in what might be called a chromatin sheet. Nuclei appear to be filling the predome from the periphery to the center. Cultures stimulated to differentiate, fixed and stained from 12 to 18 hours after the start of the process, were found to have anywhere from a few to dozens of hematoxylin-stained ovoid structures. On the basis of examining both predomes and mature domes, it can be said that although sufficient numbers of nuclei eventually form to fill the dome, nuclei do not all form at once. For this reason, and others that will appear in the discussion, it seems likely that some DNA synthesis is part of the process during the final stages of differentiation.

The predome in figure 3a was fixed and stained 18 hours after the start of differentiation. Most of the envelope is filled with nuclei, some staining more darkly than others. The arrow points to a region at the center of the predome that still

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appears to contain only the chromatin sheet, but an ordered array of non-overlapping nuclei otherwise fill the predome. Cell boundaries are not in evidence.

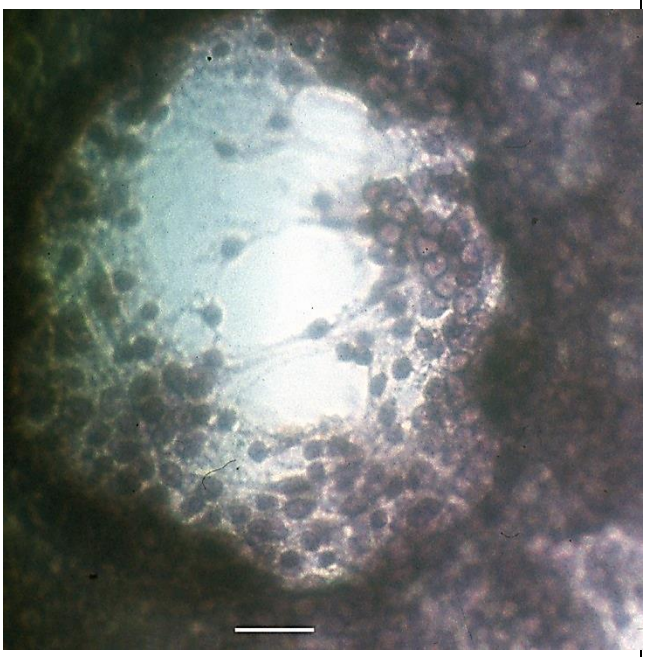

Fig. 2a Predome filling with nuclei 14 hours after the start of differentiation Bar=50um

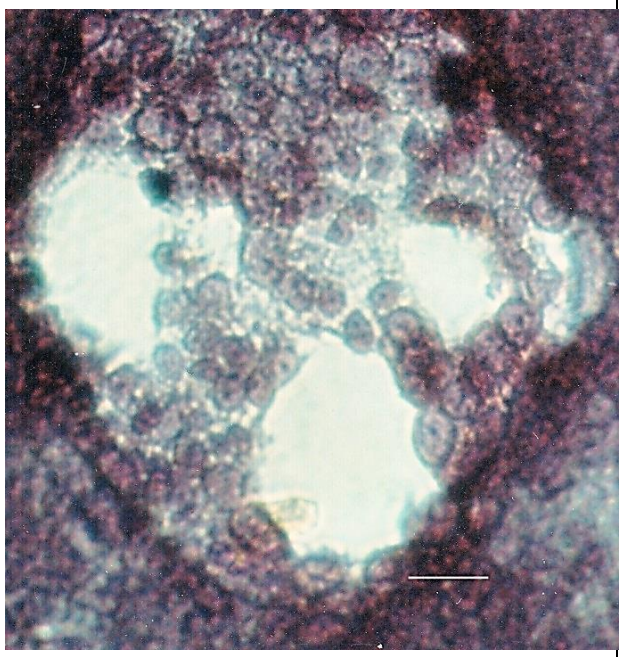

Fig. 2b Predome filling with nuclei 16 hours after the start of differentiation Bar=50microns

Dynamic Nature of the Process

As already discussed, nuclei emerge over time in the Ishikawa predome, but the apparent overlapping of nuclei in fig. 2a suggests that the rate of positioning nuclei within the envelope does not always keep up with emergence. Although unable to record nuclear migrations in real time, it was possible to fix and stain predomes within a three hour period that had as few as a dozen and as many as 80 nuclei. For the most part nuclei appear to be forming close to the predome periphery and moving up into the envelope.

It is well understood that nuclei move both short and long distances routinely, particularly during development. Starr (2011) reviewed much of what is known about nuclear movement in the nematode describing a “toolbox of motors and structural elements,” including microtubules, involved in moving nuclei. Furthermore in many systems, both actin filaments and microtubules work together to position nuclei.

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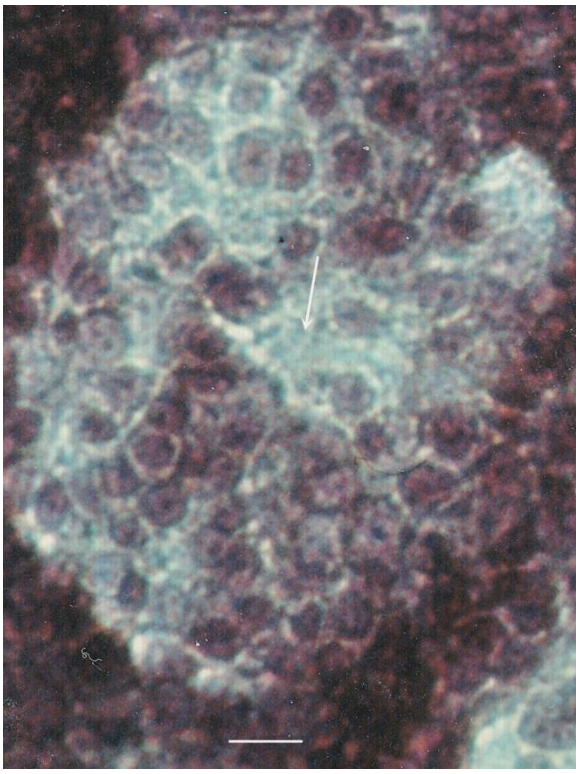

Fig. 3a Predome approximately 90% filled with nuclei, 18 hours after the start of differentiation
Bar=50 microns

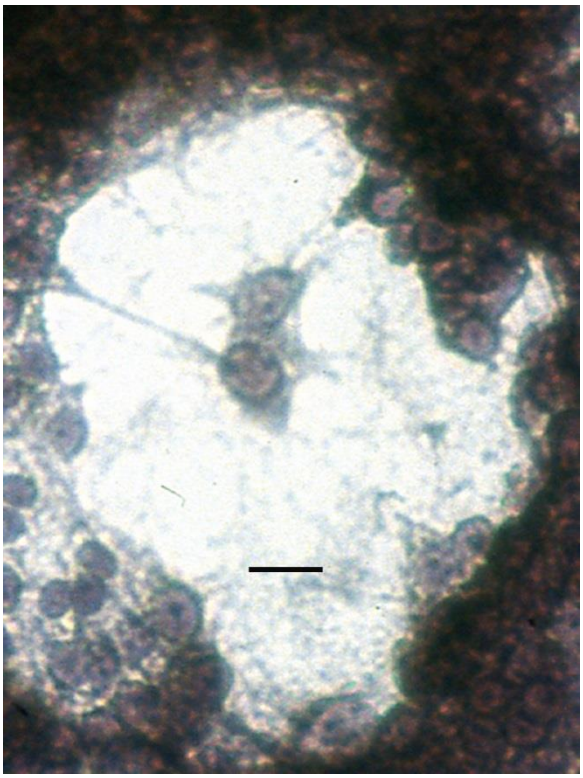

Fig. 3b Predome with only two nuclei, apparently stalled in the amitotic process.
Bar = 50 microns

Although nothing is known about the biochemistry of nuclear movement in the Ishikawa predome, the pictures in this paper leave little doubt that it happens. Certainly, the microtubular structure that is prominent when nuclei fragment during the second stage of the Ishikawa differentiation (Fleming, 2016b) could be involved. In Drosophila embryos, which have been providing insights to nuclear migration for decades, Baker, Theurkauf and Schubiger (1993) identified two distinct nuclear movements producing a syncytial blastoderm with a uniform monolayer of thousands of surface nuclei. After nuclei are positioned, cell membranes are built around them from the membrane components of intracellular organelles (Mazumdar and Mazumdar, 2002).

Fluid Passing through Predomes

In addition to dramatic changes in chromatin during dome differentiation, previously adherent cells become anchorage independent, as the syncytium forms and elevates,

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and as fluid accumulates between the basal membrane and the petri dish. It is reasonable to assume that the forces generated by the passage of fluid through the envelope could have an effect on nuclear distribution.

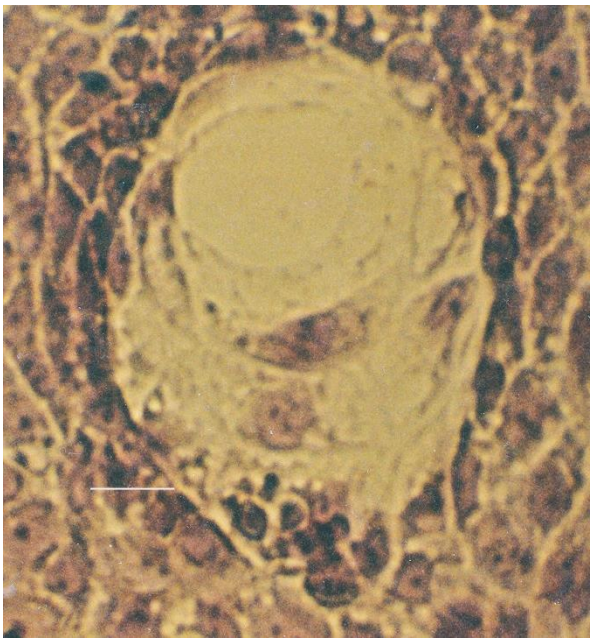

Fig. 4a Predome, with a prominent apical membrane protrusion, 14 hours after the start of differentiation.
Bar = 50 microns

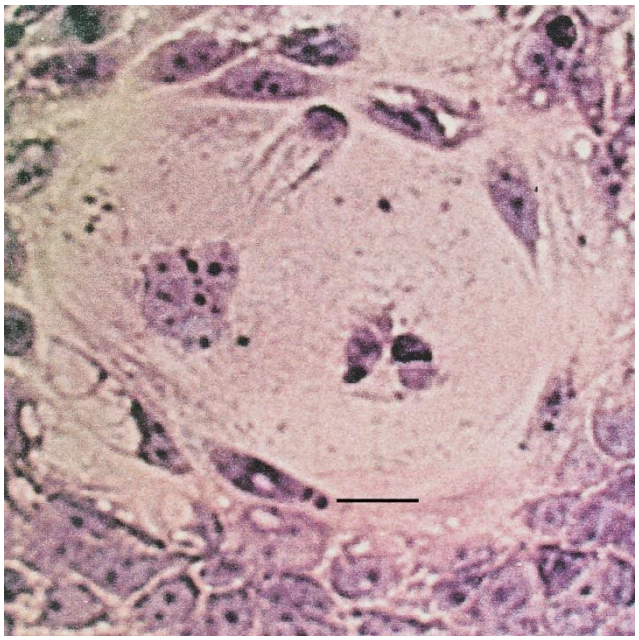

Fig. 4b Predome, 14 hours after the start of differentiation
A group of approximately 6 nuclei can be seen at 9 o'clock, presumably being repositioned.
Bar = 50 microns

Fig. 4a shows the nuclear repopulation in which reticular material fills most of the predome. Nuclear or polynuclear structures, within reticular material appear to be swirling "up," to the limits of a protrusion, as seen in figs. 1a, 2a, and 2b. Large nuclei or clusters of nuclei almost appear to be moving clockwise, as best as can be detected in these pictures. Both pictures were taken at 14 hours after the start of differentiation. It is not known how the activities of fluid accumulation and nuclear repopulation affect each other, although it seems reasonable to assume that they are not completely independent.

Fig. 5 is a picture of a 5 day old dome. The dome contains three regions with differing nuclear profiles. In the middle third of the dome, nuclei are arranged much as in fig. 3, not overlapping, but filling the apical-basal membrane. In the top third

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of the dome refractive borders around the nuclei suggest that cell membranes are forming. In the bottom third of the dome, vacuolated cells are apparent suggesting that the dome is expanding by the same process that created it, mitonucleon-generated amitosis. The arrow on the right points to small bubble-like vacuoles forming within heterochromatin, one of the earliest stages in differentiation, while the arrow on the left points to three enlarged structures whose profile is mostly vacuolated as is the "signet ring cell" described for the second phase of Ishikawa dome cell differentiation (Fleming 2015b).

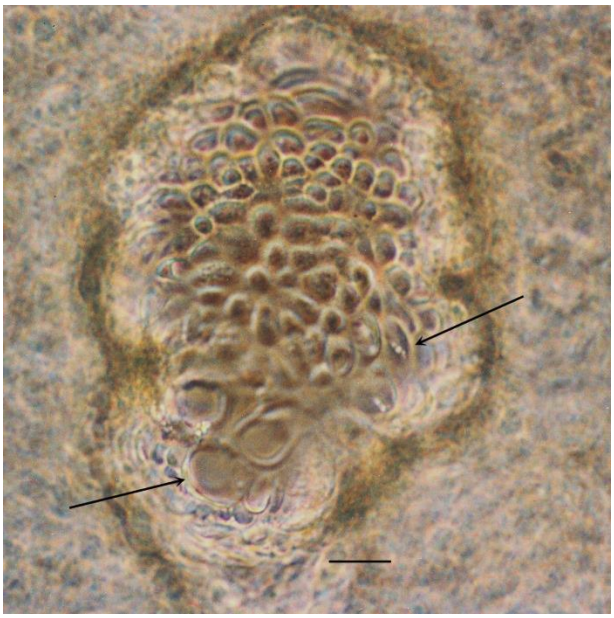

Fig. 5 Dome four days after Ishikawa monolayer was stimulated to differentiate.
Domes expand over time, and dome cells can be at multiple stages in the differentiation process.
Bar = 50 microns

Dome Differentiation, Model for Building Gland-like Structures

In considering the intriguing processes of dome formation *in vitro*, it is possible to lose sight of what this differentiation may mean *in vivo*. Cell monolayers are extremely useful for research, but their two dimensional structure and need to adhere to a surface is far from the reality of three-dimensional organisms. Any processes moving cells beyond their dependence on surface adhesion has to be of interest to researchers interested in differentiation and organ formation. Ishikawa cells showed themselves capable of detaching and moving into the third dimension, and it was our hope that this capacity might presage more complex structures. For that reason, a goal of our research was to demonstrate that more complex structures could form beyond domes, structures similar to those formed in vivo during the secretory phase of the menstrual cycle. Fig 6 demonstrates that domes can extend

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into gland-like structures without too much more intervention other than long term incubation of cells cloned for their capacity to form large domes.

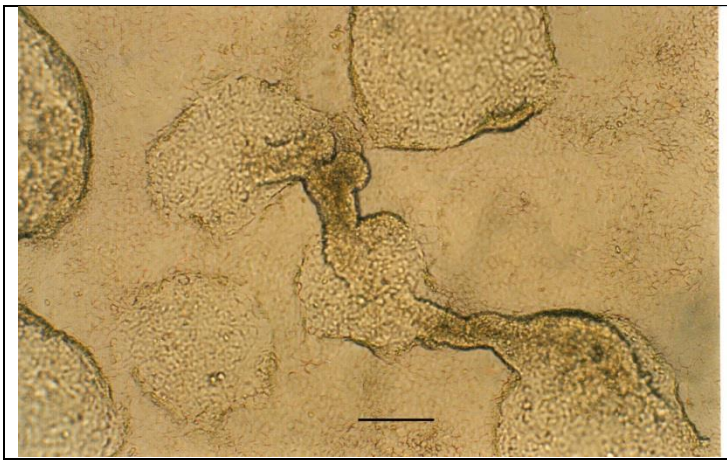

Fig. 6 Domes connected by tubules three weeks after stimulus to differentiate was added.
Bar = 100 microns

The cells were originally grown for 10 days under conditions that encourage monolayer growth. After the cells reached confluence and had clearly stopped growing, fresh medium with fetal calf serum was added. Domes formed throughout the culture and cells were left in the incubator for three more weeks. Fresh medium with fetal calf serum was added every four days. Domes continued to expand, proliferating out into tubules that linked domes.

There are actually other relevant morphological changes in "mature" domes in addition to those reported in this series of papers. Cells at the base of the dome often take on columnar morphology and significant amounts of material are secreted into the dome.

Discussion

A surprising conclusion from studying dome formation is that the process of amitosis, defined as nuclear proliferation without the formation of mitotic chromosomes, plays a substantial role in dome cell differentiation. It has been assumed by many for a long time that amitosis is not relevant in metazoans. Perhaps the orthodoxy that every daughter cell in a multicellular organism inherits the identical parental genome led to the enshrinement of mitosis with its compelling visuals as a confirmation of equal chromosome distribution. And perhaps that is why the process of amitosis seems to have been met with indifference, or even skepticism, for more than a century. What other explanation is there for the fact that so little research has been done on a process of nuclear proliferation whose existence has been known for more than a century.

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The overall lack of interest in amitosis can, perhaps, be crudely quantified by comparing searches in the Medline data base, an amazing resource covering more than a century of medically significant research. The search term "mitosis in mammalian cells," calls up more than 40,000 studies while "amitosis in mammalian cells" retrieves fewer than 50 papers. This is particularly striking when you think of the place of Cancer in the pantheon of maladies (Mukherjee, 2010) and remember that it is a disease of unchecked cell proliferation.

Suspicions about amitosis appear to be deeply rooted as evidenced by what C.C. Macklin, a pioneering cell biologist at Johns Hopkins University, wrote in 1916.

> *"During recent years, the conviction among cytologists has become more and more strongly entrenched that the problem of amitosis can not be satisfactorily solved by investigations based alone upon the study of non-living tissue, but that its successful conquest must rely principally upon the correct interpretation of the succession of morphological and physiological changes revealed by prolonged observation of the living cell, under normal and artificially varied conditions."*

That is how Macklin introduced his results (Macklin, 1916) affirming amitosis as a real phenomenon. His research involved endless hours observing living chick cells grown using newly developed cell culture conditions. Perhaps Macklin was responding to skepticism about a paper published a decade earlier naming amitosis a factor in normal and regulatory growth" (Child, 1907) or to the rumored suggestion by Walther Flemming the "father" of mitosis that both processes contribute to mammalian development. But even after his observations of amitosis in living cells, the process proved to be a "hard sell."

A resurgence (perhaps an overstatement) of interest in whether and how amitosis may play a role in cell proliferation has occurred in the last 35 years. There was, for instance, a "flurry" of reports of amitosis in placental tissue or in cells grown from placental tissue: in rats (Ferguson and Palm, 1976), in human trophoblasts (Cotte et. al.,1980), and in mouse trophoblasts (Kuhn, Therman and Susman, 1991). The manner of amitosis was reported to be by fission, a nucleus splitting into two nuclei without the involvement of chromosomes. Trophoblasts from mink have also been shown to be capable of amitotic division(Isakova GK, Shilova IE. 2000). In a follow-up, the researchers identified two different forms of amitosis which they called constriction and extrusion (Isakova and Shilova, 2002).

Amitosis by fissioning was also reported in mammalian liver cells (David and Uerlings, 1992) and human adrenal cells (Magalhães, Pignatelli, and Magalhães 1991). Chen and Wan (1986) not only reported amitosis in rat liver but also presented a mechanism for a four stage amitotic process whereby chromatin threads are reproduced and equally

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distributed to daughter cells as the nucleus splits in two. Even with a possible mechanism for equal distribution of the genome, however, amitosis still appeared to be mostly out of favor.

More recently there have been reports of amitosis-like processes although the term itself is not used. Much work has been done for instance with giant polyploid cells, an important element of placental tissue, as well as the polyploid cells that are "induced" when cells in culture are "poisoned" so that mitosis is blocked. Veteran researchers in the field (such as Erenpreisa and Zybina referenced below) have adopted the term depolyploidization to describe processes that result in smaller nuclei with diploid or near diploid complements of DNA derived from giant nuclei.

Zybina and colleagues (2001) have studied giant trophoblasts, a placental polyploid cell, in a number of different organisms for decades. Relevant to the discussion in this paper, Zybina EV and Zybina TG (2008) have suggested that the nuclear envelope of secondary giant trophoblast cells is involved in subdivision of a highly polyploid nucleus into diploid and low-ploidy nuclei.

In several different cell lines, the treatment of a population of cells with chemicals that block mitosis, induces polyploid cells. Erenpreisa and her colleagues (2000) have shown such cells capable of giving rise to "normal" diploid cells through polyploid chromatin bouquets that return to an interphase state and separate into what these researchers call secondary nuclei. In that neither example of depolyploidization involves the formation of mitotic chromosomes, and both result in an increase in nuclei, the processes seem to fall under the broadest definition of amitosis.

Walen has shown at least two different examples of amitosis arising in human cells rendered polyploid by viral transformation (2002). She found similar results in cells that had become polyploid following replicative senescence (2004). Studying mouse embryo fibroblast lines, exposed to carcinogens, Sundaram et al. (2004) have identified amitotic nuclear budding followed by asymmetric, intracellular kinesis as the mechanism that produces small mononuclear cells, a process these researchers called neosis. And in one more example, asymmetric cell fission has been reported to occur in polyploid giant cancer cells by splitting, budding or burst-like mechanisms (Zhang, Wang, and Zhang, 2012).

In fact, as recently published in a pre-print (Fleming, 2014), non-differentiating monolayer Ishikawa cells are capable of amitosis by fission as well as by asymmetric division. Furthermore, binucleated cells (assumed by many to arise from fission) could make up as much as 10% of the population (Fleming, 2014) in monolayers that are not differentiating.

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So, amitosis, a relevant process for cell division in single celled organisms and in plants, may finally be coming into its own in multicellular animals. Will more examples of amitosis be found? Is amitosis, by a variety of processes, more a partner in the production of trillions of cells in the average human being than previously suspected? Could be. In actively growing mink blastocysts, it has been estimated that 10% of newly formed cells at the onset of renewal of blastocyst growth, arise by amitosis, a fraction that is reported to increase to 20% during the stage of active growth prior to implantation (Isikova and Shilova 2003).

One more relatively recent and visually stunning example of amitosis has come out of research in fetal gut. Nuclear forms that look like hollow bells encased in tubular syncytia have been shown to divide symmetrically by an amitotic nuclear fission process. Seven other nuclear morphotypes emerge by asymmetrical amitotic nuclear fission (Gostjeva et al. 2006).

Amitosis during Ishikawa dome formation

The production of nuclei described in this paper is an example of amitosis different from fissioning, budding, and depolyploidization. Mitonucleon-generated amitosis seems particularly suited to adapting multiple nuclei to a differentiated function in a reasonably short period of time. The observations reported in this, and the two accompanying papers (Fleming, 2016a and 2016b), also add some details to the story of polyploidy, frequently a precondition for many of the examples of amitosis cited in this paper.

Polyploid cells, commonly the starting point for amitosis, are assumed to arise because of a deviation in the mitotic cycle. Edgar and Orr-Weaver (2001) described 4 different variations on the cell cycle consisting in phases omitted or shortened in a process called endoreplication by some. Lee, Davidson and Duronio in their relatively recent review (2009) discuss endoreplication as essential for normal development and physiology in many different organisms. In some cases, the cycle proceeds without cytokinesis. In other cases, sometimes called endocycling, the cycle is believed to only have two phases, the S, or DNA synthesis phase, and the G or “resting” phase. In each case, the synthesis of DNA is assumed to proceed as in a normal mitotic cycle, but then either the chromosomes are not separated, or cytokinesis does not take place. Finally when a cell is polyploid, there is the distinction between a single giant nucleus, the result of endocycling, and a cell containing multiple nuclei.

The process of cell fusion appears to result in similar morphologies for cells that spend a short period of time in a syncytial state as is the case for differentiating Ishikawa cells. The Ishikawa nuclei move relatively quickly from mononuclear, monolayer cells, to being individually present in the syncytium, to being aggregated

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into something that looks like one big nucleus in the mitonucleons that can be seen 6 or more hours after the start of differentiation. Following chromatin fragmentation and reassociation, the predome enters a new stage of polyploidy, with multiple nuclei being generated inside a single large "cell," the predome, visible after another 6 hours. Observations of a "giant nucleus" or of a multinucleated "cell" without the benefit of being able to detect the starting point when cells fuse and mitonucleons form through to the emergence of nuclei in the final 6 hours of differentiation might lead to the conclusion the endoreplication is occurring in the Ishikawa cells. It does not seem to be since fusion can explain what is observed.

Relationship of Emerging Nuclei to Phases Associated with Mitosis

Nuclei appear in predomes during the final phase of differentiation with no involvement of chromosomes. The best guess is that the amitotic process directly produces interphase nuclei, using the descriptor "interphase" not so much in its relationship with mitotic phases, but as it connotes functioning nuclei outside of the mitotic phases. Much of what happens in a cell involving the "use" of DNA (as opposed to its replication and packaging for passage into daughter cells) appears to occur in the interphase nucleus (Branco and Pombo, 2007). Remarkable progress has been made as reviewed by Dekker (2014) in comparing DNA interphase chromosomes with the far more compact DNA in mitotic chromosomes. Furthermore, Bickmore and van Steensel (2013) have demonstrated that the architecture of interphase chromosomes is important for the regulation of gene expression and genome maintenance

An important foundation for such studies was provided by the discovery that relatively discrete territories are an organizing principle for DNA in interphase nuclei (Cremer and Cremer, 2001), an observation that has been validated and extended by a number of researchers (Dehghani H, Dellaire G, Bazett-Jones DP. 2005; Bártová E, Kozubek S. 2006). Reviewing this, and much other research on interphase DNA, Rippe (2006) has concluded that this arrangement of the interphase nucleus favors "the formation of nuclear sub-compartments in a reversible self-organizing manner that is driven by entropic forces." He proposes that the process involves "the assembly of stable but plastic structures from multiple relatively weak interaction forces." Furthermore, and relevant to this discussion, Camps, Erdos, and Ried (2015) have demonstrated that lamin B1 one of the proteins in nuclear membranes is required for proper chromosome condensation in interphase nuclei.

These results suggest that interphase nuclei can "pull themselves together" and that nuclear membrane proteins are critical to the self-assembly. This appears to be what is happening in figs.1 to 4 in this paper. On the basis of Rippe's conclusions, it does

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not appear too far-fetched to suppose that when the chromosomes "find" their discrete territories, and are held in place by the accrual of multiple weak interaction forces, condensing chromatin together with protein accrual and the formation of nuclear boundaries, result in the emergence of nuclei somewhat like the resolving picture of a jigsaw puzzle as it reaches completion.

The emergence of nuclei in Ishikawa predomes fits with research on chromatin self-assembly beginning more than 30 years ago. Woodcock, Frado and Wall (1980) demonstrated that a fundamental property of nucleosomes, the 10nm chromatin unit first discovered by Olins and Olins (1974), is the ease with which it can reassemble *in vitro* from DNA and the appropriate histones. Nuclear self-assembly goes well beyond chromatin self-assembly, but in a paper in 2001, Misteli reviewed compelling evidence for self-organizing subcellular structures with the nucleus as a prime example. Such structures are assumed to be dynamic and, as Wolffe and Hansen (2001) pointed out more than a decade ago, the apparent structural fluidity of the nucleus may well be the bedrock of its functional flexibility. This appears to be an extension of the biochemical paradigm that recognizes the relationship between structure and function in biochemical molecules (Stryer, 1995)

The biochemical modifications affecting that fluidity include chemical changes in histones binding DNA, as well as the binding of other proteins to DNA, and the overall prodigious capacity for differing degrees of chromatin condensation. In drosophila, for example, it has recently been shown that the range of folding from fully extended chromatic fibers to the chromatin of interphase nuclei is 10 fold for euchromatin and up to 30 fold for heterochromatin (Eagen, Hartl, and Kornberg, 2015). Using incomplete chromatin condensation in enlarged rat myelocytic leukemia cells, Trencsenyi et al. (2012) identify 5 stages of condensation between double stranded DNA and the mitotic chromosome.

On the other hand, "nuclear clearings," (discussed in Fleming, 2015b) first detected by Roberts, Horbelt and Powell (1975) in endometrium treated with medroxyprogesterone, and later described by Mazur, Hendrickson and Kempson for endometrium exposed to trophoblasts (1983),demonstrate chromatin at the other extreme, in which the typical nuclear structure has been replaced by chromatin fibers barely visible by light microscopy filling endometrial cells in quasiparallel arrays. Both reports demonstrated that nuclei in endometrial epithelia could be, under certain circumstances, almost completely deconstructed into fibers. An oddity at the time, particularly as a "replacement" for typical interphase nuclei, the approximately 10-11 micron chromatin filament has since achieved some prominence as an essential structure. Fussner and colleagues (2012) have shown that both open and closed (with regard to transcription) chromatin domains in mouse somatic cells are composed of

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approximately 10 nm fibers. Maeshima and colleagues ( ) have suggested that interphase chromatin mainly "consists of dynamic and disordered 10 nm fibers." Derenzini, Olins, and Olins (2014) on the basis of innovative staining techniques for electron microscopy have published results on the interphase nuclei including the observation of 11nm chromatin fibers in transcriptionally active and inactive chromatin

Since the full complement of dome-filling nuclei does not appear all at once, an obvious question concerns the whereabouts of the chromatin that will eventually (a matter of 1 to 2 hours) "show up" in nuclei. Perhaps the chromatin sheet contains much of that material, capable of moving in from the periphery of the dome, as needed. Some of the material may remain beyond the power of the light microscope until self-assembly reaches the point at which something like an ovoid staining structure has emerged. But perhaps the most compelling possibility is that additional synthesis of DNA is taking place even as nuclei are forming.

Chromatic material connecting emerging nuclei

In mitonucleon-generated amitosis, emerging nuclei become bounded relatively rapidly, but not immediately. Emerging boundaries, along with nucleoli-like structures, are what is being detected in the mass of chromatic material in figs. 1a and 1b. The irregularity of the shapes in fig 1b, suggest that ovoid nuclei are still forming. Much of that irregularity is gone in fig. 2a. But it is possible to see overlapping nuclei in that predome suggesting that distribution of the nuclei does not necessarily keep pace with formation. Fig. 2b is a predome that is approximately 75% filled with nuclei at 16 hours after the start of differentiation, and the predome in fig. 3a is almost completely filled with nuclei by 18 hours after the start of differentiation.

In a few predomes, the process seems to be slowed for unknown reasons. The fixed, stained predome in fig. 3b, found at 14 hours, as was the predome in fig. 2a, appears to contain approximately a dozen nuclei in the lower left section of the predome and two prominent but apparently unfinished nuclei in the center. The irregularity of these nuclei is pronounced, so that chromatin material appears to be "bleeding." The linear chromatic material stretching up from the predome periphery to these nuclei, looks like the material connecting nuclei in fig. 2a, further suggesting that this material is involved in the process of nuclear formation. Although most clearly observed in fig. 3b, connecting chromatin is also observed when it is wrapped around an apical membrane protrusion early in emergence of nuclei, as in fig. 2a. Opportunities to detect this material are lost as the envelope becomes systematically filled with a sheet bounded, non-overlapping nuclei.

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Extra-chromosomal linear chromatin has actually been a subject of research for almost 50 years as reviewed by Olins and Olins (2003). "Envelope limited chromatin sheets" or ELCS, the name proposed by these researchers, is a structure mostly made up of approximately 10 micron chromatin fibers, bounded by nuclear envelope proteins such as laminin B (Olins et. al. 1998). ELCS have been found in many different organisms as reviewed by Davies and Hayne (1975) with the number of parallel sheets varying, as well as with differences in whether cytoplasmic material is found associated with the ELCS. On the basis of elegant electron micrographs, the envelope limited sheets are classified into at least three different types with the caveat that "ELCS can achieve breath-taking complexity." (Olins and Olins, 2003).

In the studies reported in this paper, it can only be said that structures similar to ELCS are detected by light microscopy in differentiating Ishikawa predomes (Fleming, 2016b). As remarked upon by Horowitz and Woodcock (2006) there are intrinsic challenges to integrating "information on whole nuclei obtained by light microscopy" with data on higher-order structures obtained by biophysical characterizations. Nevertheless it is an essential activity when examining delicate structures in situ. In consideration of the fact that there are some differences in the literature about the size of the fibers, a range of widths (10 to 11microns) has been used at some points in reference to that smallest of relevant chromatin structures (Olins and Olins, 1972; Derenzini M, Olins AL, Olins DE. 2014) in this paper.

The function of the structurally well-defined ELCS has proved elusive although it is reported to be formed during excessive interphase nuclear envelope growth in a variety of cells. (Eltsov M et al. 2014) Furthermore, it has been described as linking together chromatin blebs, projections, appendages and pockets (Ghadially, 1988) and to exist as "extended sheets within the cytoplasm connecting distant nuclear lobes." (Eltsov et al. 2014) Our results suggest that this ELCS-like material is part of the process of amitotic production of multiple nuclei during differentiation of Ishikawa endometrial epithelial domes.

In another example of differentiation, Olins and Olins have shown significant increases in ELCS in the leukemic cell line HL-60 stimulated to differentiate into a granulocyte by retinoic acid (1998). The differentiation involving changes in the HL-60 nucleus, was also shown to involve fragmentation of HL-60 DNA (Forzani F, Meldrum R, and Shall S., 1987). Additionally, Erenpreisa et al. (2002) have demonstrated increases in ELCS in cells in which mitosis has been blocked (Erenpreisa et al. 2011), but which nevertheless survive, suggestive of a function for ELCS in that survival, perhaps involving amitosis.

Aside from HL60, and polyploid cells that have undergone "mitotic death," the other speculations about ELCS focus on the possibility that the structures, together with

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micronuclei and nuclear buds, are indicative of pathology as reviewed by Fenech et al. (2011). As is true for observations of pyknotic heterochromatin and DNA fragmentation discussed in the second paper in this series, results from Ishikawa differentiation suggest that there may well be a role in normal cell function for what otherwise is thought to be anomalous.

The formation of nuclei by amitosis could result in variability

Since the manner of nuclear production in differentiating Ishikawa cells appears to lack the precision of mitosis, it is probably reasonable to expect that some variability may be introduced into the product genomes, especially if gene amplification occurs. While it has been understood for some time that the amount of DNA in cells can be variable, as in aneuploidy and polyploidy, the absence of a chromosome or the presence of additional chromosomes except for placental giant cells was thought to be anomalous at best, and, at worst, evidence of some kind of dysfunction, most notably in cancer.

Recently, however researchers have suggested that there might be considerable benefits to such conditions. Some examples of polyploidy may be associated with the ability of cells to adapt to external stresses (Schoenfelder and Fox, 2015). One example, as shown with some clever experimentation by Duncan et al.(2010) leads to the proposal that the genetic variations of binucleated polyploid hepatocytes, some of which may arise by amitosis (Wan and Chan, 1986, David and Uerlings, 1992) , as well as by multipolar mitoses (Duncan et al.,2010) may be part of the mechanism whereby liver is able to cope with a variety of toxins.

In the past decade, new sequencing methods, using array-based and cytogenetic approaches, have revealed more subtle and surprising variations in DNA within organisms. Freeman and colleagues (2006) reviewed much of a decade of research on variations in the number of copies of particular genes or DNA sequences, a phenomenon called “copy number variations” or CNV’s. Initially CNV’s were linked to disease with some significant exceptions (Barber et al. 1998; Engelen et al., 2000) Then in 2004, two laboratories reported widespread copy number variations among different normal individuals (Iafrate et al. 2004; Sebat et al. 2004). Each laboratory reported significant copy number differences including upwards of 70 CNV’s affecting sequences within specific genes involved in neurological function as well as in the regulation of growth and metabolism. Copy number variations were also demonstrated for mouse embryonic stem cells leading Liang et al. (2008) to suggest for mice and humans that “all somatic tissues in individuals will be mosaics composed of variants of the zygotic genome.”

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Significant progress has been made in the 10 years since the review appeared. Boone et al.(2010) reported the detection of clinically relevant exonic copy number changes. Researchers continuing studies on possible relationships between CNV and disease, as reviewed by Freed et al. (2014), have linked CNV's to abnormal brain conditions and (Iourov et al., 2010) to neurological diseases (Poduri et al. 2013).

Researchers also began to find CNV's within differentiated human tissues from a single individual (Piotrowski et al., 2008), a possibility clearly contradictory to the orthodoxy that every cell within a single organism contains an exact copy of the parental genome. Testing 34 tissue samples from 3 subjects, these researchers found at least six copy number variations (CNV's) ranging in size from 82kb to 176kb some within known genes, leading to the conclusion that CNV's must "occur commonly during somatic cell growth and division."

There were more surprises when researchers found CNV's among cells within a single organism and even differences among cells within an organ (O'Huallachain et al.,2012). Polyploid giant cells from the mouse placenta have at least 47 CNV's specifically underrepresenting sequences (Hannibal et al. 2014). Additionally analysis of human placentas in all three trimesters resulted in a report of an extensive load of somatic CNV's especially duplications (Kasak et al. 2015). And in one more example, Abyzov et al. (2012) investigating human fibroblast cell lines derived from 7 individuals, estimate that approximately 30% of the fibroblast cells have somatic CNV's in their genome concluding that there is "widespread somatic mosaicism in the human body."

What accounts for mosaicism? Researchers have known for some time that the heterochromatic regions of chromatin are under replicated in certain organisms, such as drosophila, during endoreplication. Investigating polyploidy and its reversal in liver, Duncan and colleagues conclude that the formation of polyploid liver cells by failed cytokinesis and "ploidy reversal" by multipolar mitoses results in a highly diverse population of daughter cells implying, perhaps, that multipolar mitoses is less precise than bipolar mitosis, and therefore more likely to introduce some variability. Meiotic deletions and duplications have been implicated in several genomic disorders (Turner et al. 2008). Lupski, in a review of the field, more generally concluded that mosaicism arises because of errors that occur during chromosome segregation or DNA replication (2013)

Needing to invoke such "errors" too frequently however begins to work against all that has been demonstrated about the fidelity of the mitotic process. Perhaps the production of some nuclei by an amitotic process, under certain defined circumstances, such as the process that produces nuclei for dome formation in Ishikawa endometrial cells, could explain some of the variability that presents itself

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as somatic cell mosaicism. Such a possibility might also explain some of the more unusual examples of mosaicism that are being discovered.

For instance, Mkrtchyan and his colleagues (2010) have investigated genomic mosaicism at the single cell level in 10 different individuals and for three different cell types. In the adult organism, these researchers found stability in the mosaicism that apparently had been introduced early in development in T lymphocytes, B lymphoblasts, and in skin fibroblasts. In fact, on the basis of immortalized B lymphoblastoid cell lines obtained with a 20 year difference from two subjects, they were able to prove stability in mosaicism for at least that length of time. The researchers conclude that CNV's can form early during early embryonic development, but that this mosaicism is actually highly conserved in the adult organism. A phenomenon such as this could be explained by the existence of two different kinds of nuclear reproduction: amitotic generation of nuclei out of chromatin fibers and subject to mosaicism, operative in embryonic connective tissue with mitotic proliferation of these cells occurring in the adult.

In a process such as dome formation, in which gene products from differentiated cells will be secreted into common fluid, some variability in the genome may not be problematic. In fact, in research done to identify proteins that might be synthesized in domes but not in Ishikawa monolayer cells(Fleming, 2016c). The protein leukemia inhibitory factor (LIF), the only protein that appears to be essential for implantation, is not synthesized in monolayer cells. We were delighted to discover that it was synthesized in dome cells, but puzzled that it was only detected in individual cells scattered throughout domes (Fleming, 1999). The finding that the LIF protein is only synthesized in some, but not all, dome cells could be true for many reasons such as differing expression of the gene. But it is also possible that the variability actually has to do with gene copies for LIF differentially incorporated into dome cell nuclei.

Mysteries of Mitonucleon Generated Amitotic Production of Nuclei

Of course, questions arise about the process of mitonucleon-generated amitosis. For starters what is actually happening to the aggregated nuclei surrounded by membranes containing endogenous biotin. Bubble-like vacuoles are generated within the heterochromatin, suggesting that a gas is accumulating (Fleming 2016a). Is it CO2 generated from mitochondrial enzymes oriented so that the products of metabolism end up mixing with heterochromatin? Or is it possibly a mixture of gasses? Might the vacuole also contain gasotransmitters? Is the function of these vacuoles to alter the specific density of the mitonucleon so that it pushes against the apical membrane initiating the process of elevation? And, in the process, is the DNA being fragmented so that it can be "remodeled" for the functions essential to dome cells.

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The physical reality of pyknosis, involving as it does the condensation of multiple nuclei to less than 50% of their former volume as determined from photomicrographs in the second paper of this series (Fleming, 2016b), suggests that such conditions might be right for nuclear fusion and might work against the possibility of DNA synthesis. A study done 40 years ago, in rat trophoblasts, demonstrated syncytial formation, followed first by enlargement of nuclei (aggregation perhaps) and then by compression (pyknosis, perhaps). Using tritiated thymidine, the researchers concluded that DNA replication was not occurring in the condensed syncytial nuclei. (Hernandez-Verdun and Bouteille, 1976). As will be discussed shortly, trophoblasts and placental tissue have much in common with the Ishikawa epithelial cells including the capacity to proliferate amitotically.

The central, "shocking" image in Ishikawa differentiation is the explosion of disaggregating nuclei into chromatin granules (Fleming 2016b). That effect mimics similarly fascinating *in vitro* results from Szabo's laboratory using field inversion single cell gel electrophoresis. When a single healthy nucleus in agarose was treated with a protease and elevated pH (**Székvölgyi L. et al.** 2005), the nucleus exploded into chromatin granules. Are the Ishikawa nuclear aggregates subject to proteolytic activity in the pyknotic structure that looks like a "signet ring" cell? Is there a brief elevation in pH when the vacuole gases mix with cytoplasm that interacts with fragmented DNA to effect the same explosive result? There is precedent for internal cellular alkalization (Bouyer et. al. 2007) in response to the dynamic of CO2 and the HCO3 that might result from such sudden mixing.

It is even more intriguing that the chromatin filaments come back together again in a short period of time and in a sufficiently functioning manner to be the source of amitotically produced nuclei. Filament alignment on the microtubules must be important. (Fleming, 2016b). Are the chromosomes built up completely from these filaments or is the heterochromatin seen on the edges of predome during the fragmentation phase (Fleming, 2016b) also involved? Of course, It is quite amazing, and well to remember in the context of these results, that "vegetatively growing ciliates appear to possess a mechanism for adjusting copy numbers of individual genes, which corrects gene imbalances resulting from random distribution of DNA molecules during amitosis of the macronucleus." (Prescott, 1994) Evolutionarily speaking this would appear to be at least as sophisticated a mechanism as the mechanics of mitosis. Or to put that another way, if prokaryotes can do this, perhaps so can eukaryotes!

And finally, does DNA synthesis occur at any time during this process? Multiple genomes in the chromatin of the mitonucleon were "brought into" the structure by the individual nuclei of the monolayer cells induced to differentiate. As already

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discussed, it seems unlikely that DNA is synthesized when the chromatin is in the pyknotic state. But over the period of an hour or so that DNA is arrayed throughout the apical membrane of the predome, it is certainly possible that some DNA synthesis is occurring, involving replication of the entire genome and/ or amplification of genes whose products will be secreted into the dome fluid.

For mitonucleon-generated amitosis, it is possible to approximate the number of monolayer nuclei involved in the initiation of dome differentiation. Four to eight nuclei have been observed within a single mitonucleon, and frequently 4-6 mitonucleons are observed in a predome (although dome size is variable with different Ishikawa clones). The range of input of nuclei then might be something like 24 to 48. The number of nuclei in a finished dome such as is shown in fig 3 is approximately 60 to 80 which would argue for at least one round of replication of the genomes contained in the monolayer nuclei that initiated the process.

These numbers are only approximations, but overall it does appear that more nuclei come out of the initial dome differentiation than went into it. Furthermore, domes enlarge and appear to do so by the same process that creates domes, i.e. cell fusion and the formation of vacuolated mitonucleons as seen in the bottom third of the dome in fig. 6. Domes can, in fact, enlarge into gland-like structures, as shown in fig. 7. Mitonucleon-generated amitosis appears not only to be a means of “recycling” the DNA from monolayer nuclei, but also a mechanism for proliferation just like mitosis, albeit possibly generating some genome diversity.

In this regard, it seems highly relevant that researchers from Thilly’s laboratory found that the bell shaped nuclei discovered in fetal gut segregated and doubled their DNA content coordinately during and after amitotic fission (2014). This may be true of dome cell differentiation as well, although it may not be true for all varieties of amitosis. In the amitotic process that has been called depolyploidization, multiple copies of the genome appear to have already been synthesized through endoreplication.

Furthermore in one of their earliest papers on cells with amitotically generated bell shaped nuclei, researchers from Thilly’s laboratory demonstrated cells with bell-shaped nuclei are responsible for net growth and differentiation in the embryonic gut as well as in adenocarcinomas and belong therefore to the category of post-embryonic stem cells (Gostjeva et. al. 2006). Not only is this a significant example of amitosis, but it affirms the relevance of amitosis to development. The fact that amitosis can generate stem cells should remove all doubt about the importance of the “other” processes of nuclear generation that fall under the heading of amitosis.

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What are the advantages to mitonucleon-based amitotic nuclear production

It is reasonable to wonder what advantages might be intrinsic to the process of mitonucleon-generated amitotic production of nuclei observed in Ishikawa dome differentiation. Perhaps the mechanics of the process make sense when a population of cells is being adapted and expanded for a new function. The number of nuclei in the almost mature dome shown in fig. 3a is approximately 80 and those reconstituted nuclei appear during the final (1 to 3) hours of a 16-20 hour differentiation period derived from approximately half that number of monolayer cells. Under some circumstances, there may be real advantages to an assembly line approach to nuclear production as opposed to doubling nuclei in the context of 24 hour cell cycles, even shortened cycles.

Another possible advantage to amitosis is the elimination of the mitotic phases. Neither energy nor time has to be expended lining up the chromosomes or pulling them apart, or in the conversion of chromosomes back to their typical state in the interphase nucleus.

Starting from the fascinating observation of copy number variations in pancreas, liver and intestine, O'Huallachain and colleagues (2012) speculate about whether the somatic genetic differences described in their paper play a role in the differentiation process in these organs. To that, could be added the speculation that the differentiation process itself may play a role in the generation of CNV's whose existence is either not problematic, or may actually be desirable, in somatic cells.

Are the Processes of Ishikawa Dome Differentiation Unique?

There appears to be at least one other system in which phenomena, similar to what has been shown for differentiating endometrial epithelial, are seen. As already discussed, Cotte et al. (1980) published studies of cultured human placental cells forming multinucleated cells by the process of amitosis. Researchers investigating the tissue itself in organ culture have found structures similar to what we have described during Ishikawa differentiation. The similarities include the existence of a syncytium and the aggregation of nuclei within it. Called syncytial knots by Fox (1965) these structures were described as "closely packed nuclei displaying heavily condensed chromatin, and frequently gently protruding from the villous surface" (Burton and Jones, 2009). As discussed in the first paper of this series (Fleming 2016b), it is possible to detect the apical membranes of mitonucleons protruding from a monolayer undergoing differentiation. Additionally elements called syncytial sprouts

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(Boyd and Hamilton, 1970), associated with proliferation of the villous tree are found, capable of "extending" the tree much as domes can be extended in culture into gland-like structures. Syncytial knots have not yet been shown to develop into syncytial sprouts but, by analogy, that may be the case, as mitonucleons develop into domes and gland-like structures. Interestingly enough, and not surprisingly, all of the evidence of heterochromatin compression in the knots led to speculation about programmed cell death. But researchers have found no evidence for this (Mayhew et.al. 1999; Burton and Jones, 2009; Coleman et al., 2013; Longtine et.al. 2012) as is also true for pyknotic mitonucleons (Fleming, 2015b).

Finally, the fact that Kasak et al. (2015) find "an extensive load" of CNV's, especially duplications, in placental tissue, suggests the possibility of gene amplification, a process already speculated upon as an outcome of mitonucleon-generated amitotic division. Taken together these studies suggest that not only do some trophoblasts arise by fusion and amitosis, but also that mitonucleon formation may occur during that process of fusion, a possibility that can be readily tested.

A Word About Methods

The evidence presented in this paper is not unlike evidence that has been presented by cell biologists for more than a century, using tools that extend what can be seen . Additionally, the isolation of a uterine endometrial cell line (Nishida et al.) with characteristics of the endometrial epithelial cells *in vivo* resulted in cells capable of complex "behavior." The ability of the cells to form domes that can extend into gland-like structures in a process stimulated by progesterone presented an opportunity to study at least one aspect of the way in which human endometrial cells differentiate in preparation for implantation.

The advantages of light microscopy as it applies to monolayer cells in a petri dish include the ability to examine some of the more delicate structures involved in the differentiation process such as membrane protrusions. And looking at the entire process over 16 to 20 hours provided the context for relating morphological changes one to another. Out of such a context, and especially in sectioned material, some of the results is this paper would certainly be open to different interpretations. For instance, without the opportunity to observe the process of nuclear aggregation in differentiating syncytia at the start of differentiation, the presence of multiple genomes in a cross section through mitonucleons might be thought to result from endocycling, DNA synthesis and formation of mitotic chromosomes without the final step of cytokinesis.

It might be tempting to conclude that the appearance of chromatin threads in the predome clearing has something to do with polyteny, an extremely vivid example of

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chromatin fiber array, were it not for the evidence shown in the previous paper (Fleming, 2016b) that the chromatin threads are not generated in the manner of polyteny, but instead form from fragmentation of multiple nuclei in mitonucleons. Furthermore for a short period of time, the Ishikawa predome in cross section might resemble a giant multinucleated cell, a condition also thought to arise from endoreplication. Our results demonstrate that the array of nuclei is produced amitotically within 2 to 3 hours from chromatin previously contained in mononucleated monolayer cells that has been: aggregated, vacuolized, compressed, dramatically fragmented, intriguingly arrayed, and finally pulled back together again to form interphase nuclei in a differentiated structure.

Summary of Other Possibly Useful Observations

As discussed in the second paper in this series, DNA fragmentation is not an automatic sentence to programmed cell death (Sjaste and Sjaste), nor is pyknosis. Not nearly as lopsided as mitosis v amitosis, it may be worth noting that DNA fragmentation in cell death calls up more than 20,000 citations in the Medline database while DNA fragmentation in differentiation calls up fewer than 2,000 citations. Researchers, new to the field, might want to be aware that sometimes these processes can "resemble" each other (Fleming, 2016b).

Some of the stages in Ishikawa differentiation such as vacuolization of heterochromatin and elevation of apical membranes (Fleming 2016a) result in atypical cellular structures previously thought to be characteristic of cancer such as cells with "optically clear" nuclei and "signet ring cells." (Fleming, 2016b). These results suggest that such structures represent a possibly attenuated "effort" by epithelial cancer cells to differentiate.

Finally, some of the structures associated with Ishikawa differentiation had previously aroused curiosity without a ready answer to what function they might serve. Why, for instance, do mitochondrial carboxylases associate with nuclei. In Ishikawa differentiation that association results from a "mitochondrial-like" membrane that wraps around aggregated nuclei creating a structure that can become filled with gas and move epithelial cells up into three dimensional structures. Research done on reduced cell membrane permeability to CO2, as well as on aquaphorins that exchange CO2 for H20, (as reviewed in Fleming, 2016a) provides a function, in addition to elevation, for vacuolization in differentiating Ishikawa cells.

ELCS is another well-researched structure whose function is unclear. Results in Ishikawa differentiation suggest that one function may be involvement in the process of amitosis.

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Mitonucleon-generated amitosis may, by a significant increase in efficiency, make up for its deficiency in the iconography of precise and equal distribution of genomes into newly generated nuclei. That efficiency applies to the process itself and to the fact that the bonds formed in the DNA molecule constitute an energy investment. If the molecule is no longer needed to serve the purpose for which it was originally synthesized, how much better to alter and "recyle" the molecule than to destroy and synthesize anew.

With regard to precision, it is obviously not easy to see the ways in which the process of amitosis is held to some minimum essential standards, but they must exist since dome cells are functional. And, possibly, copy number variability is not always a liability but can be a boon in certain organs such as secretory endometrium and trophoblasts which are complex, transient structures that serve functions not needed at any other time.

Nuclear clearing might be another opportunity for gene amplification, as in polytene chromosomes, whose function, at least in general terms, has been appreciated for decades. Perhaps mitonucleon-generated amitosis is "evolution's" solution for conditions in which nuclei do not need to be genomically identical to each other, or to the cells from which they originated, but do need to be produced relatively rapidly from a genome that has been strategically altered so that it will produce proteins essential for the differentiated cell.

These are, at least, some possibilities with regard to the unusual process of mitonucleon-generated amitosis during Ishikawa differentiation. The process itself is undeniable, although there are still many details to decipher. Hopefully the data in these three papers contains, quoting Macklin all these years later, "the correct interpretation of the succession of morphological and physiological changes revealed by prolonged observation" of Ishikawa dome differentiation. Together with all of the research on other forms of amitosis sited in this paper, mitonucleon-generated amitosis provides one more example that early cytologists such as Child, Macklin, and Flemming were onto something.

Materials and Methods

Ishikawa cells were cultured (Fleming 1995) in phenol red-free, Minimum Essential (MEM) supplemented with 2 mM glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin, and .25 mg amphotericin B (GIBCO, Grand Island, NY). The cells, obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital in New York, were originally derived from an endometrial adenocarcinoma line developed by Nishida et al. (1985), who demonstrated the presence of receptors for both estradiol and progesterone. Cells seeded at an approximate density of 5 x 10^5 cells/cm2, were

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grown for 1 -2 weeks in MEM containing 5% calf serum (CS), and then transferred to medium containing 1% calf serum. Cultures left in MEM with 1% CS could survive for an additional 3-5 days with little proliferation. Assays for dome formation were done in confluent cultures, although differentiation has been observed to occur, to a limited extent, in nonconfluent cultures.

Differentiation was initiated with the addition of l0-15% fetal bovine serum (FBS). Multiple dishes were fixed and stained for biotin and./or for chromatin at different times during differentiation. Structures were viewed using an Olympus inverted stage microscope at powers of 100X, 200X and 400X. As indicated in the text, differentiating structures were sometimes examined, and pictures taken without fixing and staining the cultures.

Other photomicrographs were taken of cells fixed by adding 4% paraformaldehyde in phosphate buffered saline (PBS) to the culture dish. After 10 min, the cells were washed gently four times with 5-10 ml PBS. A solution of 1% Triton X-100 was added to the cells to permeabilize the membrane. Again after 5 min, the culture was washed with successive changes of PBS. After washing, cells were exposed to a1:200 dilution of Extravidin-conjugated horse-radish peroxidase (HRP) (Signa) for 30 min. After further washing, a solution of 3-amino-9-ethylcarbazole (AEC), prepared by dissolving 20 mg of AEC in 2.5 ml of dimethylformamide and diluting with 47.5 ml of 50 mM potassium acetate adjusted to pH 5.0, was added to the cells together with .25% H2O2. This solution"was incubated at 37"C for 45 min to allow color to develop. The AEC solution was removed, and the cultures were examined and then stored in the presence of PBS at 4"C. If avidin linked to peroxidase is not added to the cultures, there is no reaction. If avidin without peroxidase is added first to the cultures, followed by avidin-linked to peroxidase, staining is not observed. Staining does not occur if avidin-HRP is not added to the cultures prior to AEC indicating that an endogenous peroxidase is not responsible for the staining. To ensure that avidin was reacting with biotin, we stained domes using streptavidin linked to horseradish peroxidase as well as primary antibody to biotin and secondary antibody-linked to horseradish peroxidase. Staining occurred under all circumstances, indicating that avidin does indeed react with biotin that is endogenously present in the cell in significant amounts.

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