Mitonucleons formed during differentiation of Ishikawa endometrial cells generate vacuoles that elevate monolayer syncytia: Differentiation of Ishikawa Domes, Part 1

Honoree Fleming

Castleton State College, Castleton Vt., Dean of Education, retired

founder CancerCellsinVitro.com

Castleton, Vermont

USA

Emails: honoree.fleming@castleton.edu or

honoree@cancercellsinvitro.com

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

Abstract

In 1998, we published a paper (Fleming et.al, 1998) describing some aspects of Ishikawa endometrial epithelial cell differentiation from monolayer cells into cells forming fluid-filled hemispheres called domes. The process begins with the dissolution of membranes within discrete regions of the monolayer. Nuclei from fused cells aggregate and endogenous biotin in particulate structures assumed to be mitochondria increase throughout the resulting syncytium. Endogenous biotin is also the distinguishing feature of a membrane that surrounds aggregates of multiple nuclei in a structure called a mitonucleon. The current paper includes additional observations on structural changes accompanying Ishikawa differentiation. Vacuoles form in the heterochromatin of the mitonucleon and within the biotin-containing double membrane surrounding heterochromatin. With the formation of vacuoles, the mitonucleon can be seen to rise along with the apical membrane of the syncytium in which it formed. The small vacuoles that form within the heterochromatin result in structures similar to "cells with optically clear nuclei" found in some cancers. The second larger vacuole that forms within the membrane surrounding the heterochromatin transforms the cell profile to one that resembles "signet ring" cells also observed in some cancers. Eventually the membrane surrounding the massed heterochromatin, generated three to four hours earlier, is breached and previously aggregated nuclei disaggregate. During this process heterochromatin in the mitonucleons undergoes changes usually ascribed to cells undergoing programmed cell death such as pyknosis and DNA fragmentation (Fleming, 2016b). The cells do not die; instead chromatin filaments appear to coalesce into a chromatin mass that gives rise to dome-filling nuclei by amitosis during the final three to four hours of the 20 hour differentiation (Fleming, 2016c).

Introduction

Endometrial epithelial cells lining the uterine cavity proliferate and differentiate in response to the hormones estradiol and progesterone in preparation for implantation of a fertilized egg in humans. More than 30 years ago, researchers including this author, began working with cultures of human endometrial epithelia hoping that some aspects of this process could be studied *in vitro* (Fleming, 1999).

Endometrial cancer cell lines retain some of the characteristics of their *in vivo* counterparts and have the distinct advantages of predictability and ready availability for experimentation. But some cell lines retain more of the organ appropriate characteristics than other lines and that proved to be true for the Ishikawa line. Started from a well-differentiated adenocarcinoma and found to contain estradiol and progesterone receptors (Nishida et al. 1985) the cells were shown capable of

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

functions characteristic of normal endometrial cells such as enhanced proliferation in response to estradiol and tamoxifen (Holinka et al.,1986a). Holinka and colleagues also demonstrated that placental alkaline phosphatase became elevated in these cells in response to hormones (1986b). Having received these cells from Dr. Erlio Gurpide's laboratory through Dr. Chris Holinka, we discovered their capacity to form fluid-filled multicellular hemispheres and decided to study what we believed might be an example of epithelial differentiation.

To characterize the process we needed to find conditions that predictably resulted in dome formation (Fleming, 1995), determine what factors enhanced the process (Fleming et al. 1998), look for the synthesis of new proteins (Fleming et.al. 1995; Fleming 1999), and finally examine the structural changes underlying differentiation.

Progesterone and a large factor in fetal calf serum stimulate dome formation in confluent monolayers of Ishikawa cells, with dimethyl sulfoxide (DMSO) and fatty acids enhancing the process (Fleming et al. 1995; 1998; 1999). The process occurs over a 16 to 20 hour period starting with the formation of syncytia in the first 4 to 6 hours when stimulating factor contained in fetal bovine serum is added to confluent, quiescent monolayers. The development of elevated predomes from syncytia occurs over the next 6 to 8 hours, and finally mature domes appear after 4 to 6 more hours. Clones could be isolated that made more and larger domes including a clone that routinely extended domes into everted gland-like structures (Fleming et al., 1998; Fleming, 1999; Fleming 2016c).

The early research demonstrated a role for mitochondria whose numbers increase dramatically in newly formed syncytia. The most unexpected involvement of endogenous biotin was its presence in membranes that envelop aggregated syncytial nuclei early in the differentiation in transient structures we have called mitonucleons. But endogenous biotin associated with nuclei had actually also been discovered for nuclei of some biopsied cancer tissues (Tsujimoto, Noguchi, and Taki, 1991; Yokoyama et al., 1993; Tanaka et. al., 1998; Gamachi et al. 2003). It turns out that the process of differentiation being studied in Ishikawa cells relates not only to what occurs in cycling endometrium but also may explain the apparent presence of nuclear endogenous biotin in some cancers. This paper explores the possible significance of endogenous biotin linked to mitochondrial carboxylases in mitonucleons in differentiating Ishikawa cells.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

Results and Discussion

Fig. 1 shows a syncytium formed in an Ishikawa monolayer in response to the stimulus to differentiate (Fleming, 1995). Levels of stainable endogenous biotin (salmon), not detectable when fusion initially occurs, increase dramatically in syncytia and ultimately characterize a membrane that envelops nuclear aggregates in transient structures (Fleming et.al. 1998) called mitonucleons (Fleming, 2014). Initially, it is possible not only to detect multiple nuclei within the mitonucleon, but even to approximate the number. As few as 4 and as many as 10 individual nuclei have been seen within mitonucleons.

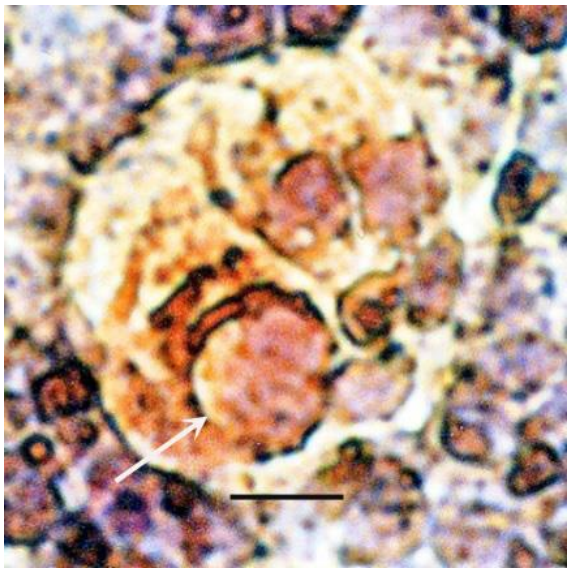

Fig. 1 Syncytium six hours after the addition of fresh medium with fetal calf serum to elicit dome formation in a confluent, quiescent monolayer of Ishikawa endometrial cells.

Endogenous biotin increases dramatically in syncytia including in a membrane that envelops aggregated nuclei. Chromatin stains blue with hematoxylin and eosin. Endogenous biotin stains salmon using avidin linked to peroxidase together with AES. For a brief period, it is possible to detect the presence of multiple nuclei within the enveloping membrane (white arrow). (bar=50μ)

Relatively rapidly, the mitonucleon becomes opaque so that individual nuclei can no longer be detected as shown in fig.2, suggesting that a second membrane, at least, has been elaborated around the structure. The chromatin will become compressed between the double membrane and the apical membrane of the syncytium as a vacuole within the double membrane grows in size. The “nucleus-like” mitonucleon stains maroon, the salmon stain of endogenous biotin in the enveloping membranes overlaying the blue hematoxylin stain of the enveloped heterochromatin.

The involvement of mitochondria in this differentiation adds to a growing list of functions for these organelles shown to be more than ovoid powerhouses over the last 20 years. They have been shown to be diverse with regard to size, structure, placement in the cell, extent of polarization and perhaps even functions (VanBlerkom et al., 2002; Liesa, Palacin, Zorzano, 2009; Wang et al., 2012). Mitochondria have

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

been shown to be involved in apoptosis (Karbowski and Youle, 2003) and it is theorized that the perinuclear positioning of mitochondria may be relevant to the quality of "stemness." (Bavister, 2006; Lonergan and Bavister, 2007; Rehman, 2010) Subplasmalemma mitochondria have also been identified in mouse oocytes (VanBlerkom et al. 2002) Finally, in cells treated with microtubule-active drugs researchers observed "perinuclear clustering of mitochondria i.e. mitochondria encircling the aggregated chromatin of the nucleus that had lost the nuclear membrane" (Kedzior et. al. 2004) These effects may be related to what has been observed during Ishikawa differentiation, although there is no mention of a membrane enveloping multiple nuclei as is true for the mitonucleons that form during Ishikawa differentiation.

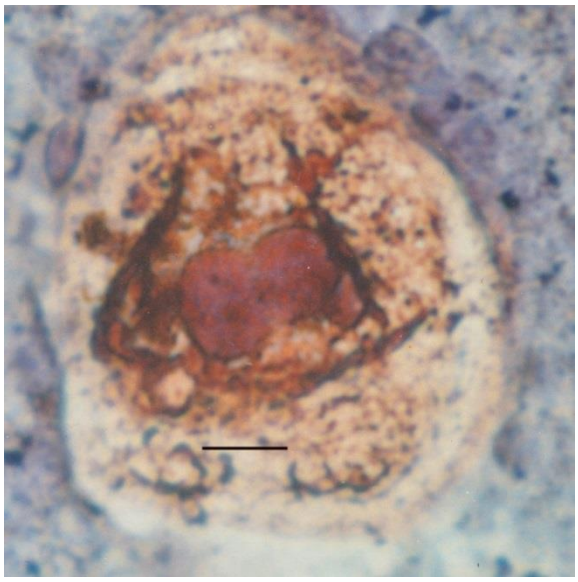

Fig. 2 Aggregated nuclei in multiple mitonucleons elevating with the apical membrane.

The mitonucleon structure becomes opaque due most probably to the elaboration of a second membrane around each of the nuclear aggregates. Mitonucleons together with apical membrane of the syncytium begins to elevate. Plane of focus for mitonucleon is above that of the monolayer.
Bar = 50 microns

On the other hand, numerous reports of membranes generated from mitochondria do exist. These include vesicles that form in HeLa cells and appear to participate in communication within the cell (Neuspiel et al 2008; Andrade-Navarro MA, Sanchez-Pulido L, McBride HM., 2009; Sugiura A. et al., 2014), as well as mitochondrial outer membranes that form autophagosomes in starved rat kidney cells (Hailey et al., 2010). Ding and collaborators have shown that, following administration of an electron transport uncoupler, whole mitochondria can become spheres engulfing various cytosolic components including other mitochondria (Ding et.al. 2012). And, in an intriguing result from non-differentiating Ishikawa cells, it was recently shown that a membrane staining for biotin can also envelop chromosomes under certain circumstances in Ishikawa monolayers. (Fleming, 2014)

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

Within 2 to 3 hours, the mitonucleons begin to elevate with the syncytial apical membrane as shown in fig. 2. Three to four mitonucleons in the center of the syncytium only come into sharp focus above the monolayer, surrounded by apparent "folds" indicating that the elevation may have been higher before the structure was fixed and stained. The pervasiveness of mostly particulate material staining for endogenous biotin throughout the syncytium is also clear at this stage of the differentiation. Fig. 3 shows that the heterochromatin of elevating mitonucleons contains vacuoles. This photomicrograph was taken of a living culture and the vacuoles only come into focus above the plane of the monolayer cells, indicating elevation of the syncytium. The vacuoles are occluded when enveloping membranes are stained for endogenous biotin as in fig. 2.

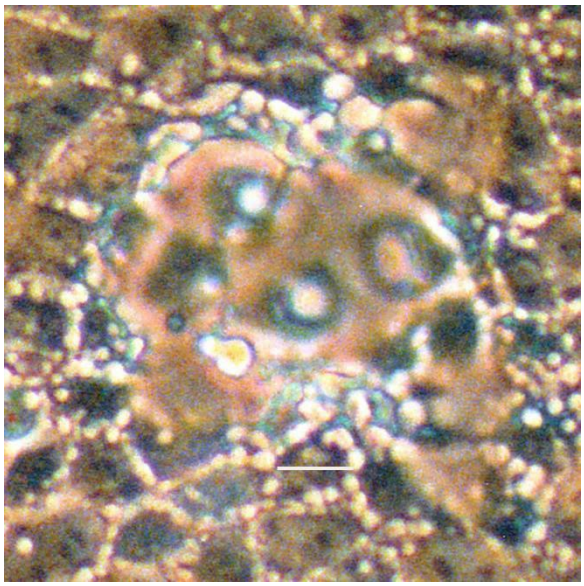

Fig. 3a Focusing on a monolayer in the midst of which a predome containing mitonucleons is beginning to elevate.
Vacuoles form in neighboring monolayer cells as well as in the mitonucleons, but vacuoles in the mitonucleons appear to be larger and have a dramatic effect on the surrounding heterochromatin. Bar=25 microns

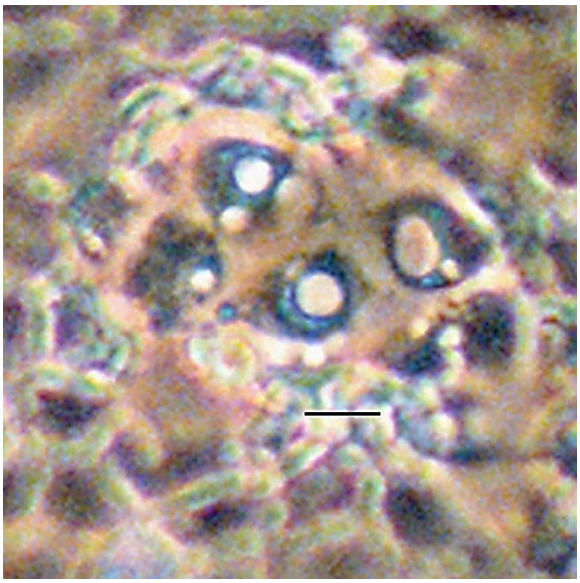

Fig. 3b Focusing on the elevating apical membrane of the elevating predome.
Vacuoles appear to be compressing the surrounding heterochromatin in a structure found in endometrium during pregnancy and in certain kinds of tumors where they have been called "optically clear" nuclei.
Bar=25 microns

As the bubble-like vacuoles enlarge within and compress the heterochromatin, the resulting structure (fig.3) comes to resemble "optically clear nuclei" identified in normal tissue (Mazur MT, Hendrikson MR, Kempson RL, 1983) as well as in cancer, and found to be associated with endogenous biotin. Such structures have been of interest

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

to oncologists since Hapke and Dehner (1979) first identified them in a papillary carcinoma of the thyroid. Aside from the "odd" look of vacuolated nuclei, Yokoyama and his colleagues (1993) reported the surprising result that endogenous biotin, thought to localize exclusively to mitochondria, could be found associated with optically clear nuclei. In a thorough review of tissues containing optically clear nuclei, Gamachi and his colleagues (2002) showed not only that endogenous biotin could be detected in 27 samples of tissue containing optically clear nuclei, but also demonstrated that the endogenous biotin is specifically associated with mitochondrial enzymes, pyruvate carboxylase and propionyl carboxylase. These otherwise unexpected results make sense if optically clear nuclei in cancerous tissues, as well as those that form in endometrium in response to pregnancy (Mazur MT, Hendrikson MR, Kempson RL, 1983), arise in the manner of the structures shown in fig. 1, that is to say nuclei become enveloped by membranes containing mitochondrial carboxylases and perhaps other mitochondrial proteins.

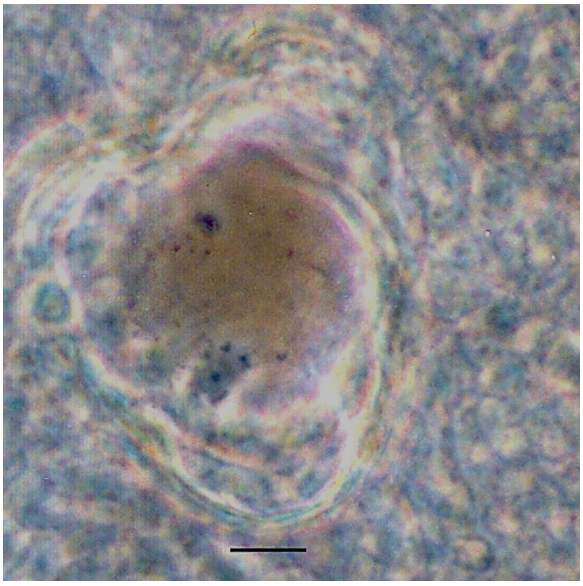

Fig. 4a Focusing on membrane telescoping upward from a syncytium in a living culture that has not been fixed or stained.
The apical membrane of the predome extends upward during the next phase of the differentiation. The nucleon is clearly flattened against the rising membrane. Bar=25 microns

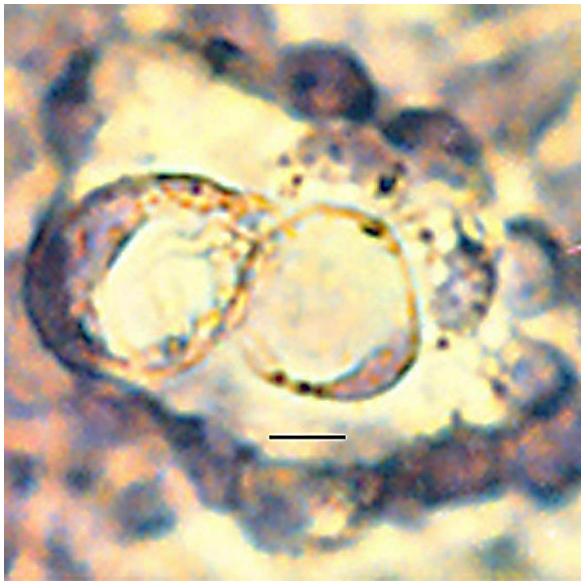

Fig. 4b Predome with two protrusions, fixed and stained.
Upward extending membranes collapse back into the syncytium where the framework of the extension can take on the appearance of a cell membrane.
Bar = 25 microns

Additionally, a large central vacuole begins to form within the double membrane elaborated around the aggregated nuclei compressing the heterochromatin even

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

164 further against the elevating apical membrane (fig. 4a). This vacuole appears to be
165 responsible for significant elevation of the apical membrane, the full extent of which
166 can only be appreciated by focusing above the monolayer in unfixed, living cultures.
167 Even then, it is clear that the boundaries of the protrusion are not all in focus. The
168 protrusions collapse if the predome is fixed and stained as in fig.4b. The collapsed
169 vacuole looks like the annulus of a "ring" whose signet stone is the heterochromatin,
170 now quite pyknotic.

171 The other prominent feature of the "rings" in fig. 4b includes dark particulate
172 structures, contained within the double membrane surrounding the vacuole. Similar
173 structures can be observed in fig 4a, outside of, but accompanying the nucleon
174 compressed against the elevating apical membrane. Subsequent deployment of
175 microtubule-like structures (Fleming, 2015b) suggests that these may be centriole-like
176 structures. While only a single protrusion can be detected in fig. 4a, multiple
177 protrusions, seen in fig 4b, are more the rule than the exception. It appears that
178 each mitonucleon is capable of generating a vacuole that will elevate a portion of the
179 apical membrane.

180 The fixed and stained predome in fig. 5 provides insight into how mitonucleons
181 disassemble. The membrane protrusion on the left in fig. 6 resembles the protrusions
182 in fig. 4b, except that at least three pyknotic nuclei appear to make up the "signet
183 stone" suggestive that the nuclear aggregate formed several hours earlier is coming
184 apart. Numerous particulate nucleoli-sized structures can be observed in the space

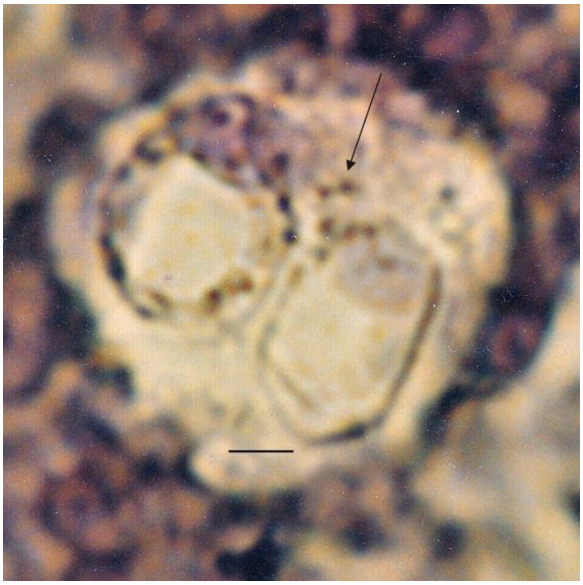

Fig.5 Fixed and stained apical membrane protrusions showing signs that the mitonucleon is falling apart.

The heterochromatin in the left-most mitonucleon looks pyknotic, almost wafer like in profile, although the aggregated nuclei appear to be disaggregating. Subtle but detectable changes have occurred in the second mitonucleon possibly due to a breach of the membrane that originally encircled chromatin. Dark structures appear to be "leaking" out of the surrounding membrane and the chromatin has begun to spread.
Bar=50 um

185

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

between the inner and outer vacuolar membranes. While in the neighboring mitonucleon, those structures are leaking into the syncytial cytoplasm (arrow) as the mitonucleon double membrane begins to break down.

The chromatin also appears to be "spreading" out from its pyknotic state, suggestive of critical changes in the chromatin as the enveloping membrane that stained for endogenous biotin disassembles. The fate of the chromatin in these structures is discussed in the accompanying paper (Fleming, 2015b).

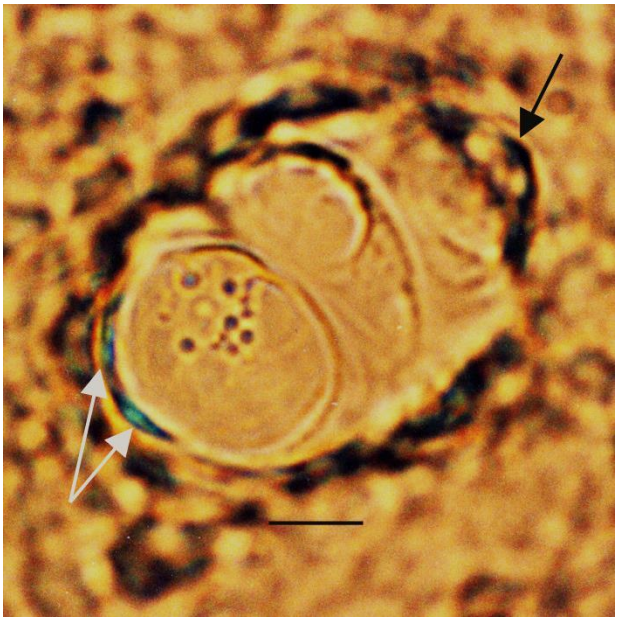

Fig. 6 Bright-field micrography focusing on the three protrusions in the apical membrane of a predome approximately half way through the process of differentiation.

An arrow points to heavily chromatic material in the protrusion furthest to the right elevating with the apical membrane. The chromatic material in the left-most protrusion appears to be spreading in the syncytial envelope, around the projection. Chromatin is not visible in the projection in the center. As the accompanying paper describes, the deconstruction of chromatin into 10 micron fibers can produce what appears to be a nuclear clearing, as in the central protrusion. The fibers then coalesce into a mass of chromatin found in the apical-basal membrane at the base of protrusions.
Bar = 25 microns

Bright field micrography of an unfixed structure allows visualization of the surfaces of the predome protrusions resulting from mitonucleon activity. Protrusions early on are characterized by dense heterochromatin moving up with the apical membrane, and flattened against it by the central vacuole (black arrow in fig 6). In the left-most protrusion in fig. 5, by contrast, a mass of spreading refractive chromatin (white arrows) formerly confined in a mitonucleon appears to be spreading, resembling the mass of chromatin that forms following fragmentation of disaggregated nuclei (Fleming, 2016b). The fact that nothing can be seen in the middle protrusion may indicate that this is the stage in differentiation when chromatin is deconstructed into 10nm fibers (Fleming, 2016b) It is tempting to speculate that the punctile discontinuities in the protrusion itself are involved in the accumulation of fluid under the structure. The mitonucleons in fig. 5 appear to be at three different stages. The

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

mitonucleon on the right, intact and elevated is the earliest stage. The mitonucleon in the middle may be at the stage of chromatin deconstruction. The 10nm filaments characteristic of that stage come together once again at the base of apical membrane protrusions, which appears to be what is being seen in profile in the protrusion to the far left. A complete description of those stages can be found in the second paper of this series (Fleming 2015b)

Commonality of structures in differentiating endometrial epithelia and in cancer tissue

Structures closely resembling those derived from mitonucleons in differentiating Ishikawa cells are frequently observed in cancer tissue. More than one term has been used to describe vacuolated nuclei resembling structures in fig. 2 including optically clear, ground glass or empty nuclei. It was suggested that these structures might serve as a diagnostic criterion for papillary carcinoma of the thyroid gland more than 30 years ago (Hapke MR, Dehner, LP, 1979) The structures have since been found in many other cancers of which the following are representative: colonic tubular adenocarcinoma (Sasaki et al. 1999), ovarian borderline endometrioid tumor (Li et.al. 2002), pancreatoblastoma (Hasegawa et al., 2003), and adenocarcinoma of the gall bladder (Kimura et.al. 2005).

Another cell structure associated with cancer is the “signet ring cell.” More than 2800 references are listed in the Medline data base as relevant to the descriptor “signet ring cell carcinoma,” from many different organs including stomach, colon, lung, and ovary, with the earliest reference discussing their appearance in bladder cancer (Rosas-Uribe and Luna, 1969). Some cancers are even named for this particular cell structure such as signet-ring cell melanoma (Grilliot, Goldblum, Liu; 2012) and signet ring cell carcinoma of the testis (Williamson et al., 2012)

On the other hand papers have also appeared reminding pathologists that not all signet ring cells are neoplastic (Iezzoni and Mills, 2001). In Ishikawa differentiation, these structures are derived sequentially from mitonucleons. An obvious question then is why haven’t mitonucleons per se been identified in tissue cross sections. One possible reason is that the structures are relatively non-descript without the vacuoles. Their dense chromatin and meager cytoplasm may look like “small cells” or even “bare nuclei” (Wright, Leiman, Burgess; 1998).

The sequential appearance of “optically clear nuclei” followed by “signet ring cells” in differentiating Ishikawa epithelia cells demonstrates that these structures represent morphological stages in a differentiation program for epithelial cells. Such a possibility would not, of course, be obvious from visual micro-inspection of a tumor at a single point in time, the necessary approach when cancerous tissue is excised. It

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

has actually been understood for some time that tumors can be classified as poorly or well- differentiated and that the prognosis of the latter is usually better than that of the former. A preponderance of cells with optically clear nuclei or of signet ring cells in a biopsy may represent gradations between poorly and well differentiated.

Membrane elevation during Ishikawa Differentiation

The physiologically significant event during the first 10 hours of Ishikawa dome differentiation is the elevation of syncytia containing mitonucleons. Vacuole formation appears to be the driving force. The rapid rise and fixation-dependent collapse of the apical protrusion suggests that the central vacuole is filled with material readily generated and easily dispersed such as a gas. The simplest, albeit unorthodox, explanation is that the mitochondrial-like membranes enveloping aggregated nuclei contain the metabolic enzymes necessary to generate CO2, and are oriented so that CO2 accumulates both within the nuclear compartment and within the double membrane surrounding the aggregated nuclei. The stimulus to differentiate was found to be most effective when delivered with fresh medium to a quiescent monolayer, which, of course, contains glucose. It is useful to note that numerous vacuoles also appear to be generated in surrounding cells but not into heterochromatin, rather at the borders of the cells that are not differentiating (an example of that can be seen in fig. 3a)

Gas vesicles, commonplace in planktonic microorganisms such as cyanobacteria where they facilitate vertical migrations (Walsby, 1994), are not generally a feature of animal cells. But that does not mean that gasses could not build up in an unusual structure such as the mitonucleon. Furthermore the century-old dogma that all lipophilic gasses, such as CO2, are so highly soluble in lipid bilayers that they always move freely in and out of cell membranes is being refined as a result of some clever

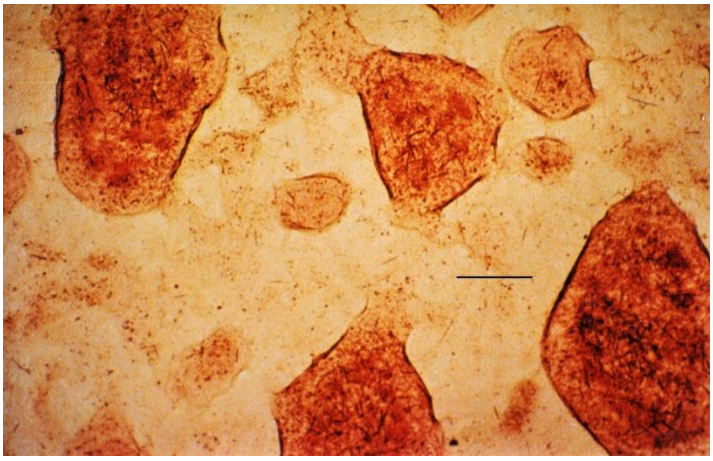

Fig. 7 Mature domes four days after the addition of fetal calf serum under conditions that stimulate dome formation.

As long as the domes are elevated, they stain brightly, although not uniformly, for endogenous biotin associated with mitochondrial carboxylases. Perhaps these are vanguard mitochondria.
Bar = 100 microns

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

experimentation as reviewed by Endeward et al. (2014). Approximately 2 decades ago, research using a single stomach gland demonstrated that while CO2 was transported across basal membranes as rapidly as might be predicted by the theory of "free movement," transport across the apical membrane (Waisbren et al. 1994) was at least two orders of magnitude slower. Similarly, Endeward and Gros (2013) demonstrated that CO2 permeability for guinea pig colon membrane is more than 100 times slower than CO2 permeability for human red cells. Subsequent research has begun to unravel the significance of cholesterol in diminishing CO2 transport through membranes, as well as the effects of proteins, particularly the water channel protein aquaporin 1, on the permeability of CO2 through membranes (Itel et.al. 2012). Nakhoul et al. (1998) demonstrated that aquaporin-1 (AQP-1), which facilitates transport of H20 across a membrane (Preston et. al. 1992), also affects CO2 permeability in Xenopus oocytes leading to the controversial proposition that protein gas channels such as aquaphorin 1 facilitate exchange of CO2 with H20. Similar reports have appeared based on other systems (Talbot et. al. 2015).

Several other aquaphorins have been found, although it is still debated whether they have a physiological significance. The system being proposed for dome formation might provide an example of significance. Do some of the cavities that form in vivo start out as gas-filled cavities with aquaphorins facilitating the exchange of gas for fluid? Even with the dissolution of the mitonucleons, mature domes continue to contain significant amounts of endogenous biotin, linked to carboxylases (Fleming, 1998), and lying close to the apical membrane surface so that domes stain brightly as in fig. 7. The abundant staining diminishes when domes collapse. This fact could be explained if CO2 generation provides for ongoing refreshment of dome fluid in exchange for that CO2. The loss of stainability and the flattening of domes appear to occur at the same time, although it is important to note that wholesale death of the cells, as might be evident by holes in the monolayer, is not seen. The organelles responsible for the staining may be similar to "vanguard mitochondria" which have been shown to occupy a circumferential domain immediately subjacent to the plasma membrane (Van Blerkom and Davis, 2006) in mouse, as well as human, oocytes where they are believed to have specialized function early development, perhaps during blastocyst formation.

Two different kinds of vacuolization are seen early in dome differentiation and have been described in this paper: small vacuoles that form in the enveloped heterochromatin itself and a large vacuole forming within the double layer of the mitonucleon membrane. Two well-known cell types, frequently but not exclusively found in cancer, are cells with optically clear nuclei and signet ring cells whose profiles are characterized by these two kinds of vacuoles. Additionally, research describes a phenomenon in excised human tissue prepared for light microscopy called

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

"pseudolipomatosis" a name that describes variably sized optically clear spaces first found in colon cross sections by Snover et al. (1985) The name arises from the fact that although the vacuoles look to be filled by lipids, they are not. It has been assumed that they are artefactually introduced during tissue preparation. Deshmukh-Rane and Li-cheng Wu (2009) looked for, and found, such vacuoles in 100% of the 50 specimens of endometrial tissue they examined. Our results suggest that the vacuoles may, like vacuolization in differentiating Ishikawa cells, be of some physiological importance.

The results suggesting a physiological role for vacuolization in differentiating Ishikawa cells raise some interesting question. Do any gasotransmitters, short-lived in aqueous solutions, mix with CO2? Does building pressure in the central vacuole contribute to chromatin pyknosis or to the break-down of the mitonucleon double membrane? What is the effect of the release of CO2 upon breakdown of the mitonucleon? Does the mixing of significant amounts of CO2 with fluid elevate the pH for a short period of time and lead to chromatin fragmentation? Buoyancy and cavity formation are essential to the differentiation of domes described in this paper and gas vacuoles, not previously considered relevant to mammalian cells may be involved. Furthermore, the biochemistry of gas vacuoles may turn out to be interesting beyond buoyancy and cavity formation.

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 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.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

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.

Bibliography

Andrade-Navarro MA, Sanchez-Pulido L, McBride HM. 2009. Mitochondrial vesicles: an ancient process providing new links to peroxisomes. Curr Opin Cell Biol. (4):560-7. doi: 10.1016/j.ceb.2009.04.005. Epub 2009 May 5.

Bavister BD 2006 The mitochondrial contribution to stem cell biology. Reprod Fertil Dev. 18(8):829-38

Deshmukh-Rane SA, Wu ML 2009. Pseudolipomatosis affects specimens from endometrial biopsies. Am J Clin Pathol. 132(3):374-7. doi: 10.1309/AJCPB5VLBIR7SKDS.

Ding WX, Li M, Biazik JM,Morgan DG, GuoF, Ni HM, Goheen M, Eskelinen EL, Yin XM, 2012 Electron microscopic analysis of a spherical mitochondrial structure. J Biol Chem. 287(50):42373-8. doi: 10.1074/jbc.M112.413674. Epub 2012 Oct 23.

Endeward V, Al-Samir S, Itel F, Gros G. 2014 How does carbon dioxide permeate cell membranes? A discussion of concepts, results and methods. J Physiol. 567(Pt 1):253-65. Epub 2005 Jun 2.

Endeward V, Gros G. 2013. Low carbon dioxide permeability of the apical epithelial membrane of guinea-pig colon. Front Physiol. 4:382. Doi10.3389/fphys.2013.00382

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

Fleming H. 1995. Differentiation in human endometrial cells in monolayer culture: dependence on a factor in fetal bovine serum. Journal of Celluar Biochemistry. 57(2): 262-270.

Fleming H. Begley M. Campi T. Condon R. Dobyns K. McDonagh JR. Wallace S 1995 Induction of alkaline phosphatase by butyrate in differentiating human endometrial cells. J.Cel Biochem 58: 509-516.

Fleming H, Condon R, Peterson G, Guck I, Prescott E, Chatfield K, Duff M. 1998. Role of biotin-containing membranes and nuclear distribution in differentiating human endometrial cells. Journal of Cellular Biochemistry. 71(3): 400-415.

Fleming H. 1999 Structure and function of cultured endometrial epithelial cells. Semin Reprod Endocrinol. 17(1):93-106

Fleming H. 2014 Unusual characteristics of opaque Ishikawa endometrial cells include the envelopment of chromosomes with material containing endogenous biotin in the latter stages of cytokinesis DOI 10.7287/peerj.preprints.772v1

Fleming H. 2016b. Pyknotic chromatin in mitonucleons elevating in syncytia undergo karyorhhexis and karyolysis before coalescing into an irregular chromatin mass: Differentiation of Ishikawa Endometrial Cells, Parts 2, submitted to PeerJ as a preprint.

Fleming H. 2016c. 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. submitted to PeerJ as a preprint.

Gamachi A, Kashima K, Daa T, Nakatani Y, Tsujimoto M, Yokoyama S. 2003 Aberrant intranuclear localization of biotin, biotin-binding enzymes, and beta-catenin in pregnancy-related endometrium and morule-associated neoplastic lesions. Mod Pathol. 16(11):1124-31.

Grilliot MA, Goldblum JR, Liu X. 2012 Signet-ring cell melanoma of the gastroesophageal junction: A case report and literature review. Arch Pathol Lab Med. 136(3):324-8.

Hailey DW, Rambold AS, Satpute-KrishnanP, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J. 2010 Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 141(4):656-67. doi: 10.1016/j.cell.2010.04.009.

Hapke MR, Dehner LP 1979. The optically clear nucleus: A reliable sign of papillary carcinoma of the thyroid? Am J Surg Pathol. (1) 31-38.

Hasegawa Y, Ishida Y, Kato K, Ijiri R, Miyake T, Nishimata S, Watanabe T, Namba I, Hayabuchi Y, Kigasawa H, et al. 2003 Pancreatoblastoma. A case report with special emphasis on squamoid corpuscles with optically clear nuclei rich in biotin. Acta Cytol 47(4):679-84.

Holinka CF, Hata H, Kuramoto H, Gurpide E. 1986a. Reponses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa). J Steroid Biochem.24:85-89

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

Holinka CF, Hata H, Kuramoto H, Gurpide E. 1986b. Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells. Cancer Res 46:2771-2774

Iezzoni JC, Mills SE 2001 Nonneoplastic endometrial signet-ring cells. Vacuolated decidual cells and stromal histiocytes mimicking adenocarcinoma. Am J Clin Pathol. 115(2):249-55.

Itel F, Al-Samir S, Öberg F, Chami M, Kumar M, Supuran CT, Deen PM, Meier W, Hedfalk K, Gros G, Endeward V. 2012. CO2 permeability of cell membranes is regulated by membrane cholesterol and protein gas channels. FASEB J. 26(12):5182-91. doi: 10.1096/fj.12-209916. Epub 2012 Sep 10.

Karbowski M, Youle RJ. 2003 Dynamics of mitochondrial morphology in healthy cells and during apoptosis. Cell Death Differ.10(8):870-80.

Kedzior J, Masaoka M, Kurono C, Spodnik JH, Hallmann A, Majczak A, Niemczy E, Trzonkowski P, Myśliwski A, Soji T, Wakabayashi T. 2004 Changes in physicochemical properties of microtubules lead to the formation of a single spherical structure of mitochondrial assembly enveloping nuclear chromatins. J Electron Microsc (Tokyo) 53(6):659-70.

Kimura Y, Kashima K, Daa T, Kondo Y, Yada K, Sasaki A, Matsumoto T, Kitano S, Kubo N, Yokoyama S. 2005 Biotin-rich intranuclear inclusions in morule-lacking adenocarcinoma of the gallbladder: A new category of "neoplastic/non-morular" lesions. Virchows Arch 446(2):194-9.

Li CC, Hirokawa M, Qian Z, Fujii Y, Ino H, Wakatsuki S, Horiguchi H, Kagawa N, Sano T. 2002 Morules with optically clear nuclei in ovarian borderline endometrioid tumor. APMIS 110(11):783-8

Liesa M, Palacín M, Zorzano A. 2009 Mitochondrial dynamics in mammalian health and disease. Physiol Rev.89(3):799-845. doi: 10.1152/physrev.00030.2008.

Lonergan T, Bavister B, Brenner C 2007 Mitochondria in stem cells.

Mitochondrion. 2007. 7(5):289-96. Epub 2007 May 23.

Mazur MT, Hendrickson MR, Kempson RL. 1983. Optically clear nuclei. An alteration of endometrial epithelium in the presence of trophoblast. Am J Surg Pathol. 7(5):415-23.

Nakhoul NL, Davis BA, Romero MF, Boron WF. 1998 Effect of expressing the water channel aquaporin-1 on the CO2 permeability of Xenopus oocytes. Am. J. Physiol. 274(2 Pt 1):C543-8.

Neuspiel M, Schauss AC, Braschi E, Zunino R, Rippstein P, Rachubinski RA, Andrade-Navarro MA, McBride HM. 2008 Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. Curr Biol. 18(2):102-8. doi: 10.1016/j.cub.2007.12.038.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

Nishida M, Kasahara K, Kaneko M, Iwasaki H. 1985. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. Acta Obstet Gynaec Japonica (In Japanese)37:1103-1111.

Preston GM, Carroll TP, Guggino WB, Agre P. 1992. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science.256(5055):385-7.

Rehman J. Empowering self-renewal and differentiation: the role of mitochondria in stem cells. J Mol Med (Berl).88(10):981-6. doi: 10.1007/s00109-010-0678-2. Epub 2010 Sep 1.

Rosas-Uribe A, Luna MA. 1969 Primary signet ring cell carcinoma of the urinary bladder. Report of two cases. Arch Pathol. 88(3):294-7

Sasaki A, Yokoyama S, Arita T, Inomata M, Kashima K, Nakayama I. 1999 Morules with biotin-containing optically clear nuclei in colonic tubular adenoma. Am J Surg Pathol (3):336-41.

Snover DC, Sandstad J, Hutton S. 1985. Mucosal pseudolipomatosis of the colon. Am J Clin Pathol. 84(5):575-80.

Sugiura A, McLelland GL, Fon EA, McBride HM. 2014 A new pathway for mitochondrial quality control: mitochondrial-derived vesicles. EMBO J 33(19):2142-56. doi: 10.15252/embj.201488104. Epub 2014 Aug 8.

Talbot K, Kwong RW, Gilmour KM, Perry SF. 2015. The water channel aquaporin-1a1 facilitates movement of CO2 and ammonia in zebrafish (Danio rerio) larvae. J Exp Biol. 218(Pt 24):3931-40. doi: 10.1242/jeb.129759.

Tanaka Y, Ijiri R, Yamanaka S, Kato K, Nishihira H, Nishi T, Misugi K. 1998 Pancreatoblastoma: Optically clear nuclei in squamoid corpuscles are rich in biotin. Mod Pathol 11(10):945-9.

Tsujimoto M, Noguchi M, Taki I. 1991 Immunohistochemical and electromicroscopic study of the intranuclear inclusion bodies containing biotin in the ovarian endometrioid carcinoma. J Clin Electron Microscopy. 24:5-6.

Van Blerkom J, Davis P, Mathwig V, Alexander S. 2002. Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. Hum Reprod.17(2):393-406.

Van Blerkom J, Davis P. 2006 High-polarized (Delta Psi m(HIGH)) mitochondria are spatially polarized in human oocytes and early embryos in stable subplasmalemmal domains: developmental significance and the concept of vanguard mitochondria. Reprod Biomed Online. 2006 13(2):246-54.

Walsby AE_1994 Gas vesicles Microbiol Rev. 58(1):94-144.

Waisbren SJ, Geibel JP, Modlin IM, Boron WF. 1994. Unusual permeability properties of gastric gland cells. Nature. 368(6469):332-5.

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016

506 Williamson SR, Kum JB, Shah SR, Einhorn LH, Eble JN, Cheng L, Ulbright TM, Idrees MT. 2012
507 Signet ring cell carcinoma of the testis: Clinicopathologic and molecular evidence for germ
508 cell tumor origin--a case report. Am J Surg Pathol 36(2):311-5.

509 Wright CA. Leiman G. Burgess SM. 1999 The cytomorphology of papillary serous carcinoma of
510 the endometrium in cervical smears. Cancer 87(1):12-8.
511
512 Yokoyama S, Kashima K, Inoue S, Daa T, Nakayama I, Moriuchi A.. 1993 Biotin-
513 containing intranuclear inclusions in endometrial glands during gestation and puerperium. Am
514 J Clin Pathol. 99(1):13-7.

515 Wang S, Xiao W, Shan S, Jiang C, Chen M, Zhang Y, Lü S, Chen J, Zhang C, Chen Q, Long M.
516 2012 Multi-patterned dynamics of mitochondrial fission and fusion in a living cell. PLoS
517 One. 7(5):e19879. doi: 10.1371/journal.pone.0019879. Epub 2012 May 23.

518

PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1728v1 | CC-BY 4.0 Open Access | rec: 9 Feb 2016, publ: 9 Feb 2016