ORIGINAL ARTICLE

Unlocking the ultrastructure of colorectal cancer cells *in vitro* using selective staining

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Abstract

AIM: To characterise differences between three widely used colorectal cancer cell lines using ultrastructural selective staining for glycogen to determine variation in metastatic properties.

METHODS: Transmission electron microscopy was used in this investigation to help identify intracellular structures and morphological features which are precursors of tumor invasion. In addition to morphological markers, we used selective staining of glycogen as a marker for neoplastic cellular proliferation and determined whether levels of glycogen change between the three different cell lines.

RESULTS: Ultrastructural analysis revealed morphological differences between the cell lines, as well as differentiation into two sub-populations within each cell line. Caco-2 cells contained large glycogen deposits as well as showing the most obvious morphological changes between the two sub-populations. SW480 cells also contained large glycogen stores as well as deep cellular protrusions when grown on porous filter membranes. HT-29 cells had trace amounts of glycogen stores with few cellular projections into the filter pores and no tight junction formation.

CONCLUSION: Morphology indicative of metastatic properties coincided with larger glycogen deposits, providing strong evidence for the use of selective staining to determine the neoplastic properties of cells.

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INTRODUCTION

Cultured human colon carcinoma Caco-2, HT-29, and SW480 cell lines have been used as model cell lines by many researchers to investigate the cell biology and metastatic properties of colorectal cancer. The main tissue of origin of these cell lines, the colon, is a structure comprising a dynamic epithelium where there is continuous

renewal of normal colonic epithelial cells that originate in the crypts (secretory cells) and migrate to the tips of the villous folds (absorptive cells)[1]. This, therefore, results in cellular heterogeneity of all epithelial cells that are derived from any human adenocarcinoma cell line. The present study is designed to examine these heterogenous features ultrastructurally within the three chosen cells lines to determine whether varying malignant characteristics are expressed.

All epithelia form a barrier made of polarized cells in which the apical and basolateral domains of the cells can be separated into functional domains[2]. Cell adhesion is critical in the organisation of normal epithelia, in particular the establishment of the adherens junction, mediated by the presence of E-cadherin, which initiates a cascade of events leading to the formation of the tight junction, gap junction, and desmosomes, all key regulators of the junctional complex[3-5] and mediators of cellular polarisation. The loss of these structures results in the breakdown of the epithelium, leading to increased permeability and pathogenesis of disease[4]. An ultrastructural investigation into the various features of the junctional complex will provide evidence of cellular communication and adhesion to determine whether cellular polarisation is present.

Additionally, the invasive properties of cells are also reflected by cell shape, whereby epithelioid cells appear to be less invasive than spindle-shaped cells[6] due to cellular polarisation and establishment of a tight junction. Additional ultrastructural assays that might help determine the differences in the invasive properties of the aforementioned cells lines is their ability to leave the compartment to which they are normally restricted and to determine whether cells have the potential to carry out invasive growth. By culturing these cells on porous membrane filters, cellular anchorage and cellular sensing movement can be investigated by analysing the degree of cellular migration into the filter pores. By examining these characteristics at an ultrastructural level, comparative invasive properties can be evaluated in each of the cell lines.

In addition to using cellular morphology as a marker for invasive properties, storage of glycogen will also be analysed. Functionally, glycogen storage in the epithelium provides an energy store for cellular proliferation[7-9]. Large quantities of glycogen stores have also been reported in a variety of cultured cells, in particular cells undergoing neoplastic transformations[9-11]. Glycogen metabolism has been widely investigated in malignant cells originating from tissues which normally store glycogen, i.e. hepatomas[11,12] and cancers of the cervix[13]. Surprisingly, elevated stores of glycogen have been reported in all malignant cell lines (in a 58 cell line culture study) irrespective of tissue of origin[9], and in particular colon adenocarcinomas, originating from human colonocytes where glycogen is normally absent[9,10]. Furthermore, the variation in the amount of glycogen storage has been utilized as a marker for malignant processes in cells, whereby increasing levels of glycogen are indicative of more malignant cells due to abnormal cellular proliferation[9]. Glycogen storage has been reported in Caco-2, HT-29, and SW480 cells *via* a glycogen quantitative assay. The highest glycogen

levels were found in Caco-2 cells, intermediate levels in HT-29 cells, and the lowest levels in SW480[10], suggesting that Caco-2 cells undergo extensive abnormal cellular proliferation compared to HT-29 and SW480 cells. These assays have not, however, yielded any information on the ultrastructural position of glycogen in the cells, as accumulation of glycogen particles are first observed in the apical cytoplasm followed by a basal distribution when glycogen particles continue to accumulate[7]. By incorporating a selective staining method to detect the presence of glycogen in the cell, this investigation will contribute to the ultrastructural localization of glycogen particles in these colorectal cancer cell lines for the evaluation of malignant properties.

Osmium tetroxide has been widely used by electron microscopists to routinely fix and stain intercellular membrane systems[14,15]; however, primary fixation in an osmium tetroxide-potassium ferrocyanide $[K_4Fe(CN)_6]$ mixture combines selective fixation and staining of various cellular components, in particular glycogen[16,17] and intracellular membranes[14,18]. Osmium-ferrocyanide has been shown to bind selectively through the C_2 and C_3 dihydroxyl groups in glycogen. Chelation of the osmium-ferrocyanide complexes by donor atoms in the tissue helps reduce the osmium to a more stable oxidation state, allowing greater osmium deposition than that observed through post-fixation with osmium tetroxide alone. Therefore the osmium-ferrocyanide complex provides excellent preservation and contrast of glycogen complexes, which in many cases might otherwise remain unstained[17]. The osmium-ferrocyanide method will be incorporated into the present study to determine whether there is an ultrastructural variation of glycogen distribution between different colorectal cancer cell lines.

This study will use three cell lines: Caco-2, HT-29 and SW480 cells. The human colon adenocarcinoma cell line, Caco-2, undergoes spontaneous differentiation in cell culture resulting in structural and functional characteristics which resembles intestinal epithelium[19-23]. The Caco-2 cell line, although derived from a tumor of the human colon epithelium (colonocytes), surprisingly exhibits characteristics of foetal ileal epithelial cells from the small intestine (enterocytes)[23]. Many reports have identified the formation of a heterogenous population of Caco-2 cells in culture[7,20,24-26]. One population consists of dome-forming simple columnar polarized epithelium with a homogenous distribution of microvilli (brush border)[27] and established junctional complexes, in particular the tight junction[4,5]. The variant population comprises multilayered cuboidal cells[28], exhibiting large intercellular spaces (cysts) characteristic of motile cells[24,29]. Due to this functional differentiation, Caco-2 cells provide a model to investigate the intestinal barrier as well as drug transport across the intestinal mucosa[19,22] and provide our study with a model cell line that exhibits a heterogenous cell population.

HT-29 cells, although not homogenous in nature, when grown in control Dulbecco's modified Eagle's medium (DMEM) remain morphologically undifferentiated and comprise of a multilayer of unpolarized cells

that contain features of mucous secreting cells[30-32]. The junctional complex and, in particular, the tight junction is absent[33], resulting in disorderly development of cells. A unique feature of HT-29 cells is their ability to undergo polarisation and reversible differentiation into enterocytes after administration of galactose into the culture medium[31]. The undifferentiated form of HT-29 cells will be analysed in this investigation to avoid confounding effects attributable to cell culture medium to allow for a comparative framework between cells lines to investigate junctional properties, glycogen content, and other microanatomy of the HT-29 cell line.

The SW480 cell line also consists of a heterogenous population of cells, but these cells do not differentiate[34]. The predominant cell type in the SW480 cell line consist of flat polygonal cells which form typical epithelial colonies (E-type); the variant R-type cells consist of round, refractile cells with a disoriented morphology. The R-type cells display anchorage-independent growth, form multilayers and are regarded as more malignant[35]. Cellular morphology, glycogen content, and desmosome formation to determine cellular contact and organisation will be used to identify whether the predominant population of cells in the investigation consist of the E- or R-type.

The comparative ultrastructural and glycogen storage data will be examined in the Caco-2, HT-29 and SW480 cell lines at day 1 and day 12 post-confluency to help promote cellular differentiation. The data collected from this investigation will contribute to the utilisation of microanatomical cellular features to determine neoplastic transformations in human adenocarcinoma cell lines and to help identify sub-populations in the chosen cell lines.

MATERIALS AND METHODS

Cell culture
Caco-2, HT-29, and SW480 cell lines were obtained from the American Type Culture Collection. Caco-2 and HT-29 cells were cultured in advanced Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 0.5 mL Glutamax, and 0.5 mL antibiotics per 40 mL of medium. SW480 cells were cultured in L15 medium supplemented with 10% fetal calf serum, 0.5 mL Glutamax, and 0.5 mL antibiotics per 40 mL of medium. The cells were incubated at 37℃ in a humidified incubator with 5% CO_2 in air and medium was changed every day after the cells reached confluency.

Transmission electron microscopy
Cells were cultured on 0.4 μm pore size cell culture inserts (BD Falcon, NSW Australia) and collected on day 1 and day 12 post-confluency. Four culture inserts per treatment day were fixed in 2.5% gluteraldehyde (ProSci Tech, Queensland, Australia) in 0.1 mol/L sodium cacodylate buffer with 0.1 mol/L sucrose pH 7.4 for 1 h. Cells were next postfixed for 1 h in a 1% osmium tetroxide solution containing 0.8% potassium ferrocyanide in 0.1 sodium cacodylate buffer (to enhance plasma membranes and

glycogen particles), dehydrated in graded alcohols, and infiltrated with 50% Spurr's resin/absolute ethanol for 1 h. The cells were then re-embedded in fresh Spurr's resin (Agar Scientific, Essex, UK) overnight under gentle agitation, re-embedded in fresh Spurr's resin, and left to polymerized at 60℃ for 24 h. Two areas of each cell culture insert ($n = 8$) were cut using a Leica ultracut UCT ultramicrotome (Leica, Heerbrugg, Switzerland) and silver-gold sections (80-90 nm) were mounted onto 200-mesh copper grids. Sections were stained with a 2% solution of aqueous uranyl acetate for 10 min, followed by Reynold's lead citrate stain for 10 min. Stained sections were then viewed using a JEOL 1400 (Tokyo, Japan) transmission electron microscope operating at 120 kV.

RESULTS
Overall, the application of the potassium-ferrocyanide methods results in selective staining of glycogen particles (approximate 20 nm in diameter), electron dense demarcation of plasma membranes, and demarcation of intranuclear indentations (intranuclear rods).

Ultrastructure of Caco-2
When grown on membrane filters, Caco-2 cells differentiated into two morphologically different populations, which will be described separately (Table 1).

Sub-population 1
On day 1 post-confluency, cells were simple squamous with irregular sparse microvilli (Figure 1A). Cells contained round euchromatic nuclei and showed evidence of all other cellular organelles, including mitochondria, rER, and Golgi (Figure 1B). Intercellular spaces between cells were evident, as well as tight junctions and desmosomes. When grown on porous membrane filters (Figure 1C), cellular processes marginally invaded into filter pores (Figure 1D). On day 12 post-confluency, cellular morphology and organisation changed to a stratified cuboidal formation and intercellular spaces increased, resulting in the loss of the tight junction, yet desmosomes were still evident (Figure 1E and F). Glycogen granules were distributed within the cytoplasm with a supranuclear predisposition (Figure 1E and G). Extensive intranuclear rods (indentations of the nuclear membrane) were evident (Figure 1F) and no invasive processes were observed projecting into the filter pores (Figure 1H).

Sub-population 2
On day 1 post-confluency, cells were simple cuboidal and contained regular microvilli (Figure 2A). No intercellular spaces were evident and a tight junction was present along the apical region of the lateral plasma membrane (Figure 2B). Small amounts of glycogen particles were evident and regularly distributed (Figure 2C). Several cellular processes projected into the filter pores (Figure 2D). On day 12 post-confluency, cells became simple columnar with a homogenous population of microvilli (Figure 1E). Glycogen accumulation increased in the cytoplasm of

Table 1 Ultrastructural and morphological features of Caco-2 (sub-population 1 and 2, HT-29 and SW480 cell lines)

Cell line	Days post-confluency	Cell type	Nucleus	Intercellular space	Microvilli	Invasive processes	Mitoch-ondria	Golgi	sER	rER	Junctions	Glycogen	Vacuoles
Caco-2 sub-pop 1	1	Simple squamous	Indented (I/N rods)	++	+/-	+/-	+	+	+	+	+Des -TJ	-	-
Caco-2 sub-pop 1	12	Stratified cuboidal	I/N rods	+++	+	+/-	+	+	+	+	+Des -TJ	+++	-
Caco-2 sub-pop 2	1	Simple squamous	Round	+	++	+/-	+	++	+	+	++Des +TJ	-	+
Caco-2 sub-pop 2	12	Simple columnar	Round	+	+++	-	++	++	+	++	+++Des +TJ	+++	+
HT29	1	Stratified cuboidal	Round	+++	+++	-	+	+	+	+	++Des -TJ	-	-
HT29	12	Stratified cuboidal	I/N rods	+++	+++	-	+	+	+	+++	++Des -TJ	+/-	+++
SW480	1	Simple squamous	Round	+	+++	+++ 4 μm	+++	++	+	+++	+/-Des -TJ	-	-
SW480	12	Simple squamous (spindle shape)	Round	+	+++	+++ 8 μm	+++	++	+	+++	+/-Des -TJ	+	-

-: Absent; +/-: Present; +: Small; ++: Intermediate; +++: Large; I/N: Intranuclear; Des: Desmosomes; TJ: Tight junction.

these cells, exhibiting a supra- and sub-nuclear distribution (Figure 2F). Mucous storing vacuoles also occupied a large volume of these cells and were located in close proximity of the glycogen particles. Intercellular spaces were evident in the basal region of the lateral plasma membrane and the tight junction and plasma membrane interdigitation was also present (Figure 2F). No invasive projections into the filter pores were evident (Figure 2H).

Ultrastructure of HT-29 cells

No morphological heterogeneity was observed in the HT-29 cell line.

On day 1 post-confluency, the cells were stratified cuboidal with microvilli projecting into large intercellular spaces between cells (Figure 3A and B). Desmosomes were evident at meeting points along the lateral plasma membrane (Figure 3C and D). The nuclei were round and euchromatic. Mitochondria, rER and Golgi bodies were all present. The apical-most cells of the multilayer contained regular microvilli; no invasive processes were present and there were no obvious glycogen stores. On day 12 post-confluency, cells remained stratified cuboidal, expressing large intercellular spaces with microvilli projecting into them (Figure 3E). Golgi bodies were more evident, as well as large accumulations of mucous secreting vacuoles (Figure 3F and G). Desmosomes were present along contact sites of the lateral plasma membrane (Figure 3G) and no significant invasive processes or glycogen stores were evident (Figure 3H).

Ultrastructure of SW480 cells

On day 1 post-confluency, cells were simple squamous with irregular microvilli (Figure 4A). Mitochondria were densely distributed through the cell cytoplasm (Figure 4B) and tight junction formation was evident when upper leaflets of the plasma membrane were in close contact (Figure 4C). Long invasive cellular projections were present in the filter pores (Figure 4D). On day 12 post-confluency, cells were simple cuboidal with irregular sparse microvilli (Figure 4E). Large numbers of mitochondria with filamentous branching were evident, as well as the presence of glycogen accumulation (Figure 4F and G). Small intercellular spaces were present, but no desmosomes were evident (Figure 4F). Extremely long invasive processes projected into the filter pores and these projections contained cellular organelles, such as mitochondria (Figure 4H).

DISCUSSION

Utilising selective staining to examine differences in metastatic properties of Caco-2, HT-29, and SW480 cells advances our understanding of the commonly used colorectal cancer cell lines. Morphologically, after reaching confluency, many differences were apparent between the cell lines in this investigation. When grown in standard culture conditions, spontaneous differentiation predominantly occurred in the Caco-2 cell line; however, ultrastructural differences suggesting heterogenous populations also became evident in the HT-29 and SW480 cell lines when cells were examined 12 d post-confluency.

Evidence of a heterogenous population already existed in the Caco-2 cells one day after reaching confluency. Although both Caco-2 cell populations were simple squamous, one population exhibited regular microvilli and tight junction formation (sub-population 2), whereas the other population was void of these features (sub-population 1). By day 12 post-confluency, this heterogeneity was even more apparent, as population 2 cells exhibited a tight junction and became a simple layer of columnar polarized cells with regular microvilli indicative of regular epithelial cell assembly. No intercellular spaces were evident between adjoining cells and there was no evidence of invasive projections into membrane pores; glycogen accumulation, however, was abundant. These features were indicative of intestinal epithelial cells[19,20] and suggested that this sub-population of cells originated from the absorptive
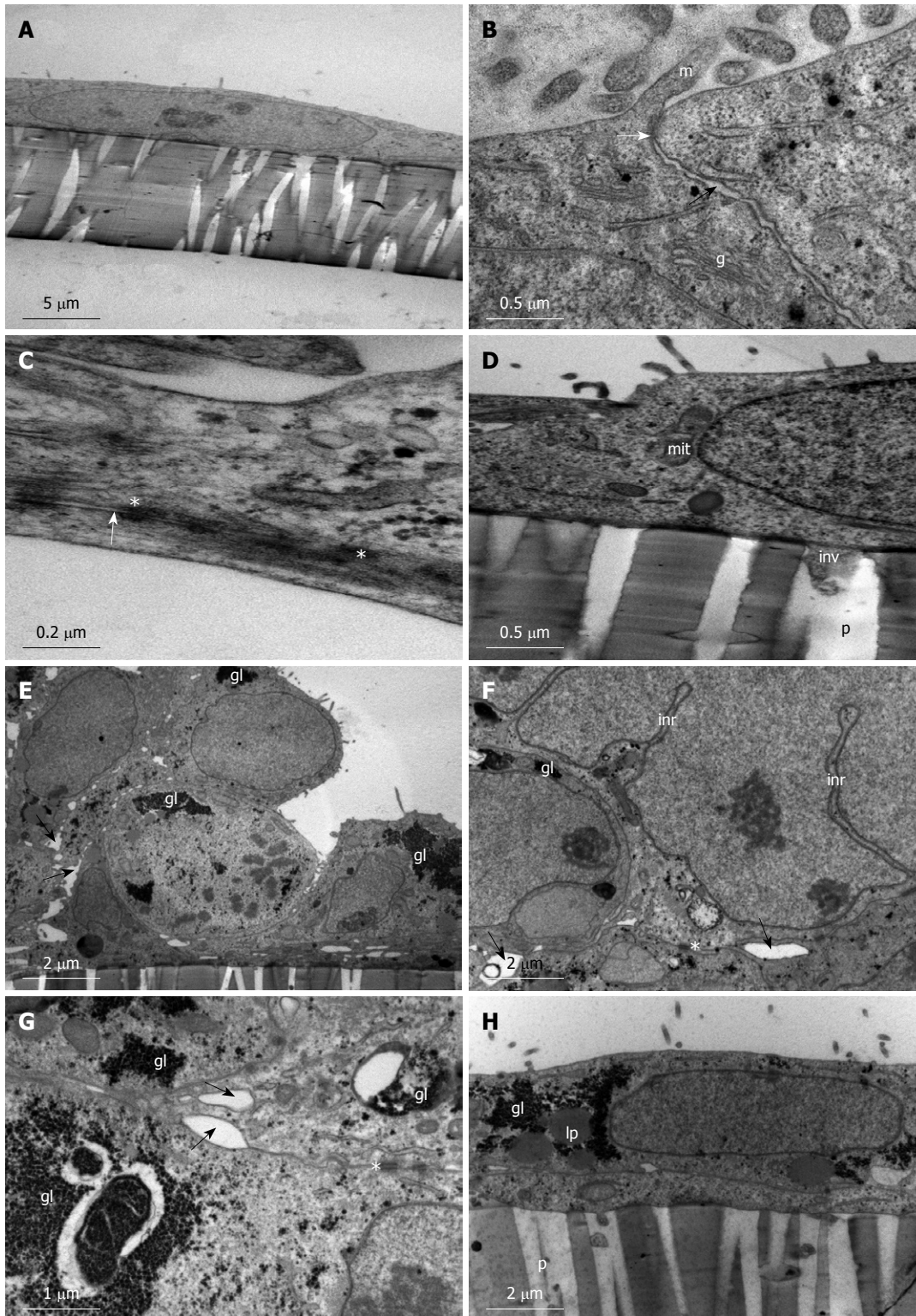
Figure 1 Spontaneous differentiation of Caco-2 into sub-population 1 of stratified cuboidal cells. A-D: Caco-2 cells grown on membrane filters examined on day 1 one post-confluency. A: Simple squamous cells with irregular sparse microvilli; B: Junction between two cells (black arrow) and establishment of a tight junction (white arrow) and the presence of microvilli (m) and golgi bodies (g); C: Desmosome (*) formation along the lateral plasma membrane between two cells (white arrow); D: Basolateral plasma membrane depicting attachment to membrane filter with evidence of small cell protrusions (inv) into the membrane pore (p). Mitochondria (mit); E-H: Caco-2 cells grown on membrane filters examined 12 d post-confluency. E: Cells appear stratified and cuboidal with large intercellular spaces (black arrows); large glycogen stores are evident (gl); F: Intercellular spaces (black arrows) and desmosomes (*) are evident between cells with glycogen deposits (gl) and indentations of the nucleus form large intranuclear rods (inr); G: High power image of intercellular spaces (black arrows) and desmosomes (*) present along the lateral plasma membrane with large glycogen deposits (gl) found in the cytoplasm; H: Basolateral plasma membrane showing attachment of the cell to the membrane filter. No invasive processes are evident projecting into the filter pores (p). Glycogen (gl) and some lipid (lp) droplets are present in the cell cytoplasm.
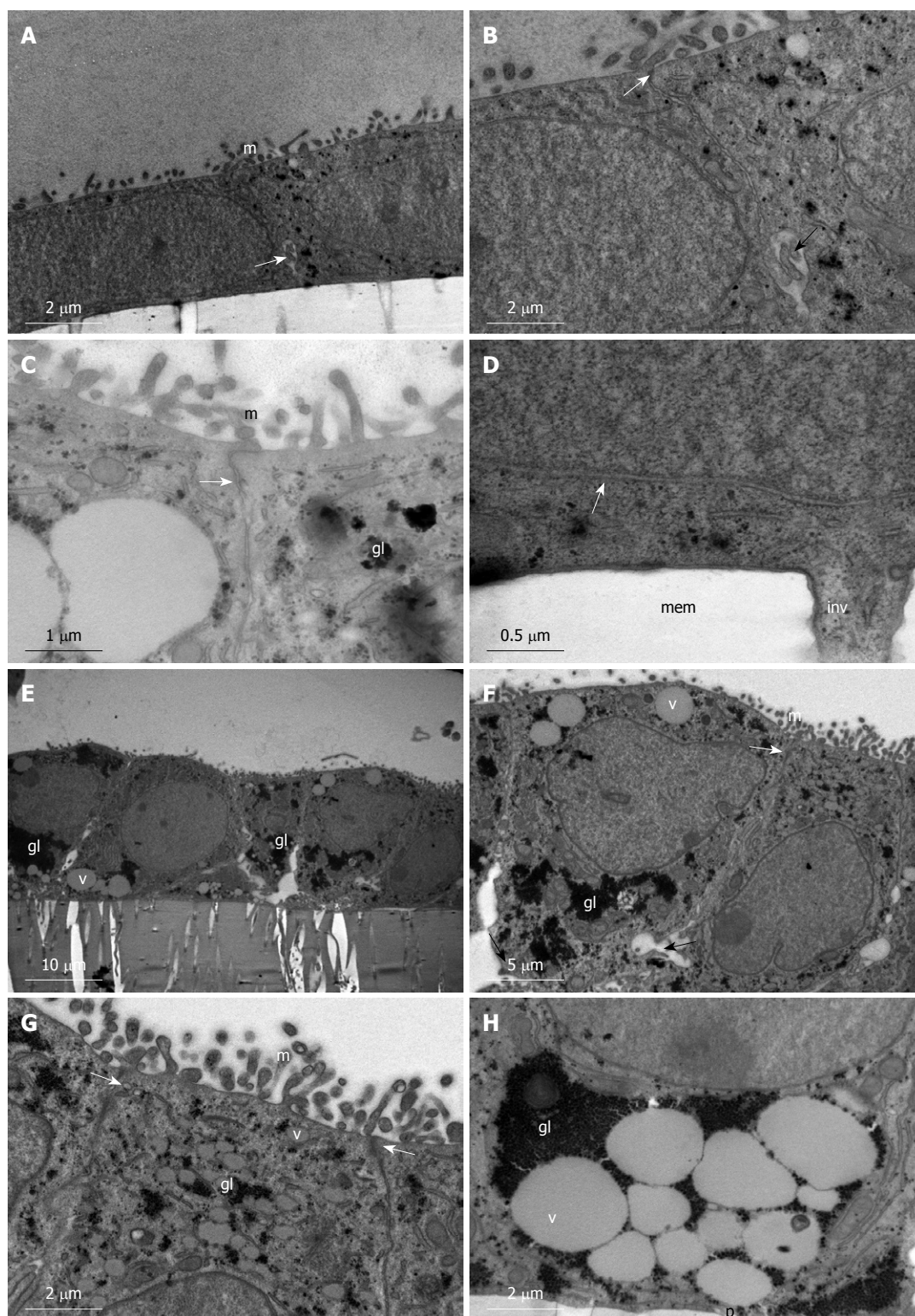
Figure 2 Spontaneous differentiation of Caco-2 into sub-population 2 of simple columnar monolayer of polarized epithelial cells. A-D: Caco-2 cells grown on membrane filters examined on day 1 post-confluency. A: Simple cuboidal epithelium with regular microvilli (m) and intercellular junctions (white arrow); B: Intercellular junction with a tight junction (white arrow) evident along the apical part of the lateral plasma membrane. A small intercellular space (black arrow) is evident near the basolateral region of the plasma membrane; C: High power image of a tight junction (white arrow) between cells showing glycogen particles (gl) and microvilli (m); D: Basolateral plasma membrane showing intercellular junction (white arrow) and small invasive projection (inv) into the filter pore. Membrane (mem); E-H: Caco-2 cells grown on membrane filters examined on day 12 post-confluency. E: Simple columnar polarized epithelium is present with large glycogen stores (gl), mucin filled vacuoles (v), and intercellular spaces along the basal part of the lateral plasma membrane; F: Junction between two columnar cells (white arrow); regular microvilli (m) are present as well as large glycogen stores (gl) and mucin filled vacuoles (v). Intercellular space (black arrow); G: High power micrograph showing the apical domain of cells with regular microvilli (m), glycogen stores (gl), mucin vacuoles (v), and tight junction formation (white arrows); H: High power image of the basal domain of cells with large mucin filled vacuoles (v) enclosed by large glycogen stores (gl). No invasive processes are evident projecting into filter pores (p).
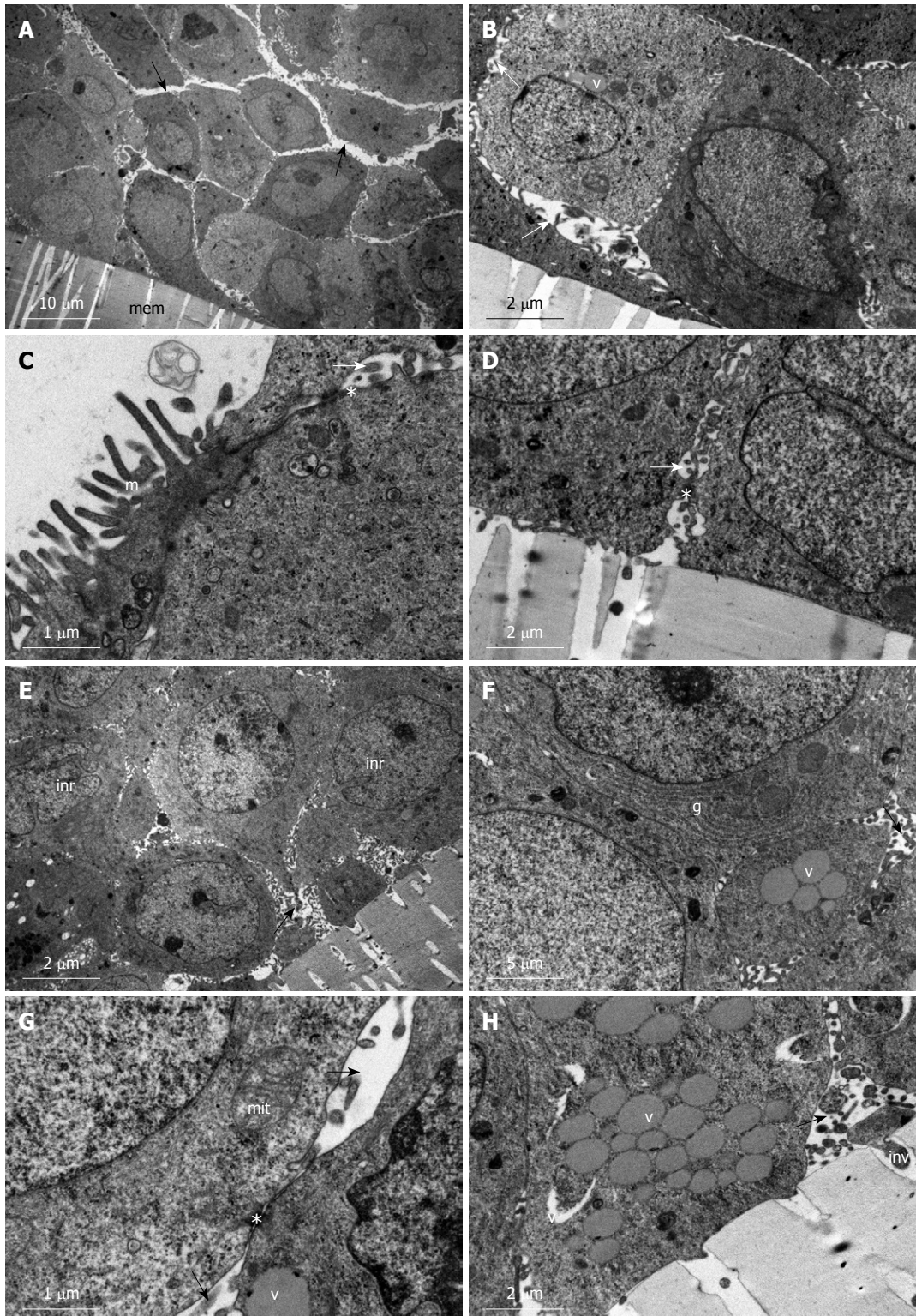
Figure 3 Undifferentiated heterogenous population of HT-29 cells. A-D: HT-29 cells grown on membrane filters (mem) and examined day 1 post-confluency. A: Stratified cuboidal cells arranged in clumps. Large intercellular spaces (black arrows) are evident with microvilli projecting into them; B: High power micrograph showing large intercellular spaces (approximate 2 μm) (white arrows) with microvillar projections and formation of small vacuoles (v); C: Microvillous (m) apical plasma membrane is present and desmosomes (*) are evident along contact sites of the lateral plasma membrane of adjoining cells and presence of intercellular spaces (white arrow); D: No invasive projections are evident along the basolateral plasma membrane. Desmosomes (*) are present at contact sites of the plasma membrane, which otherwise shows intercellular spaces (white arrow); E-H: HT-29 cells grown on membrane filters (mem) and examined day 12 post-confluency. E: Stratified cuboidal cells with microvillar projections entering the large intercellular space (black arrow). Intranuclear rods are evident (inr); F: High power micrograph of junction between two cells. Golgi bodies (g) as well as mucin filled vacuoles (v) are distributed throughout the cell cytoplasm and intercellular spaces are evident with microvilli projecting into their lumen; G: High power micrograph of a contact site between two opposing plasma membranes showing a desmosome (*). Large intercellular spaces (black arrows) are evident above and below the desmosomes contact site. Mitochondria (mit) and mucin filled vacuoles (v) are also evident; H: Basolateral attachment site showing minor invasive projections (inv) into the filter pore. Intercellular spaces (black arrow) and vacuoles (v) are evident.
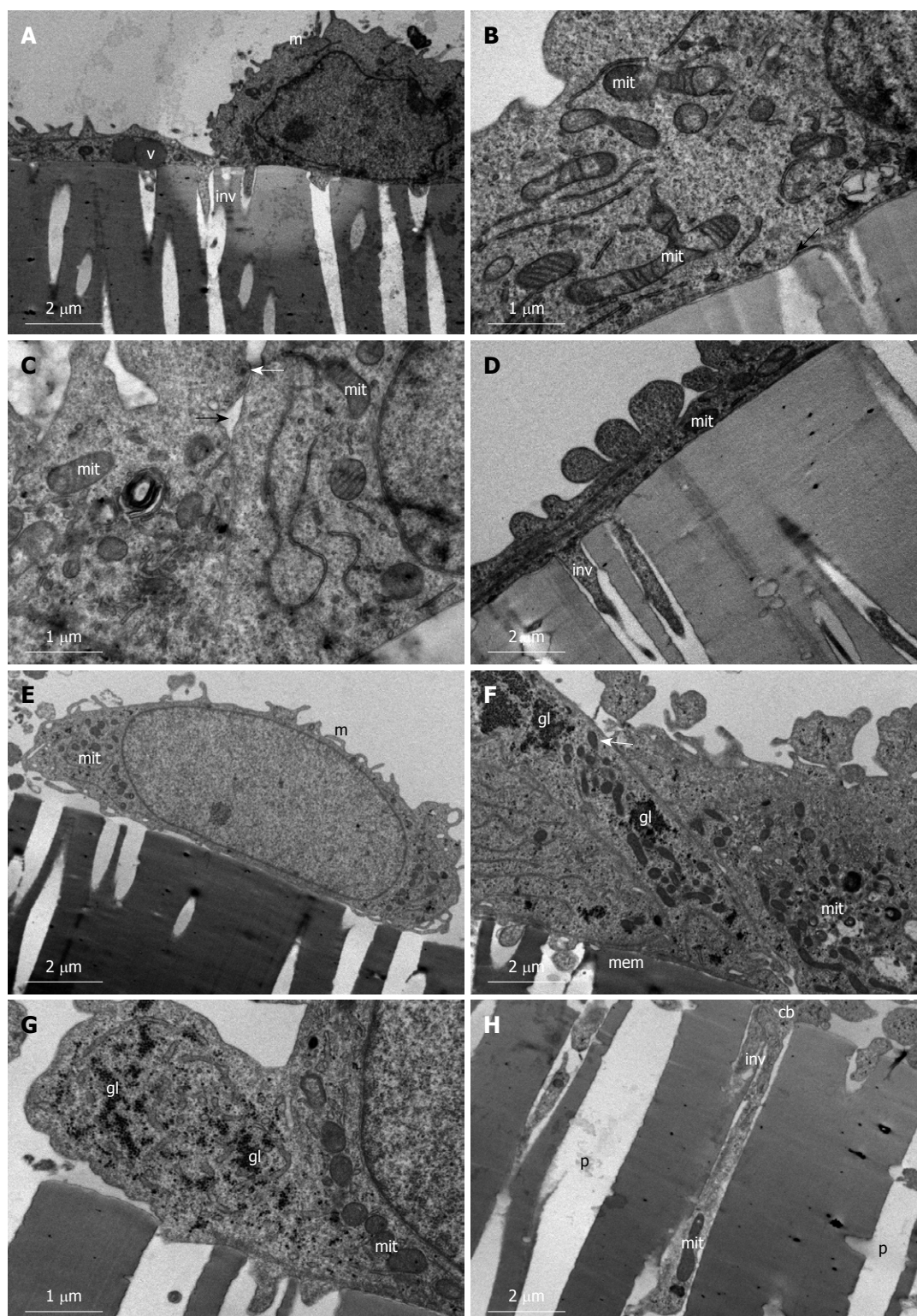
Figure 4 Undifferentiated heterogenous population of SW480 cells. A-D: SW480 cells grown on membrane filters and examined day 1 post-confluency. A: Simple squamous (left) and simple cuboidal (right) distinctions are already evident in cells on day 1 post-confluency. Cells exhibit vacuoles (v), microvilli (m), and invasive processes (inv) are evident projecting into filter pores; B: Large population of mitochondria (mit) are evident which exhibit branching characteristics; C: Intercellular junctions (black arrow) exhibit early formation of the tight junction (white arrow). Mitochondria (mit) with numerous cristae are present; D: Simple squamous cells exhibiting long invasive processes (inv) into the filter pores. Mitochondria (mit); E-H: SW480 cells grown on membrane filters and examined day 12 post-confluency. E: Cells remain a monolayer with microvillar (m) projections and abundant mitochondria (mit); F: Junction between two adjoining cells growing on membrane (mem) showing no intercellular spaces, tight junction formation (white arrow), abundant mitochondria (mit), and glycogen (gl) stores; G: High power micrograph depicting the glycogen (gl) granules in the cytoplasm in amongst mitochondria (mit); H: Basolateral region of cell body (cb) showing deep invasive processes (inv) projecting into filter pores (p). These processes extend to depths of approximately 10 μm and cellular organelles such as mitochondria (mit) are present in these processes, showing deep anchorage of cell.

enterocyte population when the original adenocarcinoma was excised. The polarisation of the Caco-2 epithelial layer indicated that epithelial organisation had been achieved[3] and the cells were contained in the epithelial monolayer without morphological indications of metastatic properties. The presence of glycogen however, suggested that neoplastic proliferation was still taking place in this population. This finding might be significant for tumor excision and treatment, as glycogen stores suggest a neoplastic cell; however, the morphological architecture indicates a contained tumor with possible clear margins and a better probability of complete surgical excision.

Sub-population 1 of Caco-2 cells did not form regular microvilli, and tight junctions did not polarise 12 d post-confluency. This cell population continued to grow in clumps of stratified cuboidal epithelium with large intercellular spaces between individual cells, suggesting high cellular motility (particularly in the apical population of cells)[24]. In the basal distribution of the cells, desmosomes were evident in areas of established cellular contact. Glycogen particles were abundant, with both an apical and basal distribution. Another additional feature that became apparent using a combined osmium tetroxide and potassium ferrocyanide selective staining, which would otherwise not have been adequately sampled, was the presence of intranuclear rods. These structures are indentations of the cytoplasm into the nucleus, whereby they increase the nucleocytoplasmic interactions[36] and carry out nucleocytoplasmic exchange, which is the most important transport phenomena in cells[37]. In the case of malignant colorectal cancer cells, intranuclear rods are suggested to carry out nucleocytoplasmic exchange of glycogen[37]. In a previous report in the Ehrlich-Lettré mouse ascites tumor, glycogen production that originated in the nucleus shifted from the nucleus to the cytoplasm *via* a transfer mechanism that allows the intranuclear glycogen to pour out into the cytoplasm *via* the intranuclear indentations (rods)[37]. Intranuclear rods are prevalent in the Caco-2 cell line (in particular sub-population 1); however, glycogen is predominantly expressed in the cytoplasm, which suggested that the nucleocytoplasmic exchange has occurred. Due to their disorganized arrangement, large intercellular spaces and lack of polarisation and tight junctional formation, sub-population 1 of Caco-2 cells did not form an impermeable monolayer or "classical epithelium". A lack of tight junction formation allows for dissociation of individual cells from the primary tumor due to a decrease in cell-cell adhesion, which consequently allows for the cancerous cells to invade the surrounding tissue, leading to metastasis[38]. Furthermore, for successive metastasis to occur, the tight junctions present in the surrounding tissue must be compromised to enable the tumor cells to penetrate[38]. In a previous investigation of colorectal cancer cells, using the tight junctional protein claudin-4 as a marker, a disruption of claudin-4 mediated tight junction formation enhanced cancer cell invasion and metastasis in colorectal cancer[3]. Finally, the morphology of the cells in sub-population 1 of the Caco-2 cell line did not provide strong evidence indicating the formation of enterocytes, and the high glycogen content signifies metastatic pro-

liferation. Thus, data associated with future functional and transport studies carried out on sub-population 1 of Caco-2 cells might be restricted.

When grown under standard conditions, the HT-29 cells, although not homogenous, did not differentiate into a monolayer of polarized cells, as there is no evidence of any tight junction formation, which again suggests that cells can break away from the primary tumor and begin a metastatic process in surrounding tissue. These results are consistent with previous reports of a heterogenous HT-29 cell population[31,33,39]. At day 1 post-confluency, the cells were arranged into a large clump of microvillous stratified cuboidal epithelium up to seven layers thick. By day 12 post-confluency, large intercellular spaces were observed suggesting that cells remain relatively motile[24], even though some desmosomes were evident at points of plasma membrane contact of cells in the basal population. No invasive cellular projections were evident in the membrane filters, and trace amounts of glycogen particles were present in HT-29 cells when compared to the abundant glycogen stores found in both populations of Caco-2 cells. Reduced glycogen storage suggested that these cells were not undergoing comparable metastatic proliferation as the two sub-populations of the Caco-2 cell line. Previous glycogen assay studies have provided comparative data, where less glycogen content was present in HT-29 cells during both the exponential and stationary growth phase than in Caco-2 cells[10], indicating that the selective staining produced by the potassium-ferrocyanide fixation as a glycogen marker yields comparative results. An additional feature of the HT-29 cell line was the presence of mucin filled vacuoles, which provides evidence that these cells potentially originated from a mucous secreting population of the intestinal epithelium. Previous investigations of the HT-29 cell line have also isolated mucous-secreting sub-clones in the population, but this was achieved by inducing cellular differentiation by the administration of galactose to the culture medium[31]. This induced differentiation also resulted in polarisation and monolayer formation of the HT-29 cells[31]. The present study did not include differentiation of HT-29 cells by adding galactose; therefore, the presence of the mucin filled vacuoles might be indicative of a population of precursor cells that later form secretory intestinal epithelial cells.

When the SW480 cells were cultured under standard conditions, a non-homogenous population of undifferentiated cells was observed. One cell type was simple squamous with irregular microvilli, and the other population of cells were simple spindle-shaped with irregular microvilli. These results were similar to those reported by previous investigations, which also described a heterogenous undifferentiated population of SW480 cells with comparable morphological features[35]. By day one post-confluency, cell-to-cell contacts have been established, with no evidence of intercellular spaces; however, no desmosomes or tight junctions were evident. The glycogen content was equivalent in both populations of SW480 cells, indicating that comparable metastatic properties were present in both sub-populations of this cell line. By day one post-confluency, abundant mitochondria were

present throughout the cell cytoplasm when compared to the cytoplasm of the other two cell lines, and some mitochondria appeared to be branching. Large numbers of mitochondria indicated that these cells were highly active.

At 12 d post-confluency, glycogen particles were also evident, representing intermediate glycogen expression when compared to the large amounts found in Caco-2 cells and trace amounts found in the HT-29 cells. The glycogen stores were distributed amongst mitochondrial populations; however, no intranuclear rods were evident projecting into the nucleus. Another striking feature of the SW480 cell line, irrespective of cell type, was their ability to send deep invasive processes into the membrane filter pores (approximate 8 μm deep). These processes often contained cell organelles, such as mitochondria, which demonstrated deep cell anchorage. Previous reports have also reported that spindle-shaped SW480 cells exhibited vast migration through uncoated membrane filters, whereas cells with a more epithelioid shape (Caco-2 and HT-29) scarcely invaded membranes, even those coated with Matrigel[6]. These potentially invasive characteristics were not observed in Caco-2 or HT-29 cells in this investigation. Invasive processes have, however, been reported in Caco-2 cells in other literature when invasion was induced by adding a collagen matrix to the membrane filters[19]. The addition of the collagen promoted cell migration through the filter pores, which eventually led to the depolarisation of the model cells[19]. No invasion inducing agents were administered in this investigation and there is clear evidence that the SW480 cells presented the most invasive capabilities of the three cell lines. These results were comparable with a previous investigation where metastatic capacities were examined in the SW480 cell line using orthotopic tumor models[40]. When SW480 cancer cells were injected or implanted into the caecum of mice, SW480 cells had a 100% take rate, when compared to a take rate of 69% in HT-29 and 40% in Caco-2 cells. The SW480 cells also had the highest growth index, which was represented by the time it took between implantation and the appearance of disease symptoms[40]. The ultrastructural features observed in this study of the SW480 cancer cell line supported other literature that considered this cell line to be highly malignant[35].

In conclusion, this was the first study to incorporate selective staining for transmission electron microscopy to help identify glycogen storage and nucleocytoplasmic exchange of glycogen by identifying intranuclear rods. Ultrastructurally, the loss of the tight junction in subpopulation 1 of Caco-2 cells, and in HT-29 and SW480 cells is indicative of highly disorganized epithelia, whereby individual tumor cells have the potential to dissociate from the primary tumor and begin a metastatic process in other tissue. Conversely, sub-population 2 of Caco-2 cells remained as a polarized monolayer of epithelia with extensive tight junction formation, suggesting organised epithelia with strong cell-cell adhesion with lower disassociation potential. Their glycogen storage, however, suggested that Caco-2 and SW480 cells were undergoing the largest amount of neoplastic transformation, and the

abundant mitochondria present in both populations of SW480 cells was also indicative of that feature. The ultrastructural features examined in this study have contributed to our already vast knowledge of colorectal cancer cells. We propose the structural parameters detailed in this systemic study can be used as a quality control in the study of the ultrastructure of Caco-2, HT-29, and SW480 cells *in vitro*. The table herein presented might serve as a morphometric guide for others who wish to utilise these cells lines. Future 3D tomographical work will further capture the nucleocytoplasmic exchange and assist in identifying other key ultrastructural regulators of the invasive process of malignant cells. The identification of glycogen stores in these cells might result in drug therapy trials targeted against these abundant intracellular features.

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COMMENTS

Background
The extensive variability that exists in the morphology, growth rate, and cell viability between different colorectal cancer cells in culture results in wide-ranging observations and the inability to standardise data. This investigation is designed to compile relevant ultrastructural data on three commonly used cancer cell lines to determine whether ultrastructural features and the presence of large glycogen stores can be used as indicators for the metastatic properties of cells.

Research frontiers
Large quantities of glycogen stores have been reported in a variety of cultured cells, particularly cells undergoing neoplastic transformations, even when the cells from the tissue of origin lack glycogen. By incorporating selective staining using an osmium tetroxide and a potassium ferrocyanide solution in routine transmission electron microscopy, these glycogen stores can be ultrastructurally identified and used as diagnostic markers for the metastatic properties of cells.

Innovations and breakthroughs
This is the first study to combined ultrastructural and functional features of colorectal cancer cells with selective staining for other neoplastic markers, such as glycogen.

Applications
The compilation of ultrastructural features and glycogen storage data from three commonly used colorectal cancer cell lines will assist future investigators in selecting appropriate cell models depending on their area of interest, particularly for drug therapy trials that might be directed against excessive intracellular glycogen stores.

Peer review
This investigation emphasizes the variability in morphology, behaviour, and metastatic properties of colorectal cancer cells and provides readers with a coherent guide on three commonly used colorectal cancer cell lines for use as a morphometric tool for future investigations.

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