Descriptive *in situ* Changes of Living Mitochondria in Human Term Trophoblast Cells Cultured for Ten Days

Addai FK[a], Henson MC[bcd]

University of Ghana Medical School, [a]Department of Anatomy, Accra, Ghana, West Africa. Tulane University Health Sciences Center, Departments of [b]Obstetrics and Gynecology, [c]Physiology, and [d]Structural and Cellular Biology, New Orleans, LA, USA.

ABSTRACT

This study sought to illuminate morphological transformation in mitochondria during terminal differentiation of trophoblast cells. Cytotrophoblast cells purified from human term placentas via density gradient centrifugation, were cultured for ten days and *in situ* mitochondrial changes monitored. At periodic intervals, some cells were vitally stained with mitochondria-specific rhodamine 123. Initially, mitochondria in mononuclear cytotrophoblast cells appeared as strands whose intracellular distribution lacked a definable pattern. By 24 hour in culture, however, cell aggregations formed and mitochondria principally surrounded nuclei. By 48 hours, mitochondria encircled cytoplasmic granules that had appeared in cells giving rise to a distinctive lattice formation. After 60 hours, in addition to their net-like intracellular pattern, minute nodular dilatations punctuated some mitochondria. By nine days in culture, large cells with multiple nuclei evinced mitochondria that were mostly devoid of nodules or lattice organization. Subjectively, organelles appeared more numerous and either slender or convoluted. Distinctively, mitochondria near the plasma membrane in these multinucleated cells were arrayed parallel to it. This contrasted with angular orientation of mitochondria to the cell membrane in mononucleated cells of similar culture age and in multinucleated cells in earlier cultures. Collectively, results suggest that changes in intracellular location and spatial distribution concomitant with altered shape, size, and numbers of mitochondria may constitute distinct stages of cytotrophoblast terminal differentiation.

Keywords: Mitochondria, Rhodamine 123, Trophoblast differentiation.

INTRODUCTION

A better knowledge of changes that mitochondria undergo in the process of trophoblast terminal differentiation is necessary for an optimal understanding of placental morphology and function. Thus, trophoblasts constitute a unique model for studying changes in mitochondria during embryogenesis. Knowledge gained from the study of such models could yield a better insight into mitochondrial development [1], and shed greater light on the still undefined process of mitochondrial growth and division [2]. It has been suggested that some alterations in mitochondrial distribution and morphology are developmentally programmed, and are evidenced by characteristic organelle migrations or morphological changes that occur at specific stages of cellular differentiation [3]. Although structural and functional differences between the mitochondria of syncytiotrophoblast and their progenitor cytotrophoblasts are acknowledged [4], how or when these come about and whether intracellular distributions are altered commensurate with differentiation are

unknown. Knowledge of this process will enhance our understanding of how cytotrophoblasts form the endocrinologically mature and immunologically distinct syncytiotrophoblast.

In respect of clinical ramifications of placental development, an anomaly of trophoblastic mitochondria has been proposed as the first step in the pathophysiological cascade culminating in pre-eclampsia [5] and other obstetric complications [6]. A definitive understanding of changes in mitochondrial shape and distribution during differentiation of normal trophoblast will, therefore, eventually allow testing of this hypothesis via comparison with pre-eclamptic placentas. In this capacity, a number of excellent model systems have been developed to study syncytiotrophoblast formation in vitro [7-11]. Little attention has been paid, however, to the specific actions of mitochondria during this vital stage of placental development. The placental synthesis of progesterone has been proposed to be directly responsible for the successful maintenance of human pregnancy [12] and mitochondria in syncytiotrophoblast house those enzymes necessary for the biosynthesis of progesterone from cholesterol. P450 side-chain cleavage cytochrome (P450scc), a rate-limiting enzyme in progesterone biosynthesis, resides in the inner mitochondrial membrane of syncytiotrophoblast, although cytotrophoblast progenitors are not endowed with significant levels of the enzyme [13].

Improved methods for isolating pure cytotrophoblast cells and maintaining them in culture permit their transformation into syncytiotrophoblast. Availability of rhodamine 123, the permeant cationic laser dye that selectively accumulates in mitochondria of aerobically respiring cells [14,15], means that studies dedicated to elucidating their role(s) in trophoblast differentiation are possible. Therefore, we employed rhodamine 123 to investigate the actions of mitochondria during formation of multinucleated cells in monolayer cultures of human trophoblast cells. Changes in morphology and intracellular/spatial distribution of mitochondria were studied over ten days of culture, during which time, multinucleated cells with distinct features formed from mononucleated cytotrophoblast cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Medium 199, Hank's Balanced Salt Solution (HBSS), Penicillin-Streptomycin, Gentamicin, and L-Glutamine were purchased from GIBCO-BRL (Gaithersburg, MD). Trypsin type 1 (T-8003), DNase (D-5025), HEPES (H-3375), and Percoll (P-4937) were purchased from Sigma Chemical Company (St. Louis, MO). Cover glasses (18 mm, round) were obtained from Fisher Scientific Company (Pittsburgh, PA), flat bottom multi-well (22 mm, circular) from PGC Scientifics (Frederick, MD), Fetal Bovine Serum (FBS) from Biowhittaker Company (Walkersville, MD), and rhodamine 123 from ICN (Costa Mesa, CA). Normal term placentas were obtained immediately after spontaneous vaginal delivery or uncomplicated cesarean section at Tulane University Hospital, New Orleans, LA. These studies were approved by Tulane University's Institutional Review Board. HBSS and Medium 199 were used with the following supplementation: 4.0 mM sodium bicarbonate, 10.0 mM HEPES, 50µg/ml gentamicin sulphate, 50 IU/ml penicillin and 50 µg/ml streptomycin; pH 7.4 at 37ºC. The DMEM, containing 25 mM glucose and 1.0 mM glutamine, was used after supplementation with 25 mM HEPES, 44 mM sodium bicarbonate, 4.0 mM L-glutamine, 50 µg/ml gentamicin sulphate, and 50 IU/ml penicillin.

Isolation and culture of cytotrophoblast cells

The procedure for isolating, purifying, and culturing human trophoblast cells [7], as performed in our laboratory, has been described previously [16]. Briefly, cytotrophoblast cells were disaggregated from placentas using trypsin/DNase in three stepwise digestions. Pellets from all three digestions were pooled and purified via a 5-70% discontinuous Percoll gradient. The purified cell pellet was

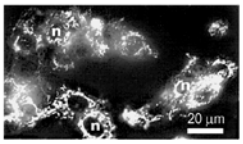

Fig. 1

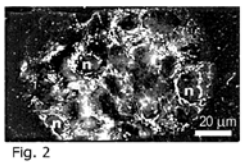

Fig. 2

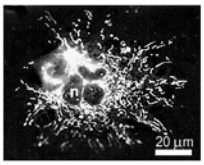

Fig. 3

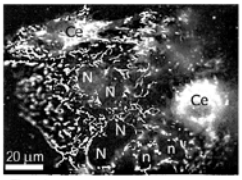

Fig. 4

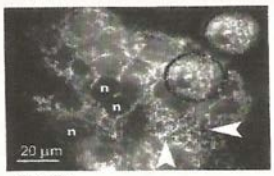

Fig. 5a

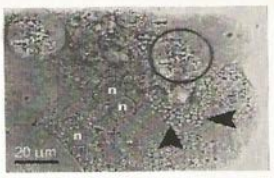

Fig. 5b

Fig. 6

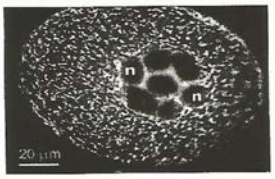

Fig. 7

Fig. 1. Fluorescence image of 12-hour living cultured human term cytotrophoblast cells, vital- stained with rhodamine 123. Mitochondria appear as white structures predominantly surrounding dye- free central areas that represent nuclei in three clusters of cells. Three of the several nuclei are labelled (n).

Fig. 2. A Large cluster of 24-hour cultured cytotrophoblast cells vitally stained with rhodamine 123 shows excludively mononuleated cells with perinuclear mitochondria. Three of the several nuclei are labelled (n).

Fig. 3. A living multinucleated cell in a 24-hour cytotrophoblast culture stained with rhodamine 123 shows mitochondria as contorted strands that generally radiate from the perinuclear region and terminate open-endedly in the periphery. One of the three nuclei is labelled (n).

Fig. 4. Rhodamine 123 vital-stained cytotrophoblast cells in a 36-hour culture show some nuclei (N) that are in intimate contact and not separated by mitochondria, while other nuclei in the same cluster (n) are separated by mitochondria. Two separate cells (Ce) adjacent to the cluster show more intensely fluorescent mitochondria. Note that the peripheral outline of cells in the cluster is not well defined.

Fig. 5 (a-b). Some mitochondria form a lattice pattern (white arrowheads) in a multinucleated cytotrophoblast in a 60-hour culture vital stained with rhodamine 123. Black arrowheads show corresponding lipid droplets encircled by the net-like mitochondria. Nodular mitochondria gibbosities (encircled) in a fluorescence micrograph (a) are superimposable with dark cytoplasmic granules (encircled) in the corresponding DIC image (b). *Use of a hand lens may enhance visualization of nodules.* Three nuclei in the cluster are labelled (n).

Fig. 6. Some mitochondria in a cluster of living cells of a 60-hour cytotrophoblast culture display nodular gibbosities (black arrowheads). Note the signet structure of mitochondria (white arrows) and net-like pattern especially obvious in the lower half of the micrograph. Some nuclei are labelled (n).

Fig. 7. A putative syncytium with several aggregated nuclei shows numerous, slender mitochondria. Note that although the nuclei are in intimate contact, their individual identity is obvious. Mitochondria in the outermost regions of the well-defined periphery of this cell are circumferentially oriented. Two nuclei are labeled (n). Nine-day culture of human term cytotrophoblast cell vitally stained with rhodamine 123.

resuspended in 10 ml supplemented DMEM, a 0.2 ml aliquot was taken for cell counting and viability assessment, and the remainder centrifuged (1000 x g, 10 minutes). The final cell pellet was resuspended in 5 ml supplemented DMEM + 10% heat inactivated FBS (hereafter referred to as growth medium, GM). Cells were counted in a haemocytometer and viability assessed by trypan blue exclusion. Live cells constituted 87% - 95% of cytotrophoblast preparations. Cytotrophoblast cells were plated a a density of 0.5 - 1.0 × 10^6 per coverslip in 12-well culture dishes. To ensure that cells settled on glass coverslips and not on plastic in culture dish wells, only 0.5 ml of cell suspension in GM was inoculated on each (coverslip). After incubating for two hours to allow cells to attach, the GM was topped with an additional 1.5 ml to yield a total of 2.0 ml per well and incubation continued. Cells were maintained in a humidified incubator with 90% air and 10% CO^2 at 37ºC. The GM was changed every 24 hours. At appropriate time intervals some cells were vital-stained for mitochondria.

Rhodamine 123 staining of living cells for fluorescent labelling of mitochondria in cultured trophoblast cells.

The procedure used was the same as previously described [17]. Briefly, cells were incubated on a coverslip in 1.5 ml of 10 µg/ml rhodamine 123 in GM for 10 minutes, under the cell-culture maintenance conditions. The coverslip was rinsed in three 5-minutes changes of rhodamine-free GM, and inverted onto a drop of dye-free GM to create a live cell observation chamber. The chamber was limited by a circular ring of pure petroleum jelly on a standard 25 × 75 mm microscope slide. Stained cells were examined under epifluorescence illumination using a dichroic filter, set to IV for FITC excitation, and fitted to a Zeiss Axioplan photomicroscope equipped with neofluar/phase contrast/DIC objective lenses and optics. Photographs were taken using either Fuji chrome Sensia II or Kodak T-Max film (ASA 400). Magnification for micrographs was determined with the help of a microscope stage graticule photographed at the same time and magnification as the cells. Results presented were obtained from cultures of four different placentas, separately cultivated. For each placenta, three (cohort) cultures on glass coverslips were stained and examined at specified time points. To ensure consistency of results, all cultures utilized the same lots of medium, enzymes, chemicals, and FBS.

RESULTS

Cytotrophoblast cells cultured for twelve hours were mononucleated with subjectively higher nuclear/cytoplasm ratio. Rhodamine 123

labelled mitochondria, in these cells were not individually discernible with the light microscope used in this study, and their intracellular distribution lacked any discernable pattern (Fig. 1). By 24 hours of culture, most cytotrophoblast cells were still mononucleated and had formed aggregations, but the shape and spatial distribution of mitochondria were unchanged from earlier time period. Mitochondria predominantly surrounded single nuclei in these cells (Fig. 2). A few multinucleated cells were observed in 24-hour cultures and evidenced mainly filamentous, but highly contorted, mitochondria (Fig. 3). Notably, mitochondria in these early multinucleated cells appeared to radiate from perinuclear regions and terminate open-endedly in the peripheral regions adjacent to the cell membrane. In 36-hour cultures, nuclei of some aggregated cells were in very close proximity and not separated by mitochondria (Fig. 4). In these cells, it was apparent that the nuclear/cytoplasm ratio had considerably reduced, as the cells had become qualitatively larger and more flattened. By 60 hours, cells showed intracytoplasmic granules (lipid droplets?) that were encircled by mitochondria, giving rise to a net-like formation (Figs. 5A and B). This spatial pattern of mitochondrial organization was observed mostly in aggregated cells, but a few solitary cells displayed similar spatial organization of mitochondria (not shown). Mitochondria still surrounded discrete nuclei in cell clusters in 60-hour cultures (Fig. 6), although some nuclei were in intimate contact. The majority of cells at this time evidenced intracytoplasmic granules, and minute localized nodular gibbosities punctuated some mitochondrial strands (Figs. 5 & 6). Some of the nodular gibbosities in mitochondria were superimposable with dark cytoplasmic granules in differential interference contrast (DIC) images (Figs. 5A and B).

After 9 days, large cells (Fig. 7) containing multiple nuclei and numerous mitochondria were seen. Only a minority of the cell population in cultures at this time period showed these characteristics, as majority of the cells were in clusters. Nuclei in the multi-nucleated cells were all grouped together and in intimate contact, but retained their individual identity. The multinucleated cells in the later cultures also had well-defined peripheral outlines (Figs. 7 and 8) compared with multi-nucleated cell aggregates in earlier cultures (Fig. 4 and 5). The spatial alignment of mitochondrial strands in the most peripheral regions of the multinucleated cells in late cultures was remarkable and consistent. Mitochondria closest to the cell membrane were arrayed circumferentially (Fig. 8) and were parallel to it. Obvious radiation of mitochondria from perinuclear regions seen in multinucleated cells in early cultures (Fig. 3) was not evident in the late cultures. Structurally, mitochondria in these multinucleated cells were variable, but generally appeared shorter (Fig. 7), or displayed a variety of convolutions (Fig. 8). In contrast, mitochondria in most of the few mononucleated cells (in the late cultures) displayed open-ended and angular terminations near their cell membranes (Fig. 9). These mononucleated cells in late cultures were subjectively larger, with majority of their mitochondria appearing longer, more prominent, and less contorted compared with those in multinucleated cells. Intra-cytoplasmic granules and associated net-like mitochondrial pattern (Figs 5 and 6) that characterised multinucleated cell clusters in earlier cultures were not observed in multinucleated cells in late cultures (Figs 7 and 8). Nodular gibbosities in mitochondria were not obvious in either early or late multinucleated cells with clearly defined boundaries, but were present in some mononucleated cells in late cultures (Fig. 10).

DISCUSSION

The observations that mitochondria; (i) encircled intracytoplasmic granules, (ii) tended to have a net-like spatial arrangement and, (iii)

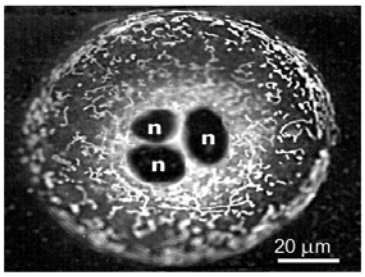

Fig. 8

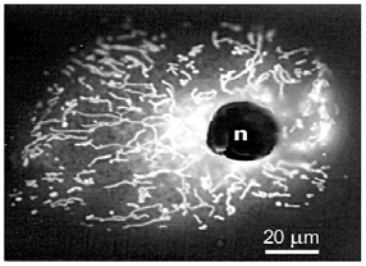

Fig. 9

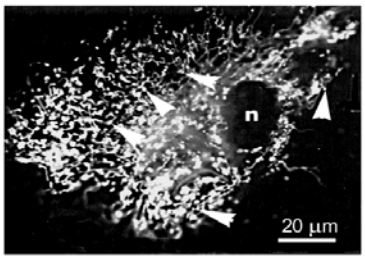

Fig. 10

Fig. 8. Mitochondrial strands in a tri-nucleated cell display variable morphology, including loops, bends, curves and a variety of convolutions. Note that strands along the periphery of the cell, immediately adjacent to the cell membrane, are circumferentially oriented and parallel to the membrane. Ten-day culture of human term cytotrophoblast cell vitally stained with rhodamine 123. The nuclei are labelled (n).

Fig. 9. A mononucleated cell in a ten-day culture displays elongate filaments of mitochondria that terminate open-endedly and at variable angles in the outermost reaches of the cell where the cell membrane is located. Note the off-center position of the nucleus (n) and the obviously low nucleus/cytoplasm ratio. Human term cytotrophoblast cell, vitally stained with rhodamine 123.

Fig. 10. An apparently mononucleated cell in a ten-day culture shows mitochondria as tangled strands with numerous nodular gibbosities (white arrow heads). Note off-center position of the single nucleus (n). Human term cytotrophoblast cells vitally stained with rhodamine 123.

were arrayed parallel to the plasma membrane in late multinucleated trophoblast cells, are perhaps being reported for the first time. Further, in respect to our observations, we propose that the intracytoplasmic granules encircled by mitochondria are lipid droplets, and their close association may serve to facilitate lipid (as cholesterol) translocation into mitochondria. This would help to meet increased lipid demand of syncytialising trophoblast, necessary as their mitochondria undergo fission and structural metamorphosis to produce smaller and more numerous organelles, characteristic of syncytiotrophoblast [4]. Speedy cholesterol transport would be required at this time to fuel the synthesis of progesterone, as the syncytialising trophoblast acquires the enzymatic machinery to do so. The need for faster cholesterol translocation to mitochondria may also explain their parallel alignment to the plasma membrane in multinucleated cells in our late cultures. Plasma membranes contain a high concentration of cholesterol that is dependent on sphingomyelin, which is contained there in far higher proportion than in mitochondria [18]. The parallel alignment of mitochondria to the plasma membrane may, therefore, facilitate the transfer of cholesterol from the latter to the former's outer membrane. Transposition of cholesterol from the outer membrane to the inner mitochondrial membrane, and P450scc, at contact sites [19] may thus be enhanced. In this regard, it is noteworthy that in cultured Leydig and adrenal cortical cells, cholesterol content of the plasma membrane decreased after stimulation of steroidogenesis, with increased plasma membrane cholesterol concentrations being associated with increased steroid synthesis [20].

Previously, nodular swellings in mitochondria were cited as evidence, that in cultured first trimester placental cells, organelles were not under tension or being stretched [17]. In the present purified term cytotrophoblast cultures, however, superpositioning of the nodules with phase-dense granules suggests that they may be (enlarged) lysosomes. Ongoing efforts are aimed at establishing the identity of these granules at the electron microscopic level. Jefcoate et al [18] asserted that in most steroidogenic cells, cholesterol esters are principally provided via endocytic uptake of low-density lipoprotein (LDL). The LDL is then processed in lysosomes, where it is hydrolysed by acidic cholesterol esterase to yield cholesterol [21]. Since the transfer of LDL-cholesterol from lysosomes to other cell membranes is comparatively slow [22], the punctuation of mitochondrial strands with lysosomes may serve to further reduce the transfer distance of substrate for steroid synthesis during terminal differentiation of non-steroidogenic cytotrophoblast into steroidogenic syncytiotrophoblast. We propose that these distinctive, albeit transient, gibbosities can be termed mitochondrial nodules. Furthermore, the great number of apparently smaller-sized mitochondria observed in multinucleated cells in late cultures agrees with prior reports on syncytiotrophoblast mitochondria [4]. We also agree with others, that inherent morphological and numerical changes in mitochondria may serve to provide a larger surface to volume ratio, necessary for enhancing the direct uptake of cholesterol from the cytosol for steroidogenesis. We might further suggest that acquisition of the P450scc enzyme by syncytiotrophoblast mitochondria, combined with their structural alteration to facilitate a faster rate of cholesterol utilisation, may account for the paucity of lipid droplets in multinucleated cells in our late cultures. A question regarding the fate of residual (superfluous) membranes resulting from fusion of cytotrophoblast cells may have a speculative answer from the present study. In addition to the mobilization of cholesterol from the plasma membrane for steroidogenesis (vide supra), the otherwise superfluous membranes may be utilised in producing more mitochondria.

Although LDL-cholesterol is the preferred substrate for human placental progesterone biosynthesis [11,23], HDL-cholesterol is taken into some steroidogenic cells by binding to their plasma membrane, followed by direct transfer to the cytosol where lipid droplets form following sufficient accumulation. In the same manner, placental

progesterone synthesis might be augmented by HDL-cholesterol [24] and might mirror the intermediate stages of multinucleated-cell formation in our cultures, when lipid droplets were surrounded by mitochondria to give rise to the net-like pattern described above. It is worthy of note, that both lipid droplets and the distinctive mitochondrial lattice were seen in both single mononucleated cells and aggregated cells, but not in multinucleated cells (presumptive syncytiotrophoblast). Similarly, in the cardiac tissue of some mammals, mitochondria envelop lipid droplets and are thought to be an adaptation to provide the energy required for variable rates of cardiac function [25]. Perhaps a similar variability in energy requirements is imposed on the terminally differentiating trophoblast during the metamorphosis of their mitochondria.

Multinucleated cells with distinctive spatial distribution of mitochondria (our putative syncytia), were not observed in our cultures until after nine days. This contrasts with previous reports of syncytial formation after two days [7, 26], four days [15,27,28] and five days [10]. Perhaps cells similar to the multinucleated ones seen as early as 24 hours in our cultures, represent the basis for the determination of early syncytial formation in some studies, although differences in cell growth attributable to enzymes, FBS, and other chemicals used in the culturing protocol cannot be discounted. However, our results support the assertion that phase-contrast microscopic examination alone is unsatisfactory to monitor the formation of multinucleated cells in cytotrophoblast cultures [10]. The need exists, therefore, to use other known markers or methodologies to confirm the identities of multinucleated cells. Since the spatial arrangement differed dramatically in early compared to later cultures, it is suggested that rhodamine 123 vital staining may be a useful tool in distinguishing cyto- and syncytiotrophoblast in early cultures. When confirmed, this must be of particular interest to endocrinologists interested in the two cell types because they produce the major placental hormones and growth factors [29].

ACKNOWLEDGMENTS

We are grateful to Robert D Yates, Ph D (Chairman) and the staff of the Department of Structural and Cellular Biology, and April G. O'Quinn, MD (Chairman) and the staff of the Department of Obstetrics and Gynecology at the Tulane University Health Sciences Center for financial and administrative support for this work. The invaluable assistance of Lloyd Jollibois, PhD with the cell culture technique is also acknowledged. We are deeply indebted to the labor and delivery nurses of the Tulane University Hospital for clinical co- ordination and appreciate the contributions of Mrs Nathlynn Dellande and Mr. Kevin Adutwum Ofosu in manuscript preparation.

REFERENCES

1. Shepard TH, Muffley LA & Smith LT (1998) Ultrastructural study of mitochondria and their cristae in embryonic rats and primate (*N. nemistrina*). *Anat Rec*, 252, 3830392.
2. Beech PL, Nheu T, Schultz T, Herbert S, Lithgrow T, Gilson PR & McFadden GI (2000) Mitochondrial FtsZ in a chromophyte alga. Science, 287, 1276-1279.
3. Yaffe MP (1999) The machinery of mitochondrial inderitance and behavior. *Science*, 283, 1493-1497.
4. Martinez F, Kiriakidou M & Strauss III JF (1997) Structural and functional changes in mitochondria associated with trophoblast differentiation: methods to isolate enriched preparations of syncytiotrophoblast mitochondria. *Endocrinology*, 138, 2172-2183.
5. Widschwendter M, Schrocksnadel H & Morti MG (1998) Pre-eclampsia: a disorder of placental mitochondria? *Molec Med Today*, 4(7), 286-291.
6. Rakheja D, Bennet MJ, Foster BM, Domiati-Saad R, & Rogers BB (2002) Evidence for fatty acid oxidation in human placenta, and

the relationship pf fatty acid oxidation enzyme activities with gestational age. *Placenta,* 23(5), 442-450.

7. Kliman HJ, Nestler JE, Serman E, Sanger JM & Strauss III JF (1986) Purification, characterization and invitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology,* 118, 1567-1582.
8. Douglas GC & King BF (1990) Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei. *J Cell Sci,* 96, 131-141.
9. Coutifaris C, Kao L-C, Sehdev HM, Chin U, Babalola GO, Blaschuk OW & Strauss III JF (1991) E-cadherin expression during the differentitation of human trophoblasts. *Development,* 113, 767-777.
10. Douglas GC & King BF (1993) Colchicine inhibits human trophoblast differentiation in vitro. *Placenta,* 14, 187-201.
11. Bloxam DL, Bax CMR & Bax E (1997) Culture of syncytiotrophoblast for study of human placental transfer. Part 1: isolation and purification of cytotrophoblast. *Placenta,* 18, 93-98.
12. Pepe GJ & Albrecht ED (1995) Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr Rev,* 16, 608-648.
13. Beaudoin C, Blomquist CH, Bonenfant M & Tremblay Y (1997) Expression of the genes for 3β-hydroxysteroid dehydrogenase type 1 and cytochrome P450scc during synctium formation by human placental cytotrophoblast cells in culture and the regulation by progesterone and estradiol. *J Endocrinol,* 154, 379-387.
14. Johnson LV, h Johnson LV, Walsh ML & Chen LB (1980) Localization of mitochondria in living cells with rhodamine 123. *Proc Nat Acad Sci USA,*. 77, 910-994.
15. Kagan T, Davis C, Lin L & Zakeir Z (1999) Coenzyme Q10 can in some circumstances block apoptosis, and this effect is mediated through mitochondria. *Ann NY Acad Sci,* 887, 31-34.
16. Henson MC, Shi W, Greene SJ & Reggio BC (1996) Effects of pregnant human, non-pregnant human, and fetal bovine sera on human chorionic gonadotropin, estradiol and progesterone release by cultured human trophoblast cells. *Endocrinology,* 137(15), 2067-2074.
17. Addai FK & Ockleford CD (1986) Mitochondria in living cells cultured from human chorionic villi: effects of colchicine on numbers and distribution. *J Anat,* 147, 219-233. *Chem,* 264, 8141-8150.
18. Jefcoate CR, McNamara C, Artemenko I & Yamazaki T (1992) Regulation of cholesterol movement to mitochondrial cytochrome P450scc in steroid hormone synthesis. *J Steroid Biochem Molec Biol,* 43(8), 751-767.
19. Papadopoulos V, Amri H, Boujrad N, Cascio C, Culty M, Garnier M, Hardwick M, Li H, Vidic B, Brown AS, Reversa JL, Bernassau JM & Drieus K (1997). Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids,* 62, 21-28.
20. Papadopoulos V (1993) Peripheral type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenesis. Cell function. *Endocr Rev*, 14(2), 222-240.
21. Fowler SD & Brown WJ (1984) Lysosomal acid lipases. In *Lipases* (Ed.) Bergstrom B & Brockman HL, pp. 329-364. New York, Elsevier Sciences.
22. Johnson WJ, Chacko GK, Phillips MC & Rothblat GH (1990) Efflux of lysosomal cholesterol from cells. *J Biol Chem* 265, 5546-5553.
23. Gywnne JT & Mahaffee DD (1989) Rat adrenal uptake and metabolism of high density lipoprotein cholesterol ester. *J Biol* 264, 8141-8150.
24. Lasuncion MA, Bonet B & Knopp RH (1991) Mechanism of the HDL2 stimulation of progesterone secretion in cultured placental trophoblast. *J Lipid Res* 32, 1073- 1087.
25. Navaratnam V, Ayettey AS, Addai F, Kesse K & Skepper JN (1986) Ultrastructure of the ventricular myocardium of the bat *Eidolon helvum. Acta Anatomica*, 126, 240-243.

26. Lobo J, Bellino F & Sergei A (1987) Characterization of isolated cells in primary culture from human term placenta by electron microscopy and immunohistochemistry. *Troph Res*, 2, 477- 486.
27. Jolibois LS, Shi W, George WJ, Henson MC & Anderson MB (1999a) Cadmium accumulation and effects on progesterone release by cultured human trophoblast cells. *Reprod Toxicol,* 13, 215-221.
28. Jolibois LS, Burow ME, Swan KF, George WJ, Anderson MB & Henson MC (1999b) Effects of cadmium on cell viability, trophoblast development, and expression of low density lipoprotein receptor transcripts in cultured human placental cells. *Reprod Toxicol,* 13, 473-480.
29. Cross JC, Anson-Cartwright L, & Scott IC (2002) Transcription factors underlying the development and endocrine functions of the placenta. Recent Prog Horm Res. 57, 221-234