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Comparative Analysis of Trophoblasts and Angiogenesis in Human Placental Compartments

Análisis Comparativo de Trofoblastos y Angiogénesis en Compartimentos Placentarios Humanos

Pinar Ayran Fidan & Attila Dagdeviren

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SUMMARY: Trophoblasts perform different functions depending on their location. This study aimed to obtain structural clues about the functions of villous and extravillous trophoblasts by using light and electron microscopy. Term placenta samples were obtained from 10 healthy pregnant women following cesarean sections. Frozen sections were stained with hematoxylin-eosin, semi-thin sections were stained with toluidine blue and examined with a light microscope, while thin sections were contrasted using uranyl acetate-lead citrate and evaluated under an electron microscope. Fine structural features of villous trophoblasts overlapped some villous stromal cells. In addition to the usual appearance of mature capillaries in villous stroma, we demonstrated and reported maturational stages of angiogenetic sprouts in term placenta. Extravillous trophoblasts were classified according to their location: fibrinoid, chorion, trophoblastic, column, maternal vascular endothelium, or decidua. All of these trophoblasts shared some ultrastructural features but also were distinct from each other. In decidua, it was noted that the endothelial lining of some vessels was invaded by a few endovascular trophoblasts with irregular microvilli. These cells shared some ultrastructural properties with both villous trophoblasts and stromal cells. Examination showed that angiogenesis was still present in term placentas and that trophoblasts, endothelial and stromal cells have very similar properties ultrastructurally, suggesting they represent transformational forms.

KEY WORDS: Angiogenetic sprouts; Electron microscope; Extravillous trophoblast; Fibrinoid; Placenta.

INTRODUCTION

The placenta is the crucial organ which maintains normal gestation and fetal development. Despite numerous studies, the structure-function relationships within the human placenta are not yet fully understood.

During implantation trophoblasts rapidly convert into two distinct cell types, cytotrophoblasts and syncytiotrophoblast. Cytotrophoblasts proliferate at ad-embryonic pole, fusing to neighboring syncytiotrophoblast toward maternal tissues (Guttmacher *et al*., 2014). Primary, secondary and finally tertiary (floating) villi develop, in the core of villi, there are villous cytotrophoblasts just beneath the syncytiotrophoblast, stroma with several types of cells, and capillaries (Burton & Fowden, 2015). Cytotrophoblasts leaving villous tree are called extravillous trophoblasts (EVTs) and some of them are in direct contact with maternal stroma as they invade the endometrium. These cells are located in: a) fibrinoid, b) chorion (trophoblastic shell), c) trophoblastic column, d) maternal vascular endothelium (endovascular trophoblast) and e) decidua basalis (Borbely *et al*., 2014). The aim of this study was to compare the fine structure of trophoblastic cell lineage located at different placental compartments using light and electron microscopes to obtain further evidence about their function.

MATERIAL AND METHOD

This study was approved by Baskent University Institutional Review Board and Ethics Committee (Project no. KA14/110) and supported by Baskent University Research Fund. Before the samples were collected, patients were informed about the study and the procedure for signing the “Informed Consent Form for Scientific Research”. Ten placenta samples were obtained from healthy pregnant

Baskent University Faculty of Medicine, Department of Histology and Embryology, Ankara, Turkey.

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women aged 24 to 40, and between 38 and 40 weeks of gestation, following cesarean sections. Only patients without a known chronic disease and/or complications, followed up in Baskent University Faculty of Medicine, Obstetrics and Gynecology were included in the study. Placenta samples were picked up just after delivery. To ensure standardization during sampling, tissue samples were dissected and coded as shown in Figure 1.

Of the placenta samples, some pieces were snap frozen in liquid nitrogen, sectioned using a cryostat and stained with hematoxylin-eosin. Others were added to 2 % glutaraldehyde solution and prepared for examination by using a routine plastic embedding method (Fidan *et al*., 2019). Semi-thin sections were stained with toluidine blue, examined under a light microscope and photographed with a Leica DM3000 digital camera. Thin sections were stained with uranyl acetate-lead citrate and photographed under a LEO-500 electron microscope.

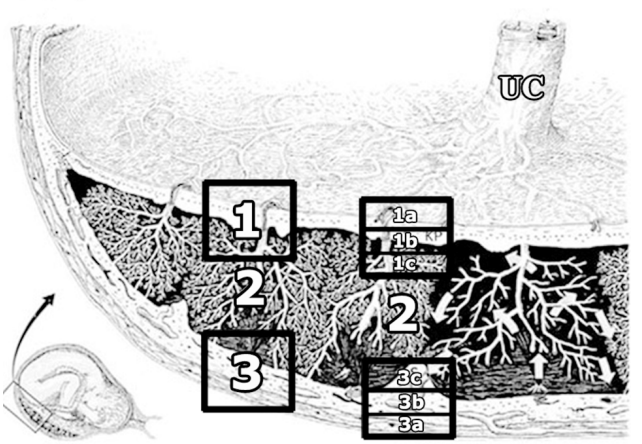

Fig. 1. Placenta tissue sampling. 1: Fetal (chorionic) side as 1A-1B-1C, 2: villous tree core, 3: maternal (decidual) side as 3C, 3B, 3A. UC; umblical cord, A; indicates the outermost, C; indicates the innermost samples.

RESULTS

Villous tree, syncytiotrophoblast, cytotrophoblasts, villous stromal cells, extravillous trophoblasts (EVTs) in fibrinoid, chorion, trophoblastic column, maternal vessels and decidua were observed through the light microscope (Fig. 2A). These examinations revealed conventionally recognized structural properties of the organ.

Certain areas were examined in detail using an electron microscope. Syncytiotrophoblast surrounding the villi had an electron-dense cytoplasm, rich in ribosomes, granular endoplasmic reticulum, numerous mitochondria of variable size and shape, a heterochromatin rich nucleus, and numerous irregularly oriented microvilli. Deep in the syncytiotrophoblast, cytotrophoblasts with relatively less electron dense cytoplasm, containing active nuclei, prominent nucleoli and abundant free ribosomes were located. The basal face of the cytotrophoblasts rests on the trophoblastic basement membrane. This basement membrane was in direct contact with the syncytiotrophoblast where cytotrophoblasts were missing. In these sections, invaginations of basal cell membranes packed with many projections resembling microvilli were also detected in the syncytiotrophoblast. (Figs. 2B, 2C, 4A-C). Similar structures were also observed between cytotrophoblasts and the syncytiotrophoblast at certain areas (Figs. 2C, 4B). However, cytotrophoblast membranes, which were in direct contact with basal lamina were rather smooth lacking such invaginations (Figs. 2B, 2C, 4A, 4B). Desmosomes were also frequently observed between syncytiotrophoblast and cytotrophoblasts supporting structural integrity. Associated with villi, syncytial knots, sprouts and bridges were also observed both at light and electron microscopic levels. In villous stroma two types of cells were mainly detected, namely mesenchymal cell and Hofbauer cells. Four stromal cell types: fibroblasts, reticulum cells, pericytes and myofibroblasts were identified as mesenchymal cell derivatives according to their location and ultrastructural properties. Elongated fibroblasts with thick processes had active nuclei, prominent nucleoli, abundant organels and cytoskeletal elements in their cytoplasm (Fig. 2C). Relatively inactive fibroblasts (reticulum cells) were distinguished by thinner processes with a slender cytoplasm extending between collagen fiber bundles. Myofibroblasts, reflecting the ultrastructural features of protein synthesizing cells were clearly distinguished from the previous two cell groups by the presence of caveola/pinocytotic vesicles and being surrounded by an external lamina (Fig. 2D). Pericytes sharing similar structural properties to myofibroblasts were distinguished by their locations along the capillaries. Pericytes were also observed to be surrounded by an external lamina which was continuous with the basal lamina of endothelial cells. Hofbauer cells, the second major cell group in stroma, were identified by the presence of numerous phagocytic vacuoles in their cytoplasm, their relatively irregular shape and peripherally located indented nuclei (Fig. 2B).

Fetal capillaries and collagen fibers were the major components of the villus stroma. Capillaries were lined by typical squamous endothelial cells sitting on a basal lamina in most cases (Fig. 3A). These cells were attached to each other by tight junctions and had pinocytotic vesicles at their luminal and basal cell membranes. We also

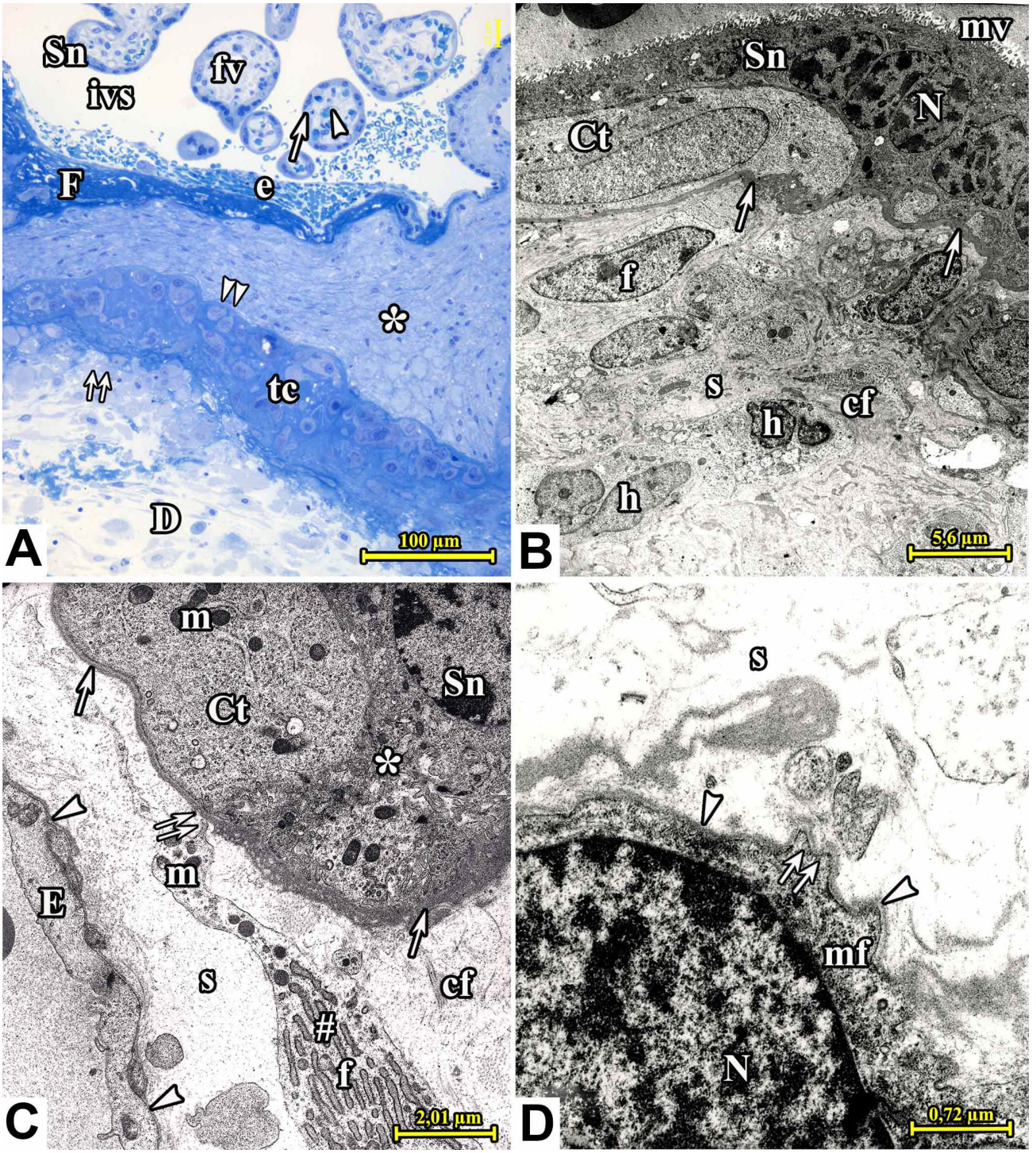

Fig. 2. A) In the light micrograph (LM) of placental villi adjacent to decidual (basal) plate, floating villi (fv), an anchoring villus (*), trophoblastic column (tc) and decidua (D) are seen. Sn;syncytiotrophoblast, arrow;cytotrophoblast, arrowhead;fetal capillary, F;Fibrinoid, e;erythrocytes, ivs;intervillous space (Semi-thin section, Toluidin blue (TB), X20). 2B) Electron micrograph (EM) of a floating villus. N;nuclei, mv; microvilli, Ct; cytotrophoblast, arrow; trophoblast basement membrane, f;fibroblast, h;Hofbauer cell, cf;collagen fiber, s;villous stroma (Uranyl acetate-Lead citrate (UALC), X2156). 2C) *;microvillous projections, #;granular endoplasmic reticulum, m;mitochondria, E;endothelial cell, arrow head;basal lamina. A process of fibroblast contacts (double arrow) with the trophoblast basement membrane (arrow) (UALC, X6000). 2D) EM of a myofibroblast (mf). Arrowhead;external lamina, double arrow; pinocytotic vesicles (UALC, X 16700).

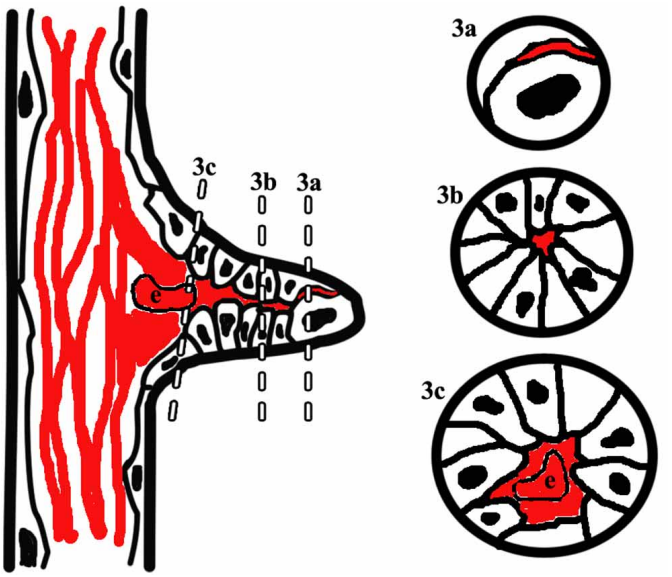

Fig. 3. Schematic view of sprouting angiogenesis. Figures a, b, c show the cross sections of the areas marked with dashed lines (e: eritrocyte).

observed cross sections of angiogenetic sprouts at the peripheral compartments of stroma reflecting the maturational stages of newly arising capillaries (Figs. 3 and 4). Those with the most primitive appearance at the tip of the sprouts were composed of moderately flattened endothelial cells with a centrally located large cell (Figs. 3A, 4A-C). Though these intermediate endothelial cells had an immature appearance, intercellular junctions and pinocytotic vesicles at their basal surface were present similar to those of classical capillary endothelial cells. In some of the sprout sections the lumen was occupied by a large cell with migratory protrusions (Fig. 4C). Lumina of these vessels were represented by tiny clefts between neighboring cells which were limited by junctional complexes (Figs. 3A, 4A-C). When these vessels were seen surrounded by taller endothelial cells, their slightly enlarged lumina usually contained 1-2 erythrocytes. These tall endothelial cells also had intercellular junctions at their basolateral surface and pinocytotic vesicles. On the outside of endothelial cells there was usually a laminated basal lamina and pericytes were present (Figs. 3B, 3C, 4D-G). It was noted that the tall endothelial cells shared ultrastructural features with cytotrophoblasts. Basically, there were two types of large endothelial cells depending on their cytoplasmic components. The first one was rich in cytoplasmic filaments while the latter was rich in organelles (Figs. 4E-G).

EVTs at different locations in the placenta exhibited unique fine structural features and were accordingly identified. Trophoblasts forming the outer margins of trophoblastic shell were attached to a distinct basal lamina. Opposite membranes of the cells were embedded with granular material rich in irregularly oriented microvilli. Between the individual trophoblasts forming the shell there were also desmosomes. Shape and structures of these cells were most similar to villous trophoblasts (Figs. 5A, 5B). Two types of fibrinoid were identified as fibrin type and matrix type. The fibrin type fibrinoid was rich in granular substance formed from remnants of coagulation material lacking cells. However, the matrix type fibrinoid contained EVTs embedded in an extracellular matrix. There were basically two types of EVTs within the matrix type fibrinoid, one which was oval-shaped with rounded cells and a second which was elongated with several processes (Figs. 5C, 5D). The rounded cells had euchromatin rich nuclei with their cytoplasm having two distinct compartments. The darker perinuclear areas of the cells were observed to be rich in granular endoplasmic reticulum, while peripheral cytoplasm was moderately electron lucent. These cells were either scattered as single cells in the fibrinoid or sometimes formed small neighboring groups. Their facing cell membranes were extremely rich in microvillous lateral protrusions and some desmosomes. At higher magnifications these intermingling lateral protrusions and bud-like larger protrusions toward the matrix were identifiable in more detail. These cells were embedded in a mildly electron dense, homogenous amorphous material. Additionally, small degenerated cell particles were observed in some areas within the matrix between the cells described above. The second type of EVTs in the matrix type fibrinoid were elongated cells rich in organelles with several processes (Figs. 5C, 5D).

EVTs moving from the trophoblastic column into the decidua were moderately electron dense, their nuclei were rich in euchromatin, and their cytoplasm was rich in organelles. These cells were surrounded by a matrix of dense granular material containing cell debris. EVTs deeper into the decidua were similarly divided into two main groups, rounded ones and those with processes (Figs. 6A, 6B). The rounded cells had euchromatin rich nucleus with a prominent nucleolus and they were surrounded by an external lamina. Their cytoplasm was moderately electron dense and rich in ribosomes and mitochondria (Fig. 6A). EVTs with processes also had euchromatin rich nucleiwith prominent nucleoli and organelle-rich cytoplasm, however their external lamina was incomplete (Fig. 6b). As we progressed deeper into the decidua, maternal vessels were observed. Some of this microvasculature was lined by taller endothelial-like cells with numerous microvilli in addition to regular flattened endothelial cells. These cells were distinguished as endovascular EVTs (Figs. 6C, 6D).

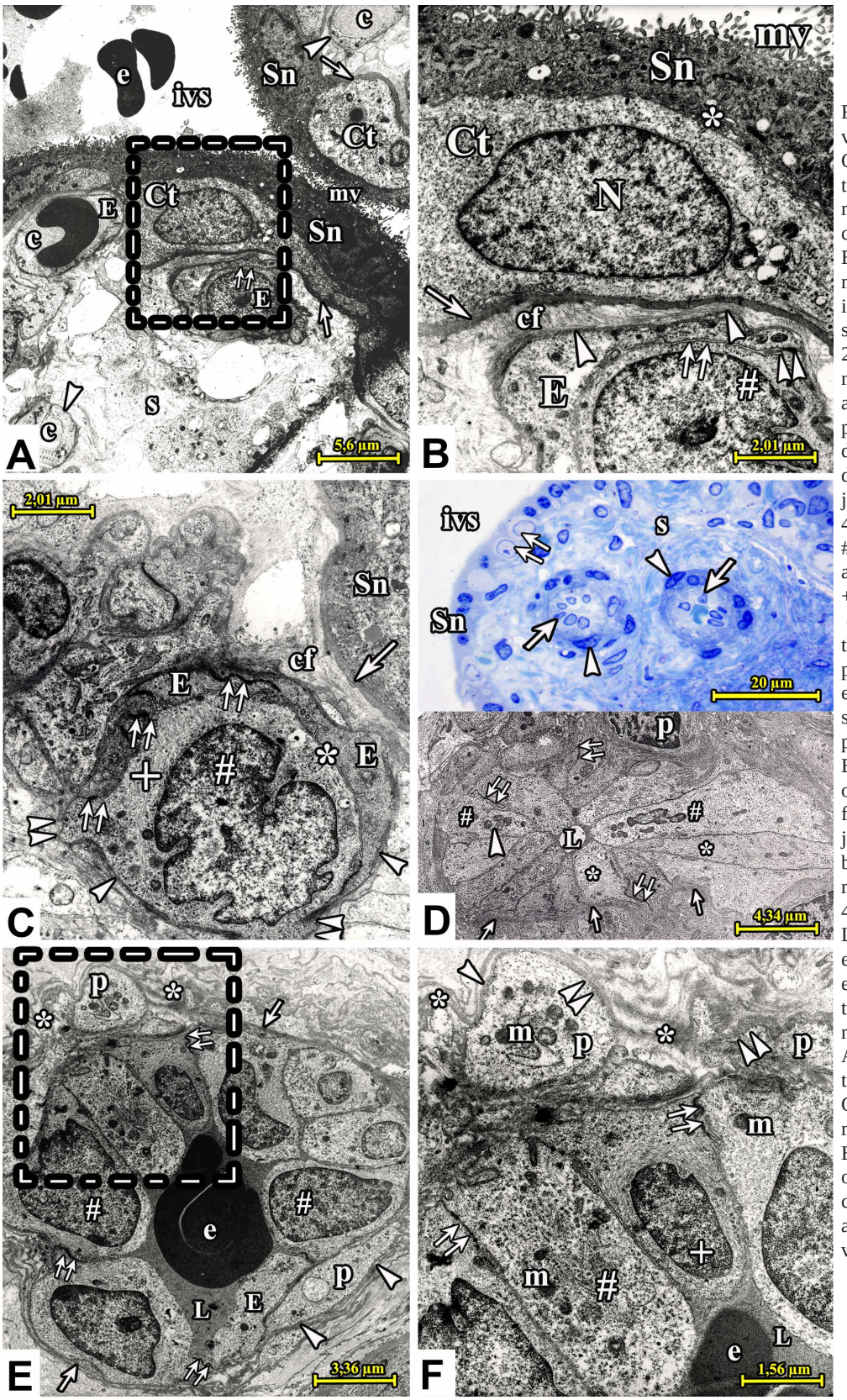

Fig. 4. A) EM of two floating villi. Sn;syncytiotrophoblast Ct;cytotrophoblasts, arrow; trophoblast basement membrane, arrowhead; capillary basal lamina, E:endothelial cell, mv: microvillus, e: erythrocytes, ivs: intervillous space, s: stroma, c: capillary (UALC, X 2156). 4B) Higher magnification of the marked area at fig. 4a. *;microvillous projections, #;rounded cell, douuble arrow; cleft like lümen, double arrowhead; intercellular junctions. (UALC, X 6000). 4C) Tip of the sprout *;cleft, #;luminal cell, double arrowhead;pseudopod +;cytoplasmic filaments (UALC, X 6000). 4D) LM of the floating villus. Arrowhead; pericytes, arrow; taller endothelial cells, (Semi-thin section, TB, X100). 4E) Middle part of the sprout in figure 4d. Endothelial cells (#;rich in organelle, *; rich in cytoplasmic filament). double arrow; tight junction,p; pericyte, arrow; basal lamina, arrowhead; mitochondria (UALC X 2784). 4F) Proximal part of the sprout L; lumen e; erythrocyte, #; tall endothelial cells, E; flattened endothelial cell, double arrow; tight junction, arrow; basal lamina, *; lamination, Arrowhead: external lamina of the pericyte (p) (UALC X3597) G) Higher magnification of the marked area at figure 4f. Endothelial cells (#;rich in organelle, +; rich in cytoplasmic filament). Double arrowheads: pinocytotic vesicles (UALC X 7750).

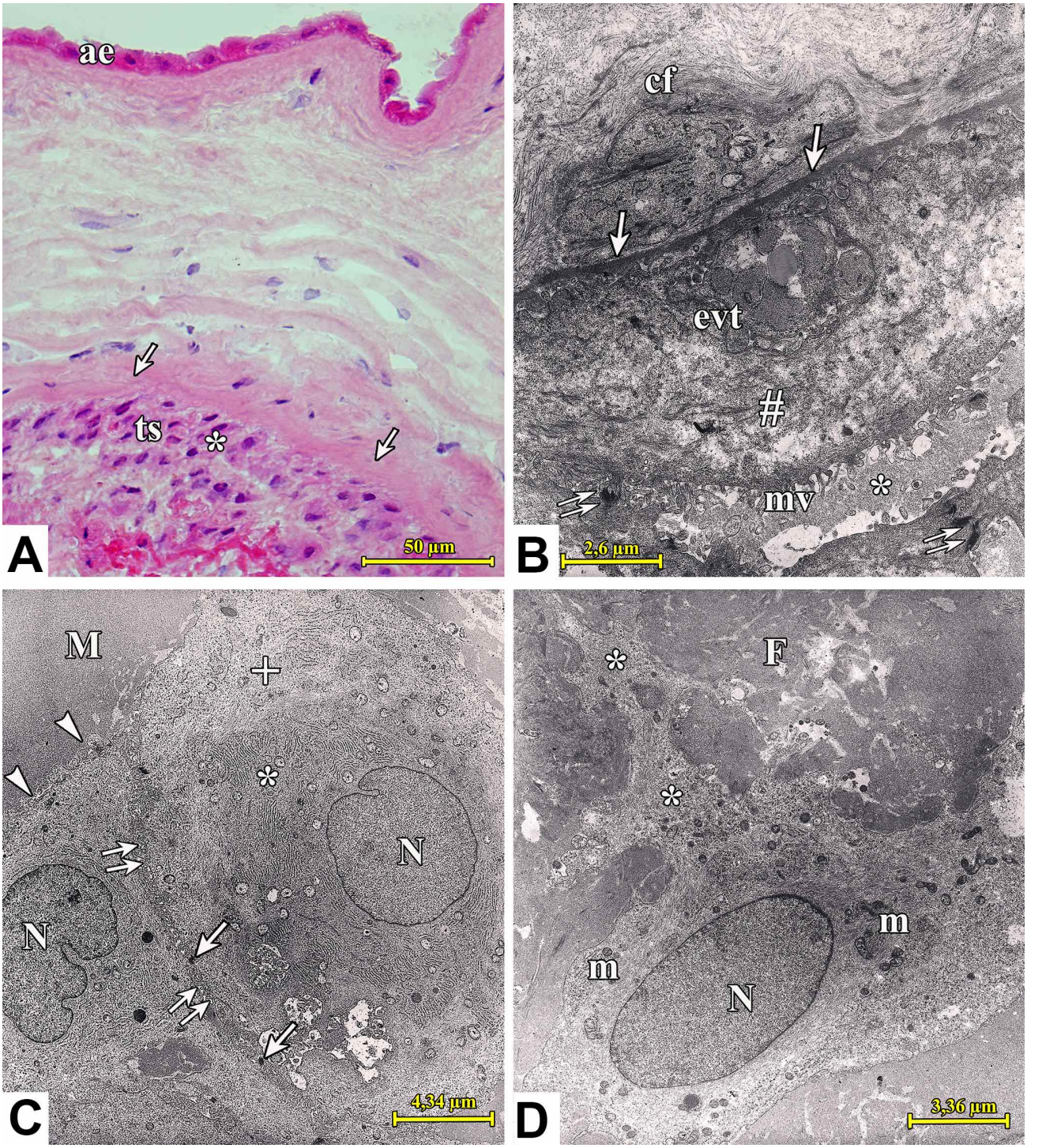

Fig. 5. A) LM of the amniochorionic membrane. ae; amniotic epithelium, arrow; trophoblast basement membrane, *;EVT, ts; trophoblastic shell (Frozen section, H&E,X40). B) EM of the EVT seen in fig 5a. cf; collagen fiber, *; granular material, mv; microvilli, double arrow; desmosomes, #; filaments (UALC X 4646). C) EM of the rounded trophoblasts embedded in fibrinoid. N; nucleus, *; darker perinuclear compartment, +; peripheral cytoplasm, double arrows; microvillous lateral protrusions, arrows; desmosomes, M; homogenous amorphous material, arrowheads; bud like larger protrusions (UALC, X 2784). D) EM of the elongated trophoblast embedded in fibrinoid (F). *; processes, N; nucleus, m; mitochondria (UALC, X 3597).

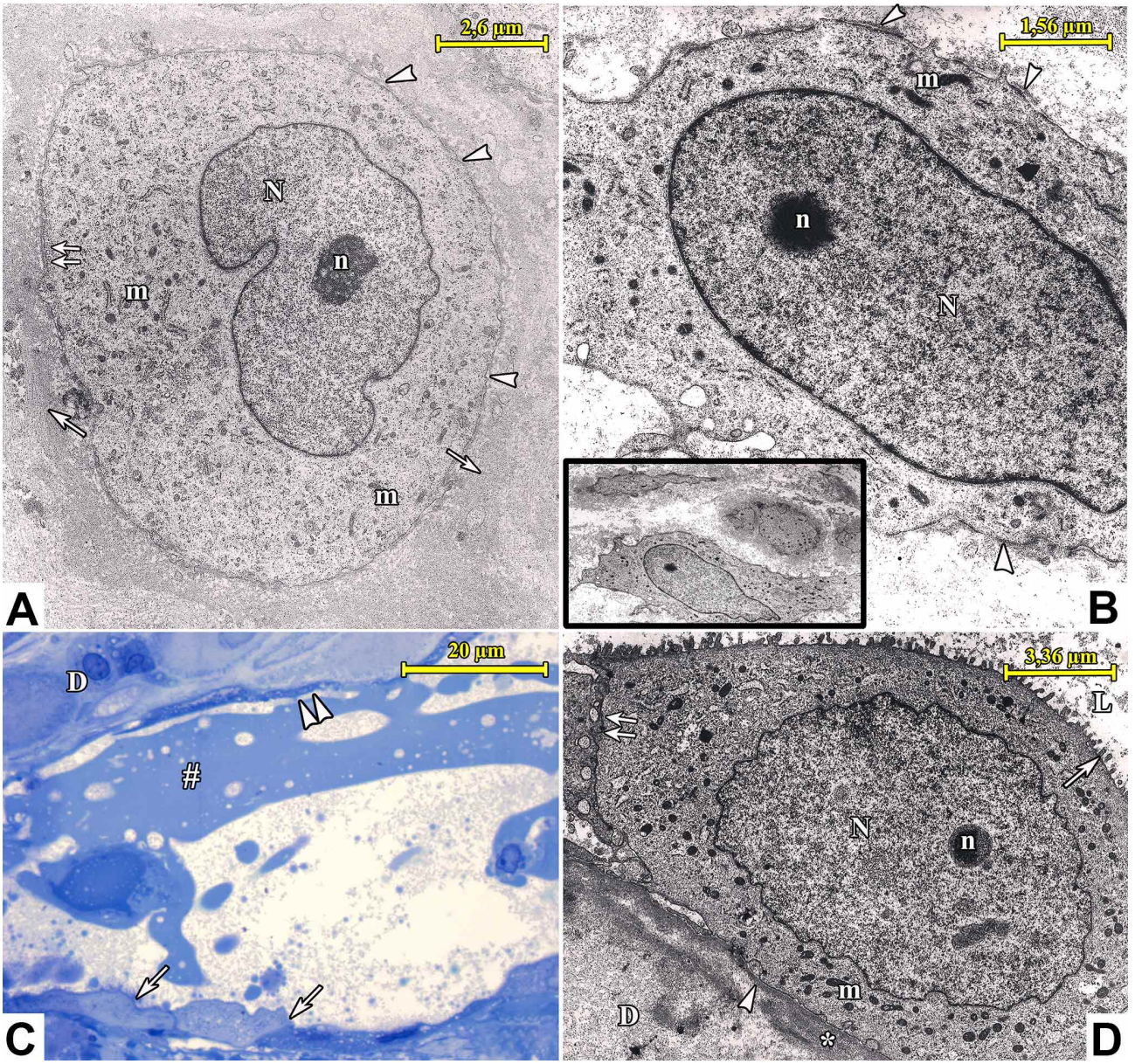

Fig. 6. A) EM of a decidual rounded EVT. N; nucleus, n; nucleolus, arrowhead; external lamina, m; mitochondria, double arrow; pinocytotic vesicles, arrows; granular material (UALC, X4646). B) EM of a decidual elongated EVT. Inset: lower magnification (UALC, X 7750). C) LM of a maternal vessel in decidua. D). double arrowhead; simple squamous endothelial cell, arrows; endovascular EVT, #; coagulation material (Semi-thin section, TB, X100). D) EM of endovascular EVT is seen in fig 6c. Arrow; microvilli, L; lumen, double arrow; tight junction, arrowhead; vascular basal lamina, *: laminations, D: decidua (UALC, X 3597).

DISCUSSION

The structure of the human placenta is still being explored since it is critical to the developing the fetus and there are still many aspects of its components which are not yet understood. In this study, we have obtained additional information on the fine structure of human placental components, mainly cells of trophoblastic lineage. We observed well- known structural components in the examination of placental villi consistent with the literature data (Guttmacher *et al.*, 2014; Burton & Fowden, 2015).

The placental capillary network gradually enlarges by angiogenesis and angiogenic growth is stimulated by angiogenic factors produced by stromal cells as is commonly accepted (Pavlov *et al*., 2014; Aplin *et al*., 2015; Guerra *et al*., 2021). Along the villous vasculature two types of endothelial cells were reported. The first group of the cells were rich in cytoplasmic filaments, while the others were rich in organelles. We determined two types of endothelial cells similarly in villous capillaries. Vascular sprouts were previously reported at early placentas. However, we could detect detailed information about the ultrastructure of vascular sprouts in term placentas. In cross sections of angiogenic sprouts, the maturational stages of newly arising capillaries were clearly distinguishable in our study. Our observations reflect three angiogenic sprout stages, the first (proximal) part arising from the mature vessel was composed of tall endothelial cells with enlarged lumen usually containing few erythrocytes. The middle section of the sprout was also composed of tall endothelial cells but with a narrower lumen lacking erythrocytes. The tip (distal compartment) of the sprout was composed of relatively flattened endothelial cells surrounding a large cell separated by a cleft which would form the future lumen. We suggest that the tall endothelial cells will retract down to the level of basolateral intercellular junctions during further maturation as blood flow is established. Another interesting finding to us was the presence of rounded cells with pseudopod- like protrusions filling the entire lumen in some sprouts. The tip cells and their podosomes were previously reported for sprouting angiogenesis (Galaris *et al*., 2021; Kuo *et al*., 2021). These vessels had an intact basal lamina and the luminal cells were also in contact with the basal lamina extending between neighboring endothelial cells. These findings suggest that described cells possibly originate from blood and they were locating to within the endothelial lining to expand its wall. The cytoplasmic appearance of this cell group was very similar to that of villous trophoblasts. In summary, we suggest that the tall endothelial cells of the angiogenic sprouts possibly originate from trophoblast/ mesenchymal cell clusters and they also use the general circulation as a path to the sprout area.

In villous tree unhealthy and/or degenerated villi were usually surrounded by fibrinoid. Fibrinoid has conventionally been divided into matrix type and fibrin type (Kim *et al*., 2019). Our observations also revealed both types of fibrinoid in term placenta. Fibrin type fibrinoid surrounding villi was lacking cells however matrix type fibrinoid did contain cells, mainly EVTs. EVTs within the matrix type fibrinoid reflected the structure of healthier, active cells so we suggest that these active cells are present in these locations for repair in response to hypoxia. Some other researchers have also suggested the role of oxygenation on the differentiation of cytotrophoblasts, syncytiotrophoblast and EVTs (Wakeland *et al*., 2017; He *et al*., 2019). Interaction of fetal cells with their maternal counterparts is believed to be essential for a successful gestation (Burton & Jauniaux, 2017; Pollheimer *et al*., 2018; Knofler *et al*., 2019; Menkhorst *et al*., 2019). The main source of the EVTs in the decidua is the trophoblastic column. The first type of these cells was quite similar to cytotrophoblasts however the second group resembled myofibroblasts. Subtypes of EVTs are previously described depending on their location, overall shape and ultrastructural features (Jones *et al*., 2014). In our study we observed cells with caveola usually surrounded by a continuous external lamina similar to pericytes and myofibroblasts of the villi also in decidua. Additionally, we observed endovascular trophoblasts within the endothelial lining of decidual vasculature as previously reported in several studies (Huppertz *et al*., 2014; Moser *et al*., 2015, 2017; Windsperger *et al*., 2017; Duzyj *et al*., 2018). Researchers described such cells as having the ultrastructural features of cytotrophoblasts in general. However, we found that the cytoplasm of these individual cells resembled syncytiotrophoblast cytoplasm rather than cytotrophoblasts. Endovascular EVTs and endothelial cells were both located on the basal lamina and inter-connected by junctional complexes (Rai & Cross, 2014, Almasry *et al*., 2015). Additionally, these cells had basal and lateral foldings which were previously seen in syncytiotrophoblast.

The findings of this study; 1) Trophoblastic cells exhibit structural features that overlap with the stromal cell types depending on their environmental conditions suggesting they represent transformational forms. 2) Maturational stages of sprouting angiogenesis were observed in detail so sprouting angiogenesis still continue in term placentas. 3) Matrix type fibrinoid develops secondarily to fibrin type as a reactive response to local damage in the term placenta

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RESUMEN: Los trofoblastos dependiendo de su ubicación realizan diferentes funciones. Este estudio tuvo como objetivo obtener pistas estructurales sobre las funciones de los trofoblastos vellosos y extravellosos mediante el uso de microscopía óptica y electrónica. Se obtuvieron muestras de placenta a término de 10 mujeres embarazadas sanas después de cesáreas. Las secciones congeladas se tiñeron con hematoxilina-eosina, las secciones semidelgadas se tiñeron con azul de toluidina y se examinaron con un microscopio óptico, mientras que las secciones delgadas se contrastaron con acetato de uranilo-citrato de plomo y se evaluaron con un microscopio electrónico.

Las finas características estructurales de los trofoblastos vellosos se superponen a algunas células estromales vellosas. Además de la apariencia habitual de capilares maduros en el estroma velloso, demostramos e informamos etapas de maduración de brotes angiogenéticos en la placenta a término. Los trofoblastos extravellosos se clasificaron según su localización: fibrinoide, corion, trofoblástico, columna, endotelio vascular materno o decidua. Todos estos trofoblastos compartían algunas características ultraestructurales, pero también eran distintos entre sí. En decidua se observó que el revestimiento endotelial de algunos vasos estaba invadido por unos pocos trofoblastos endovasculares con microvellosidades irregulares. Estas células compartían algunas propiedades ultraestructurales tanto con los trofoblastos vellosos como con las células del estroma. El examen mostró que la angiogénesis todavía estaba presente en las placentas a término y que los trofoblastos, las células endoteliales y estromales tienen propiedades ultraestructurales muy similares, lo que sugiere que representan formas de transformación.

PALABRAS CLAVE: Brotes angiogenéticos; Microscopio electrónico; Trofoblasto extravelloso; Fibrinoide; Placenta.

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Corresponding author:
P. Ayran Fidan
Baskent University Faculty of Medicine
Department of Histology and Embryology
Ankara
TURKEY

E-mail: pinarfidan@baskent.edu.tr

ORCID: P. Ayran Fidan: 0000-0003-3047-0305
ORCID: A. Dagdeviren: 0000-0001-8990-8282