Review

Villous trophoblast of human placenta: a coherent view of its turnover, repair and contributions to villous development and maturation

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Summary. A coherent view of human villous trophoblast as a continuously renewing epithelium is presented. Epithelia undergoing continuous renewal (e.g. intestinal mucosa, epidermis) display clonogenic cells which pass through several transit divisions before migrating out of proliferation zones and into zones of maturation/differentiation. Quantitative relations (e.g. relative numbers of cells) between proliferation and differentiation zones help to define the steady state and this may vary in response to physiological and pathological circumstances. From the differentiation compartment, cells or cell fragments are eventually extruded by mechanisms which may involve apoptosis. All these features are seen in trophoblastic epithelium. Cytotrophoblast cells (CT, proliferation zone) divide continuously throughout gestation and post-mitotic cells are recruited into syncytiotrophoblast (ST, differentiation zone) after membrane fusion. Evidence of fusion events includes localised confluence of CT and ST cytoplasms, and intrasyncytial plasma membrane segments bearing desmosomal remnants. During differentiation, nuclei undergo changes in shape, chromatin condensation and packing density. Densely-clustered nuclei are associated with cytokeratin intermediate filaments and annulate lamellae. Both clustered and non-clustered nuclei show ultrastructural features of pre-apoptosis and apoptosis. Normally, apoptosis is triggered only when nuclei are in the syncytium. Some (pre-)apoptotic nuclear aggregates are sequestered in syncytial knots, extruded as trophoblast fragments into the intervillous space and then deported into the maternal circulation to be phagocytosed at extraplacental sites. During gestation, there is some constancy in the numerical ratios between CT and ST nuclei pointing to a normal steady state. The steady state may be perturbed when the epithelium is damaged locally. Where the epithelium is denuded, fibrin-type fibrinoid from the intervillous space plugs the

discontinuity and, with CT proliferation, facilitates re-epithelialisation. Features of normal villous development (e.g. sprouting, intervillous bridge formation, bridge abruption, syncytial knot formation) are explicable in the context of trophoblast turnover with early CT proliferation being mainly for growth and later proliferation for renewal and repair. Adaptive re-settings of the epithelial steady state may also occur in abnormal pregnancies.

Key words: Placental villi, Trophoblast, Proliferation, Recruitment, Differentiation, Apoptosis, Extrusion, Repair

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Introduction

During pregnancy, fetal well-being depends critically on the regulated growth and activity of villous arborisations in the placenta. Each arborisation comprises tertiary (mature) villi which are divisible topologically into stem, intermediate and terminal villi. Three main tissues contribute to the structure of tertiary villi: an outer trophoblastic epithelium surrounds a mesodermal connective tissue core in which run fetal blood vessels. Early in gestation, trophoblast proliferation contributes to formation and expansion of new villous sprouts by a process of random segmental branching. Sprouting of terminal villi is dramatic from the second trimester onwards and this is driven mainly by growth of fetal vessels in intermediate villi (Kaufmann et al., 1987; Arnholdt et al., 1991; Jauniaux et al., 1991; Simpson et al., 1992; Kosanke et al., 1993; Benirschke and Kaufmann, 2000; Castellucci et al., 2000). These changes increase enormously the volume and surface area of trophoblast and fetal vessels and contribute to the enhanced functional capacity of the placenta (Aherne and Dunnill, 1966; Teasdale, 1980; Boyd, 1984; Burton, 1987; Jackson et al., 1992; Benirschke and Kaufmann, 2000).

Growth of villi increases their packing density and probably contributes to formation of transient

intervillous bridges and fusions. True bridges are sites at which trophoblast passes between adjacent villi but tissue sectioning may produce images of artefactual bridges (Burton, 1986; Cantle et al., 1987; Benirschke and Kaufmann, 2000). Growth is also accompanied by villous maturation. From a morphological viewpoint, maturation depends on several influential factors. These include the increase in trophoblast volume, the vascularisation of growing villi and the peripheralisation of fetal vessels within them. Another important factor is the gradual thinning of villous trophoblast and a greater variability in its local thicknesses (Jackson et al., 1992). This variability arises by the creation of thin regions of trophoblast called vasculosyncytial membranes and thick regions called syncytial knots. The former optimise the efficiency of passive diffusion by reducing distances between the apposed maternal and fetal circulations. Syncytial knots are sites of clustering of trophoblast nuclei and some of these clumps are shed into the maternal intervillous space (Benirschke and Kaufmann, 2000).

A more integrated perspective on these events requires an appreciation of the way in which trophoblast itself grows. This in turn requires an understanding of trophoblast structure and turnover. The aim here is to review the principal features of trophoblast morphology, including cytotrophoblast cells, non-syncytial knot (thin) and syncytial knot (thick) regions of the syncytium. Villous development and maturation are re-examined in light of the notion that trophoblastic epithelium exhibits
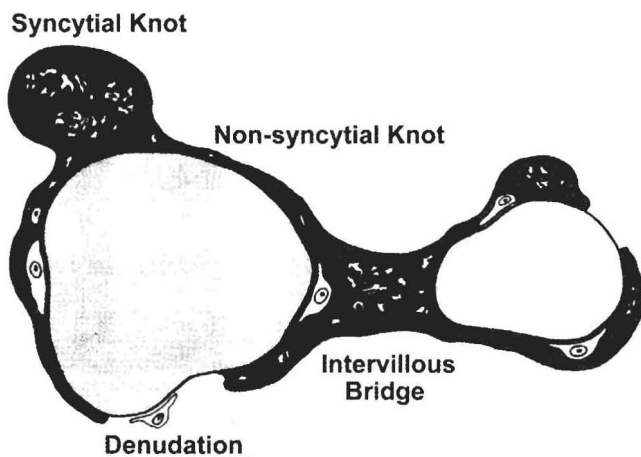
Syncytial Knot

Fig. 1. Basic morphology of the trophoblast of a mature tertiary villus at term. A central mesodermal core (light shading) contains fetal blood vessels and other connective tissue elements. The core is covered by a chorionic epithelium which has an inner zone of scattered cytotrophoblast cells (white) and an outer syncytial zone (dark shading). Cytotrophoblast nuclei tend to be euchromatic with prominent nucleoli. Nuclei in syncytium are more heterochromatic and cluster in thicker regions (syncytial knots and intervillous bridges). The latter term embraces true bridges as well as villous branchpoints. Non-syncytial knot regions of syncytium are thin and often overlie dilated fetal vessels in the core. Due to various forms of trauma, epithelium may be lost and form denudation sites which may yet retain cytotrophoblasts sitting on the epithelial basal lamina.

continuous turnover with a regulated steady state between phases of proliferation, recruitment, maturational differentiation, terminal differentiation (apoptosis) and extrusion which occur in different spatial compartments.

Villous trophoblast is compartmentalized

Trophoblastic epithelium has two compartments in which spatial sub-compartments can be distinguished (Figs. 1, 2). The inner compartment (Langhans cell layer or cytotrophoblast, CT) is proliferative and transforms from a continuous to an incomplete cellular layer as gestation advances. By fusion, post-mitotic uninucleate CT cells are recruited into an outer non-mitotic terminally-differentiating compartment, the syncytiotrophoblast (ST), which is a syncytial continuum (Kliman et al., 1986; Muhlhauser et al., 1993; Mayhew et al., 1999a; Benirschke and Kaufmann, 2000). Within ST, there are regions of attenuation, thickening and loss. Syncytium is thickened due to clustering of nuclei with a relatively high content of heterochromatin (Jones and Fox, 1977; Burton, 1986; Cantle et al., 1987; Mayhew et al., 1999a). Two broad classes of nuclear clustering can be seen on sections: syncytial knots (SK) and syncytial bridges. The former represent sites at which nuclei are sequestered prior to extrusion into the maternal circulation within trophoblast fragments. Syncytial bridges are a mixture of true bridges plus artefactual bridges. Some of the latter are villous branchpoints. Trophoblast attenuation arises in two ways: first, peripheralisation of fetal vessels and their obtrusion into the syncytium and, second, streaming of nuclei and intracytoplasmic organelles away from obtruding vessels (Amaladoss and Burton, 1985; Jackson et al., 1988b). Finally, there are regions at which epithelium may be lost due to the damaging effects of various mechanical or chemical agents.

Trophoblast is a continuously-renewing epithelium

Trophoblast is in continuous turnover with a regulated steady state between phases of proliferation, recruitment, maturation, apoptosis and extrusion. These phases operate in defined spatial compartments (see Fig. 3). Until recently, trophoblast was regarded as an initially renewing epithelium in which continued expansion became constrained by a gradually shrinking pool of less proliferative CT cells (e.g. Arnholdt et al., 1991; Jones and Fox, 1991). This interpretation is understandable but erroneous. It was based on viewing 2D images of microscopical thin sections in which profiles of CT nuclei are seen less frequently after the first trimester. However, 3D (stereological) estimates of total numbers of nuclei using disector pairs of sections (Mayhew and Gundersen, 1995) have demonstrated that there is continuous proliferation of CT cells and recruitment into ST. Because growth in trophoblast surface area outpaces that in volume, the epithelium

becomes thinner and CT cells become more widely dispersed on the epithelial basal lamina (Jackson et al., 1992; Simpson et al., 1992; Mayhew and Simpson, 1994; Mayhew et al., 1994). These changes also have greater impact in the second trimester (after roughly the 20th week) of pregnancy.

Not only are CT cells not depleted by continuous recruitment into ST but there are remarkable structural constancies between these compartments (Sen et al., 1979; Simpson et al., 1992; Mayhew et al., 1994, 1999a). Firstly, at different sites along a villous tree, the proportion of trophoblast volume occupied by CT displays little variation. Secondly, 3D counts have shown that, over most of gestation, the ratio of ST:CT nuclei remains constant at about 10:1. Thirdly, a fixed volume of trophoblast per nucleus is maintained from at least the beginning of the second trimester. These relationships point to a continuously renewing epithelium (like epidermis and small intestine) in which recruitment and extrusion are in steady state (Mayhew et al., 1999a). They occur despite regular shedding of trophoblast debris into the intervillous space as SK fragments which are relatively rich in pre-apoptotic and apoptotic nuclei (Jones and Fox, 1977; Cantle et al., 1987; Huppertz et al., 1998; Mayhew et al., 1999a). Evidence suggests that the normal steady state may alter in other types of pregnancy. For example, although CT proliferation and SK formation are both enhanced in placentas from high-altitude pregnancies, the ratio between nuclear numbers in CT and ST alters significantly owing to relatively greater proliferation (Ali, 1997).

Trophoblast compartments are not homogeneous in size or composition (Martin and Spicer, 1973; Jones and Fox, 1977, 1991; Dearden and Ockleford, 1983; Palmer et al., 1997; Huppertz et al., 1998; Mayhew et al., 1999a; Benirschke and Kaufmann, 2000). Differences involve the nucleus and cytoplasm with nuclear changes affecting their packing density, shape, size and contents of heterochromatin and nucleoli. Some nuclei in ST show morphological and immunochemical signs of apoptosis (Jones and Fox, 1977; Nelson, 1996; Smith et al., 1997a; Huppertz et al., 1998; Mayhew et al., 1999a). At first sight, this heterogeneity appears to be incompatible with constant ratios between compartments but the apparent inconsistency can be resolved if the extrusion of SK fragments is an integral part of the normal turnover of epithelium.

Later in gestation, the proliferative activity of CT declines, cells become more dispersed, levels of apoptosis increase and apoptotic nuclei are often aggregated in SK regions (Arnholdt et al., 1991; Mayhew and Simpson, 1994; Smith et al., 1997a; Huppertz et al., 1998; Mayhew et al., 1999a). The incidence of SK regions increases during gestation and their combined volumes correlate positively with the total numbers of apoptotic and pre-apoptotic nuclei in the trophoblast (Fox, 1965; Jones and Fox, 1977; Cantle et al., 1987; Mayhew et al., 1999a).
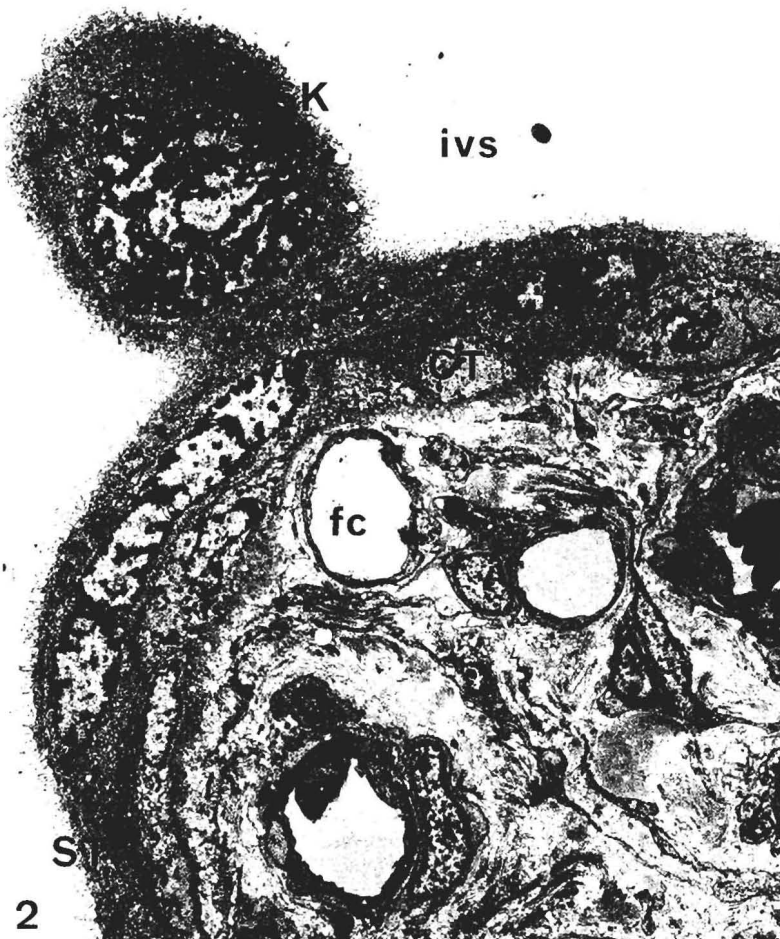

Fig. 2. Ultrastructural appearance of trophoblast compartments on a tertiary villus. A basal lamina separates the trophoblast from the mesodermal core in which some fetal capillaries (fc) can be seen. Scattered cytotrophoblast cells (CT) lie on the basal lamina and below the syncytiotrophoblast (ST). Within the latter are nuclei at different stages of differentiation evidenced by varying degrees of chromatin condensation and changes in nuclear shape and aggregation. Some highly pleiomorphic nuclei with relatively condensed chromatin are aggregated in a syncytial knot (SK). Eventually, this will detach and pass into uterine veins via the maternal intervillous space (ivs). x 2,200

Proliferation is confined to cytotrophoblast

Proliferation occurs in CT cells and not in the overlying syncytium (see Kliman et al., 1986; Muhlhauser et al., 1993; Benirschke and Kaufmann, 2000). Mitotic activity in CT is observed in the first trimester and continues, albeit to a gradually lesser degree, to term (Arnholdt et al., 1991; Blankenship and King, 1994; Sakuragi et al., 1997). By themselves, decreases in mitotic index or the incidences of PCNA-positive or Ki67-positive cells must not be regarded as clear evidence of reduced proliferative capacity. This is so because the size of the proliferative pool is determined also by the total complement of CT cells and this pool increases at least 8-fold after the first trimester (Simpson et al., 1992; Mayhew et al., 1994). Using cultured CT cells isolated from term placenta, Kliman et al. (1986) estimated that about 15% of cells were actively dividing or ready to divide. In vivo, this would represent a substantial proliferative pool of some 9×10^8 cells because term placenta is estimated to contain about 6×10^9 CT cells (Mayhew et al., 1994). Because of this much more influential factor, the total production rate of CT cells is probably accelerating towards term (Simpson et al., 1992). Taking this into account with published figures on the frequencies of BrdU- and Ki67-labelled cells (Arnholdt et al., 1991) suggests that the pool of S phase CT cells doubles, and that of cycling cells almost trebles, between first and third trimesters.

Placental growth also involves other villous compartments (Mayhew et al., 1994) and is monophasic (solely proliferative) rather than biphasic (early proliferation is not followed by a late hypertrophic phase). Total numbers of nuclei (trophoblastic, stromal

Extrusion and Deportation

Terminal Differentiation

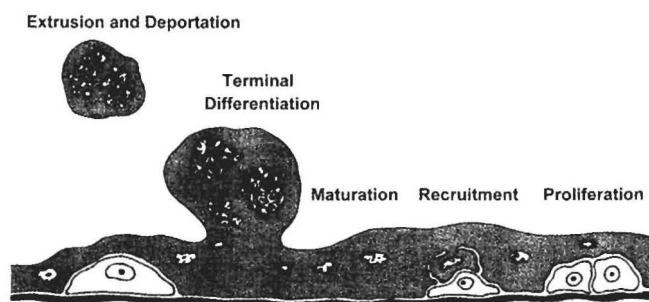Maturation   Recruitment   Proliferation

Fig. 3. The spatiotemporal compartments of villous trophoblast involved in continuous epithelial turnover. Cytotrophoblast cells are proliferative and some of their post-mitotic daughter cells are recruited into the overlying syncytiotrophoblast by fusion. Once in the syncytium, they are programmed to undergo a sequence of maturational differentiation followed by a short terminal differentiation (apoptosis) phase. Pre-apoptotic and apoptotic nuclei are sequestered in syncytial knots and may form multiple clusters each surrounded by cytokeratin filament bundles. These knots eventually detach, are extruded into the intervillous space and then deported from the uteroplacental bed. Normally, these events exist in a steady state but this can be perturbed in complicated pregnancies.

and endothelial) increase exponentially and roughly in parallel, outstripping changes in placental volume and suggesting that growth in different compartments is regulated. This is further emphasised by the fact that not all CT cells fuse into the ST. A reservoir of clonogenic cells is maintained until term and findings on numbers of trophoblast nuclei are compatible with CT cells experiencing up to four transit divisions before passing into ST. Growth constants for endothelial nuclei may be greater than those for CT and stromal cells, supporting the idea that genesis and growth of terminal villi are affected by the linear growth of fetal microvessels (Benirschke and Kaufmann, 2000). Immunocyto-chemistry has confirmed the existence of separate centres of proliferation for epithelium, stroma and vascular endothelium (Kaufmann et al., 1991; Blankenship and King, 1994). However, proliferation overall may be fairly constant across different levels of villous arborisations since the volume fraction of CT cells within trophoblast is 11-16% at all levels (Sen et al., 1979).

Two types of CT cell have been identified on ultrastructural criteria: undifferentiated cells and others intermediate in appearance between undifferentiated cells and ST (Jones and Fox, 1991; Benirschke and Kaufmann, 2000). Undifferentiated cells predominate in the first trimester and tend to be cuboidal with a large spheroidal euchromatic nucleus containing a prominent nucleolus. The cytoplasm is comparatively electron-lucent and harbours few organelles. Intermediate cells are more flattened, the nucleus is more irregular in shape and heterochromatic, and the cytoplasm has a larger complement of organelles. In the third trimester, some intermediate cells can be distinguished from syncytium only by virtue of their delimiting plasma membranes. In both types of CT cell, interdigitations of adjacent plasma membranes occur where cells abut the syncytium and bear desmosomal and other junctional complexes (Dearden and Ockleford, 1983; Jones and Fox, 1991; Benirschke and Kaufmann, 2000). Immunocytochemical studies have revealed punctate staining of the epithelial adhaerens junction molecules E-cadherin and ß-catenin on the apical surface of CT cells (Mayhew and Leach, 1998).

Syncytialisation requires recruitment of cytotrophoblasts

In the human context, the epithelial syncytium is unique and may have evolved to allow invasion of maternal tissues without breaching the intervascular barrier. However, syncytium confers certain other adaptive advantages. For example, it facilitates quick-response spatial redistributions of ST mass which permit very economical improvements in the diffusive conductances of gases and nutrients (Jackson et al., 1985). The effectiveness of ST in this regard depends on its mean thickness, variability of thickness and surface area. During pregnancy, surface area increases

enormously by the formation of new terminal villi whilst the harmonic mean thickness of trophoblast declines (Aherne and Dunnill, 1966; Sen et al., 1979; Teasdale, 1980; Boyd, 1984; Burton, 1987; Jackson et al., 1992; Mayhew et al., 1993).

ST grows and is rejuvenated by cell recruitment. Once intermediate CT cells resemble syncytium, they begin to express connexin-43 in their plasma membranes and display transient gap-junctional communication with each other and with overlying ST (Cronier, 2001). These events seem to be a precondition, and may even initiate, fusion of CT cells into syncytium (Benirschke and Kaufmann, 2000; Cronier et al., 2001). Another membrane event implicated in fusion is the phosphatidylserine (PS) flip. PS is a phospholipid normally confined to the inner layer of the plasma membrane but, prior to fusion, this translocates to the outer layer. This positional flip facilitates CT-ST intermembrane fusion, cytoplasmic confluence and syncytialisation (Lyden et al., 1993; Adler et al., 1995; Huppertz et al., 1998). Recruitment probably involves the ectoplasmic type of membrane fusion which is mediated also by lipid-based pores in the fusing cell membranes (Monck and Fernandez, 1996).

Whilst occasional evidence is seen of pre-fusion or recent fusion events, investigation of the phenomenon might benefit from further studies using rapid-freezing preparations (Chandler and Heuser, 1980; Ornberg and Reese, 1981). Evidence of fusion (see Fig. 4) includes confluence of the cytoplasms of CT cells and ST and traces of intrasyncytial plasma membranes with associated desmosomes (Dearden and Ockleford, 1983; Jones and Fox, 1991). Studies on cultured term CT cells (Morrish et al., 1997) suggest that there may be one path of differentiation that leads to a post-mitotic cell and a second path, or a later stage, which requires epidermal growth factor (EGF) for extensive syncytialisation. EGF receptor is present on plasma membranes of CT cells in first- and third-trimester placentas (Muhlhauser et al., 1993).

The presence in syncytium of membrane segments which are remnants of fusion events raises the possibility of confusion in electron microscopical thin sections with so-called transtrophoblastic channels. True transtrophoblastic channels would form a continuum from the apical to basal membranes of ST and provide an effectively paracellular route for solute transfer (Stulc, 1989; Benirschke and Kaufmann, 2000). More recent studies suggest that the function of such paracellular permeability channels might be performed
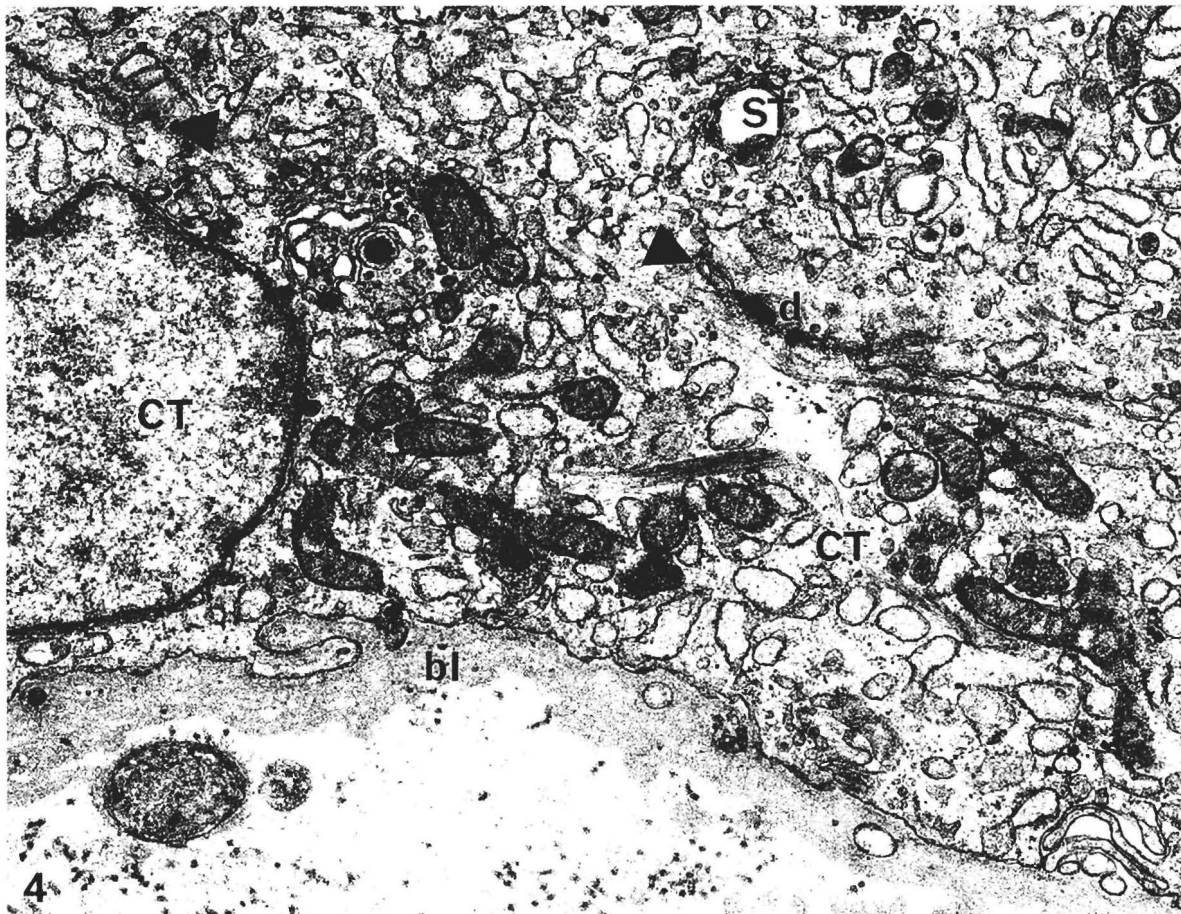

Fig. 4. Ultrastructural appearance of early fusion events. An intermediate cyto-trophoblast cell (CT) rests on the basal lamina (bl). Its apical plasma membrane shows desmosome-like junctions (d) attaching it to the adjacent membrane of the syncytio-trophoblast (ST). At one site (between the arrowheads), the plasma membranes have fused and there is confluence of cytosol and cytoplasmic organelles between CT and ST. x 22500

instead by sites of trophoblast denudation (Brownbill et al., 2000).

Intrasyncytial differentiation is maturational and terminal

Recruitment into the syncytium commits the recruited nucleus and cytoplasm to a coordinated process of differentiation which is coupled to an apoptotic cascade. The transformation from CT to ST is a complex process in which many regulatory factors, including oestrogen, are implicated (see Babischkin et al., 2001; Cronier et al., 2001). Initially, differentiation produces a mature syncytium which combines the activities of endocrine gland, endothelium and secretory/transporting epithelium. Mature regions are rich in organelles and membrane-bound compartments which indicate that this is metabolically active tissue with steroidal and protein synthetic capabilities. Eventually, however, maturation is succeeded by terminal differentiation and sequestration of heterochromatic nuclei in SK regions.

Qualitative descriptions (Jones and Fox, 1977, 1991; Dearden and Ockleford, 1983; Palmer et al., 1997) and quantitative analyses (Mayhew et al., 1999a) have indicated that trophoblast compartments show a CT-to-SK gradient of differences in size and composition. Marked changes in morphology and activity involve the nucleus, cytoplasmic organelles and plasma membrane. Near term, CT occupies about 15% of trophoblast volume whilst non-SK regions account for 53% and SK regions for about 33% (Sen et al., 1979; Mayhew et al., 1999a). In CT, nuclei account for about 24% of cell volume, vary in size and, in undifferentiated cells, tend to be rounded (surface:volume ratio, 1.1 $\mu m^2/\mu m^3$) and euchromatic (euchromatin accounting for 83% of nuclear volume) with a prominent nucleolus (2% of nuclear volume). In non-SK regions of ST, nuclear size seems to be smaller and less variable and nuclei are indented as well as rounded whilst the chromatin pattern is more heterochromatic. Nuclei are irregular in shape and have relatively dispersed chromatin with occasional clumps of heterochromatin near the nuclear membrane. SK nuclei are even more pleiomorphic (surface:volume ratio, 1.7 $\mu m^2/\mu m^3$), densely packed (volume fraction 49%) and heterochromatic with scattered islands of peripheral and central euchromatin occupying only 37% of nuclear volume. Nucleoli have almost fully regressed (0.02% of nuclear volume). Densely-clustered nuclei sometimes appear to interlock like pieces of a jigsaw puzzle.

The ratio of CT:ST nuclear volumes (about 1:8) underestimates the relative number of ST nuclei (about 1:10) because the mean volume of these nuclei is about 23% smaller in ST (Simpson et al., 1992; Mayhew et al., 1994, 1999a). In addition, the average ST nucleus harbours 98% less nucleolar material and 56% less euchromatin. By contrast, it has more than twice as much heterochromatin and roughly the same, or slightly more, envelope surface area. This implies that nuclei

decrease in size and become more convoluted mainly by losing volume whilst conserving or expanding membrane surface area.

Some regions of ST display apoptotic nuclei (Jones and Fox, 1977, 1991; Nelson, 1996; Smith et al., 1997a) identifiable by the condensation and peripheralisation of heterochromatin at the inner membrane, a central island of euchromatin and convolution of the nuclear envelope (Huppertz et al., 1998; Mayhew et al., 1999a). Associated features may include blebbing of the apical ST surface with loss of microvilli and absence of inflammatory infiltration (Gerschenson and Rotello, 1992; Nelson, 1996). Apoptotic nuclei are seen in non-SK and SK regions suggesting that changes are not a consequence of aggregation itself (Mayhew et al., 1999a). Annulate lamellae are sometimes seen within nuclear clusters (Jones and Fox, 1977, 1991; Mayhew et al., 1999a). These structures have been noted in cells undergoing rapid division or protein synthesis, e.g. oocytes, embryonic and neoplastic cells (Kessel, 1992). They are usually located near the nucleus and comprise parallel stacks of membranes with intermittent annuli resembling the pore complexes of the nuclear envelope. In placenta, annulate lamellae are found in non-SK regions and in SK regions associated with cytokeratin filaments and (pre-) apoptotic nuclei (Jones and Fox, 1977; Mayhew et al., 1999a).

Given the geographical proximity of annulate lamellae to ST nuclei, it is conceivable that they are either contributing to synthesis of nuclear pore complexes or acting as foci for shed nuclear pores. Nuclear membrane area changes during differentiation could help to elevate protein synthesis by allowing incorporation of more pore complexes and, hence, greater scope for bidirectional molecular trafficking (RNA export, protein import) between nucleus and cytosol. In fact, the packing density of pore profiles in nuclear membranes does not decrease as nuclei transform from the euchromatic CT phenotype to the heterochromatic SK phenotype (Mayhew et al., 1999a). Moreover, pore complexes were difficult to detect in the membranes of apoptotic nuclei. Clearly, more systematic quantitative studies are required on the relationships between nuclei, their pore complexes and annulate lamellae at different stages of differentiation.

It has been suggested that pore density in the nuclear membrane increases in the more transcriptionally active nuclei but this relationship is not universal (Kessel, 1992). We have found that heterochromatic nuclei may have a greater packing density of pore complexes and this implies that formation of annulate lamellae does not depend on loss of nuclear pores from pre-apoptotic nuclei. Clustering of nuclear pore complexes has been observed in apoptotic cells (Reipert et al., 1996) but not in overtly apoptotic ST nuclei. The discrepancy may be due to alternative patterns of chromatin condensation in different types of nucleus undergoing apoptosis (Mayhew et al., 1999a). In some cells, pore complexes migrate to regions of the nuclear envelope which are free

of subjacent heterochromatin (Earnshaw, 1995). In ST, however, the envelope of apoptotic nuclei almost always has subjacent condensed chromatin (Huppertz et al., 1998; Mayhew et al., 1999a). Immunochemical studies have also shown that lamin B (a nuclear membrane protein associated with pore complexes) is decreased in ST compared with CT, probably as a consequence of caspase-dependent degradation which accompanies the apoptotic cascade (Huppertz et al., 1998).

The quantitative data confirm that the morphophenotype of trophoblast elements alters as they pass from the proliferative compartment, via the non-SK regions, and into the SK regions. Changes include a decrease in nuclear volume with attendant nucleolar regression and greater heterochromatisation and convolution of the nuclear envelope. These changes seem to be pre-apoptotic because at least part of the differentiation process involves formation of apoptotic nuclei in which euchromatin is confined eventually to a small central island surrounded by condensed heterochromatin. The changes are consistent with the proposition that this epithelium exhibits continuous turnover with fixed relationships between proliferating and differentiating compartments. To achieve this, the fusion of uninucleate CT cells must be balanced by the formation (and subsequent loss) of SK fragments rich in highly heterochromatic (pre-)apoptotic nuclei.

Terminal differentiation involves apoptosis and nuclear sequestration

Tissue turnover depends on the steady state between cell proliferation and cell death. Whilst mitosis is the usual method of cell proliferation, forms of cell death may vary but the principal forms are necrosis and apoptosis. Apoptosis is evidenced by morphological (nuclear fragmentation, chromatin condensation) and biochemical (endonuclease-catalysed internucleosomal DNA fragmentation; various cleavage events affecting nucleus and cytoplasm) changes and may be mediated by intracellular repressors and inducers (Gavrieli et al., 1992; Gerschenson and Rotello, 1992; Huppertz et al., 1998; Mayhew et al., 1999a). In primary human CT cells, apoptotic changes can be triggered by cytokines which include tumour necrosis factor-alpha and gamma interferon (Yui et al., 1994) and blocked by EGF (Garcia-Lloret et al., 1996). The cytokine-induced apoptosis is not mediated by reactive nitrogen or oxygen intermediates such as nitric oxide, peroxynitrites and superoxide anions (Smith et al., 1999). Moreover, although CT and ST elements constitutively express Fas ligand, an unknown mechanism inactivates the pro-apoptogenic Fas response (Payne et al., 1999). These and other studies suggest that diverse pro-apoptotic signalling pathways are present in villi but the mechanisms of regulation remain poorly understood (Levy and Nelson, 2000).

Ultrastructural features of apoptosis are found in ST nuclei (Nelson, 1996; Smith et al., 1997a; Huppertz et al., 1998; Mayhew et al., 1999a) but have yet to be seen in vivo in CT cells. ST nuclei of similar appearance are seen in early micrographs (Martin and Spicer, 1973; Jones and Fox, 1977) but were not identified at the time as being apoptotic. TUNEL-labelling has also demonstrated that apoptotic nuclei are not uncommon in ST and are found in first-trimester and term placentas (Thiet et al., 1995; Yasuda et al., 1995; Sakuragi et al., 1997).

Quantitative findings on nuclear morphology suggest that some convoluted heterochromatic nuclei are pre-apoptotic or in the earliest stages of apoptosis (Mayhew et al., 1999a). This is consistent with the findings of Huppertz et al. (1998) who used immunochemical methods to study the spatiotemporal distribution of a panel of proteins involved in apoptosis. They found that part of the machinery for apoptosis is present in CT cells but not activated until nuclei enter the syncytium. We have observed that, at the latest stages of differentiation, euchromatin seems to be confined almost entirely to a central location and surrounded by peripherally condensed heterochromatin. Such nuclei are not restricted to SK regions but may be detected in non-SK areas where, on section, they appear as linear arrangements of nuclear profiles (see Fig. 5). It may be that cohorts of such nuclei are pulled into SK regions by an actin-based mechanism which is also involved in the extrusion process (Mayhew et al., 1999a). Active drawing of nuclear cohorts into SK regions might account for the finding of multiple nuclear clusters in such regions. Often, each cluster resembles a fascicle or a layer and is delimited from its neighbours by bundles of intermediate filaments (Mayhew et al., 1999a).

TUNEL-labelled nuclei have been observed singly or in clusters in ST together with unlabelled nuclei (Yasuda et al., 1995; Sakuragi et al., 1997). Moreover, the TUNEL method labels some cells which do not exhibit apoptotic morphology and this raises the possibility that it may also label pre-apoptotic nuclei (Gavrieli et al., 1992; Negoescu et al., 1996).

Though definitive studies are required, it seems likely that the complement of apoptotic nuclei increases substantially during gestation. Smith et al. (1997a) have indicated that the frequency of apoptotic nuclei within villi does not vary with placental region but alters from 0.07% during the first trimester to 0.14% in the third trimester. At first sight, this seems a very modest increase. However, the total complement of nuclei in villi over the same period changes from about 1.7×10^{10} to 11.4×10^{10} (Mayhew et al., 1994) and this implies that apoptotic nuclei increase dramatically from about 1.2×10^7 to 1.6×10^8. If 50% of all apoptoses are in the trophoblast (Smith et al., 1997a), the figures imply an increase from 6.1×10^6 to 8.0×10^7 in the numbers of apoptotic nuclei within trophoblast. They also imply that the ratio of CT:apoptotic nuclei drops during gestation from about 115:1 in the first trimester to 55:1 in the third. If apoptotic nuclei are lost as part of the normal

process of SK extrusion, the increasing proportion of the apoptotic pool could also contribute to the gradual thinning of trophoblast (Jackson et al., 1992; Mayhew et al., 1993). However, the data obtained by Smith et al. (1997a) were for histological (haematoxylin and eosin) preparations and TUNEL-labelling yielded higher estimates of the apoptotic fractions. Indeed, TUNEL-based methods of labelling (Yasuda et al., 1995) have suggested not only that labelled nuclei may not be detected histologically but also that so-called apoptotic nuclei may be undetectable at 20-33 weeks and include necrotic nuclei.

Using TUNEL-labelling, Sakuragi et al. (1997) found that the incidence of apoptotic nuclei in trophoblast declined during gestation whilst that of Bcl-2 (a proto-oncogene which promotes cell survival by blocking apoptosis) increased. Given these contradictory early findings on the relative incidences of apoptosis, more definitive studies need to be performed on larger samples and on separate tissue compartments using different identification methods. Despite these reservations, it seems unlikely that all nuclei in SK fragments are apoptotic. Some may be pre-apoptotic and this conclusion is confirmed by the results of ultrastructural studies and supported by those of TUNEL studies. Whatever their status, studies using propidium iodide to identify nuclei with condensed chromatin (Fig. 5) have shown that the numbers of such nuclei correlate positively with the global volume of SK regions (Mayhew et al., 1999a). A prediction which follows from this is that SK fragments should be rich in (pre-)apoptotic nuclei. Clearly, there is a need to study intravascular SK fragments for evidence of apoptosis. Recent in vitro findings suggest that induced fragments are associated with apoptosis (Kumar et al., 1999).

Sequestered nuclei are extruded and deported in intravascular syncytial fragments

SK regions contain densely-packed, pleiomorphic nuclei of high heterochromatin content some of which detach from the trophoblast surface and pass into the intervillous space whence they are probably rapidly deported from the placental site. As many as 150,000 SK fragments per day may enter the maternal systemic circulation (Nelson et al., 1990; Chua et al., 1991; Hawes et al., 1994) where they vary widely in size (20-600 μm), nuclear content (1-60) and packing density (0 to 6000 per litre of blood). In many other tissues, apoptotic nuclei are interiorised by mononuclear phagocytes (or other neighbouring cells) and rapidly removed. As yet, there is no evidence that this method of removal operates in placental villi (e.g. via the intermediary of mesodermal macrophages or maternal phagocytes). Indeed intravillous phagocytosis is unnecessary given that fragments lost into the intervillous space are then deported. In the small intestines of some mammals, a similarly economical mechanism also operates: apoptotic epithelial cells are shed into the intestinal lumen (Mayhew et al., 1999b). Of course, the loss of SK fragments into maternal blood does not preclude the possibility that circulating emboli are phagocytosed at extraplacental sites.

The functional significance of SK regions has been the subject of much speculation (Benirschke and Kaufmann, 2000). They have been implicated in the processes of senescence, degeneration, amoeboid activity, structural support and adaptation to ischaemic or hypoxic damage (Fox, 1965; Jones and Fox, 1977). Their incidence increases throughout gestation and, at term, they are seen on 10-30% of profiles of terminal villi (Fox, 1965). We have proposed that SK regions merely represent the extrusion component of normal continuous epithelial renewal (Mayhew et al., 1999a). In other epithelia of this type, cell recruitment is in steady state with cell extrusion. For example, in mammalian small intestine, recruitment of cells from the proliferative compartment (crypts) is accompanied by

Fig. 5. Using propidium iodide as a DNA fluorochrome to label condensed chromatin reveals that a syncytial knot, and adjacent regions of syncytiotrophoblast, are rich in nuclei with highly condensed chromatin. Such nuclei may stream into the bases of knots from various directions by an actin-mediated process. This probably contributes to the formation of nuclear 'fascicles' which are sometimes seen in ultrastructural thin sections of syncytial knots. x 1,000

cell loss at or near the villous tips. At least two mechanisms of cell loss involve apoptosis as a consequent of which cells, or cell fragments, may be extruded into the intestinal lumen or ingested by lamina propria macrophages (Mayhew et al., 1999b).

The circumstances in which SK fragments are lost from trophoblast may vary. It is expected that, normally, this occurs by a budding process which maintains the integrity of the trophoblastic epithelium. Occasionally, however, epithelial continuity is breached by local injury and this produces denudation of ST and release of SK fragments.

Trophoblast damage-and-repair involves de-epithelialisation, fibrin deposition and re-epithelialisation

Local injury to trophoblast probably occurs via the direct or indirect effects of mechanical or chemical events. Recently, we advanced the notion that at least some denudation sites arise as a consequence of SK formation and extrusion (Mayhew et al., 2000). However, this is not the sole mechanism since immunochemical studies suggest that extrusion may be mediated by actin microfilaments in a process reminiscent of cytokinesis in dividing cells (Mayhew et al., 1999a). Another physical trauma capable of generating denudation sites is the avulsion or rupture of large (> 10 μm diameter) intervillous bridges (Burton, 1986, 1987). This is more likely to occur in an accidental rather than regulated fashion and could be influenced by the increasing vigour of fetal movements as pregnancy nears completion.

Apoptosis of ST nuclei may be another way of initiating trophoblast de-epithelialisation (Nelson, 1996) but necrotic features have also been seen in cultured syncytium in relation to local ischaemic or hypoxic degeneration and subsequent syncytial regeneration (MacLennan et al., 1972; Kaufmann, 1985; Palmer et al., 1997). Necrosis within ST has also been noted in placentas from diabetic women (Fox and Jones, 1983). Finally, degeneration may happen following loss of local cell players or factors involved in promoting epithelial growth. For example, it has been proposed that the gradual loss of ST from stem villi during gestation is due to depletion of mesodermal macrophages and the consequent loss of factors which stimulate CT proliferation (Demir et al., 1997).

Once created, denudation sites provide possible routes of transient local paracellular permeability, particularly for macromolecules which are known to cross the placenta (Brownbill et al., 2000). Denuded areas also provide sites for preferential deposition of perivillous fibrin-type fibrinoid (Mayhew et al., 2000), a loose network of coagulum products of the maternal vascular bed (Frank et al., 1994; Kaufmann et al., 1996). Its preferential distribution suggests that its deposition is both targetted - designed to limit local epithelial damage - and regulated – influenced by systemic and trophoblastic haemostatic factors. Trophoblast-associated factors include annexin-V, thrombomodulin, nitric oxide and plasminogen activators and their inhibitors (for references, see Mayhew et al., 2000).

Denudation sites are pro-coagulatory and fibrin deposits act as a matrix for CT proliferation followed by syncytialisation and re-epithelialisation (Nelson et al., 1990). Scanning EM of denudation sites created by detached intervillous bridges show CT cells scattered on the underlying basal lamina (Burton, 1986, 1987). If re-epithelialisation occurs where trophoblast on adjacent villi is contiguous, it is possible that this could lead to formation of new intervillous bridges by shared re-epithelialisation. Therefore, we may envisage the periodic formation and rupture of intervillous bridges as an ongoing process linked to villous morphogenesis, trophoblast damage and trophoblast repair.

Perturbing the epithelial steady state: effects of oxygen tensions

Normal pregnancy

In normal term placenta, vasculosyncytial membranes account for 8-20% of total villous surface area (e.g. Noack et al., 1983). The incidences of thin (vasculosyncytial) and thick (SK) regions are related since they are both formed partly by the same process of redistribution of syncytial mass (Jackson et al., 1988b). A high incidence of SK regions tends to be associated with a low incidence of vasculosyncytial membranes (Fox, 1965). There is evidence that the frequency of these regions is also related to proliferative activity in CT and correlates with placental location, directions of blood flow and local oxygen tensions (Sala et al., 1983; Schuhmann, 1985; Sala and Matheus, 1986; Benirschke and Kaufmann, 2000). Tritiated-thymidine labelling of CT cells and the frequency of SK regions are greater in the periphery of placentones (where oxygen tensions are lower) than in the centre (where tensions are higher) but vasculosyncytial membranes are more frequent centrally.

These findings emphasise the links between proliferation and extrusion and open up the possibility that the epithelial steady state varies with placental site and oxygen partial pressure. Site-dependency is seen in other continuously renewing epithelia. For example, studies on the epithelial steady state in small intestine have revealed that the number of villous epithelial cells per crypt cell (the intestinal proliferon) varies in a proximo-distal gradient (Zoubi et al., 1994).

Pregnancies associated with hypoxic stress

Pregnancies can be associated with alternative forms of fetal hypoxia including preplacental, uteroplacental and postplacental (Kingdom and Kaufmann, 1997). These different forms of hypoxia are accompanied by differences in villous branching, capillarisation and development. The clearest quantitative evidence that

hypoxia might also alter the epithelial steady state comes from studies on pregnancies associated with high altitude, i.e. hypobaric hypoxia (an example of preplacental hypoxia).

In high-altitude pregnancies, villous growth is impoverished but the trophoblast is thinner (Jackson et al., 1987, 1988a). In contrast, the volume fraction of trophoblastic bridges and SK regions in villi is increased (Ali et al., 1996). Moreover, Ali (1997) found that whilst the mean volume of a CT cell is unaltered, there is a differential increase in number of CT nuclei such that the ST:CT ratio shifts from 15:1 to 6:1. The shift in steady state might be due to increased proliferation coupled with accelerated extrusion because the incidence of SK regions also increases in highland placentas (Chabes et al., 1968). Another possible factor is that hypoxia stimulates CT proliferation but compromises recruitment and syncytialisation (Alsat et al., 1996). It would be of interest to test whether or not the frequency of apoptosis is also altered in villi from highland placentas.

Clearly, further quantitative studies on trophoblast relationships and the incidence of apoptosis is required in abnormal pregnancies associated with fetal hypoxic stress. For example, in toxaemic pregnancies, villous surface area is also reduced and SK regions are more common (Fox, 1965; Alvarez et al., 1967). Women with pre-eclampsia have more trophoblast fragments in uterine vein blood (Chua et al., 1991) and this suggests accelerated terminal differentiation but the incidence of apoptosis in pre-eclampsia is unknown. Interestingly, the proportion of apoptotic nuclei in villous sections increases in intrauterine growth restriction (Smith et al., 1997b).

Conclusions

The changes which occur during villous development and morphogenesis lead to the idea that proliferation of CT cells early in gestation is principally for trophoblast growth whilst that later in gestation is for renewal and repair. This is consistent with reported differences in indices of proliferation: these tend to be lower at later stages of gestation. During the first trimester, villous sprouting is initiated at sites of CT proliferation and these sites produce nascent fungiform villous outgrowths rich in ST nuclei. Later in gestation, when most villous branches are established, sprouts are linked to capillary growth and probably represent sites of nuclear clustering within a heterogeneous mix of true sprouts, intervillous bridges, villous branchpoints and SK regions. The larger bridges may arise in part by PS-mediated membrane fusions at SK regions on touching villi since membranes associated with these regions may exhibit the PS flip phenomenon. Rupture or abruption of bridges, detachment of SK regions, and other sources of damage may lead to de-epithelialisation and deposition of fibrin-type fibrinoid in the breach. Re-epithelialisation occurs by CT proliferation and syncytialisation and may lead to formation of new bridges by shared re-

epithelialisation. Villous development is linked to maturation and changes in the trophoblast and villous stroma help to thin the trophoblast and make it more efficient at diffusive transport.

Many of these events in normal pregnancy may be related to local oxygen tensions. Lower oxygen levels stimulate CT proliferation but compromise recruitment and, thereby, damage syncytium. Perturbations of these events are features of pregnancies associated with various types of fetal hypoxia and villous maldevelopment.

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