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REVIEW

Development of the human placenta

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ABSTRACT

The placenta is essential for normal *in utero* development in mammals. In humans, defective placental formation underpins common pregnancy disorders such as pre-eclampsia and fetal growth restriction. The great variation in placental types across mammals means that animal models have been of limited use in understanding human placental development. However, new tools for studying human placental development, including 3D organoids, stem cell culture systems and single cell RNA sequencing, have brought new insights into this field. Here, we review the morphological, molecular and functional aspects of human placental formation, with a focus on the defining cell of the placenta – the trophoblast.

KEY WORDS: Development, Maternal, Placenta, Trophoblast

Introduction

The placenta is the largest fetal organ and the first to develop. It plays a central role in the health of both the fetus and its mother, and has a lifelong impact on their future wellbeing. Indeed, disordered placental development is the primary defect in major diseases of pregnancy, such as pre-eclampsia, fetal growth restriction, recurrent miscarriage and still-birth (Brosens et al., 2011). The links between the *in utero* environment and susceptibility to chronic disease in adults are also well recognised (Barker, 1995). However, despite its importance in reproductive success, there is still limited understanding of how the human placenta develops. The obvious ethical and logistical obstacles in investigating early human pregnancy, and the lack of long-term physiologically relevant *in vitro* models, make experimentation problematic. This is further compounded by the great diversity of strategies used by different eutherian mammals to build a placenta, which make extrapolating data from other species to humans difficult (Carter and Enders, 2004).

Here, we provide a broad overview of current knowledge of human placental development, based on morphological studies of archival specimens, immunohistochemical and bulk transcriptome analyses, and *in vitro* studies using primary cells, cell lines and villous explants. We also highlight recent technological advances, such as comparative genomic and transcriptomic studies at the single cell level, organoid culture systems, and systematic analyses of biomarkers for placental function, that now allow us to study the early stages of human placental development in more detail.

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Anatomy of human placental development

The human placenta develops from the trophectoderm (TE), the outer layer of the pre-implantation embryo, which forms at ∼5 days post fertilisation (dpf). At this stage, the pre-implantation embryo (termed a blastocyst) is segregated into two lineages: the inner cell mass (ICM) and the TE. The polar TE (the part of the TE that is contiguous with the underlying ICM) attaches to the surface epithelium of the uterine mucosa: the endometrium (Fig. 1A). Although the earliest stages of implantation have not been visualised in humans, morphological observations of early pregnant hysterectomy specimens and higher primates suggest that, following attachment to the uterine surface epithelium at ∼6-7 dpf, the TE fuses to form a primary syncytium. This is the prelacunar phase of placental development (Boyd and Hamilton, 1970).

Following implantation, the primary syncytium quickly invades through the surface epithelium into the underlying endometrium, which is transformed during pregnancy into a specialised tissue known as decidua (Fig. 1B) (Schlafke and Enders, 1975). By the time of the first missed menstrual period (∼14 dpf), the blastocyst is completely embedded in the decidua and is covered by the surface epithelium (Fig. 1C) (Hertig et al., 1956). Fluid-filled spaces (lacunae) then appear within the syncytial mass that enlarge and merge, partitioning it into a system of trabeculae. This is the lacunar stage. The syncytium also erodes into decidual glands, allowing secretions to bathe the syncytial mass (Hertig et al., 1956).

The trophoblast cells beneath the syncytium (termed cytotrophoblast cells) are initially not in direct contact with maternal tissue but rapidly proliferate to form projections that push through the primary syncytium to form primary villi (a cytotrophoblast core with an outer layer of syncytiotrophoblast, SCT); this is the villous stage of development (Fig. 1D). The villous trees are formed by further proliferation and branching, and the lacunae become the intervillous space. Cytotrophoblast cells eventually penetrate through the primary syncytium and merge laterally to surround the conceptus in a continuous cytotrophoblast shell between the villi and the decidua (Fig. 1D). The blastocyst is now covered by three layers: the inner chorionic plate in contact with the original cavity; the villi separated by the intervillous space; and the cytotrophoblast shell in contact with the decidua.

Soon afterwards, around day 17-18, extraembryonic mesenchymal cells penetrate through the villous core to form secondary villi. By day 18 dpf, fetal capillaries appear within the core, marking the development of tertiary villi. The villous tree continues to rapidly enlarge by progressive branching from the chorionic plate to form a system of villous trees. Where the cytotrophoblast shell is in contact with the decidua (the maternal-fetal interface), individual cytotrophoblast cells leave the shell to invade into decidua as extravillous trophoblast (EVT) in a process closely resembling epithelial-mesenchymal transition (EMT). In this way, by the end of the first trimester, the blueprint of the placenta is established.
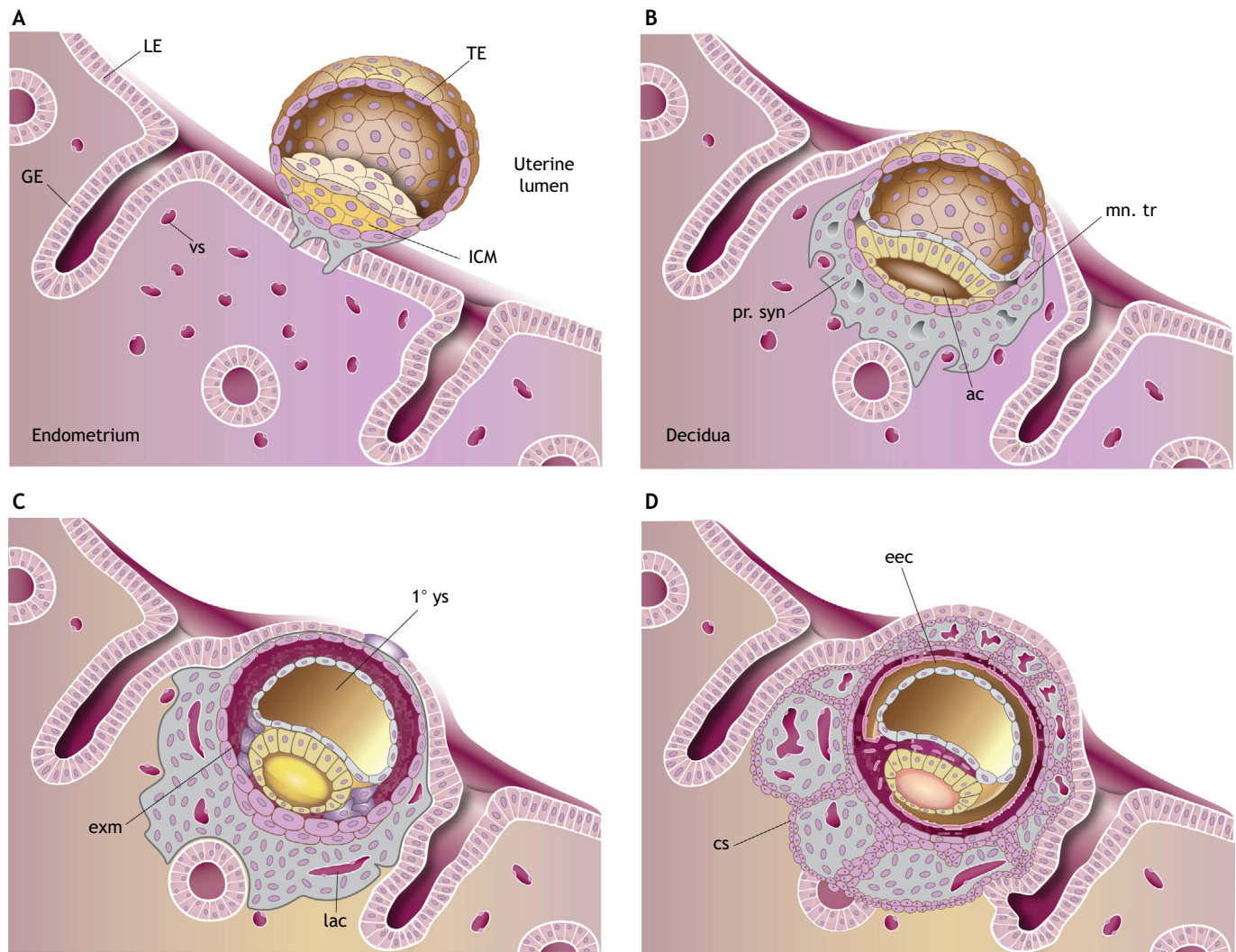
Fig. 1. The early stages of human placental development. Diagram depicting the early steps in placenta formation following blastocyst implantation. (A,B) The pre-lacunar stages. (C) The lacunar stage. (D) The primary villous stage. 1° ys, primary yolk sac; ac, amniotic cavity; cs, cytotrophoblastic shell; eec, extra-embryonic coelom; exm, extra-embryonic mesoderm; GE, glandular epithelium; ICM, inner cell mass; lac, lacunae; LE, luminal epithelium; mn. tr, mononuclear trophoblast; pr. syn, primary syncytium; TE, trophectoderm; vs, blood vessels.

Cell types of the human placenta

Trophoblast cells

The major functions of the placenta are performed by trophoblast cells. The appearance of the trophoblast is an important evolutionary advance that defines placental mammals. The term 'trophoblast' was first used by the Dutch embryologist Ambrosius Arnold Willem Hubrecht in 1889 to describe cells that transport nutrients and form the protective barrier between mother and fetus (Pijnenborg and Vercruysse, 2013). He also observed that the trophoblast is inherently highly invasive or 'corrosive', and dependent on decidua to support its development (Hubrecht, 1908). Since these early studies, a variety of human trophoblast subtypes have been identified. These include the SCT, the villous cytotrophoblast (VCT) and subtypes of the EVT.

The SCT is the outer lining of the placental villi that is in direct contact with maternal glandular secretions and, later, with maternal blood flowing into the intervillous space (Fig. 2). It is the main site of maternal/fetal exchange of gases and nutrients necessary for the growth of the feto-placental unit. The SCT has a highly polarised epithelial layer densely covered with microvilli, which increases its

surface area five- to seven-fold (Teasdale and Jean-Jacques, 1985). Microinjection of fluorescein-labelled dextrans into the SCT confirms that it is multinucleated with no cell borders, presumably to facilitate diffusion across its entire structure and protect the fetus from pathogens (Gaunt and Ockleford, 1986). Proteomic analyses of SCT membranes show that the microvilli are rich in receptors for growth factors and hormones (Robinson et al., 2009). Both the apical and basal membranes of the SCT are packed with transporter proteins for amino acids and glucose, as well as those that efflux xenobiotics. The SCT is also a major endocrine organ, secreting hormones and proteins into the maternal circulation to drive the physiological and metabolic adaptations to pregnancy. Furthermore, SCT functions as a protective immunological barrier because it never expresses any human leukocyte antigen (HLA) molecules, meaning that, despite the presence of the allogeneic fetus, circulating immune cells will not detect the SCT as 'non-self' (Moffett and Loke, 2006). The SCT also expresses the neonatal Fc receptor (FcRn) that allows transport of maternal IgG antibodies to the fetal circulation (Roopenian and Akilesh, 2007). The antibodies that preferentially bind to FcRn on the SCT are digalactosylated
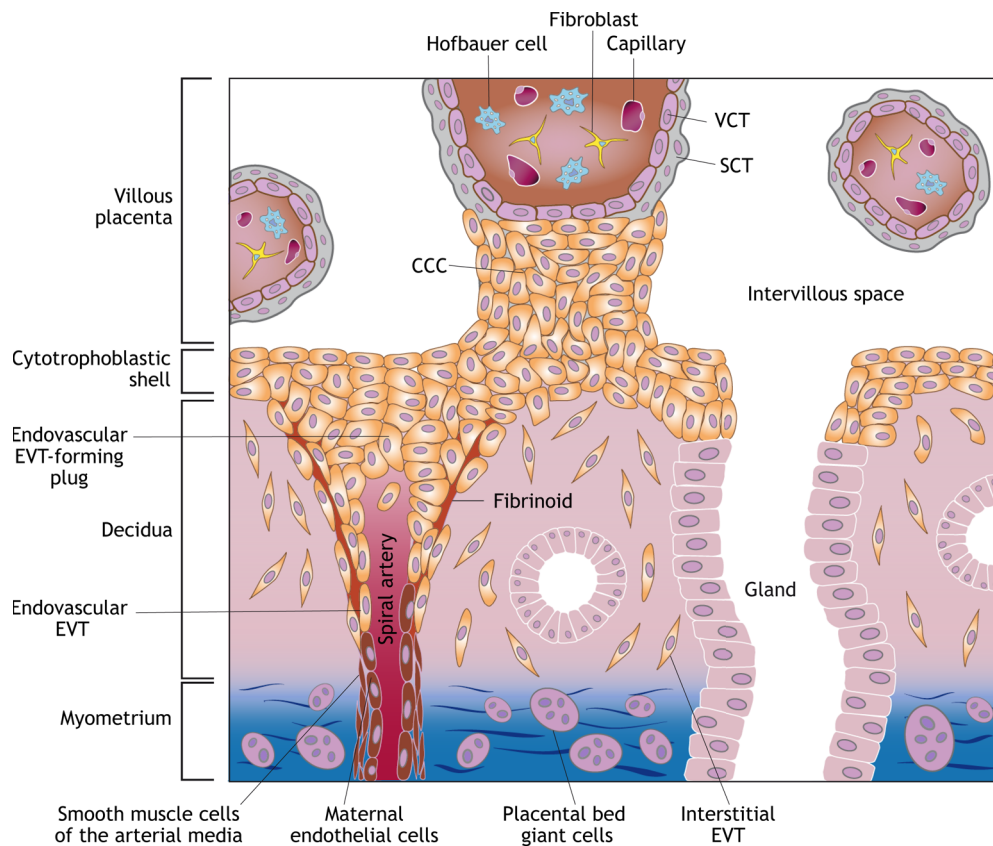
Fig. 2. The maternal-fetal interface and trophoblast subtypes. Cells contained within the villi of the early first trimester placenta and the major trophoblast subtypes in relation to the decidua are represented. The decidual region has been illustrated to include the myometrium. Synctiotrophoblast (SCT, grey), villous cytotrophoblast (VCT, pink), the cytotrophoblast cell column (CCC) and extravillous trophoblast (EVT) populations (endovascular and interstitial EVT, orange) are indicated. The endpoint of EVT differentiation, placental bed giant cells, are also indicated.

IgG1 molecules that are effective at activating fetal natural killer (NK) cells to protect the neonate before birth (Jennewein et al., 2019).

The mononuclear VCT lies beneath the SCT on a basement membrane (Fig. 2). Historically, the VCT have been considered the 'germinative' layer of trophoblast because they are mitotic and express proliferative markers (Simpson et al., 1992). In early pregnancy, when they form a continuous layer, the VCT are cuboidal cells with a large nucleus:cytoplasm ratio. As the villous trees expand, the VCT layer becomes discontinuous and covers only 25% of the villous surface by term, when only a thin syncytial layer separates most of the villous core from maternal blood (Benirschke et al., 2012).

As the placenta enlarges, the cytotrophoblast shell becomes discontinuous and cytotrophoblast cell columns (CCCs) emerge from the distal tips of the anchoring villi in contact with the decidua (Fig. 2). The cells in the columns are rounded, cohesive and rich in glycogen. From the shell, and later the anchoring villi, the EVT migrate into the decidua along two differentiation pathways: the interstitial EVT (iEVT) migrate through the decidual stroma towards the maternal spiral arteries, while the endovascular trophoblast EVT (eEVT) moves down the inside of the spiral arteries (Pijnenborg et al., 1980). In the decidua, the iEVT have a pleomorphic and fusiform morphology, tetraploid nuclei, and are non-cycling and show changes in senescence (Velicky et al., 2018). They home towards the spiral arteries to form a cuff of surrounding cells. This is associated with loss of actin in smooth muscle cells of the arterial media, which is replaced by amorphous eosinophilic material, resulting in the histological appearance known as 'fibrinoid' change (Pijnenborg et al., 2006). This trophoblast-mediated transformation of the artery results in loss of vasoactivity

and conversion into a vessel that is capable of high conductance at low pressure, an essential adaptation for normal pregnancy (Brosens et al., 1967). The iEVT invade as far as the inner third of the myometrium (the muscular layer of the uterine wall), where it is thought they fuse to form placental bed giant cells (Pijnenborg et al., 1980). After the arterial transformation occurs, the eEVT move in a retrograde manner down the artery to form a plug that prevents blood entering the intervillous space until towards the end of the first trimester, when the full haemochorial circulation is established (Boyd and Hamilton, 1970; Burton et al., 1999; Hustin and Schaaps, 1987).

Other placental cells

Besides trophoblast cells, the placenta contains a range of cells present within the stromal core of the villi, including fibroblasts, immune and vascular cells (Fig. 2). These cells are all thought to be generated from the extra-embryonic mesenchyme, the origin of which in humans is uncertain. Suggestions are that it arises from the VCT that undergoes EMT, or that it originates from the hypoblast, the endodermal derivative of the ICM, with contribution from the embryonic mesoderm after gastrulation (Boss et al., 2018).

Single cell RNA-sequencing (scRNA-seq) of first-trimester villi shows there are at least two fibroblast populations distinguished by the presence or absence of the imprinted gene *DLK1* (Kagami et al., 2012; Vento-Tormo et al., 2018). DLK1$^+$ cells have similarities to pericytes and may be involved in vascular development (Liu et al., 2018; Suryawanshi et al., 2018). The fibroblasts link up to form a network of canals that feed into the extra-embryonic coelom. These canals contain placental macrophages termed Hofbauer cells (Burton, 1987; Castellucci et al., 2000; Kaufmann et al., 1977). Because Hofbauer cells are the only immune cells in the placenta

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and appear in the villous core from around day 18 postcoitum, before there is any vascular connection to the embryo itself, they are likely to be generated from haemangioblastic cells in extra-embryonic tissue (Boyd and Hamilton, 1970; Castellucci et al., 1987). Their functions have been little investigated but they are likely to play roles in protecting the fetus from vertical infections, in influencing trophoblast and placental vascular development, and in transferring nutrients to the extra-embryonic coelom. The vascular system also develops from haemangioblastic populations in the mesenchyme and connects to the fetus via the umbilical cord by the end of the first trimester (Dempsey, 1972; Robin et al., 2009). These immature endothelial cells have a specific pattern of gene expression, e.g. they express EGFL7, which is known to regulate vascular morphogenesis (Parker et al., 2004).

Models for the study of human placental development
Animal models
Mammals exhibit an enormous diversity of placental strategies, particularly with regard to the degree of trophoblast invasion into uterine tissues and the number of cell layers between the maternal and fetal circulations (Roberts et al., 2016). Both the laboratory mouse and primates have a haemochorial type of placenta, where the trophoblast invades through the uterine epithelium, stroma and maternal arterial walls to come into direct contact with maternal blood (Georgiades et al., 2002). Lesser degrees of invasion are seen in carnivores with an endotheliochorial placenta, where trophoblast cells contact maternal endothelial cells. In the least invasive form, an epitheliochorial placenta, which is characteristic of ruminants and ungulates, the trophoblast remains superimposed on the uterine epithelium (Carter and Enders, 2013).

There is no perfect experimental model to investigate human placentation, even in species with haemochorial placentas (e.g. the laboratory mouse and non-human primates) (Carter and Pijnenborg, 2011). An additional problem is that, from the vantage point of an obstetrician, disorders such as pre-eclampsia in which the primary defect is failure of placentation are found only in humans and possibly in great apes (Carter, 2011). The differences between mouse and human placentation are considerable. In humans, the polar TE attaches to the uterus, whereas, in mice, it is the mural TE (which lies opposite the ICM) that implants into the uterus first, followed by the polar TE that will give rise to the ectoplacental cone (EPC) (Georgiades et al., 2002). Moreover, EPC cells are mononuclear, and their invasion into the decidua occurs almost halfway through murine gestation, unlike the initial invasion by primary syncytium in humans. The EPC in mice forms the spongiotrophoblast and, although this is viewed as equivalent to the EVT in humans, there is no deep interstitial invasion of the decidua. This type of invasion is characteristic only of humans and great apes (gorillas and chimpanzees), and is not seen in other primates (Pijnenborg et al., 2011). Unlike the human villous placenta, the site of placental exchange in rodents is a labyrinth that has a complex tightly packed arrangement of maternal and vascular channels. These substantial differences in trophoblast development between mouse and humans mean that findings obtained in studies of the mouse must be treated with a certain degree of healthy scepticism. Indeed, genome-wide expression profiling of mouse and human placentae across gestation has revealed clusters of genes with very different co-expression patterns (Soncin et al., 2018).

Despite these caveats, important insights can be made from studying murine genetic knockout models of in utero fetal loss (Rossant and Cross, 2001). In ~70% cases, the phenotype is attributable to placental abnormalities and several of the genes identified using this approach are known to play a role in EMT (Perez-Garcia et al., 2018). A better rodent model to study arterial transformation is the laboratory rat as, in this system, the trophoblast extends deeply into the uterine wall to remodel the artery feeding the placentation site (Soares et al., 2012).

In vitro models
Until recently, the study of human placental development and trophoblast specification and differentiation had been hampered by the lack of reliable, physiologically relevant and reproducible in vitro models. While various cell lines and models are available, they do not all retain key features of the equivalent cells in vivo. However, recent advances have led to the development of more robust in vitro systems that can be used to model human placentation.

Trophoblast cell lines
While a number of trophoblast cell lines exist, a major problem with using these has been the lack of consensus about how to define the human trophoblast in vitro, with a wide range of features being used in each case (King et al., 2000a). This is the issue for the various cell lines that have been derived from first-trimester or term placentas, so their identity is still not clear (Feng et al., 2005; Genbacev et al., 2011, 2016; Graham et al., 1993; James et al., 2007, 2015; Perez-Garcia et al., 2018; Straszewski-Chavez et al., 2009; Takao et al., 2011; Zdravkovic et al., 2015). Care must be taken, as contaminating maternal epithelial and fetal mesenchymal cells can outgrow trophoblast isolated from the placenta (Heazlewood et al., 2014; Turco et al., 2018). We recently proposed that four criteria could together be used to characterise human first-trimester (post-implantation) trophoblast: the expression of genes highly expressed in trophoblast, such as TFAP2C, GATA3 and cytokeratin (KRT7); a unique pattern of HLA expression, either HLA null (VCT and SCT) or HLA-G+, HLA-C+ but HLA-A– and HLA-B– (EVT); very high expression of the C19MC microRNA complex; and hypomethylation of the ELF5 promoter (Lee et al., 2016a). Indeed, the analysis of ELF5 methylation and HLA class I expression indicates that most trophoblast cell lines do not share the profile of in vivo first trimester trophoblast and may instead be mesenchymal cells (Abou-Kheir et al., 2017; Hemberger et al., 2010; King et al., 2000a). Undoubtedly, further improvements in defining and characterising human trophoblast in vitro will be made in the future.

Choriocarcinoma cell lines
Although trophoblast cell lines derived from choriocarcinomas (malignant tumours of trophoblast), such as JEG3, JAR and BeWo cells, do fulfil the four criteria defining trophoblast and have been widely used as in vitro models, their genetic signatures are quite unlike that of normal trophoblast (Apps et al., 2009; Poaty et al., 2012). BeWo cells are derived from metastatic deposits of a gestational choriocarcinoma that have been cultured by passaging within the hamster cheek pouch more than 300 times. The JEG-3 line was subcloned from BeWo; both are hypertriploid with ~70 chromosomes (Hertz, 1959; Kohler and Bridson, 1971; Pattillo and Gey, 1968). JAR cells are HLA null, like VCT; however, although JEG3 cells, like EVT, do express HLA-G and HLA-C, the HLA-G dimers characteristic of normal trophoblast are absent and only HLA-G monomers are expressed (Apps et al., 2007). This limits the experimental use of JEG3 cells as targets for maternal uterine immune cells (NK cells and myeloid cells) that express the LILRB1 receptor for the dimeric form of HLA-G dimers (Shiroishi et al.,

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2006). Moreover, genome-wide DNA methylation studies comparing primary trophoblast to many of these cell lines show significant variability in their profiles that are likely to contribute to differential expression profiles (Apps et al., 2011; Novakovic et al., 2011). This highlights the caution needed for interpreting results using such cell lines and the importance of validating findings by using primary cells. Indeed, much of the controversy in the literature has arisen from using cell lines that are not representative of bona fide trophoblast cells *in vivo*.

Human embryonic stem cell-derived trophoblast cells

Another approach has been to differentiate human embryonic stem cells (hESCs) into trophoblast cells by culturing them in the presence of BMP4 and inhibitors of FGF2 and TGFβ signalling (Amita et al., 2013; Horii et al., 2016; Xu et al., 2002). A comprehensive analysis of all reports using this method, and a discussion of how the culture conditions (including the source of suppliers for BMP4) and different hESC lines can affect trophoblast differentiation, is available (Roberts et al., 2018). Characterisation of such hESC-derived trophoblast cells using our four putative trophoblast criteria is incomplete, but it has been shown that these cells exhibit partial (but not complete) hypomethylation of the *ELF5* promoter and downregulate some of the C19MC complex microRNAs (Lee et al., 2016a). The formation of SCT that secretes human chorionic gonadotropin (hCG) and expresses the EVT marker, HLA-G, as well as other genes expressed by trophoblast, such as KRT7, GATA2/3 and TCFAP2A/C, has also been reported (Amita et al., 2013; Horii et al., 2016; Krendl et al., 2017). However, transcriptomic analysis of such hESC-derived trophoblast reveals that these cells are different from both term trophoblast and mesoderm lineages; this has been a subject of discussion as some markers are expressed in both cell types (Bernardo et al., 2011; Jain et al., 2017). A defining feature of trophoblast – the absence of HLA-A and HLA-B – has not yet been analysed in these cells. Thus, although this approach seems to promote some transition towards the trophoblast lineage, the identity of the trophoblast cells generated and the cells they are most similar to *in vivo* is still unclear. One possibility is that hESC-derived trophoblast cells represent an early post-implantation trophoblast population (Roberts et al., 2018).

Placental explants or primary trophoblast cells

Many labs have used explants of placenta or isolates of primary placental cells. Explants are prepared from the villous placenta and adhere to plastic or a defined matrix. Extravillous HLA-G+ cells then move away from the tips of the villi. Preparations of primary isolates of trophoblast and other placental cells can be obtained from early gestation or at term. At term, because the VCT is not easily visible by light microscopy and only a thin layer of the SCT covers the villi, any placental isolates may potentially contain other cells from the villous core (Hofbauer cells, fibroblast and endothelial cells) as well as maternal and fetal blood cells, together with attached decidual cells. In the first trimester, the villi are covered by the SCT and an inner layer of the VCT; the latter can be isolated by enzymatic digestion and density gradient sedimentation (Male et al., 2012). However, the cells need to be plated onto an ECM matrix as they adhere poorly to plastic; after overnight culture they are all HLA-G+. The cells can be used within 3-4 days but within 1 week become overgrown by mesenchymal contaminants, as the trophoblast cells do not proliferate *in vitro*. Other placental cells from the villous core can also be isolated, and phenotypic markers such as CD34 for endothelial cells can be used to verify purity. The

problems with using primary cells for experimentation are obvious; ethical permission is essential, limiting use to certain countries and requiring good liaison with clinical staff. There are also several difficulties relating to cell viability, degeneration of SCT and reproducibility. Indeed, the innate variability between samples, which depends on factors such as gestational age, parity, intercurrent disease and area of placenta sampled, means that multiple experiments are needed to ensure validity of results.

The recent development of human embryo culture has allowed the visualisation of human embryo reorganisation and development beyond the stage of implantation, although this is only possible to observe up to day 13 (owing to ethical constraints). The trophoblast lineages that emerge in this culture system and how well they recapitulate *in vivo* events remain still to be understood.

Trophoblast stem cells and organoids

Progress has been now made in generating bona fide human trophoblast stem cells (hTSCs) from both trophectoderm and first-trimester placentas (Okae et al., 2018). hTSCs are grown on collagen IV and can be cultured long term in a medium that stimulates WNT and MAPK (through EGF) signalling, and inhibits TGFβ/activin A, HDAC and Rho kinase. These cells are genetically stable and fulfil all four characteristics of first-trimester trophoblast. They cannot be derived from term placentas, in keeping with the dramatic loss of proliferative potential of the VCT after ~10 weeks gestation and the scarcity of the VCT in term villi (Mayhew, 2014). By modifying the culture conditions, hTSCs can be induced to differentiate along either syncytial or extravillous lineages. They also have clonal ability and a clone can generate both the SCT and EVT, providing the first solid evidence of the bipotentiality of hTSCs, a subject of ongoing debate (Baczyk et al., 2006; Haider et al., 2016; James et al., 2005; Lee et al., 2018). Comparison of the transcriptomes of hTSCs and the first-trimester trophoblast shows close similarity to the VCT. However, the exact anatomical location of hTSCs *in vivo* in both the early placenta and the TE is still unclear. Likewise, while mouse TSCs can be derived from both the TE and the chorion prior to E11.5, and can be differentiated to all trophoblast populations, further investigation is needed to understand which murine trophoblast population they resemble *in vivo* and how they relate to human TSCs (Natale et al., 2017; Tanaka et al., 1998). Single cell RNA-sequencing has now been performed on the mouse mid-gestation placenta and on human term placenta, as well as on these placentas early in their development (Liu et al., 2018; Nelson et al., 2016; Pavličev et al., 2017; Suryawanshi et al., 2018; Vento-Tormo et al., 2018). These studies may help elucidate the identities of the various trophoblast cultures and their relation to trophoblast *in vivo*.

hTSCs lines grow as a monolayer and therefore cannot recapitulate the complex branching morphology of early placental villi. Recently, 3D cultures of human trophoblast cells, termed trophoblast organoids, that closely model the villous placenta and can be differentiated into EVT have been established (Haider et al., 2018; Turco et al., 2018). Like other organoid culture systems, these organoids are likely to be transformative in advancing our understanding of the physiology and disease of human tissues (Clevers, 2016; Huch and Koo, 2015). Trophoblast organoids grow for >1 year, are genetically stable and fulfil all trophoblast criteria (Turco et al., 2018). They also secrete placental hormones and proteins that alter maternal metabolism, appetite and preparation for lactation. The ability to biobank hTSC cells/organoids from individuals with a range of possible pregnancy problems also offers great potential. We anticipate these trophoblast culture

systems can be used to dissect the genetic and epigenetic mechanisms underlying the identity of trophoblast lineages, the differentiation of SCT and EVT, and the regulation of HLA expression. Furthermore, it will be possible to test the role of many genes directly in hTSCs using knockdown or knockout approaches (e.g. using siRNAs or gene editing with CRISPR/Cas9).

Bioengineering-based approaches

Although hTSCs and organoids represent a major step forward in studying human trophoblast *in vitro*, there are several limitations; they model only the trophoblast component of the placenta and are heterogenous. Furthermore, because other cellular components of the placenta play an important role in regulating their development and function, future work is necessary to build more complex models. Microfluidics approaches that have been previously used to model human placental functions, such as transport and invasion, have also shown promise (Abbas et al., 2017; Lee et al., 2016b). More recently, a variety of bioengineering systems such as the use of biomimetic scaffolds and synthetic hydrogels are being implemented for other organoid systems to generate improved tissue-like models (Takebe and Wells, 2019). These approaches have the added advantage of providing mechanical and signalling cues that further control cell behaviour and function while improving reproducibility (Yin et al., 2016). These systems could be adapted to study the interaction of the trophoblast with placental stromal components and maternal cells (such as glands or uterine NK cells) to investigate the maternal-fetal dialogue of early pregnancy. For example, our long-term organoid culture system of endometrial glands will provide a tool for studying the effect of glands on the trophoblast and also provides an *in vitro* system for studying glandular function in women with implantation failure (Turco et al., 2017).

Molecular mechanisms underpinning human placental development

Trophoblast cells have many unique features that set them apart from all other cell types, including global hypomethylation, differential expression of microRNAs, unusual patterns of HLA molecule expression and expression of endogenous retroviral products (Macaulay et al., 2017; Sadovsky et al., 2015). Imprinted genes, preferentially expressed from one parental allele, are also important in placental development and, out of the 92 human imprinted genes, 75 are expressed in the placenta (Monk, 2015). The C19MC cluster, which encodes microRNAs, is paternally expressed in the trophoblast. Although these miRNAs are expressed in hESCs, expression is much higher in the trophoblast (Lee et al., 2016a; Malnou et al., 2019; Noguer-Dance et al., 2010). Apart from the C19MC cluster, several other genes that are involved in reproduction are located on chromosome 19q13.4 (Moffett and Colucci, 2015). These include genes encoding choriogonadotropin subunit β (CGB), pregnancy-specific glycoproteins (PSGs), killer-cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs) and nucleotide-binding oligomerisation domain, and leucine-rich repeat and pyrin domain-containing family (NLRP) members. Moreover, many X chromosome genes escape X inactivation in the human placenta, leading to transcriptional differences between female and male pregnancies that may explain the sex-based differences in pregnancy disorders (Gong et al., 2018; Moreira de Mello et al., 2010; Vatten and Skjaerven, 2004). There are also striking similarities between trophoblast and tumour cells. For example, trophoblast cells exhibit invasive proclivities and an ability to avoid destructive inflammatory or immune responses, as well as widespread hypomethylation and focal hypermethylation at CpG islands, including at promoters of tumour suppressor genes (Nordor et al., 2017).

Studies of the molecular changes occurring during human placental development have been mainly descriptive due to the lack of, until recently, long-term, genetically normal human trophoblast cell lines with which to perform functional studies. Nonetheless, these studies have provided insights into the molecular factors that contribute to trophoblast development and placentation. Below, we discuss these studies, highlighting relevant mouse data and, when available, data in humans, with a focus on papers that have validated the work using primary trophoblast cells. If other *in vitro* tools were used, this is specified.

Establishment of the trophoblast lineage

In mice, a hierarchy of transcription factors (TFs) drives TE lineage commitment. This process begins when, at the morula stage of preimplantation development, Hippo signalling upregulates *Tead4* in a subset of cells, which then drives *Cdx2* expression in the future TE (Nishioka et al., 2009; Yagi et al., 2007). Tcfap2c is already expressed at the eight-cell stage and also regulates *Cdx2* expression by binding to its enhancer (Cao et al., 2015). Cdx2 represses *Oct4* to maintain its own expression while Gata2/Gata3 act in parallel to ensure trophoblast development (Home et al., 2017; Ralston et al., 2010; Strumpf et al., 2005). The identity of the trophoblast lineage is locked in place through the epigenetic regulation of *Elf5*, the expression of which is reinforced by a positive-feedback loop involving Cdx2 and Eomes (Ng et al., 2008).

In human embryos, the timing and expression pattern of these TFs is different, suggesting other regulatory mechanisms are already in place from the earliest stage of TE specification. In mice, there is clear differential expression of *Oct4* and *Cdx2* in the ICM and TE, respectively, while in humans, lineage segregation between the ICM and TE occurs later in development; OCT4 is still expressed in the TE until day 6, whereas CDX2 is only expressed at the blastocyst stage (Blakeley et al., 2015; Boroviak et al., 2018; Niakan and Eggan, 2013). Some ICM cells even retain TE identity at day 7 (Petropoulos et al., 2016; Stirparo et al., 2018). Thus, there seems to be more plasticity in the human TE lineage leading up to implantation. In a pioneering study of gene editing of human embryos by CRISPR/Cas9, deletion of *OCT4* at the diploid zygote stage compromised the development of both the ICM and the TE, highlighting the molecular differences between humans and mice during these early fate decisions (Fogarty et al., 2017).

VCT and CCC formation

The molecular events occurring between implantation and villous formation in humans are obviously still a black box. Presumably, trophoblast populations arise from the polar TE as these are the cells that initially implant. VCT is the proliferative compartment of the placenta and expresses the trophoblast TFs found in mouse TSCs – GATA2, GATA3, TFAP2C/A and TEAD4 – but not EOMES (Mirkovic et al., 2015; Soncin et al., 2018). Indeed, four of these factors, GATA2, GATA3, TFAP2A and TFAP2C can induce cells purported to be of the trophoblast lineage in human pluripotent stem cells (Krendl et al., 2017). In humans, *ELF5*, the promoter of which is hypomethylated, is expressed in the VCT, similar to mouse (Hemberger et al., 2010; Lee et al., 2016a). Although the *ELF5* promoter remains hypomethylated in the EVT, no ELF5 protein is present so other mechanisms must regulate its expression. TP63, a TF that is characteristic of epithelial stem cells, is also widely expressed in the early first-trimester VCT all the way to term (Lee et al., 2007). A proportion of TP63+ cells close to the chorionic

plate also express CDX2 (Horii et al., 2016; Li et al., 2014). CDX2 expression is then downregulated in the VCT throughout the bulk of the placenta during the first trimester, apart from in those cells close to the chorionic plate, where residual expression is seen (Soncin et al., 2018). Notably, hTSCs lack CDX2 expression, questioning whether this is a marker of trophoblast stem cells in the human placenta.

The VCT also express a range of surface markers, such as EGFR, MET and specific members of the Wnt family (Jokhi et al., 1994; Kauma et al., 1997; Sonderegger et al., 2007), that distinguishes it from other trophoblast populations. The importance of these signalling pathways for VCT proliferation has been confirmed by the isolation of proliferative trophoblast (Haider et al., 2018; Okae et al., 2018; Turco et al., 2018). Proliferative cells are also present in CCCs early in the first trimester, and these gradually decrease in number and are mostly localised to the base of CCCs by ∼12 weeks. This 'generative niche' was noticed >50 years ago and more recently trophoblast cells in this location were shown to have a distinct transcriptome compared with the VCT and EVT, expressing Notch1, ITGA2 and CD31 (Haider et al., 2014; Lee et al., 2018; Pattillo et al., 1968). Notch1 expression becomes restricted to the base of the CCC by the end of the first trimester (Haider et al., 2016). Whether cells in this niche generate EVT alone or both EVT and VCT is unknown.

SCT differentiation

SCT covering the villi is terminally differentiated and SCT fragments are continuously shed into the maternal circulation. Placental explants, primary trophoblast cells that can fuse *in vitro* and the BeWo choriocarcinoma line have been used to study the process of SCT regeneration from the underlying VCT. These studies have revealed human endogenous retroviral proteins (HERVs), such as SYNCYTIN-1 (HERV-W env), as key regulators of this process (Mi et al., 2000). Retroviral elements are viral gene remnants that have been integrated into the human genome and co-opted as either promoters or enhancers and, in the case of placenta, encode retroviral proteins. Around 8% of the human genome contains HERVs (McPherson et al., 2001). The placenta is a particularly favourable site for HERV expression and different species have co-opted different retroviral families for the same function (Frendo et al., 2003a; Harris, 1991).

A key step in syncytialisation is the acquisition of fusion competence, which requires the VCT to exit the cell cycle. Whether the signal that triggers this event originates from the SCT or VCT, and whether the same process occurs after syncytial damage, is unknown. VCT cells upregulate fusion genes, and this is followed by disintegration of cell membranes and incorporation of the cytoplasm into the SCT. Using choriocarcinoma lines, a crucial event occurring during this process was identified as an increase in cyclic AMP through protein kinase A (PKA) signalling, which upregulates GCM1 expression and in turn induces expression of the fusion proteins syncytin 1 and syncytin 2 (Knerr et al., 2005; Liang et al., 2010). The final step in syncytialisation requires fusion of the cell membranes and intermingling of the cellular contents (Gerbaud and Pidoux, 2015).

Syncytins use specific receptors, ASCT2 (for syncytin 1) and MFSD2 (for syncytin 2), to allow fusion of the VCT to the SCT (Esnault et al., 2008; Hayward et al., 2007). ASCT2 is restricted to VCT while MFSD2 is expressed on the SCT. Furthermore, SCT produces hCG, which, after binding to the LH-CG receptor, induces cyclic AMP signalling and thereby promotes the upregulation of syncytial genes, as well as that of hCG (Pidoux et al., 2007; Shi

et al., 1993). Thus, the SCT itself reinforces the process in a positive-feedback loop. Activation of PKA signalling through hCG is also coupled to phosphorylation of connexin 43 (Cx43) through ezrin, which induces the opening of gap junctions and transfer of fusogenic signals between the VCT and SCT (Frendo et al., 2003b; Pidoux et al., 2014). Several other factors may also be involved in syncytialisation, such as the MAPK signalling pathway, which acts through EGF and EGFR, GM-CSF signalling and activin A signalling, which acts through SMAD4 (Garcia-Lloret et al., 1994; Gerbaud et al., 2011; Morrish et al., 1987). On the other hand, TGFβ produced by the SCT seems to provide a signal that balances the syncytialisation process by inhibiting fusion (Morrish et al., 1991). Notably, interferons induced by infection act to prevent infection of other neighbouring cells. One interferon-stimulated gene, IFITM3, inhibits syncytin-mediated fusion of trophoblast into SCT and this may cause pregnancy failure in infections such as CMV or rubella (Buchrieser et al., 2019).

EVT differentiation

Global transcriptomic studies show clear differences between the VCT and EVT (Apps et al., 2011; Lee et al., 2018; Tilburgs et al., 2015). When the EVT migrates out from the CCCs into the decidua, features characteristic of EMT are observed. These include: a loss of epithelial characteristics, including downregulation of E-cadherin and tight junction proteins such as ZO-1; a change in apical-basal polarity; a switch in integrin expression, from laminin-binding integrin α6β4 to fibronectin-binding integrins α1β1 and α1β5; an increase in cell size; and an accumulation of glycogen (Damsky et al., 1992, 1994; Marzioni et al., 2001). The EVT also upregulates endothelial markers such as the laminin α4 receptor MCAM, VE-cadherin and the metalloproteinases MMP2, MMP3 and MMP9 (Fisher, 1989; Xu et al., 2000; Zhou et al., 1997). A defining feature of EVT differentiation is upregulation of the non-classical MHC class I molecule HLA-G, as well as HLA-C (King et al., 1996, 2000b; Kovats et al., 1990). The expression of several growth factor receptors also changes: EGFR, MET (the receptor for HGF), prolactin receptor (PRLR) and the BMP receptor BMPR1A are downregulated, while ERBB2 is upregulated (Jokhi et al., 1994; Kauma et al., 1999; Ladines-Llave et al., 2013; Stefanoska et al., 2013; Tilburgs et al., 2015). All of these changes are accompanied by loss of proliferation, as assessed by Ki67 expression, and a change in expression of cell cycle regulators (Chan et al., 1999; Genbacev et al., 2000). EVT differentiation is also characterised by endoreduplication, resulting in tetraploid cells (Velicky et al., 2018).

EVT cells themselves produce several proteins and hormones: TGFβ1, follistatin and hyper-glycolsylated hCG. Activin A has a stimulatory effect on the differentiation on EVT differentiation in placental explants, in complete contrast to the mouse, where it maintains trophoblast stem cell proliferation (Caniggia et al., 1997; Erlebacher et al., 2004).

The endpoint of iEVT differentiation is fusion to placental bed giant cells. These large multinucleated cells produce human placental lactogen and are present deep in the decidua basalis and the inner one-third of the myometrium – the normal limit of iEVT infiltration (Al-Lamki et al., 1999; Boyd and Hamilton, 1970; Brosens et al., 1967; Kurman et al., 1984). The signals and TFs driving EVT differentiation into either eEVT or iEVT are unknown. The cells in the EVT plugs in the spiral arteries are morphologically very different from iEVT cells (Pijnenborg, 1996). Only two clear phenotypic differences have been defined so far: eEVT express CD56 (NCAM) and, because occasional CD56+ cells appear within the shell overlying the openings of the arteries, it is thought that

signals from maternal blood may stimulate eEVT differentiation (Burrows et al., 1994; Kam et al., 1999). In contrast, iEVT specifically express placenta-specific protein 8, PLAC8, which is known to be involved in EMT (Chang et al., 2018).

Owing to the presence of eEVT plugs in the spiral arteries, the conceptus is in a physiologically low-oxygen environment before ~10 weeks gestation; this is not be confused with hypoxia, which refers to a non-physiological state of insufficient oxygen. The metabolic consequences of this are a reliance on glycolysis, preservation of carbon skeletons used for synthesis of cellular components and protection from damage by free radicals (Burton et al., 2017). The subsequent dissolution of the eEVT plugs (at ~10 weeks) and the onset of the maternal circulation correlate with changes in oxygen levels, from 2.5% to around 8% (Jauniaux et al., 2000; Rodesch et al., 1992). The onset of circulation occurs more in the centre than at the periphery of the placentation site because of the more pronounced trophoblast invasion and arterial transformation centrally. The villi regress at the periphery, resulting in formation of the chorion laeve (fetal membranes composed of amnion and chorion superimposed on decidua parietalis) and the definitive placenta (Burton, 2009). Disturbance of this orderly process can result in miscarriage and abnormal placental membranes, predisposing to preterm labour and placental abruption (Burton and Jauniaux, 2017; Jauniaux et al., 2000). The hypoxic environment of the first few weeks of gestation might induce EVT differentiation through HIF1A; indeed, more HLA-G+ EVT are generated *in vitro* when cultured in 2% O_2 with upregulation of the imprinted bHLH factor ASCL2, a WNT target involved in EMT (Wakeland et al., 2017).

Regulation of placental development by the decidua

The human placenta develops within the endometrium, which is transformed into decidua during pregnancy under the influence of progesterone secreted by the corpus luteum (Gellersen and Brosens, 2014). The endometrium is highly dynamic and undergoes cyclical regeneration, differentiation and shedding during the menstrual cycle under the control of hormones of the ovarian-pituitary axis. In humans, features of decidualisation (pre-decidual change) are seen after the mid-secretory phase of the menstrual cycle and begin around the spiral arteries (Kurman, 2002). Following implantation, proper decidualisation of the endometrium then plays a key role in the development of the placenta and is likely to involve all the major cellular elements of the endometrium: glands, vessels, stromal cells and immune cells.

Endometrial glands and stromal cells

The endometrial glands play a key role in embryo implantation and development of the placenta in mice and domestic species (Spencer et al., 2019). Glands become hypersecretory in early pregnancy, exhibiting a characteristic appearance known as the Arias-Stella reaction (Arias-Stella, 1954). In humans, the conceptus depends on glandular secretions as the source of histotrophic nutrition during the early weeks of pregnancy, when the endovascular plugs only allow seepage of maternal blood into the intervillous space (Burton et al., 2002, 2007).

The stromal cells of the endometrium also produce a wide range of growth factors that stimulate the glands. As they decidualise, the stromal cells secrete a rim of basement membrane proteins, fibronectin and laminin, which provides a scaffold for the EVT to move through (Aplin et al., 1988). Exactly what defines the characteristics of a receptive decidualised endometrium that will support the developing placenta is still unclear (Koot et al., 2016;

Young, 2017). This is an important issue because evidence to suggest that defective decidualisation is an antecedent of pregnancy disorders is growing (Conrad et al., 2017; Garrido-Gomez et al., 2017). Maternal nutrition, extremes of reproductive life, low or high BMI, endocrine disorders (e.g. thyroid disease) and diabetes can all affect the cycling of a healthy endometrium but it is unclear how these affect decidualisation and embryo receptivity.

Uterine leukocytes and other immune cells

The dominant immune cells in the first trimester are a type of innate lymphoid cell known as uterine natural killer (uNK) cells (Bulmer and Lash, 2015; Moffett and Colucci, 2015; Vacca et al., 2019). These cells make up ~70% of immune cells in the uterine environment, while macrophages account for ~20% and T cells for ~10%. B cells and mast cells are virtually absent, and neutrophils are also sparse (Vento-Tormo et al., 2018). This is therefore an unusual immune environment where cells of the innate rather than the adaptive (T and B cells) immune system predominate. Our recent scRNA-seq study of first trimester decidual cells defined three sub-populations of uNK cells, all with specific immunomodulatory signatures and expressing a range of receptors for the EVT (Vento-Tormo et al., 2018). Their functional roles await investigation.

As mentioned, EVT cells have been compared to tumour cells due to their invasive properties. However, unlike tumour cells, the behaviour of trophoblast cells within the decidual microenvironment is controlled. Accordingly, necrosis of the decidua is not seen as the EVT migrates deep into the tissue, apart from in the enigmatic Nitabuch's layer, a thin rim of fibrinoid tissue subjacent to the shell at the maternal-fetal boundary (Boyd and Hamilton, 1970). Furthermore, although placentation is often considered as an inflammatory process (Chavan et al., 2017), the defining classical features of inflammation (neutrophil infiltration followed by granulation tissue, inflammatory cells, capillary angiogenesis and fibrosis) are always absent. Our scRNA-seq study predicts several mechanisms that could explain why inflammatory or adaptive immune responses are less likely to occur in this specialised environment (Vento-Tormo et al., 2018).

Abnormal placental development and pregnancy complications

Many complications of pregnancy have their origins in abnormal development of the placenta in the first trimester (Smith, 2010). These include pre-eclampsia, fetal growth restriction (FGR), unexplained stillbirth, placental abruption and preterm labour; these complications are known collectively as the great obstetric syndromes (GOSs) (Brosens et al., 2011). These conditions are responsible for a high proportion of maternal and neonatal morbidity and mortality seen in all populations, but particularly in sub-Saharan Africa (Graham et al., 2016).

Defective trophoblast invasion is the ultimate cause of the GOSs. Trophoblast cells invade into the decidua to gain access to the maternal blood supply and successful EVT transformation of ~30-40 spiral arteries deep into the myometrium is essential for normal fetal growth and development (Burton et al., 2009; Collins et al., 2012). If the arteries are not sufficiently converted and retain their contractile media, there is disordered perfusion of blood flow into the intervillous space. This, together with an inadequate supply of nutrients and oxygen, reduces the progressive branching of the villous tree as gestation proceeds, reducing the surface area available for exchange, with the possible outcome of FGR and stillbirth. In addition, if the process of regression of the chorion frondiosum to
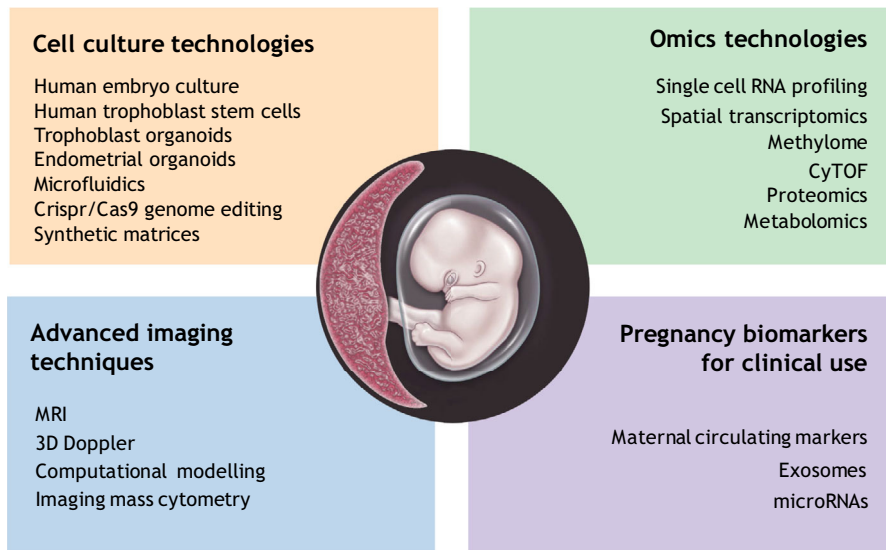
Fig. 3. Emerging technologies for the study of human trophoblast biology and placental development. New techniques that can be applied to investigate the fundamental biology of human placentation are shown. These include various cell culture technologies, omics techniques, advanced imaging approaches and analyses of biomarkers for clinical use.

form the chorion laeve does not occur correctly, the chorionic membranes can separate prematurely, resulting in placental abruption or preterm labour. Pre-eclampsia results from the release of products from the poorly perfused and stressed placenta into the maternal circulation, triggering a systemic endothelial disorder (Burton et al., 2019; Roberts and Redman, 1993). Thus, the exact clinical outcome of defective trophoblast invasion depends on the extent of arterial invasion and the number of arteries invaded.

Because deep trophoblast invasion into the uterus is a feature seen only in humans and great apes, defective transformation of arteries has been hard to characterise and hence diagnose early in pregnancy. A number of clinical measurements are being developed to overcome this, such as uterine artery Doppler velocimetry, which measures the resistance to blood flow and is thus an indirect readout of the degree of spiral artery remodelling (O'Gorman et al., 2017). As the EVT moves deeper, expression of pregnancy-associated plasma protein-A (PAPPA-A) increases, and measurement of this protein in maternal serum in the first trimester is a useful predictor of a GOS (Gaccioli et al., 2018). The ratio of soluble fms-like tyrosine kinase 1 (Sflt1) to placental growth factor (PlGF) is also elevated in women before they develop the clinical symptoms of pre-eclampsia (Zeisler et al., 2016); as such, a low ratio can be used to predict the women who will not go on to develop the syndrome in the following week. Another approach is to screen maternal blood for biomarkers that reflect placental function. These include miRNAs, exosomes, free DNA, proteins and short non-coding RNAs (Barchitta et al., 2017; Gaccioli et al., 2018; Rolnik et al., 2018; Tsang et al., 2017; Yoffe et al., 2018). The benefits of these screening tests in low risk populations are still not obvious, particularly as the only intervention possible at present is early delivery with obvious risk to the neonate; moreover, some reports suggest that these screening tests may actually be harmful (Monier et al., 2015).

As defective trophoblast invasion is the ultimate cause of the GOS, it is important to understand how EVT invasion into the uterus is regulated. A role for decidua in preventing placental cells from invading too far is clear from clinical reports where the placenta implants on a site where decidua is deficient or absent (Jauniaux et al., 2018). This can occur in the lower segment of the uterus close to the cervix or over a caesarean section scar from a previous pregnancy. In these situations, the EVT penetrates into the myometrium and destroys the smooth muscle cells with a similar 'fibrinoid' appearance to that seen when the trophoblast transforms the spiral arterial media. Furthermore, fusion to placental bed giant cells, which is normally observed at the end of EVT migration, is drastically reduced (Hannon et al., 2012). Together, these findings highlight that the territorial boundary between placenta and mother needs to be finely controlled and that this balancing act is mediated by the decidua.

Memory and specificity are features of immune responses. These have resonance in pre-eclampsia and point to a role of the immune system in its pathogenesis (Wikström et al., 2012). Pre-eclampsia exhibit some level of memory, whereby immune cells 'remember' encounters with specific pathogens and subsequently respond differently to the same pathogen (Sun et al., 2014). Indeed, the incidence of pre-eclampsia is highest in first pregnancies and then falls if the second pregnancy is with the same partner. There are also partner-specific effects on pre-eclampsia, as seen in pregnancies in women who change partners (Wikström et al., 2012). These epidemiological findings led us to explore the interactions between KIR and their HLA-C ligands in uNKs. This study revealed that both the maternal KIR and fetal HLA-C ligands are highly polymorphic, and particular KIR/HLA-C combinations are found in pregnancies complicated by pre-eclampsia and FGR (Moffett and Colucci, 2015). Exactly how these genetic findings translate into functions of uNK cells is still unknown; the uNK cells might affect EVT invasion directly or act indirectly on the arteries or glands.

Conclusions and future directions

Although much has been learnt recently, there are still many questions to answer about human placental development: the specification of the TE lineage, the identity of TSCs, differentiation into the two main lineages and the impact of the maternal environment on both the VCT and EVT. Although recent studies have revealed specific and unique features of human trophoblast and the decidual microenvironment, it is clear that the development of the human placenta is complex and challenging to study. However, new techniques are now being used to study human placental development to overcome these challenges. These include scRNA-seq, spatial transcriptomics, epigenetics, miRNA expression, advanced imaging techniques and organoid cultures (Fig. 3). This is therefore an exciting time for placental research as we begin to address the fundamental issue of how the human placenta develops in normal and abnormal pregnancies.

DEVELOPMENT

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