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GRAND THEME REVIEW

New era of trophoblast research: integrating morphological and molecular approaches

Shingo Io [1,2,3,*], Eiji Kondoh[2], Yoshitsugu Chigusa[2], Kaoru Kawasaki[2], Masaki Mandai[2], and Shigehito Yamada[4,5]

[1]Department of Life Science Frontiers, Center for iPS Cell Research & Application, Kyoto University, Kyoto, Japan [2]Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine, Kyoto, Japan [3]Japan Society for the Promotion of Science, Tokyo, Japan [4]Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan [5]Congenital Anomaly Research Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

*Correspondence address. Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel: +81-75-751-3269; Fax: +81-75-761-3967; E-mail: shinio@kuhp.kyoto-u.ac.jp
orcid.org/0000-0001-8769-134X

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ABSTRACT: Many pregnancy complications are the result of dysfunction in the placenta. The pathogenic mechanisms of placenta-mediated pregnancy complications, however, are unclear. Abnormal placental development in these conditions begins in the first trimester, but no symptoms are observed during this period. To elucidate effective preventative treatments, understanding the differentiation and development of human placenta is crucial. This review elucidates the uniqueness of the human placenta in early development from the aspect of structural characteristics and molecular markers. We summarise the morphogenesis of human placenta based on human specimens and then compile molecular markers that have been clarified by immunostaining and RNA-sequencing data across species. Relevant studies were identified using the PubMed database and Google Scholar search engines up to March 2020. All articles were independently screened for eligibility by the authors based on titles and abstracts. In particular, the authors carefully examined literature on human placentation. This review integrates the development of human placentation from morphological approaches in comparison with other species and provides new insights into trophoblast molecular markers. The morphological features of human early placentation are described in Carnegie stages (CS), from CS3 (floating blastocyst) to CS9 (emerging point of tertiary villi). Molecular markers are described for each type of trophoblast involved in human placental development. We summarise the character of human trophoblast cell lines and explain how long-term culture system of human cytotrophoblast, both monolayer and spheroid, established in recent studies allows for the generation of human trophoblast cell lines. Due to differences in developmental features among species, it is desirable to understand early placentation in humans. In addition, reliable molecular markers that reflect normal human trophoblast are needed to advance trophoblast research. In the clinical setting, these markers can be valuable means for morphologically and functionally assessing placenta-mediated pregnancy complications and provide early prediction and management of these diseases.

Key words: Carnegie stages / cell surface markers / early pregnancy / embryogenesis / extra-embryonic tissue / gene expression / trophoblast / trophoblast differentiation / placenta / placental development

Introduction

The placenta plays a vital role in the development of the foetus and in the maintenance of pregnancy, including pregnancy-specific hormone production, nutrient transfer, gas exchange and immunotolerance. Abnormalities in the trophoblast, the functional cell type of the placenta, can cause inadequate placentation, leading to preeclampsia, the birth of small-for-gestational age neonates, placental abruption and late pregnancy loss. These placenta-mediated pregnancy complications are estimated to occur in 15% of pregnancies and are associated with the morbidity and mortality of the mother and foetus (Wilcox *et al.*, 1988; Wang *et al.*, 2003; Froen *et al.*, 2004; Kuklina *et al.*, 2009; Damodaram *et al.*, 2011; Larsen *et al.*, 2013; Lees *et al.*, 2013; Rodger *et al.*, 2016; Hiersch *et al.*, 2017; Skeith and Rodger, 2017). However, the pathogenic mechanisms of these diseases remain unclear, and there are no effective treatments for placental dysfunction at the present time.

Placental dysfunction begins in the first trimester of pregnancy, before symptom occurrence (Burton and Jauniaux, 2004; Redman, 2014; Khong *et al.*, 2015). In order to elucidate the pathogenesis of various pregnancy complications, it is important to understand the development of normal placenta. Placental development has been mainly described based on mouse embryogenesis and mouse trophoblast stem cells, but human placenta has unique morphology and trophoblast differentiation. This review elucidates this uniqueness from the aspect of structural development and molecular markers.

Cellular differentiation of the placenta

Cellularly, the placenta is primarily constituted of trophoblasts, but also includes stroma cells, macrophages and foetal endothelial cells (FECs). In this review, we focus on trophoblasts, which are responsible for the main function of the placenta. Trophoblast lineage, which describes the developmental sequence of the placenta, consists of trophectoderm (TE), cytotrophoblast (CT), syncytiotrophoblast (ST) and extravillous trophoblast (EVT; also called intermediate trophoblast) (Fig. 1A) (Lee *et al.*, 2016). These cell types are morphologically and functionally distinct. The TE, which is the outermost layer of the blastocyst (Fig. 2A), is the precursor of all trophoblasts. It contributes largely to implantation. The CT is a uninuclear cell that is mitotically active and the progenitor of ST and EVT. The ST is a multinucleated cell that is generated from the fusion of uninuclear CT and is mitotically inactive. The ST serves as a place for nutrient transport, gas exchange and pregnancy hormone secretion. The microvilli on the surface of ST increase the efficiency of nutrient and gas exchange (Teasdale and Jean-Jacques, 1985). The EVT, also a uninuclear cell, is involved in the remodelling of maternal spiral arteries depending on the blood supply.

Human placental development in Carnegie stages

The differentiation and histological maturation of placental cells occur over a fixed time course. Morphological changes of human embryo development are described using Carnegie stages (CS) (O'Rahilly and Müller, 1987). CS can also be applied to human placental development, especially the earlier stages of human embryo development, such as CS5a-c. Here we review early CS and the foetal period (Fig. 2).

Carnegie stages 1 and 2 (Days 1–3)

The zygote starts from one cell fertilised oocyte. Cleavage begins, and finally the morula is formed. Differentiation into the placental lineage has not started yet.

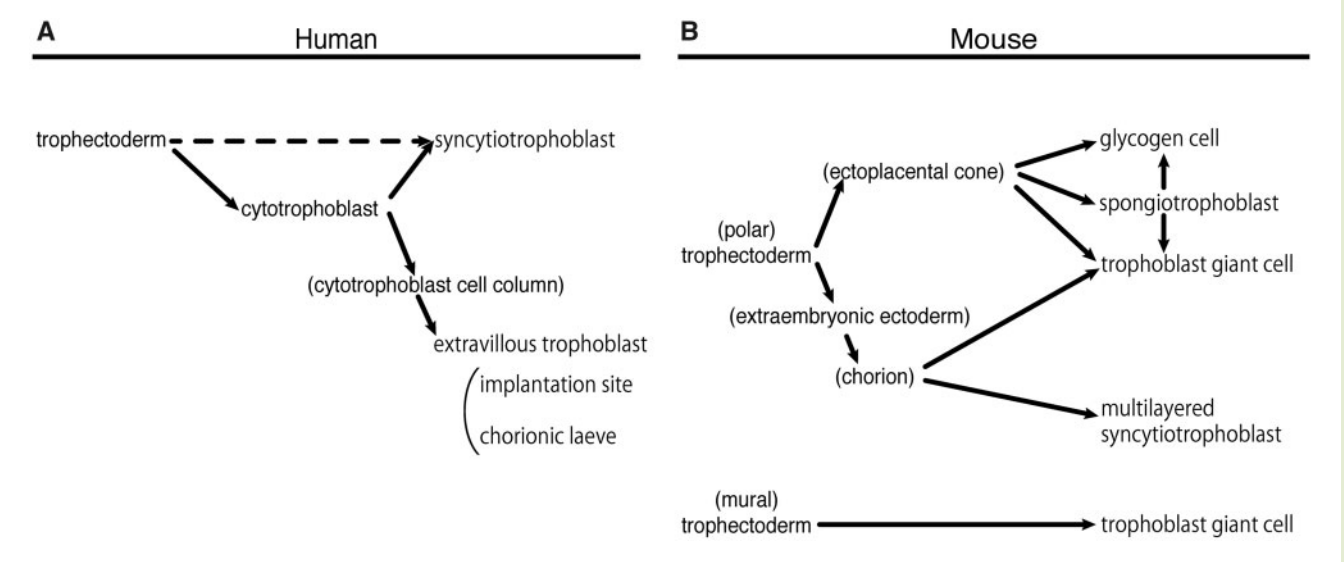

Figure 1. Flow chart of trophoblast cell lineage from trophoblast origin to terminal differentiation between human and mouse. (**A**) Human. TE, the origin of all trophoblasts, differentiates into cytotrophoblast. Cytotrophoblasts, which have high-proliferation capacity, can differentiate into syncytiotrophoblasts and extravillous trophoblasts (EVT). EVTs are mainly categorised by location, such as implantation site EVT and chorionic laeve EVT. Whether trophectoderm differentiates into primitive syncytiotrophoblast though the stage of cytotrophoblast is controversial. The dashed lines indicate the differentiation pathway from trophectoderm to primitive syncytiotrophoblast. (**B**) Mouse. Polar trophectoderm differentiates into the main part of mouse placenta. Spongiotrophoblast is the putative mouse counterpart of human cytotrophoblast in cytotrophoblast cell columns. Spongiotrophoblast differentiates into both mouse TGCs and glycogen cells (glycogen trophoblasts), which are analogous to human EVT. Mural trophectoderm stops proliferating and forms TGCs.

Carnegie stage 3 (Days 4–6)

CS3 describes the blastocyst stage. The blastocyst is still enclosed in the zone pellucida, where it floats freely and 'hatches' from this surrounding extracellular matrix before implantation. At the early blastocyst stage, two cell groups, the inner cell mass (ICM) and outer cell layer, or TE, are visible. At the late blastocyst stage (expanded blastocyst), the ICM segregates into epiblast and primitive endoderm (hypoblast).

Carnegie stage 4 (Days 6–7)

At CS4, the blastocyst is anchored to the endometrium, and interaction between the embryo and maternal tissue begins (Fig. 2A) (Hill, 2020). The TE, which is the source of all trophoblast subtypes, shows a distinct polarisation. The TE plays an important role in embryo implantation and interaction with the maternal endometrium.

Carnegie stage 5 (Days 7–12)

At CS5, implantation is complete, and development of the trophoblast progresses. Based on the original classification of CS, CS5 is further subdivided into stages 5a, 5b and 5c based on trophoblast differentiation.

Carnegie stage 5a (Days 7–8)

At CS5a, the trophoblast exists as a mass of cells and is called solid trophoblast (Fig. 2B). Two distinct layers of cells (bilaminar germ disc), epiblast and primitive endoderm, appear. The amniotic cavity appears between the epiblast and TE and the yolk sac appears between primitive endoderm and TE.

Carnegie stage 5b (Days 9–10)

At CS5b, the trophoblast has two morphologically and functionally distinct layers of cells; the inner layer (i.e. CT) and outer layer (i.e. ST) (Fig. 2C). Lacunae appear in primitive ST, in which small vacuoles fuse with each other, thus leading to CS5b also being called 'lacunar stage' or 'primitive syncytium' (Fig. 2C) (Hertig *et al.*, 1956; Boyd and Hamilton, 1970; James *et al.*, 2012). Tissue specimens at the time of implantation indicate that CT and ST emerge concurrently (O'Rahilly and Müller, 1987), however, whether TE differentiates into primitive ST through a CT stage is controversial (Boyd and Hamilton, 1970; Knofler and Pollheimer, 2013).

Carnegie stage 5c (Days 11–12)

In CS5c, the primitive ST continues to penetrate into the endometrium, maternal capillaries connect with lacunae in the primitive ST, and maternal bloods flow into the lacunae. The ST forms about three quarters of the total trophoblastic shell, and the CT constitutes the remaining one-quarter (O'Rahilly and Müller, 1987).

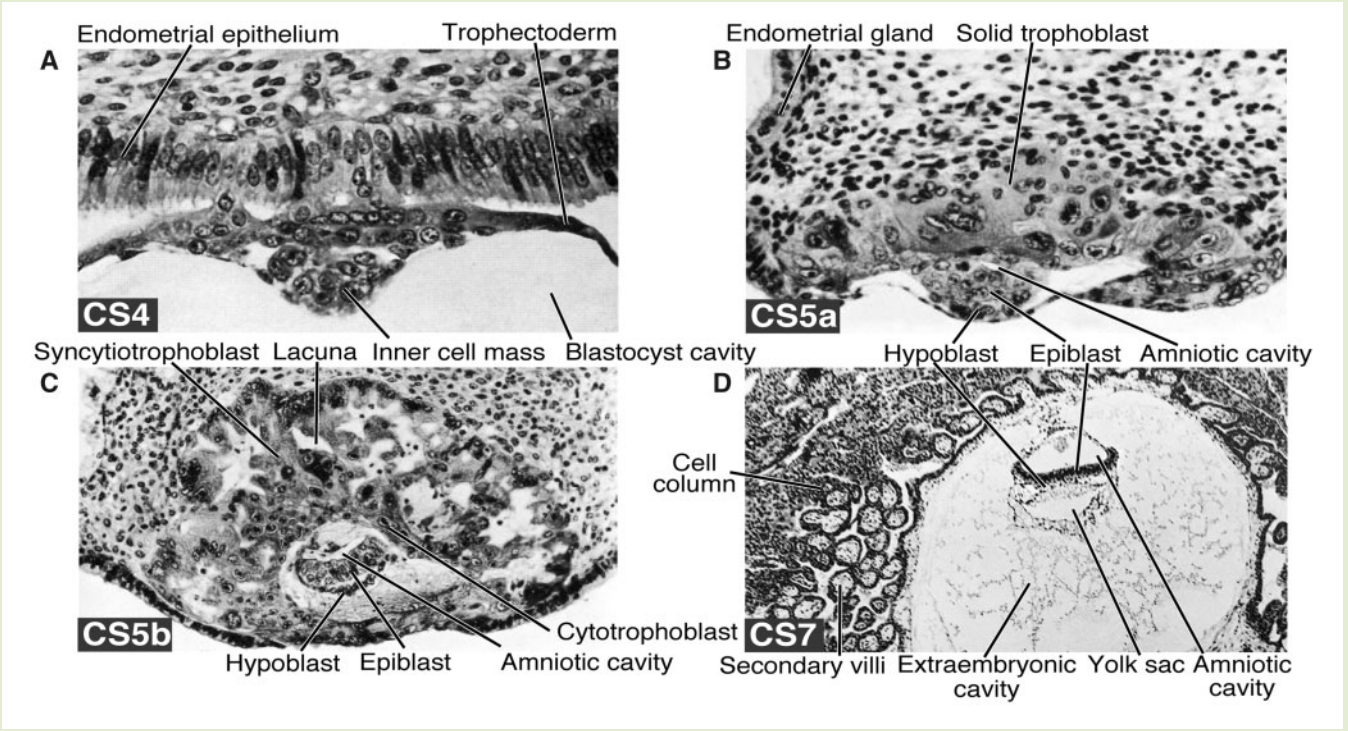

Figure 2. Early development of human placenta based on Carnegie stages. (**A**) Carnegie stage 4 (embryonic Days 6–7). Blastocyst is anchored on the endometrium. (**B**) Carnegie stage 5a (embryonic Days 7–8). Blastocyst invades the endometrium, and trophectoderm becomes solid trophoblast. In addition, the bilaminar germ disc and amniotic cavity appear. (**C**) Carnegie stage 5b (embryonic Days 9–10). There are two distinct layers of trophoblast: cytotrophoblast layer and syncytiotrophoblast layer. (**D**) Carnegie stage 7 (embryonic Days 15–17). Secondary villi consist of cytotrophoblast, syncytiotrophoblast and extra-embryonic mesoderm. Images published from https://embryology.med.unsw.edu.au with permission from Dr. Mark Anthony Hill (Hill, 2020).

Carnegie stage 6 (Days 13–14)

CS6 marks the first stage of chorionic villi development, when the CT grows externally and penetrates into the primitive ST to form cellular columns. The columns are called primary villi and have two cellular layers (Fig. 3A); the outer layer is ST, and the inner layer is CT. The embryonic body at this stage is now about 0.2 mm diameter in size. Furthermore, extra-embryonic cavities coalesce to form the chorionic cavity.

Carnegie stage 7 (Days 15–17)

At the second stage of chorionic villi development, i.e. CS7, extra-embryonic mesoderm proliferates and penetrates into the core of the primary villi. This structure, in which extra-embryonic mesoderm is surrounded by CT and ST, is called the 'secondary villi' (Figs 2D, 3B). The embryonic body is now about 0.4 mm in size at the diameter. Laterality is established, gastrulation starts and germ layers are formed from the epiblast.

Carnegie stages 8 and 9 (Days 18–21) and later embryonic stages

Extra-embryonic mesoderm in the secondary villi differentiates, and blood cells and vessels are developed. The vessels of the villi connect with the vessels of the embryonic body, and the maternal-placental (uteroplacental) blood circulation begins. This structure is called the 'tertiary villi' and is completed by the end of Week 8 after fertilisation (Fig. 3C). The third stage of chorionic villi development (from Weeks 3 to 8 after fertilisation) is quite long; the embryonic body has three germ layers and organogenesis begins. Furthermore, at the end of Week 8 after fertilisation, the tertiary villi have been completed, and organogenesis has ended.

Cellular differentiation and structural development of the placenta occur during the early embryonic stages, and CS5b-9 is important for these phenomena (O'Rahilly and Müller, 1987). They are not only the periods of organogenesis in the embryonic body, but also the periods of placental differentiation. It is very difficult to obtain samples of embryo and placental specimens, thus, most direct observations of CS depend on old serial sections from a few collections of human embryos.

The foetal embryonic stage

At Week 8 after fertilisation, when the tertiary villi have developed, EVT emerges at the front edge of the villi. In other words, EVT is differentiated from CT cell columns. EVT forms plugs in the maternal spiral arteries to occlude these arteries and block maternal blood from

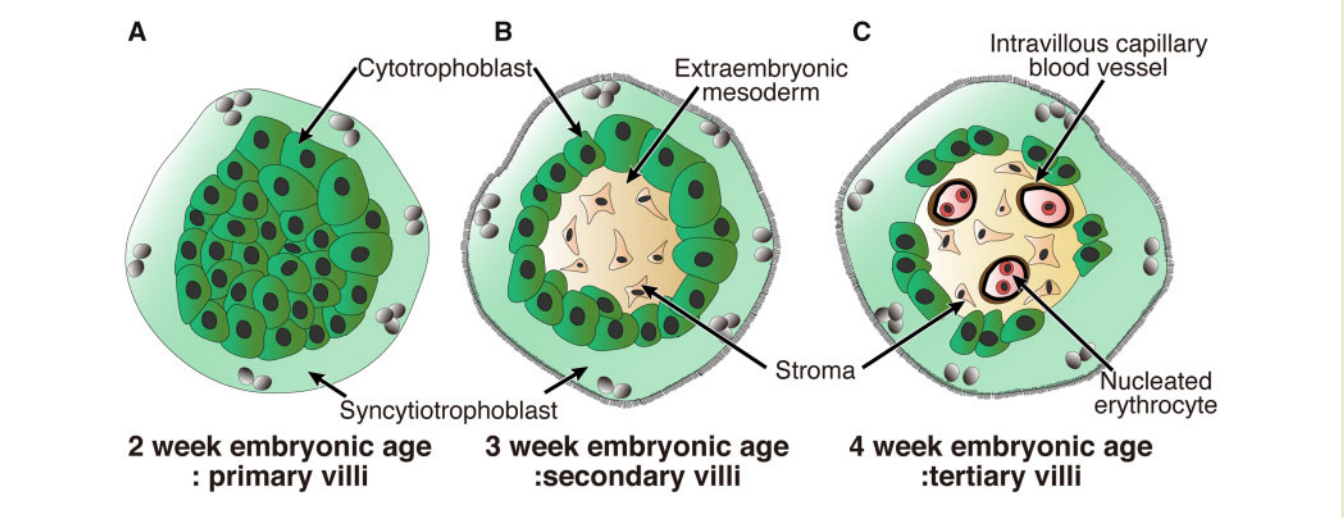

Figure 3. Structure of chorionic villi. The development of chorionic villi proceeds through three stages: primary, secondary and tertiary villi. The stages are defined by the number of layers and composite cell type. (**A**) A transverse section of primary chorionic villi. The villi consist of a cytotrophoblast core and syncytiotrophoblast. (**B**) A transverse section of secondary chorionic villi. Extra-embryonic mesoderm grows into the core of the villi. (**C**) A transverse section of tertiary chorionic villi. Extra-embryonic mesoderm differentiates into red blood cells and small blood vessels, forming the villous capillary system.

flowing into intervillous spaces, although whether the plugs obstruct these arteries completely is controversial (Kurjak and Kupesic, 1996; Valentin *et al.*, 1996; Weiss *et al.*, 2016; Roberts *et al.*, 2017). The oxygen tension of the intervillous space is at a low concentration at 6–8 weeks after fertilisation, otherwise known as the plugged stage (Rodesch *et al.*, 1992; James *et al.*, 2006). At Week 12 after fertilisation, the EVT plugs dissolve, and the oxygen tension changes from low to normal oxygen concentration (Hustin *et al.*, 1988; Jauniaux *et al.*, 1991; Rodesch et al,. 1992; Coppens *et al.*, 1996; James *et al.*, 2006a). Accordingly, low oxygen tension stimulates CT proliferation, and normal oxygen tension stimulates CT differentiation into ST and EVT (Genbacev *et al.*, 1997; James *et al.*, 2006b; Tuuli *et al.*, 2011). Recently, it was reported that low oxygen tension promotes immature EVT differentiation (Wakeland *et al.*, 2017; Chang *et al.*, 2018; Treissman *et al.*, 2020); therefore, the role of oxygen concentration in EVT differentiation needs to be examined in detail. EVT replaces arterial endothelial and vascular smooth muscles, and this remodelling transforms maternal spiral arteries into low-resistance, high-flow conduits. Consequently, the intervillous space is filled with high amounts of maternal blood (Pijnenborg *et al.*, 2006).

The ST forms the outer layer of the chorionic villi that contacts directly with the maternal vascular space and functions to exchange gases and metabolites between the mother and foetus (Benirschke *et al.*, 2000; Lee *et al.*, 2016). The other main function of ST is to produce most pregnancy-specific proteins and hormones. CT and ST are located on the villi; therefore, these cell types are called villous trophoblasts. In contrast, EVT infiltrates the decidua, myometrium and maternal spinal arteries. The functions of the EVT are to anchor the foetus to the uterine wall, maximise oxygen delivery and establish the maternal–foetal interface (Graham and Lala, 1992; Benirschke *et al.*, 2012b; Lee *et al.*, 2016). The EVT is largely composed of uninuclear cells (Kurman *et al.*, 1984) with various morphologies including round, polyhedral or spindle shape (Kaufmann and Castellucci, 1997; Gersell and Kraus, 2011). In humans, the placenta is mainly constituted of CT, ST and EVT (Fig. 1A).

Human placental development with molecular markers

The anatomical boundary of the placenta has not been defined well, which has motivated a search for new molecular markers. The placenta includes cells that originate from TE and ICM and ultimately from the fertilised oocyte. The TE gives rise to all placental epithelium (CT, ST and EVT), whereas the ICM gives rise to stroma cells (placental connective tissue cell), Hofbauer cells (placental villous macrophage) and FECs. Whether the placenta is also comprised of maternal-derived cells is controversial. We therefore exclude maternal-derived cells, such as decidua, decidual natural killer cells, dendritic cells, decidual macrophages, maternal endothelial cells and epithelial glandular cells, from our definition in this review (Fig. 4A). Details of each trophoblast subtype are described in the sections 'Cellular differentiation of the placenta' and 'Human placental development in Carnegie stages' above.

Trophoblasts can be identified in accordance with their anatomical location, cellular morphology and expression of particular markers *in vivo* (Hsi *et al.*, 1991). On the other hand, stage-specific molecular markers are very important for the characterisation because there is no spatial information *in vitro*. Below we summarise cellular stage-

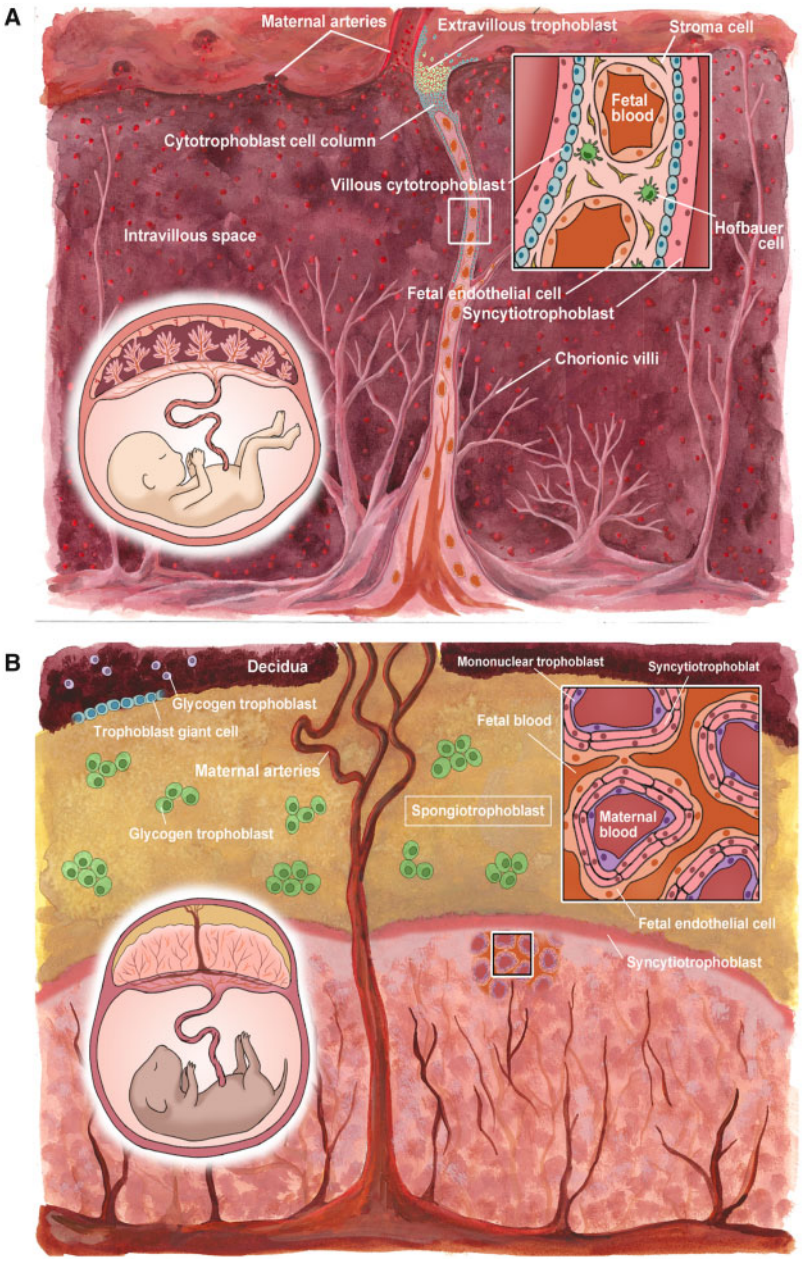

Figure 4. Illustration of human and mouse placental structures. (**A**) Human placental structures (villous type). Chorionic villi consist of a mesenchymal core, inner layer of cytotrophoblast and outer layer of syncytiotrophoblast. The chorionic villi protrude into the intervillous space and are bathed directly in maternal blood. Syncytiotrophoblast-derived extracellular vesicles and pregnancy-specific hormones are released directly into the maternal circulation. Hofbauer cells are macrophages in the mesenchymal core of chorionic villi and these often localise close to foetal endothelial cells and villous cytotrophoblasts. Cytotrophoblasts are located in cell columns and differentiate into extravillous trophoblasts (EVT). EVT invade into the maternal decidua and maternal spiral arteries. (**B**) Mouse placental structures (labyrinth type). The polar trophectoderm gives rise to the ectoplacental cone and extra-embryonic ectoderm. Mouse trophoblast stem cells can be derived from either polar trophectoderm or extra-embryonic ectoderm. The precursors for multilayered syncytiotrophoblasts and TGCs reside within the extra-embryonic ectoderm. In addition, precursors within the ectoplacental cone differentiate into spongiotrophoblasts (SpTs), TGCs and glycogen cells (glycogen trophoblasts). SpTs are located in the junctional zone, and TGCs exist at the borderline between the maternal decidua and SpT layer. Glycogen cells exist in the SpT layer and maternal decidua. In the mouse, there are three layers of trophoblasts between the maternal and foetal blood: the trichorial trophoblast layers consist of two syncytial layers and a single mononuclear layer; the mononuclear layer is in proximity to the maternal blood. Adjacent to the mononuclear trophoblast layer are two layers of syncytiotrophoblasts which are in contact with the foetal blood.

specific molecular markers based on comprehensive immunostaining, microarray and RNA-sequencing analyses (Table I).

Pan-trophoblast

GATA3, TFAP2C and KRT7 are the consensus markers of 'first trimester trophoblasts' *in vivo* and *in vitro* (Lee *et al.*, 2016). Importantly, Lee *et al.* confirmed the above consensus markers with post-implantation placenta, which assures GATA3, TFAP2C and KRT7 are expressed in CT, ST and EVT (Table I) (Lee *et al.*, 2016; Liu *et al.*, 2018; Okae *et al.*, 2018; Vento-Tormo *et al.*, 2018). Therefore, CT, ST and EVT can be distinguished using these consensus markers in combination with other markers. Overall, Lee *et al.* summarised the criteria of trophoblast as (i) the demethylation of ELF5 promoter, (ii) protein expression of GATA3, TFAP2C and KRT7, (iii) negative expression of HLA-A and HLA-B and (iv) high expression of microRNA in the chromosome 19 microRNA cluster (C19MC).

In addition to the consensus markers described above, GATA2 and KRT19 are persistently expressed markers for TE, CT, ST and EVT (Table I) (Shorter *et al.*, 1993; Aghajanova *et al.*, 2012; Assou *et al.*, 2012; Yan *et al.*, 2013; Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016; Liu *et al.* 2018; Okae *et al.*, 2018; Stirparo *et al.*, 2018). GATA3 and TFAP2C are also consensus markers and expressed in pre-implantation trophoblast (Yan *et al.*, 2013; Blakeley *et al.*, 2015; Nakamura *et al.*, 2016; Petropoulos *et al.*, 2016; Deglincerti *et al.*, 2016a; Stirparo *et al.*, 2018). Therefore, these four genes mark trophoblast in the first trimester (gestational Weeks 1–12).

On the other hand, KRT7 expression is controversial in human TE. KRT7 expression was observed in human TE with immunostaining (Niakan and Eggan, 2013), but single-cell RNA-sequencing (scRNA-seq) data of pre-implantation embryo revealed TE expresses KRT7 at the same low level as epiblast and primitive endoderm (Yan *et al.*, 2013; Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016; Stirparo *et al.*, 2018). Peri-implantation *in vitro* culture of human embryo demonstrated KRT7 expression in CT just after implantation (Table I) (Shahbazi *et al.*, 2016; Deglincerti *et al.*, 2016a). scRNA-seq data during peri-implantation *in vivo* have not been published, because specimens for this stage are rare. For these reasons, in this review, we assume KRT7 is expressed just after implantation.

Negative molecular markers are also useful. HLA-A, HLA-B, THY1 (CD90) and VIM are non-trophoblast markers (Shorter *et al.*, 1993; Yan *et al.*, 2013; Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016; Liu *et al.*, 2018; Okae *et al.*, 2018; Stirparo *et al.*, 2018). In addition to these positive and negative markers, evaluating trophoblast cell subtypes in combination with specific molecular markers is recommended.

Trophectoderm

TE is trophoblast during the pre-implantation stage. Cdx2 is specifically expressed in mouse TE and is an important cell lineage determinant between mouse ICM and TE (Niwa *et al.*, 2005). Previous reports also used this gene as a TE marker in human (Table I) (Deglincerti *et al.*, 2016b; Shao *et al.*, 2017; Iwasawa *et al.*, 2019). However, other reports showed that CDX2 is expressed only partially in human TE (Chen *et al.*, 2009; Niakan and Eggan, 2013; Petropoulos *et al.*, 2016; Stirparo *et al.*, 2018), and CDX2 positive cells were contained in first-trimester placenta as residual TE (Hemberger *et al.*, 2010; Horii *et al.*, 2016; Haider *et al.*, 2018; Soncin *et al.*, 2018). To date, there are no specific markers of human TE.

Cytotrophoblast

For the assessment of CT, we can use multiple consensus markers of trophoblast. The identification of CT-specific markers can distinguish CT from other cell types *in vitro* or *in vivo*. CT expresses TP63, VGLL1 and ITGA6 (CD49f) specifically (Table I) (Damsky *et al.*, 1992; Shih and Kurman, 2004; Kalhor *et al.*, 2009; Knofler and Pollheimer, 2013; Okae *et al.*, 2018; Soncin *et al.*, 2018). ITGA6 is a cell surface protein that constitutes the principal adhesion receptors for laminin. Stem cell niches have abundant laminin, and ITGA6 plays a role in stemness (cell proliferation and self-renewal) (Krebsbach and Villa-Diaz, 2017). Reflecting the proliferative capacity of CT, a subset of CT expresses Ki67 (MKI67) (Haider *et al.*, 2018; Lee *et al.*, 2018), the positive ratio of which decreases with passing gestational weeks (Arnholdt *et al.*, 1991).

Syncytiotrophoblast

Lee's consensus markers of trophoblast, such as GATA3, TFAP2C and KRT7, are all expressed in ST (Liu *et al.*, 2018; Okae *et al.*, 2018), although they also are considered CT and EVT markers (Lee *et al.*, 2016). On the other hand, the representative cell surface marker of ST is SDC1 (CD138) (Jokimaa *et al.*, 1998; Okae *et al.*, 2018). A combination of these four markers and ST-specific markers (Table I) may provide a more robust definition of ST *in vitro* and *in vivo*. ST cells are mitotically inactive (Ki67 negative) (Turco *et al.*, 2018) and secrete placental specific hormones such as hCG (human chorionic gonadotropin), hPL (human placental lactogen) and PSG (pregnancy-specific glycoprotein) into the maternal systemic circulation. The fusion of CT to make ST is initiated by the upregulation of ERVW-1 (Syncytin-1) (Mi *et al.*, 2000; Yu *et al.*, 2002; Frendo *et al.*, 2004).

Extravillous trophoblast

EVT also expresses the consensus markers GATA3, TFAP2C and KRT7 (Lee *et al.*, 2016; Liu *et al.*, 2018; Okae *et al.*, 2018), and HLA-G is widely accepted as a specific cell surface marker of mature EVT (Table I) (Heap *et al.*, 1988; Tarrade *et al.*, 2001; Shih and Kurman, 2004; Mao *et al.*, 2007; Kalhor *et al.*, 2009; Benirschke *et al.*, 2012c; Knofler and Pollheimer, 2013; Liu *et al.*, 2018; Okae *et al.*, 2018). Although CT and ST express neither Class I (HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-G) nor Class II (HLA-DR, HLA-DQ and HLA-DP) HLA on their surfaces, EVT expresses HLA-C and HLA-E Class I molecules (Loke, 1989). HLA-C and HLA-G binds maternal killer immunoglobulin-like receptors and leukocyte immunoglobulin-like receptors, respectively (Sharkey *et al.*, 2008; Parham and Moffett, 2013; Vento-Tormo *et al.*, 2018), therefore, these HLA ligands facilitate immune tolerance (Hunt *et al.*, 2005). ITGA5 is a cell surface protein that constitutes the principal adhesion receptors for fibronectin, which is one of the main constituents of the extracellular matrix near

Table I Characterisation of human first-trimester trophoblast stage-specific markers with immunostaining and RNA-sequencing data.

	Trophecto-derm	Cytotropho-blast	Syncytiotroph-oblast	Extravillous trophoblast		Reference (immunostaining of *in vivo* tissues or cells)	Reference (RNA-seq of *in vivo* tissues or cells)
				Implantation site	Chorionic laeve		
GATA2 GATA3 TFAP2C KRT19	+	+	+	+	+	Mühlhauser *et al.* (1995); Challier *et al.* (2005); Biadasiewicz *et al.* (2011); Genbacev *et al.* (2011); Lee *et al.* (2016); Paul *et al.* (2017)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018); Liu *et al.* (2018)
KRT7	±	+	+	+	+	Mühlhauser *et al.* (1995); Blaschitz *et al.* (2000); Frank *et al.* (2001); Hemberger *et al.* (2010); Biadasiewicz *et al.* (2011); Niakan and Eggan (2013); Lee *et al.* (2016)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018); Liu *et al.* (2018)
HLA-A[a] HLA-B[a] THY1[a] VIM	–	–	–	–	–	Loke (1989); Frank *et al.* (2001); Biadasiewicz *et al.* (2011)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018); Liu *et al.* (2018)
CDX2	+	– (subset +)	–	–	–	Hemberger *et al.* (2010); Niakan and Eggan (2013); Horii *et al.* (2016); Haider *et al.* (2018); Soncin *et al.* (2018)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
TP63 VGLL1	–	+ (subset -)	–	–	+	Shih and Kurman (2004); Kalhor *et al.* (2009); Soncin *et al.* (2018)	Yan *et al.* (2013); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
ITGA6[a]	–	+	–	–	–	Damsky *et al.* (1992)	Yan *et al.* (2013); Knofler and Pollheimer (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
SDC1[a]	–	–	+	–	–	Jokimaa *et al.* (1998)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
CGB	–	–	+	partial +	–	Shih and Kurman (2004); Tarrade *et al.* (2001); Handschuh *et al.* (2007); Cole (2010)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
CSH1	–	–	+	+	+	Tarrade *et al.* (2001); Shih and Kurman (2004)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
HLA-G[a] ITGA5[a] MCAM[a]	–	–	–	+	+	Damsky *et al.* (1994); Tarrade *et al.* (2001); Shih and Kurman (2004); Mao *et al.* (2007); Kalhor *et al.* (2009)	Yan *et al.* (2013); Knofler and Pollheimer (2013); Blakeley *et al.* (2015); Tilburgs *et al.* (2015); Petropoulos *et al.* (2016); Lee *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)
MUC4	–	–	–	+	–	Mao *et al.* (2007)	Yan *et al.* (2013); Blakeley *et al.* (2015); Petropoulos *et al.* (2016); Okae *et al.* (2018); Stirparo *et al.* (2018)

[a]Cell surface marker.

the cell column (Humphries *et al.*, 2006). EVT deposits high doses of fibronectin, and the expression of ITGA5 facilitates cell-matrix binding (Earl *et al.*, 1990; Damsky *et al.*, 1992). In addition, EVT invades the decidual layer when it expresses ITGA1, and ITGA1 is essential for the invasion (Damsky *et al.*, 1992; 1994). CD146 (also known as melanoma cell adhesion molecule, MCAM) is another representative cell surface marker of EVT (Table I) (Shih, 1999; Pujades *et al.*, 2002; Wang and Yan, 2013; Lee *et al.*, 2018; Turco *et al.*, 2018), but it is also expressed by neural crest cells, ganglion cells and activated T lymphocytes (Shih, 1999; Pujades *et al.*, 2002; Wang and Yan, 2013). EVT is classified as two types based on its location and immunohistochemical profiles: implantation site EVT and chorionic laeve EVT. The major function of implantation site EVT is to establish the maternal–foetal circulation by remodelling the maternal spiral arteries during early pregnancy, while the function of chorionic laeve EVT is unknown (Shih *et al.*, 2018). MUC4 is expressed in implantation site EVT, but not chorionic laeve EVT (Table I) (Mao *et al.*, 2007). The significance of discriminating implantation site EVT and chorionic laeve EVT is the classification of gestational trophoblastic diseases (GTDs). Immunohistochemistry has revealed that the origin of several GTDs differs with the EVT type. For example, exaggerated placental site and placental site trophoblastic tumour arise from implantation site EVT, and placental site nodule and epithelioid trophoblastic tumour arise from chorionic laeve EVT (Heller, 2018; Shih *et al.*, 2018; Kaur and Sebire, 2019). It should be noted that MUC4 cannot identify GTD subtypes.

Stroma cell

Stroma cells, which are derived from extra-embryonic mesenchyme, are located in the placental villous core (Fig. 4A) (Kaufmann *et al.*, 1977). They provide mechanical support to the villous structures and control intervillous blood flow (Demir *et al.*, 1997). Stroma cells express HLA-A, HLA-B, THY1 (CD90) and VIM, in contrast to trophoblasts, which do not express these molecular markers. The high expression of HLA-A and HLA-B in stroma cells indicates that these cells are derived from ICM, not TE. Furthermore, stroma cells do not express the trophoblast markers GATA2, GATA3, TFAP2C, KRT7 or KRT19 (Liu *et al.*, 2018; Okae *et al.*, 2018).Despite the above knowledge, the development of stroma cells over gestation is unclear at this time.

Hofbauer cell

Hofbauer cells are macrophages in the stroma of chorionic villi (Seval *et al.*, 2007; Schliefsteiner *et al.*, 2017; Reyes and Golos, 2018), which often localise close to FECs and CT (Fig. 4A) (Cervar *et al.*, 1999; Khan *et al.*, 2000; Seval *et al.*, 2007; Loegl *et al.*, 2016; Reyes and Golos, 2018). These macrophages are derived from foetal mesenchymal cells, not trophoblast. Hofbauer cells are characterised by general macrophage markers, such as CD14, FCGR1A (CD64), CD68, CD163 and LYVE1 (Seval *et al.*, 2007; Schliefsteiner *et al.*, 2017; Reyes and Golos, 2018; Vento-Tormo et al., 2018).

Hofbauer cells secrete the pro-angiogenic molecules VEGF and FGF2 (Loegl *et al.*, 2016). They also secrete EGF, which promotes trophoblast proliferation (Maruo *et al.*, 1995; Leach *et al.*, 2004; Barber *et al.*, 2005; Johnstone *et al.*, 2005; Vento-Tormo *et al.*, 2018). In addition, they express sprouty proteins that regulate branching morphogenesis (Anteby *et al.*, 2005). Therefore, Hofbauer cells can play a role in vascular conditioning, the proliferation of CT and the branching morphogenesis of placental villi during development by paracrine signals and cell-to-cell crosstalk in the placental villi. (Cervar *et al.*, 1999; Khan *et al.*, 2000; Seval *et al.*, 2007; Vento-Tormo *et al.*, 2018). Like stromal cells, the development of Hofbauer cells over gestation is unclear at this time.

Foetal endothelial cell

FECs are of mesenchymal origin and lie between circulating foetal blood and the placental stroma (Fig. 4A). In short, they are vascular endothelial cells in the chorionic villi and are characterised by general endothelial markers such as PECAM1 (CD31) and CD34 (Mutema and Stanek, 1999; Vento-Tormo *et al.*, 2018). However, FECs are characterised by no specific marker and can only be distinguished from other cells based on their location in the chorionic villi.

Summary

The majority of molecular markers regarding placental development are intracellular markers, and few cell surface markers have been identified (Table I). Cell surface markers are valuable for trophoblast isolation from placenta and the evaluation of cell lines, because they allow live trophoblasts to be detected with antibodies. Cell surface antibodies also provide qualitative and defined endpoints, and they can be used to elucidate the timing and order of molecular changes. Combinations of cell surface markers can be used to identify the trophoblast state, and thus provide a quality standard for trophoblast experiments.

Models for human trophoblast research

Common *in vitro* models of human trophoblast include primary cultures, placental villous explants, choriocarcinoma cell lines and immortalised cell lines derived from placenta. Additionally, BMP-treated human embryonic and induced pluripotent stem cells (ESCs and iPSCs, respectively) have been established, and recent studies have reported the long-term culture system of human CT, both monolayer and spheroid. The characterisation of human trophoblast cell lines is summarised in Table II.

Conventional human trophoblast cell lines

As conventional human trophoblast cell lines, primary trophoblast cell culture, placental villous explant, trophoblast tumour-derived cell lines (choriocarcinoma cell lines) and immortalised trophoblast cell lines have all been used in placental research because of their expected similarities with *in vivo* trophoblast (Table I). The first two models are culture systems and cannot be expanded for a long time. Choriocarcinoma cell lines, such as BeWo, JEG-3 and JAR, have been around for five decades

Table II Commonly used *in vitro* human trophoblast models for investigating placental development.

Cellular models	Origin	Immortalisation	Differentiation potency	Advantages	Limitations	References
Primary cultures						
Placental villous explants, Primary two-dimensional culture	Placenta	–	ST, EVT	Bipotency of differentiation Patient-specific samples can be used	Difficulty of manipulation Mesenchymal contamination Maintenance for only a short period Donor consent or ethical review permit	Greenwood *et al.* (1996); Sooranna *et al.* (1999); Potgens *et al.* (2001); Newby *et al.* (2005); Orendi *et al.* (2011)
Two-dimensional cell lines						
BeWo	Choriocarcinoma	–	ST, EVT	Easy culture and gene manipulation Bipotency of differentiation	Tumour-derived	Pattillo and Gey (1968)
JEG-3, JAR	Choriocarcinoma	–	EVT	Easy culture and manipulation	Tumour-derived Unipotency of differentiation	Pattillo *et al.* (1971); Kohler and Bridson (1971)
HTR8/SVneo, Swan71	First-trimester placenta	+	EVT	Easy culture and manipulation	Immortalization using virus Unipotency of differentiation	Graham *et al.* (1993); Straszewski-Chavez *et al.* (2009)
TCL-1	Term placenta	+	EVT	Easy culture and manipulation	Immortalization using virus Unipotency of differentiation	Lewis *et al.* (1996)
TBPC (trophoblast progenitor cells)	Chorion	–	ST, EVT	Easy culture and manipulation	No investigation of first-trimester trophoblast criteria OCT4, GATA4, EOMES positive cells, which is unlike cytotrophoblast	Genbacev *et al.* (2011)
BMP-treated conventional hPSCs	Conventional hPSCs	–	ST, EVT (mixed trophoblast)	Good manipulation Bipotency of differentiation Application to disease modeling	HLA-A and HLA-B positive Low expression of C19MC microRNA Transient CT-like state	Xu *et al.* (2002); Sudheer *et al.* (2012); Li *et al.* (2013); Amita *et al.* (2013); Yang *et al.* (2015); Yabe *et al.* (2016); Horii *et al.* (2016); Horii *et al.* (2019)
TGFb1-treated expanded potential stem cells	Expanded potential stem cells	–	ST, EVT (mixed trophoblast)	Bipotency of differentiation Application to disease modelling	Difficult to derive expanded potential stem cells No investigation of first-trimester trophoblast criteria	Gao *et al.* (2019)
Blastomere-derived trophoblast stem-like cell lines	Pre-implantation embryo	–	ST, EVT	Good manipulation Bipotency of differentiation	No investigation of first-trimester trophoblast criteria	Zdravkovic *et al.* (2015)
Trophoblast stem cells	Pre-implantation embryo First-trimester placenta	–	ST, EVT	Good manipulation Bipotency of differentiation Accordance with criteria of first-trimester trophoblast	Unproven derivation from second- or term-trimester placenta for patient-specific cell lines	Okae *et al.* (2018)
Three-dimensional cell lines						
Trophoblast organoid	First-trimester placenta	–	ST, EVT	Bipotency of differentiation Accordance with the criteria of first-trimester trophoblast Semiphysiologic representation (with ST)	Unproven derivation from second- or term-trimester placenta for patient-specific cell lines	Haider *et al.* (2018); Turco *et al.* (2018)

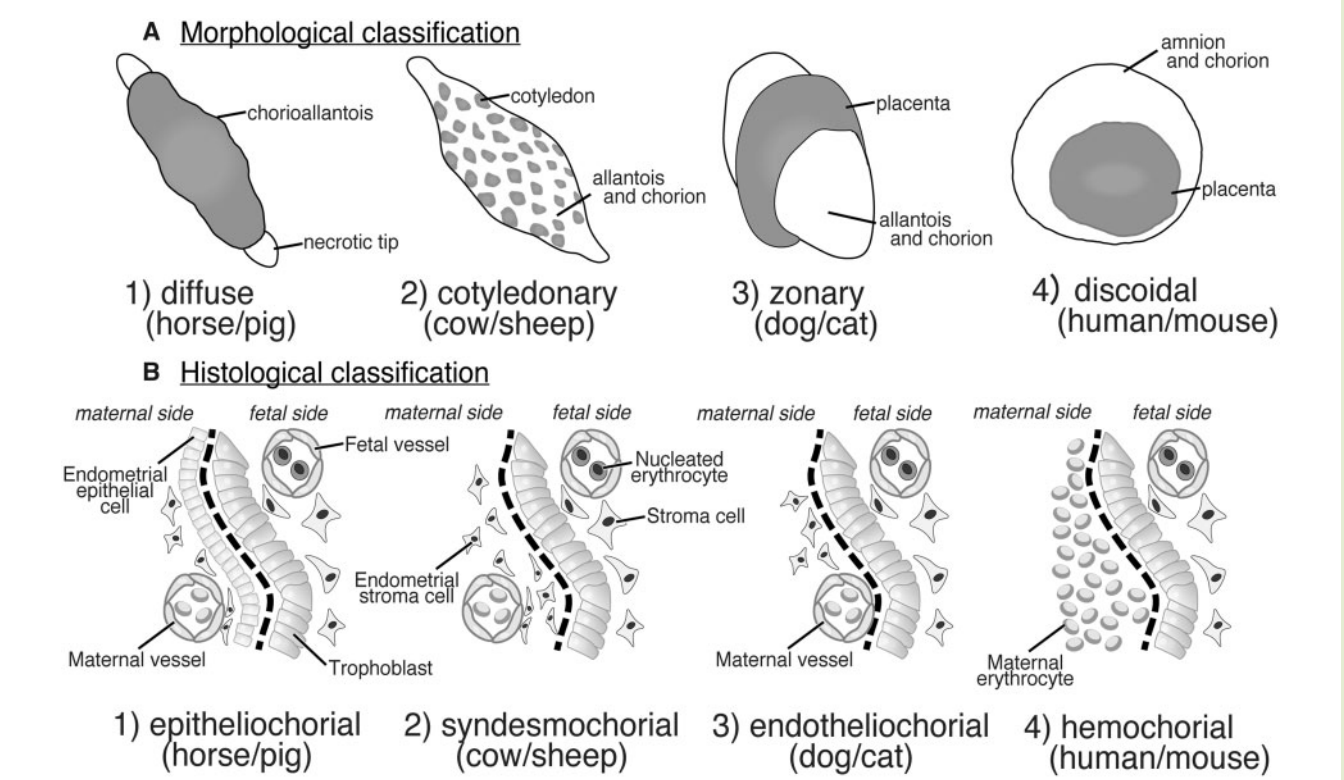

Figure 5. Classification of placenta. (**A**) Morphological classification. (1) Diffuse placenta, seen in horses and pigs, has a large surface area attached to the uterine wall. (2) Cotyledonary placenta, seen in goats and cows, is characterised by many 'polka dot' like cotyledons. (3) Zonary placenta, seen in dogs and cats, is characterised by an equatorial placenta band surrounding the chorionic sac. (4) Discoid placenta, seen in humans, monkeys, mice, rats and rabbits, is characterised by a single (discoid) or double (bidiscoid) disc. (**B**) Histological classification. Dashed lines indicate the decidual membrane, which peels off during delivery. (1) Epitheliochorial placenta, seen in horses and pigs, is the most superficial type, lacking significant invasion to the maternal tissues. In epitheliochorial placenta, there are six layers: maternal endothelia, endometrial stroma cells, endometrial epithelial cells, trophoblasts, foetal stroma cells and foetal endothelia. (2) In syndesmochorial placenta, seen in goats and cows, among the six layers in epitheliochorial placenta, endometrial epithelial cells are absent. (3) In endotheliochorial placenta, seen in dogs and cats, endometrial epithelial cells and endometrial stroma cells are lost between the maternal and foetal sides. (4) Haemochorial placenta, seen in humans, monkeys, mice, rats and rabbits, is the most invasive type. There are no maternal endothelia, endometrial stroma cells or endometrial epithelial cells. Therefore, the maternal erythrocytes are in direct contact with foetal tissues.

(Pattillo and Gey, 1968; Pattillo *et al.*, 1968a,b, 1971a,b; Kohler and Bridson, 1971), but they have various chromosomal aberrations (Poaty *et al.*, 2012). Owing to widespread transfection methods, immortalised cells, such as HTR-8/SVneo, Swan 71 and TCL-1, have also been established (Graham *et al.*, 1993; Lewis *et al.*, 1996; Straszewski-Chavez *et al.*, 2009). Choriocarcinoma and immortalised cell lines overcome the problem of long-term passage, but their genome-wide DNA methylation patterns are clearly different from primary trophoblast (Novakovic *et al.*, 2011). The poor differentiation potency and transcriptome discrepancy are important limitations for human placental research (Bilban *et al.*, 2010; Apps *et al.*, 2011; Ji *et al.*, 2013).

Pluripotent stem cell-derived trophoblasts

Human ESCs and iPSCs differentiate into trophoblast lineage in a species-dependent manner (Xu *et al.*, 2002; Sudheer *et al.*, 2012; Amita *et al.*, 2013; Li *et al.*, 2013; Yang *et al.*, 2015; Horii *et al.*, 2016; Lee *et al.*, 2016; Yabe *et al.*, 2016; Horii *et al.*, 2019). The conventional way to induce trophoblast lineage is to expose these pluripotent stem cells (PSCs) to bone morphogenetic protein-4 (BMP4) (Xu *et al.*, 2002), and the induced trophoblasts were recently shown to express the distinctive placental signature (Roberts *et al.*, 2018). Several efficient differentiation protocols for trophoblast have been published: (i) BAP treatment (BMP4 plus the activin A signalling inhibitor A83-01 and the FGF2 signalling inhibitor PD173074) (Amita *et al.*, 2013; Yang *et al.*, 2015; Yabe *et al.*, 2016), and (ii) BMP4 plus a Wnt/beta-catenin inhibitor (Horii *et al.*, 2019). The PSCs can thus terminally differentiate into hCGβ^{positive} ST-like cells and HLA-G^{positive} EVT-like cells. However, whether they differentiate into trophoblast remains controversial. First, BMP-treated PSCs do not agree with the criteria of first-trimester trophoblast, that is, they express HLA-A and HLA-B and have a low expression of C19MC microRNA (Lee *et al.*, 2016). In addition, these models are

contradictory in developmental biology, as conventional human PSCs correspond to the post-implantation stage, which is different from mouse ESCs (Nakamura *et al.*, 2016), and the lineage segregation between ICM and TE takes place at the blastocyst stage (De Paepe *et al.*, 2013; Petropoulos *et al.*, 2016; Rossant and Tam, 2018; Leng *et al.*, 2019). To solve these problems, a new type of stem cell has been established; expanded potential stem cells retain the ability to differentiate into any embryonic lineage as well as trophoblasts (Gao *et al.*, 2019), although this model has not been fully evaluated.

Trophoblast stem cells and trophoblast organoids

New culture systems for human trophoblast stem cells (monolayer, 2D) and trophoblast organoids (spheroid, 3D) allow for long-term expanding trophoblast, thus providing the next generation of human trophoblast cell lines and human placental embryology (Table II) (Haider *et al.*, 2018; Okae *et al.*, 2018; Turco *et al.*, 2018). Since both trophoblast stem cells and organoids meet the first-trimester trophoblast criteria, these culture systems are considered improvements to previous trophoblast cell lines (Haider *et al.*, 2018; Okae *et al.*, 2018; Turco *et al.*, 2018). Trophoblast stem cells derived from human blastocyst and first-trimester placenta behave as long-term expanding CT and can differentiate into ST and EVT with high purity (Okae *et al.*, 2018). ST specification from human trophoblast stem cells is induced by forskolin (cAMP activator), whereas EVT specification is induced by Neuregulin-1, ALK4/ALK5/ALK6 inhibitor and Matrigel (Okae *et al.*, 2018). Trophoblast organoids derived from first-trimester placenta behave as long-term expanding 3D villous trophoblasts, consisting of CT and ST (Haider *et al.*, 2018; Turco *et al.*, 2018). In these organoids, ST are surrounded with CT without stroma cells (Haider *et al.*, 2018; Turco *et al.*, 2018). This reversed polarity is characteristic of ordinary organoid culture systems (Sato *et al.*, 2009, 2011; Bartfeld and Clevers, 2015; Bartfeld *et al.*, 2015). Trophoblast organoids can differentiate into HLA-G^{positive} EVT cells under Okae's EVT differentiation medium (Turco *et al.*, 2018) or regulation of Wnt signalling (Haider *et al.*, 2018). Although the proliferative capacity of CT decreases prominently after 10 weeks of gestation (Mayhew, 2014), trophoblast stem cells and trophoblast organoids from second trimester or term placenta are strongly needed to model placenta-mediated pregnancy complications, because complications do not appear until these later stages.

Placental classification by animal taxonomical approaches

Species with placenta-like structures

Animals can be grouped as oviparous species, which lay eggs, and viviparous species, which have placentas and include most mammals. One major characteristic of oviparity is the constant volume of the egg during development. To excrete nitrogen, the egg produces uric acid, which maintains the constant egg volume (Baggott, 2009). In contrast, viviparous species produce urine, which is excreted through the placenta. The placenta itself is classified into yolk-sac placenta and chorio-allantoic placenta. Yolk-sac placenta is developed from the yolk sac, in which the allantoic membrane does not adhere to the chorion, and material exchange occurs between the mother and the foetus through a part of the yolk sac. Fish, reptiles and most marsupials have yolk-sac placenta (Freyer and Renfree, 2009). Chorio-allantoic placenta includes the human placenta. Here, the allantoic membrane and serosa coalesce to form the chorio-allantoic membrane, develop blood vessels in the mesodermal part of the chorio-allantoic membrane and adhere closely to the maternal endometrium. *Placentalia (Eutheria)* have chorio-allantoic placenta as too does the marsupial bandicoot. The subclade *Euarchontoglires* in *Placentalia* consists of *Glires* and *Euarchonta*; *Glires* includes *Rodentia*, and *Euarchonta* includes *Primates*.

Structural classification of the placenta

Chorio-allantoic placenta is grossly classified as the following four types (Fig. 5A). (i) Diffuse placenta: the placenta is scattered throughout the surface of the chorion (horses and pigs). (ii) Cotyledonary placenta: the placenta has multiple small placentae isolated on the amniotic membrane in a mottled pattern (cows and sheep) (Furukawa *et al.*, 2014). (iii) Zonary placenta: the placenta is shaped like a band around the centre of the amniotic membrane (dogs and cats). (iv) Discoidal placenta: the placenta forms a discoid region in the uterus (humans, macaques, mice and brown bears).

The placenta is responsible for nutrient and gas exchange between the mother and foetus at the surface where the chorion and allantoic membrane are facing. Although the structure on the foetal side typically has an epithelial cell layer (trophoblast cell layer), connective tissue and capillary vessels, the structure on the maternal side varies across species. Therefore, the placenta is also classified histologically into the four types below (Fig. 5B) (Wagner *et al.*, 2014). (i) Epitheliochorial placenta: the maternal side is composed of capillaries, connective tissue and an endometrial epithelium, and the endometrium is in contact with the maternal–foetus boundary (horses and pigs). (ii) Syndesmochorial placenta: there is no epithelium on the maternal side, and the connective tissue is in contact with the maternal–foetus boundary (cows and sheep). (iii) Endotheliochorial placenta: there is no connective tissue on the maternal side, and the endothelial cells of the capillary are in contact with the maternal–foetus boundary (dogs and cats). (iv) Haemochorial placenta: there are no capillary vessels on the maternal side, thus, maternal blood is in direct contact with the foetus (humans, macaques and mice).

Notably, epitheliochorial placenta has three layers (capillaries, connective tissue and an endometrial epithelium), and haemochorial placenta has no cell layer between the maternal blood and trophoblast. These histological differences suggest that maternal damage during delivery is relatively large in the haemochorial placenta (Abrams and Rutherford, 2011). In contrast, the extent of maternal damage has not been established for epitheliochorial and syndesmochorial placenta because they have endometrial epithelium or endometrial stroma cells next to the decidua membrane.

Table III Characterisation of human, macaque (subfamily of old world monkey) and mouse placenta.

	Human	Macaque	Mouse
Gestation time (days)	280	155	20
Gross morphology	Monodiscoid	Bidiscoid	Monodiscoid
Historical morphology	Villous haemochorial	Villous haemochorial	Labyrinth haemochorial
Layers between maternal and foetal blood	2	2	3
Site of attachment of TE to uterus	Embryonic, polar	Embryonic, polar	Abembryonic, mural
Implantation type	Interstitial	Superficial	Superficial
Endometrial reaction	Massive	Moderate	Little
Trophoblast invasion	Deeper	Superficial	Superficial
Interstitial trophoblast	Ordinary	Rare	Rare
Primitive syncytium	+	+	−
Ectoplacental cone	−	−	+
Villous/labyrinth function	Maternal–foetal transport Hormone production	Maternal–foetal transport Hormone production	Maternal–foetal transport
Pre-implantation TE marker (consistent)		CDX2↑, GATA2↑, GATA3↑, TFAP2C↑	
Pre-implantation TE marker (different)	KLF5↓, EOMES↓, ETS2↓, ELF5↓	KLF5↑, EOMES↓, ETS2↓, ELF5↓	KLF5↑, EOMES↑, ETS2↑, ELF5↑

TE, trophectoderm.

Differences in placentation between humans and other species

Research on placental development with other species

Our understanding of trophoblast has been refined with long-time research using different animal models, but mainly mouse and rat. Studies with mouse trophoblast stem cells (Tanaka *et al.*, 1998), which were established 20 years ago, model mouse trophoblast development *in vivo* with remarkable accuracy (Perez-Garcia *et al.*, 2018). An analysis of mutant mouse trophoblast stem cells and conditional knockouts suggests that early miscarriage (embryonic Days 9.5–14.5) is usually associated with severe placental malformations (Perez-Garcia *et al.*, 2018). Although mouse and human placentation are not exactly the same, mouse trophoblast stem cells provide a useful platform for placental research, because experiments *in vivo* are easy and the gestational period is short.

Other popular animal model is macaques (a subfamily of old world monkey) and marmoset (a subfamily of new world monkey). In general, these monkeys have served as valuable models for human diseases and treatments due to their close similarities to humans in terms of genetic and physiological features (Chan, 2013). Monkeys have also been studied for many years in placental research (Myers, 1972; Hearn *et al.*, 1988; Enders, 1995, 2000; Carter and Pijnenborg, 2011; Carter *et al.*, 2015). Past studies were mainly based on morphological approaches, although scRNA–seq approaches have begun recently (Nakamura *et al.*, 2016). In addition, the *in vitro* culture of macaque embryos has been established (Ma *et al.*, 2019; Niu *et al.*, 2019). Great apes (gorillas, chimpanzees, bonobo and orangutans) are closely related to humans and have very similar placenta structures (Carter and Pijnenborg, 2011; Pijnenborg *et al.*, 2011). However, many governments ban or severely restrict research on great apes, because great apes are cognitively similar to humans (Knight, 2008).

As useful as these models are, placenta formation between humans and other animal species differs in many ways. Below we summarise some of these differences.

Gross and histological differences of placenta

As described above, humans, other primates and mice all have discoidal placenta, but the shapes are different. Humans, great apes and mice have a single placental disc, but macaques and marmosets have bidiscoidal placenta (Table III) (Myers, 1972). Some researchers have observed that a secondary attachment generates bidiscoidal placenta (Carter *et al.*, 2015), but the true cause is a matter of debate. In terms of histomorphology, primates and mice have the same haemochorial placenta among the four types of histological classification in Fig. 5B. Implantation begins at the blastocyst stage in human (Day 7) (O'Rahilly and Müller, 1987) and mouse (Day 4) (Theiler, 1972), but at the embryonic period in cow (Day 20) (King *et al.*, 1980; Wathes and Wooding, 1980) and horse (Day 35) (Bowen and Burghardt, 2000; Allen and Stewart, 2001). Since mouse placenta shares more similarities to human placenta compared with other species in early placental development except those whose experimental use is greatly restricted, like great apes, toxicity studies with mouse have been used to evaluate teratogenicity and developmental toxicity (Pijnenborg *et al.*, 1981).

Nevertheless, mouse and human placenta have significant structural differences. The haemochorial placenta is classified into villous type (humans, apes and old world monkeys) and labyrinth type (mice, rats and new world monkeys) (Fig. 5, Table III). Human villous haemochorial placenta has two layers of trophoblasts that separate maternal blood from foetal blood (Fig. 5A, Table III). The bichorial trophoblast layers consist of a single syncytial layer and a single cytotrophoblastic layer (Rossant and Cross, 2001; Soncin *et al.*, 2015). The cytotrophoblastic layer becomes discontinuous and covers 20% of chorionic villi in term placenta, whereas the syncytial layer does not change (Benirschke *et al.*, 2012a). On the other hand, mouse labyrinth haemochorial placenta has three layers of trophoblasts between the maternal and foetal blood (Table III) (Enders and Blankenship, 1999). The mouse placenta is called labyrinth because of its maze-like appearance in cross-section (Fig. 5B). The trichorial trophoblast layers consist of two syncytial layers and a single mononuclear layer of unknown function. The labyrinth zone of mouse placenta does not have a proliferative cytotrophoblastic layer (Simmons, 2013). These species differences may be related to the much shorter duration of mouse pregnancies than human. From the perspective of drug and nutrient diffusion, the ability for molecules to cross the placenta is strongly influenced by the number or the thickness of cell layers between the maternal and foetal blood (Mihaly and Morgan, 1983; Schroder, 1995; Pere, 2003; Furukawa *et al.*, 2014). It is important to consider the diversity of the histological structures for drug metabolism and placental transfer, although active and facilitated transport also have effects.

Another difference between mouse and human is the supplied blood volume. In the villous haemochorial placenta (human), maternal spiral arteries open directly into the intervillous spaces. The chorionic villi protrude into the intervillous space and are bathed directly in maternal blood (Furukawa *et al.*, 2014). Interestingly, maternal blood flow regularly spouts into the intervillous space, as if washing the foetal villi (Dancis and Schneider, 1986; Burton *et al.*, 2009). In contrast, maternal blood is exposed to a large amount of foetal blood indirectly through the trophoblast layers in labyrinth haemochorial placenta (mouse).

In addition, several articles have reported that placental drug transfer is determined largely by placental blood flow (Mihaly and Morgan, 1983; Schroder, 1995; Furukawa *et al.*, 2014). Circulation between the mother and foetus also affects the transfer ratio of nutrients ([concentration in foetus]/[concentration in maternal plasma]) (Tarui *et al.*, 2018). In placental circulation, the flow rate and the flow direction affect the transfer ratio (Faber *et al.*, 1992; Faber, 1995; Perazzolo *et al.*, 2017). Mouse placenta has countercurrent blood flow (opposite directions) through the maternal and foetal vessels (Faber *et al.*, 1992; Adamson *et al.*, 2002). On the other hand, human chorionic villi are exposed to the maternal pool of blood in the intervillous space (Costa *et al.*, 1992; Acharya *et al.*, 2016; Plitman Mayo, 2018), resulting in a lower transfer ratio than the countercurrent system (Faber *et al.*, 1992). Although monkeys and humans share more similar placental development compared with mice, it is difficult to conduct large-scale toxicity tests with monkeys. At the same time, when using mouse models, the structural differences between species must be considered.

Morphology during implantation

The first morphological difference between primates (human and monkey) and rodents (mouse and rat) is the direction of implantation. The polar TE overlying ICM is the site of primate implantation (Herzog, 1909), whereas rodent implantation is initiated in the opposing mural TE (Table III) (Wimsatt, 1975). Despite the difference in the direction of implantation, the main component of the placenta originates from polar TE in both groups. The distinct behaviour of polar and mural TE has been proposed to result from differential signal transduction from the epiblast (Gardner and Johnson, 1972; Gardner *et al.*, 1973; Papaioannou, 1982).

The blastocysts of macaque and mouse remain in the uterine cavity; in other words, the blastocysts embed partially within the uterine cavity. This type of implantation is called superficial implantation. On the other hand, the implantation type of human is interstitial (Table III) (Chapman *et al.*, 2013). That is, the human blastocyst embeds entirely within the endometrial connective tissue and there is a massive endometrial reaction. Thereafter, EVT invades through the decidua to the inner one-third of the myometrium (interstitial invasion) (Ferretti *et al.*, 2007).

The primitive syncytium forms lacunae filled with maternal blood (Hertig *et al.*, 1956; Enders, 1989). The lacunae coalesce with each other gradually and become one large intervillous space. The primitive syncytium is observed only in primates and guinea pig (Enders and Schlafke, 1969; Schlafke and Enders, 1975; James *et al.*, 2012; Soares *et al.*, 2018). In contrast, the ectoplacental cone is a specific structure in mice and rats (Table III). The ectoplacental cone is a cellular mass formed by intensive proliferation of polar TE (Amoroso, 1952; Snell and Stevens, 1966; Barlow and Sherman, 1972; Gardner *et al.*, 1973; Enders and Blankenship, 1999). It is located in the maternal endometrium and contains progenitors for a number of trophoblast subtypes (Simmons *et al.*, 2007; El-Hashash *et al.*, 2010; Mould *et al.*, 2012).

Predominantly expressed genes of TE

A comprehensive analysis of the expressed genes in mouse, monkey and human embryos is valuable for understanding trophoblast specification. These three animals share certain similarities in that their trophoblasts express GATA2, GATA3 and TFAP2C (Nakamura *et al.*, 2016). The main signalling pathway that governs TE specification in the mouse is HIPPO signalling (Nishioka *et al.*, 2008, 2009), but in human this is unknown at this time. In addition, unlike in the mouse, the expressions of EOMES, ETS2 and ELF5, three transcription factors for TE specification, were undetectable in the human pre-implantation embryo (Table III) (Petropoulos *et al.*, 2016; Kubaczka *et al.*, 2017; Roberts *et al.*, 2018). In particular, EOMES is not expressed in any human trophoblast subtype. In contrast, Dab2, a primitive endoderm marker in mouse (Morrisey *et al.*, 2000; Yang *et al.*, 2002; Moore *et al.*, 2013), is expressed in human TE (Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016; Stirparo *et al.*, 2018). Thus, some master regulators in mouse trophoblast lineage are not expressed in human TE, and the expression timing of other regulators differs in the two animals. Consequently, it is unclear whether the mechanisms of trophoblast differentiation and maintenance are similar among mouse, monkey and human. In mice, about 70 knockout lines have been

shown to cause lethality with placenta and placental dysmorphologies (Perez-Garcia *et al.*, 2018), but these genes have not been examined in humans. Knockout experiments in human trophoblast cell lines would therefore be useful. Gene expression has been investigated in cymolgous monkey *in vivo*, showing different kinetic gene expression before and after implantation (Nakamura *et al.*, 2016).

Cell types of trophoblast

Spongiotrophoblasts in mouse are the putative mouse counterpart of human CT in CT cell columns. Both mouse trophoblast giant cells (TGCs) and glycogen cells (glycogen trophoblasts), which are derived from spongiotrophoblasts, are analogous to human EVT (Georgiades *et al.*, 2002; Cross, 2005; El-Hashash *et al.*, 2010; Soncin *et al.*, 2015) (Fig. 1B). TGCs have their name because of their 'giant' nuclei, which is caused by DNA synthesis without nuclear division. Human EVT do not have giant nuclei (Soncin *et al.*, 2015). Although mouse TGCs and human EVT commonly function as an anchor for the placenta to the uterus, some functional features of these cells are different. TGCs are the main endocrine cells producing hormones in mouse placenta, whereas in humans those cells are ST, and human EVT secrete a small amount of hormones (e.g. human placental lactogen) (Tarrade *et al.*, 2001). Another difference between human and mouse trophoblast is the invasive capacity. Both TGCs and glycogen cells have little invasive capacity (Simmons *et al.*, 2007). Human EVT, on the other hand, contributes largely to vascular remodelling of the spiral arteries, which then dilate (Soncin *et al.*, 2015). It has been reported that all trophoblast cell types among monkeys, apes and humans are similar morphologically and functionally (Myers, 1972; Enders, 1995, 2000; Carter *et al.*, 2015).

Peri-implantation stage research with experimental animals

Ethics, including the use of experimental animals, will continue to limit placental research and prevent the use of *in vivo* human specimens from reaching the implantation phase. Therefore, placental research using other animals, such as macaques, for insights of early trophoblast development is required. Recently, the specification of primordial germ cells was identified by immunostaining and *in situ* hybridisation using macaque embryos during the peri-implantation stage (Sasaki *et al.*, 2016). Other studies revealed the development trajectories of epiblast, primitive endoderm, TE and primordial germ cells (Ma *et al.*, 2019; Niu *et al.*, 2019). In total, our understanding of early human placental development will depend on a combination of (i) human cells *in vitro*, (ii) non-human primates *in vivo* and *in vitro* and (iii) mice *in vivo* and *in vitro*.

Future perspectives

In the past several years, new models of *in vitro* trophoblast, such as trophoblast stem cells and trophoblast organoids, have been developed. Trophoblast organoids still lack blood vessels, stroma cells and Hofbauer cells compared to *in vivo* chorionic villi, but in the future, innovative bioengineering systems will be used to create chorionic villi-like models or blood-placental barrier-like models (Takebe and Wells, 2019). In addition, crosstalk between the endometrium and trophoblasts will be elucidated using endometrial organoids (Boretto *et al.*, 2017; Turco *et al.*, 2017; Ezashi *et al.*, 2019).

Owing to the advent of high throughput technology, scRNA-seq is enabling single-cell transcriptome analysis at high resolution (Wagner *et al.*, 2018), including placental development. Quantifying the transcriptome of individual cells can lead to the discovery new cell types, reveal cellular heterogeneity (Islam *et al.*, 2014) of the developmental trajectory (Cheng *et al.*, 2019), identify gene regulatory mechanisms and dissect cell–cell interactions. In the past several years, scRNA-seq has been performed with trophoblasts at the pre-implantation stage (Yan *et al.*, 2013; Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016) and peri-implantation stage with *in vitro* culture systems to study the human embryo (Zhou *et al.*, 2019; Xiang et al., 2020) and post-implantation stage (Liu *et al.*, 2018; Vento-Tormo *et al.*, 2018). These data identified new maternal–fetal interactions and epigenetic programmes. In this way, scRNA-seq can be a powerful tool for the study of early embryogenesis and reproductive medicine.

Other new methods for placental research, such as single-cell proteomics (Su *et al.*, 2017; Lundberg and Borner, 2019; Marx, 2019) and single-cell epigenetics (Clark *et al.*, 2016; Kelsey *et al.*, 2017; Zhou *et al.*, 2019), are also progressing. Differences in gene expression among individual cells can be controlled by epigenetic diversity. Single-cell epigenome methods provide high resolution profiling of DNA modifications, such as transcription factor binding, histone modifications and DNA accessibility (Kelsey *et al.*, 2017). These new techniques can reveal trophoblast cell-fate decisions, identity and function in normal placental development (Kelsey *et al.*, 2017). Additionally, single-cell western blotting can identify cell-to-cell variations in protein-mediated cell functions (Hughes *et al.*, 2014).

Conclusion

Cell biological research of the human placenta began five decades ago with the use of choriocarcinoma and immortalised cell lines (King *et al.*, 2000; Shiverick *et al.*, 2001). However, these cell lines have transcriptome discrepancy and poor differentiation potency compared to *in vivo* CT (Bilban *et al.*, 2010), thus demanding other cells lines. To evaluate cell lines, new criteria for first-trimester trophoblast were made (Lee *et al.*, 2016). In addition, more molecular markers, epigenetic features and morphological appearance of trophoblast subtypes are also required when selecting new cell lines (Gamage *et al.*, 2018a,b). In parallel, it is important to recognise species differences in various features, such as placental structure, early embryonic shape, implantation type, trophoblast type and gene expression. This review deals with basic knowledge of various aspects of placental development. Compared to other organs, the placenta exhibits great biodiversity among species, but also common features across species. Although there are ethical and technical constraints, continued efforts to elucidate human placental development in normal and abnormal pregnancies are encouraged.

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Authors' roles

S.I. and S.Y. contributed to the conceptual planning of the manuscript and drafted the manuscript. S.I. designed the figures and tables. Y.C., K.K., E.K., M.M. and S.Y. edited the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

References

Abrams ET, Rutherford JN. Framing postpartum hemorrhage as a consequence of human placental biology: an evolutionary and comparative perspective. *Am Anthropol* 2011;**113**:417–430.

Acharya G, Sonesson SE, Flo K, Rasanen J, Odibo A. Hemodynamic aspects of normal human feto-placental (umbilical) circulation. *Acta Obstet Gynecol Scand* 2016;**95**:672–682.

Adamson SL, Lu Y, Whiteley KJ, Holmyard D, Hemberger M, Pfarrer C, Cross JC. Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev Biol* 2002;**250**:358–373.

Aghajanova L, Shen S, Rojas AM, Fisher SJ, Irwin JC, Giudice LC. Comparative transcriptome analysis of human trophectoderm and embryonic stem cell-derived trophoblasts reveal key participants in early implantation. *Biol Reprod* 2012;**86**:1–21.

Allen WR, Stewart F. Equine placentation. *Reprod Fertil Dev* 2001;**13**:623–634.

Amita M, Adachi K, Alexenko AP, Sinha S, Schust DJ, Schulz LC, Roberts RM, Ezashi T. Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proc Natl Acad Sci USA* 2013;**110**:E1212–E1221.

Amoroso EC Placentation. In Parkes AS (ed). *Marshall's Physiology of Reproduction*. London, UK: Longman, Green and Co, 1952, 127–311.

Anteby EY, Natanson-Yaron S, Greenfield C, Goldman-Wohl D, Haimov-Kochman R, Holzer H, Yagel S. Human placental Hofbauer cells express sprouty proteins: a possible modulating mechanism of villous branching. *Placenta* 2005;**26**:476–483.

Apps R, Sharkey A, Gardner L, Male V, Trotter M, Miller N, North R, Founds S, Moffett A. Genome-wide expression profile of first trimester villous and extravillous human trophoblast cells. *Placenta* 2011;**32**:33–43.

Arnholdt H, Meisel F, Fandrey K, Lohrs U. Proliferation of villous trophoblast of the human placenta in normal and abnormal pregnancies. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1991;**60**:365–372.

Assou S, Boumela I, Haouzi D, Monzo C, Dechaud H, Kadoch IJ, Hamamah S. Transcriptome analysis during human trophectoderm specification suggests new roles of metabolic and epigenetic genes. *PLoS One* 2012;**7**:e39306.

Baggott GK. Development of extra-embryonic membranes and fluid compartments. *Avian Biol Res* 2009;**2**:21–26.

Barber KJ, Franklyn JA, McCabe CJ, Khanim FL, Bulmer JN, Whitley GS, Kilby MD. The in vitro effects of triiodothyronine on epidermal growth factor-induced trophoblast function. *J Clin Endocrinol Metab* 2005;**90**:1655–1661.

Barlow PW, Sherman MI. The biochemistry of differentiation of mouse trophoblast: studies on polyploidy. *Development* 1972;**27**:447–465.

Bartfeld S, Bayram T, van de Wetering M, Huch M, Begthel H, Kujala P, Vries R, Peters PJ, Clevers H. In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology* 2015;**148**:126–136.e6.

Bartfeld S, Clevers H. Organoids as model for infectious diseases: culture of human and murine stomach organoids and microinjection of helicobacter pylori. *J Vis Exp* 2015;**105**:53359.

Benirschke K, Burton GJ, Baergen RN. Characterization of the Developmental Stages. In: Benirschke K, Burton GJ, Baergen RN (eds). *Pathology of the Human Placenta*. New York, NY: Springer, 2012a, 145–155.

Benirschke K, Burton GJ, Baergen RN. Early development of the human placenta. In: Benirschke K, Burton GJ, Baergen RN (eds). *Pathology of the Human Placenta*. New York, NY: Springer, 2012b, 41–53.

Benirschke K, Burton GJ, Baergen RN. Nonvillous parts and trophoblast invasion. In: Benirschke K, Burton GJ, Baergen RN (eds). *Pathology of the Human Placenta*. New York, NY: Springer, 2012c, 157–240.

Benirschke K, Kaufmann P, Baergen R. *Pathology of the Human Placenta*. New York, NY: Springer, 2000.

Biadasiewicz K, Sonderegger S, Haslinger P, Haider S, Saleh L, Fiala C, Pollheimer J, Knöfler M. Transcription factor AP-2α promotes EGF-dependent invasion of human trophoblast. *Endocrinology* 2011;**152**:1458–1469.

Bilban M, Tauber S, Haslinger P, Pollheimer J, Saleh L, Pehamberger H, Wagner O, Knöfler M. Trophoblast invasion: assessment of cellular models using gene expression signatures. *Placenta* 2010;**31**:989–996.

Blakeley P, Fogarty NME, del Valle I, Wamaitha SE, Hu TX, Elder K, Snell P, Christie L, Robson P, Niakan KK. Defining the three cell

lineages of the human blastocyst by single-cell RNA-seq. *Development* 2015;**142**:3613.

Blaschitz A, Weiss U, Dohr G, Desoye G. Antibody reaction patterns in first trimester placenta: implications for trophoblast isolation and purity screening. *Placenta* 2000;**21**:733–741.

Boretto M, Cox B, Noben M, Hendriks N, Fassbender A, Roose H, Amant F, Timmerman D, Tomassetti C, Vanhie A *et al*. Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability. *Development* 2017;**144**:1775–1786.

Bowen JA, Burghardt RC. Cellular mechanisms of implantation in domestic farm animals. *Semin Cell Dev Biol* 2000;**11**:93–104.

Boyd JD, Hamilton WJ. *The Human Placenta*. Cambridge, UK: Macmillan Press, 1970.

Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. *J Soc Gynecol Investig* 2004;**11**:342–352.

Burton GJ, Woods AW, Jauniaux E, Kingdom JC. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta* 2009;**30**:473–482.

Carter AM, Enders AC, Pijnenborg R. The role of invasive trophoblast in implantation and placentation of primates. *Philos Trans R Soc Lond B Biol Sci* 2015;**370**:20140070.

Carter AM, Pijnenborg R. Evolution of invasive placentation with special reference to non-human primates. *Best Pract Res Clin Obstet Gynaecol* 2011;**25**:249–257.

Cervar M, Blaschitz A, Dohr G, Desoye G. Paracrine regulation of distinct trophoblast functions in vitro by placental macrophages. *Cell Tissue Res* 1999;**295**:297–305.

Challier JC, Galtier M, Cortez A, Bintein T, Rabreau M, Uzan S. Immunocytological evidence for hematopoiesis in the early human placenta. *Placenta* 2005;**26**:282–288.

Chan AWS. Progress and prospects for genetic modification of non-human primate models in biomedical research. *ILAR J* 2013;**54**:211–223.

Chang CW, Wakeland AK, Parast MM. Trophoblast lineage specification, differentiation and their regulation by oxygen tension. *J Endocrinol* 2018;**236**:R43–R56.

Chapman MG, Grudzinskas JG, Chard T. *Implantation: Biological and Clinical Aspects*. London, UK: Springer, 2013.

Chen AE, Egli D, Niakan K, Deng J, Akutsu H, Yamaki M, Cowan C, Fitz-Gerald C, Zhang K, Melton DA *et al*. Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell* 2009;**4**:103–106.

Cheng Y, Luo H, Izzo F, Pickering BF, Nguyen D, Myers R, Schurer A, Gourkanti S, Bruning JC, Vu LP *et al*. m(6)A RNA methylation maintains hematopoietic stem cell identity and symmetric commitment. *Cell Rep* 2019;**28**:1703–1716.e6.

Clark SJ, Lee HJ, Smallwood SA, Kelsey G, Reik W. Single-cell epigenomics: powerful new methods for understanding gene regulation and cell identity. *Genome Biol* 2016;**17**:72.

Cole LA. Hyperglycosylated hCG, a review. *Placenta* 2010;**31**:653–664.

Coppens M, Loquet P, Kollen M, De Neubourg F, Buytaert P. Longitudinal evaluation of uteroplacental and umbilical blood flow changes in normal early pregnancy. *Ultrasound Obstet Gynecol* 1996:7;114–121.

Costa A, Costantino ML, Fumero R. Oxygen exchange mechanisms in the human placenta: mathematical modelling and simulation. *J Biomed Eng* 1992;**14**:385–389.

Cross JC. How to make a placenta: mechanisms of trophoblast cell differentiation in mice—a review. *Placenta* 2005;**26 Suppl A**:S3–S9.

Damodaram M, Story L, Kulinskaya E, Rutherford M, Kumar S. Early adverse perinatal complications in preterm growth-restricted fetuses. *Aust N Z J Obstet Gynaecol* 2011;**51**:204–209.

Damsky CH, Fitzgerald M, Fisher S. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J Clin Invest* 1992;**89**:210–222.

Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK, Fisher SJ. Integrin switching regulates normal trophoblast invasion. *Development* 1994;**120**:3657–3666.

Dancis J, Schneider H. Physiology of the placenta. In: F. Tanner JM Falkner (eds). *Human Growth*. Boston, MA: Springer, 1986, 221–244.

De Paepe C, Cauffman G, Verloes A, Sterckx J, Devroey P, Tournaye H, Liebaers I, Van de Velde H. Human trophectoderm cells are not yet committed. *Hum Reprod* 2013;**28**:740–749.

Deglincerti A, Croft GF, Pietila LN, Zernicka-Goetz M, Siggia ED, Brivanlou AH. Self-organization of the in vitro attached human embryo. *Nature* 2016a;**533**:251–254.

Deglincerti A, Etoc F, Guerra MC, Martyn I, Metzger J, Ruzo A, Simunovic M, Yoney A, Brivanlou AH, Siggia E *et al*. Self-organization of human embryonic stem cells on micropatterns. *Nat Protoc* 2016b;**11**:2223–2232.

Demir R, Kosanke G, Kohnen G, Kertschanska S, Kaufmann P. Classification of human placental stem villi: review of structural and functional aspects. *Microsc Res Tech* 1997;**38**:29–41.

Earl U, Estlin C, Bulmer JN. Fibronectin and laminin in the early human placenta. *Placenta* 1990;**11**:223–231.

El-Hashash AH, Warburton D, Kimber SJ. Genes and signals regulating murine trophoblast cell development. *Mech Dev* 2010;**127**:1–20.

Enders AC. Trophoblast differentiation during the transition from trophoblastic plate to lacunar stage of implantation in the rhesus monkey and human. *Am J Anat* 1989;**186**:85–98.

Enders AC. Transition from lacunar to villous stage of implantation in the macaque, including establishment of the trophoblastic shell. *Acta Anat (Basel)* 1995;**152**:151–169.

Enders AC. Trophoblast-uterine interactions in the first days of implantation: models for the study of implantation events in the human. *Semin Reprod Med* 2000;**18**:255–263.

Enders AC, Blankenship TN. Comparative placental structure. *Adv Drug Deliv Rev* 1999;**38**:3–15.

Enders AC, Schlafke S. Cytological aspects of trophoblast-uterine interaction in early implantation. *Am J Anat* 1969;**125**:1–29.

Ezashi T, Schust DJ, Schulz LC. Modeling the placenta with stem cells. *N Engl J Med* 2019;**381**:1681–1683.

Faber JJ. Review of flow limited transfer in the placenta. *Int J Obstet Anesth* 1995;**4**:230–237.

Faber JJ, Thornburg KL, Binder ND. Physiology of placental transfer in mammals. *Am Zool* 1992;**32**:343–354.

Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update* 2007:13;121–141.

Frank H-G, Morrish D, Pötgens A, Genbacev O, Kumpel B, Caniggia I. Cell culture models of human trophoblast: primary culture of trophoblast—a workshop report. *Placenta* 2001;**22**:S107–S109.

Frendo JL, Guibourdenche J, Pidoux G, Vidaud M, Luton D, Giovangrandi Y, Porquet D, Muller F, Evain-Brion D. Trophoblast production of a weakly bioactive human chorionic gonadotropin in trisomy 21-affected pregnancy. *J Clin Endocrinol Metab* 2004;**89**: 727–732.

Freyer C, Renfree MB. The mammalian yolk sac placenta. *J Exp Zool B Mol Dev Evol* 2009;**312**:545–554.

Froen JF, Gardosi JO, Thurmann A, Francis A, Stray-Pedersen B. Restricted fetal growth in sudden intrauterine unexplained death. *Acta Obstet Gynecol Scand* 2004;**83**:801–807.

Furukawa S, Kuroda Y, Sugiyama A. A comparison of the histological structure of the placenta in experimental animals. *J Toxicol Pathol* 2014;**27**:11–18.

Gamage TK, Schierding W, Hurley D, Tsai P, Ludgate JL, Bhoothpur C, Chamley LW, Weeks RJ, Macaulay EC, James JL. The role of DNA methylation in human trophoblast differentiation. *Epigenetics* 2018a;**13**:1154–1173.

Gamage TKJB, Schierding W, Tsai P, Ludgate JL, Chamley LW, Weeks RJ, Macaulay EC, James JL. Human trophoblasts are primarily distinguished from somatic cells by differences in the pattern rather than the degree of global CpG methylation. *Biol Open* 2018b;**7**:bio034884.

Gao X, Nowak-Imialek M, Chen X, Chen D, Herrmann D, Ruan D, Chen ACH, Eckersley-Maslin MA, Ahmad S, Lee YL *et al*. Establishment of porcine and human expanded potential stem cells. *Nat Cell Biol* 2019;**21**:687–699.

Gardner R, Papaioannou V, Barton S. Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. *Development* 1973; **30**:561–572.

Gardner RL, Johnson MH. An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J Embryol Exp Morphol* 1972;**28**:279–312.

Genbacev O, Donne M, Kapidzic M, Gormley M, Lamb J, Gilmore J, Larocque N, Goldfien G, Zdravkovic T, McMaster MT *et al*. Establishment of human trophoblast progenitor cell lines from the chorion. *Stem Cells* 2011;**29**:1427–1436.

Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. *Science* 1997;**277**:1669–1672.

Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. *Placenta* 2002;**23**:3–19.

Gersell DJ, Kraus FT. Diseases of the placenta. In: Kurman RJ (ed). *Blaustein's Pathology of the Female Genital Tract*. New York, NY: Springer, 2011, 999–1073.

Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N, Lala PK. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 1993;**206**:204–211.

Graham CH, Lala PK. Mechanisms of placental invasion of the uterus and their control. *Biochem Cell Biol* 1992;**70**:867–874.

Greenwood SL, Clarson LH, Sides MK, Sibley CP. Membrane potential difference and intracellular cation concentrations in human placental trophoblast cells in culture. *J Physiol* 1996;**492** (Pt 3):629–640.

Haider S, Meinhardt G, Saleh L, Kunihs V, Gamperl M, Kaindl U, Ellinger A, Burkard TR, Fiala C, Pollheimer J *et al*. Self-renewing trophoblast organoids recapitulate the developmental program of the early human placenta. *Stem Cell Reports* 2018;**11**:537–551.

Handschuh K, Guibourdenche J, Tsatsaris V, Guesnon M, Laurendeau I, Evain-Brion D, Fournier T. Human chorionic gonadotropin expression in human trophoblasts from early placenta: comparative study between villous and extravillous trophoblastic cells. *Placenta* 2007;**28**:175–184.

Heap RB, Fleet IR, Finn C, Ellis ST, Yang CB, Whyte A, Brigstock DR. Maternal reactions affecting early embryogenesis and implantation. *J Reprod Fertil Suppl* 1988;**36**:83–97.

Hearn JP, Gidley-Baird AA, Hodges JK, Summers PM, Webley GE. Embryonic signals during the peri-implantation period in primates. *J Reprod Fertil Suppl* 1988;**36**:49–58.

Heller DS. Update on the pathology of gestational trophoblastic disease. *APMIS* 2018;**126**:647–654.

Hemberger M, Udayashankar R, Tesar P, Moore H, Burton GJ. ELF5-enforced transcriptional networks define an epigenetically regulated trophoblast stem cell compartment in the human placenta. *Hum Mol Genet* 2010;**19**:2456–2467.

Hertig AT, Rock J, Adams EC. A description of 34 human ova within the first 17 days of development. *Am J Anat* 1956;**98**:435–493.

Herzog M. A contribution to our knowledge of the earliest known stages of placentation and embryonic development in man. *Am J Anat* 1909;**9**:361–400.

Hiersch L, Shinar S, Melamed N, Aviram A, Hadar E, Yogev Y, Ashwal E. Recurrent placenta-mediated complications in women with three consecutive deliveries. *Obstet Gynecol* 2017;**129**:416–421.

Hill MA. *Embryology Main Page*. 2020, March 23. https://embryology.med.unsw.edu.au/embryology/index.php/Main_Page (23 March 2020, date last accessed).

Horii M, Bui T, Touma O, Cho HY, Parast MM. An improved two-step protocol for trophoblast differentiation of human pluripotent stem cells. *Curr Protoc Stem Cell Biol* 2019;**50**:e96.

Horii M, Li Y, Wakeland AK, Pizzo DP, Nelson KK, Sabatini K, Laurent LC, Liu Y, Parast MM. Human pluripotent stem cells as a model of trophoblast differentiation in both normal development and disease. *Proc Natl Acad Sci USA* 2016;**113**:E3882–E3891.

Hsi BL, Hunt JS, Atkinson JP. Differential expression of complement regulatory proteins on subpopulations of human trophoblast cells. *J Reprod Immunol* 1991;**19**:209–223.

Hughes AJ, Spelke DP, Xu Z, Kang CC, Schaffer DV, Herr AE. Single-cell western blotting. *Nat Methods* 2014;**11**:749–755.

Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. *J Cell Sci* 2006;**119**:3901–3903.

Hunt JS, Petroff MG, McIntire RH, Ober C. HLA-G and immune tolerance in pregnancy. *FASEB J* 2005;**19**:681–693.

Hustin J, Schaaps JP, Lambotte R. Anatomical studies of the uteroplacental vascularization in the first trimester of pregnancy. In: Kaufmann P, Miller RK (eds). *Placental Vascularization and Blood Flow*. Boston, MA: Springer, 1988, 49–60.

Islam S, Zeisel A, Joost S, La Manno G, Zajac P, Kasper M, Lonnerberg P, Linnarsson S. Quantitative single-cell RNA-seq with unique molecular identifiers. *Nat Methods* 2014;**11**:163–166.

Iwasawa T, Takahashi K, Goto M, Anzai M, Shirasawa H, Sato W, Kumazawa Y, Terada Y. Human frozen-thawed blastocyst morphokinetics observed using time-lapse cinematography reflects the number of trophectoderm cells. *PLoS One* 2019;**14**:e0210992.

James JL, Carter AM, Chamley LW. Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta* 2012;**33**:327–334.

James JL, Stone PR, Chamley LW. The regulation of trophoblast differentiation by oxygen in the first trimester of pregnancy. *Hum Reprod Update* 2006a;**12**:137–144.

James JL, Stone PR, Chamley LW. The effects of oxygen concentration and gestational age on extravillous trophoblast outgrowth in a human first trimester villous explant model. *Hum Reprod* 2006b; **21**:2699–2705.

Jauniaux E, Jurkovic D, Campbell S. In vivo investigations of the anatomy and the physiology of early human placental circulations. *Ultrasound Obstet Gynecol* 1991;**1**:435–445.

Ji L, Brkic J, Liu M, Fu G, Peng C, Wang YL. Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia. *Mol Aspects Med* 2013;**34**:981–1023.

Johnstone ED, Sibley CP, Lowen B, Guilbert LJ. Epidermal growth factor stimulation of trophoblast differentiation requires MAPK11/14 (p38 MAP kinase) activation. *Biol Reprod* 2005;**73**:1282–1288.

Jokimaa V, Inki P, Kujari H, Hirvonen O, Ekholm E, Anttila L. Expression of syndecan-1 in human placenta and decidua. *Placenta* 1998;**19**:157–163.

Kalhor N, Ramirez PT, Deavers MT, Malpica A, Silva EG. Immunohistochemical studies of trophoblastic tumors. *Am J Surg Pathol* 2009;**33**:633–638.

Kaufmann P, Castellucci M. Extravillous trophoblast in the human placenta: a review. *Placenta* 1997;**18**:21–65.

Kaufmann P, Stark J, Stegner HE. The villous stroma of the human placenta. I. The ultrastructure of fixed connective tissue cells. *Cell Tissue Res* 1977;**177**:105–121.

Kaur B, Sebire NJ. Gestational trophoblastic tumours and non-neoplastic trophoblastic lesions: morphology and immunocytochemistry to refine the diagnosis. *Diagn Histopathol* 2019;**25**: 53–65.

Kelsey G, Stegle O, Reik W. Single-cell epigenomics: recording the past and predicting the future. *Science* 2017;**358**:69–75.

Khan S, Katabuchi H, Araki M, Nishimura R, Okamura H. Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation in vitro. *Biol Reprod* 2000;**62**: 1075–1083.

Khong SL, Kane SC, Brennecke SP, da Silva Costa F. First-trimester uterine artery Doppler analysis in the prediction of later pregnancy complications. *Dis Markers* 2015;**2015**:679730.

King GJ, Atkinson BA, Robertson HA. Development of the bovine placentome from days 20 to 29 of gestation. *J Reprod Fertil* 1980; **59**:95–100.

King A, Thomas L, Bischof P. Cell culture models of trophoblast II: trophoblast cell lines—a workshop report. *Placenta* 2000;**21** Suppl A:S113–S119.

Knight A. The beginning of the end for chimpanzee experiments? *Philos Ethics Humanit Med* 2008;**3**:16.

Knofler M, Pollheimer J. Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling. *Front Genet* 2013;**4**:190.

Kohler PO, Bridson WE. Isolation of hormone-producing clonal lines of human choriocarcinoma. *J Clin Endocrinol Metab* 1971;**32**:683–687.

Krebsbach PH, Villa-Diaz LG. The role of integrin alpha6 (CD49f) in stem cells: more than a conserved biomarker. *Stem Cells Dev* 2017;**26**:1090–1099.

Kubaczka C, Kaiser F, Schorle H. Breaking the first lineage barrier - many roads to trophoblast stem cell fate. *Placenta* 2017;**60** Suppl 1:S52–S56.

Kuklina EV, Ayala C, Callaghan WM. Hypertensive disorders and severe obstetric morbidity in the United States. *Obstet Gynecol* 2009; **113**:1299–1306.

Kurjak A, Kupesic S. Doppler proof of the presence of intervillous circulation. *Ultrasound Obstet Gynecol* 1996;**7**:463–464.

Kurman RJ, Main CS, Chen H-C. Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. *Placenta* 1984;**5**:349–369.

Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. *BMC Med* 2013;**11**:154.

Leach RE, Kilburn B, Wang J, Liu Z, Romero R, Armant DR. Heparin-binding EGF-like growth factor regulates human extravillous cytotrophoblast development during conversion to the invasive phenotype. *Dev Biol* 2004;**266**:223–237.

Lee CQ, Gardner L, Turco M, Zhao N, Murray MJ, Coleman N, Rossant J, Hemberger M, Moffett A. What is trophoblast? A combination of criteria define human first-trimester trophoblast. *Stem Cell Reports* 2016;**6**:257–272.

Lee CQE, Turco MY, Gardner L, Simons BD, Hemberger M, Moffett A. Integrin alpha2 marks a niche of trophoblast progenitor cells in first trimester human placenta. *Development* 2018;**145**:dev162305.

Lees C, Marlow N, Arabin B, Bilardo CM, Brezinka C, Derks JB, Duvekot J, Frusca T, Diemert A, Ferrazzi E *et al*. Perinatal morbidity and mortality in early-onset fetal growth restriction: cohort outcomes of the trial of randomized umbilical and fetal flow in Europe (TRUFFLE). *Ultrasound Obstet Gynecol* 2013;**42**:400–408.

Leng L, Sun J, Huang J, Gong F, Yang L, Zhang S, Yuan X, Fang F, Xu X, Luo Y *et al*. Single-cell transcriptome analysis of uniparental

embryos reveals parent-of-origin effects on human preimplantation development. *Cell Stem Cell* 2019;**25**:697–712.e6.

Lewis MP, Clements M, Takeda S, Kirby PL, Seki H, Lonsdale LB, Sullivan MH, Elder MG, White JO. Partial characterization of an immortalized human trophoblast cell-line, TCL-1, which possesses a CSF-1 autocrine loop. *Placenta* 1996;**17**:137–146.

Li Y, Moretto-Zita M, Soncin F, Wakeland A, Wolfe L, Leon-Garcia S, Pandian R, Pizzo D, Cui L, Nazor K *et al*. BMP4-directed trophoblast differentiation of human embryonic stem cells is mediated through a DeltaNp63+ cytotrophoblast stem cell state. *Development* 2013;**140**:3965–3976.

Liu Y, Fan X, Wang R, Lu X, Dang YL, Wang H, Lin HY, Zhu C, Ge H, Cross JC *et al*. Single-cell RNA-seq reveals the diversity of trophoblast subtypes and patterns of differentiation in the human placenta. *Cell Res* 2018;**28**:819–832.

Loegl J, Hiden U, Nussbaumer E, Schliefsteiner C, Cvitic S, Lang I, Wadsack C, Huppertz B, Desoye G. Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis. *Reproduction* 2016;**152**:447–455.

Loke Y. Human trophoblast antigens. In: Stern CM (ed). *Immunology of Pregnancy and its Disorders*.Dordrecht, Netherlands: Springer, 1989,61–89.

Lundberg E, Borner GHH. Spatial proteomics: a powerful discovery tool for cell biology. *Nat Rev Mol Cell Biol* 2019;**20**:285–302.

Ma H, Zhai J, Wan H, Jiang X, Wang X, Wang L, Xiang Y, He X, Zhao ZA, Zhao B *et al*. In vitro culture of cynomolgus monkey embryos beyond early gastrulation. *Science* 2019;**366**:eaax7890.

Mao T-L, Kurman RJ, Huang C-C, Lin M-C, Shih I-M. Immunohistochemistry of choriocarcinoma: an aid in differential diagnosis and in elucidating pathogenesis. *Am J Surg Pathol* 2007;**31**:1726–1732.

Maruo T, Matsuo H, Otani T, Mochizuki M. Role of epidermal growth factor (EGF) and its receptor in the development of the human placenta. *Reprod Fertil Dev* 1995;**7**:1465–1470.

Marx V. A dream of single-cell proteomics. *Nat Methods* 2019;**16**:809–812.

Mayhew TM. Turnover of human villous trophoblast in normal pregnancy: what do we know and what do we need to know?. *Placenta* 2014;**35**:229–240.

Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S *et al*. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 2000;**403**:785–789.

Mihaly GW, Morgan DJ. Placental drug transfer: effects of gestational age and species. *Pharmacol Ther* 1983;**23**:253–266.

Moore R, Cai KQ, Tao W, Smith ER, Xu XX. Differential requirement for Dab2 in the development of embryonic and extraembryonic tissues. *BMC Dev Biol* 2013;**13**:39.

Morrisey EE, Musco S, Chen MY, Lu MM, Leiden JM, Parmacek MS. The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially regulated by GATA-6 and GATA-4 in the visceral endoderm. *J Biol Chem* 2000;**275**:19949–19954.

Mould A, Morgan MA, Li L, Bikoff EK, Robertson EJ. Blimp1/Prdm1 governs terminal differentiation of endovascular trophoblast giant cells and defines multipotent progenitors in the developing placenta. *Genes Dev* 2012;**26**:2063–2074.

Mühlhauser J, Crescimanno C, Kasper M, Zaccheo D, Castellucci M. Differentiation of human trophoblast populations involves alterations in cytokeratin patterns. *J Histochem Cytochem* 1995;**43**:579–589.

Mutema G, Stanek J. Numerical criteria for the diagnosis of placental chorangiosis using CD34 immunostaining. *Placenta* 1999;**20**:443–452.

Myers RE. The gross pathology of the rhesus monkey placenta. *J Reprod Med* 1972;**9**:171–198.

Nakamura T, Okamoto I, Sasaki K, Yabuta Y, Iwatani C, Tsuchiya H, Seita Y, Nakamura S, Yamamoto T, Saitou M. A developmental coordinate of pluripotency among mice, monkeys and humans. *Nature* 2016;**537**:57–62.

Newby D, Marks L, Cousins F, Duffie E, Lyall F. Villous explant culture: characterization and evaluation of a model to study trophoblast invasion. *Hypertens Pregnancy* 2005;**24**:75–91.

Niakan KK, Eggan K. Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. *Dev Biol* 2013;**375**:54–64.

Nishioka N, Inoue K, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N *et al*. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Dev Cell* 2009;**16**:398–410.

Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mech Dev* 2008;**125**:270–283.

Niu Y, Sun N, Li C, Lei Y, Huang Z, Wu J, Si C, Dai X, Liu C, Wei J *et al*. Dissecting primate early post-implantation development using long-term in vitro embryo culture. *Science* 2019;**366**:eaaw5754.

Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* 2005;**123**:917–929.

Novakovic B, Gordon L, Wong NC, Moffett A, Manuelpillai U, Craig JM, Sharkey A, Saffery R. Wide-ranging DNA methylation differences of primary trophoblast cell populations and derived cell lines: implications and opportunities for understanding trophoblast function. *Mol Hum Reprod* 2011;**17**:344–353.

O'Rahilly R, Müller F. *Development Stages in Human Embryos: Including a Revision of Streeter's "Horizons" and a Survey of the Carnegie Collection*. Washington, DC: Carnegie Institution of Washington, 1987.

Okae H, Toh H, Sato T, Hiura H, Takahashi S, Shirane K, Kabayama Y, Suyama M, Sasaki H, Arima T. Derivation of human trophoblast stem cells. *Cell Stem Cell* 2018;**22**:50–63.e6.

Orendi K, Kivity V, Sammar M, Grimpel Y, Gonen R, Meiri H, Lubzens E, Huppertz B. Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. *Placenta* 2011;**32** Suppl:S49–S54.

Papaioannou VE. Lineage analysis of inner cell mass and trophectoderm using microsurgically reconstituted mouse blastocysts. *J Embryol Exp Morphol* 1982;**68**:199–209.

Parham P, Moffett A. Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nat Rev Immunol* 2013;**13**:133–144.

Pattillo RA, Gey GO. The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro. *Cancer Res* 1968; **28**:1231–1236.

Pattillo RA, Gey GO, Delfs E, Huang WY, Hause L, Garancis DJ, Knoth M, Amatruda J, Bertino J, Friesen HG *et al*. The hormone-synthesizing trophoblastic cell in vitro: a model for cancer research and placental hormone synthesis. *Ann N Y Acad Sci* 1971a;**172**:288–298.

Pattillo RA, Gey GO, Delfs E, Mattingly RF. Human hormone production in vitro. *Science* 1968a;**159**:1467–1469.

Pattillo RA, Gey GO, Delfs E, Mattingly RF. In vitro identification of the trophoblastic stem cell of the human villous placenta. *Am J Obstet Gynecol* 1968b;**100**:582–588.

Pattillo RA, Hussa RO, Garancis JC. Glycogen metabolism in human hormone-producing trophoblastic cells in continuous culture. I. Regulation of glycogen metabolism by glucose. *In Vitro* 1971b;**7**:59–67.

Paul S, Home P, Bhattacharya B, Ray S. GATA factors: master regulators of gene expression in trophoblast progenitors. *Placenta* 2017;**60** Suppl 1:S61–S66.

Perazzolo S, Lewis RM, Sengers BG. Modelling the effect of intervillous flow on solute transfer based on 3D imaging of the human placental microstructure. *Placenta* 2017;**60**:21–27.

Pere MC. Materno-foetal exchanges and utilisation of nutrients by the foetus: comparison between species. *Reprod Nutr Dev* 2003;**43**:1–15.

Perez-Garcia V, Fineberg E, Wilson R, Murray A, Mazzeo CI, Tudor C, Sienerth A, White JK, Tuck E, Ryder EJ *et al*. Placentation defects are highly prevalent in embryonic lethal mouse mutants. *Nature* 2018;**555**:463–468.

Petropoulos S, Edsgard D, Reinius B, Deng Q, Panula SP, Codeluppi S, Plaza Reyes A, Linnarsson S, Sandberg R, Lanner F. Single-cell RNA-seq reveals lineage and X chromosome dynamics in human preimplantation embryos. *Cell* 2016;**165**:1012–1026.

Pijnenborg R, Robertson WB, Brosens I, Dixon G. Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. *Placenta* 1981;**2**:71–91.

Pijnenborg R, Vercruysse L, Carter AM. Deep trophoblast invasion and spiral artery remodelling in the placental bed of the lowland gorilla. *Placenta* 2011;**32**:586–591.

Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta* 2006;**27**: 939–958.

Plitman Mayo R. Advances in human placental biomechanics. *Comput Struct Biotechnol J* 2018;**16**:298–306.

Poaty H, Coullin P, Peko JF, Dessen P, Diatta AL, Valent A, Leguern E, Prevot S, Gombe-Mbalawa C, Candelier JJ *et al*. Genome-wide high-resolution aCGH analysis of gestational choriocarcinomas. *PLoS One* 2012;**7**:e29426.

Potgens AJ, Gaus G, Frank HG, Kaufmann P. Characterization of trophoblast cell isolations by a modified flow cytometry assay. *Placenta* 2001;**22**:251–255.

Pujades C, Guez-Guez B, Dunon D. Melanoma cell adhesion molecule (MCAM) expression in the myogenic lineage during early chick embryonic development. *Int J Dev Biol* 2002;**46**:263–266.

Redman C. The six stages of pre-eclampsia. *Pregnancy Hypertens* 2014;**4**:246.

Reyes L, Golos TG. Hofbauer cells: their role in healthy and complicated pregnancy. *Front Immunol* 2018;**9**:2628.

Roberts RM, Ezashi T, Sheridan M, Yang Y. Specification of trophoblast from embryonic stem cells exposed to BMP4. *Biol Reprod* 2018;**99**:212–224.

Roberts VHJ, Morgan TK, Bednarek P, Morita M, Burton GJ, Lo JO, Frias AE. Early first trimester uteroplacental flow and the progressive disintegration of spiral artery plugs: new insights from contrast-enhanced ultrasound and tissue histopathology. *Hum Reprod* 2017;**32**:2382–2393.

Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* 1992;**80**:283–285.

Rodger MA, Gris J-C, de Vries JIP, Martinelli I, Rey É, Schleussner E, Middeldorp S, Kaaja R, Langlois NJ, Ramsay T *et al*. Low-molecular-weight heparin and recurrent placenta-mediated pregnancy complications: a meta-analysis of individual patient data from randomised controlled trials. *Lancet* 2016;**388**:2629–2641.

Rossant J, Cross JC. Placental development: lessons from mouse mutants. *Nat Rev Genet* 2001;**2**:538–548.

Rossant J, Tam PPL. Exploring early human embryo development. *Science* 2018;**360**:1075–1076.

Sasaki K, Nakamura T, Okamoto I, Yabuta Y, Iwatani C, Tsuchiya H, Seita Y, Nakamura S, Shiraki N, Takakuwa T *et al*. The germ cell fate of cynomolgus monkeys is specified in the nascent amnion. *Dev Cell* 2016;**39**:169–185.

Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, Van Houdt WJ, Pronk A, Van Gorp J, Siersema PD *et al*. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011;**141**:1762–1772.

Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ *et al*. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009;**459**:262–265.

Schlafke S, Enders AC. Cellular basis of interaction between trophoblast and uterus at implantation. *Biol Reprod* 1975;**12**:41–65.

Schliefsteiner C, Peinhaupt M, Kopp S, Logl J, Lang-Olip I, Hiden U, Heinemann A, Desoye G, Wadsack C. Human placental hofbauer cells maintain an anti-inflammatory M2 phenotype despite the presence of gestational diabetes mellitus. *Front Immunol* 2017;**8**:888.

Schroder HJ. Comparative aspects of placental exchange functions. *Eur J Obstet Gynecol Reprod Biol* 1995;**63**:81–90.

Seval Y, Korgun ET, Demir R. Hofbauer cells in early human placenta: possible implications in vasculogenesis and angiogenesis. *Placenta* 2007;**28**:841–845.

Shahbazi MN, Jedrusik A, Vuoristo S, Recher G, Hupalowska A, Bolton V, Fogarty NM, Campbell A, Devito LG, Ilic D *et al*. Self-organization of the human embryo in the absence of maternal tissues. *Nat Cell Biol* 2016;18:700–708.

Shao Y, Taniguchi K, Townshend RF, Miki T, Gumucio DL, Fu J. A pluripotent stem cell-based model for post-implantation human amniotic sac development. *Nat Commun* 2017;**8**:208.

Sharkey AM, Gardner L, Hiby S, Farrell L, Apps R, Masters L, Goodridge J, Lathbury L, Stewart CA, Verma S *et al*. Killer Ig-like receptor expression in uterine NK cells is biased toward recognition of HLA-C and alters with gestational age. *J Immunol* 2008; **181**:39–46.

Shih IM. The role of CD146 (Mel-CAM) in biology and pathology. *J Pathol* 1999;**189**:4–11.

Shih I-M, Kurman RJ. p63 expression is useful in the distinction of epithelioid trophoblastic and placental site trophoblastic tumors by profiling trophoblastic subpopulations. *A J Surg Pathol* 2004;**28**: 1177–1183.

Shih I-M, Ronnett BM, Mazur M, Kurman RJ. Gestational trophoblastic tumors and related tumorlike lesions. In: RJ Kurman, LH Ellenson, BM Ronnett (eds). *Blaustein's Pathology of the Female Genital Tract*. New York, NY: Springer, 2018,1–71.

Shiverick K, King A, Frank H-G, Whitley GSJ, Cartwright J, Schneider H. Cell culture models of human trophoblast II: trophoblast cell lines—a workshop report. *Placenta* 2001;**22**:S104–S106.

Shorter S, Starkey P, Ferry B, Clover L, Sargent I, Redman C. Antigenic heterogeneity of human cytotrophoblast and evidence for the transient expression of MHC class I antigens distinct from HLA-G. *Placenta* 1993;**14**:571–582.

Simmons DG. Postimplantation placental development of the chorioallantoic placenta. In BA Croy, AT Yamada, FJ DeMayo, SL Adamson (eds). *The Guide to Investigation of Mouse Pregnancy*. Cambridge, MA: Academic Press, 2013, 143–161.

Simmons DG, Fortier AL, Cross JC. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. *Dev Biol* 2007;**304**:567–578.

Skeith L, Rodger M. Anticoagulants to prevent recurrent placenta-mediated pregnancy complications: is it time to put the needles away? *Thromb Res* 2017;**151**:S38–S42.

Snell GD, Stevens LC. Early embryology. *Biol Lab Mouse* 1966;**2**: 205–245.

Soares MJ, Varberg KM, Iqbal K. Hemochorial placentation: development, function, and adaptations. *Biol Reprod* 2018;**99**:196–211.

Soncin F, Khater M, To C, Pizzo D, Farah O, Wakeland A, Arul Nambi Rajan K, Nelson KK, Chang CW, Moretto-Zita M *et al*. Comparative analysis of mouse and human placentae across gestation reveals species-specific regulators of placental development. *Development* 2018;**145**:dev156273.

Soncin F, Natale D, Parast MM. Signaling pathways in mouse and human trophoblast differentiation: a comparative review. *Cell Mol Life Sci* 2015;**72**:1291–1302.

Sooranna SR, Oteng-Ntim E, Meah R, Ryder TA, Bajoria R. Characterization of human placental explants: morphological, biochemical and physiological studies using first and third trimester placenta. *Hum Reprod* 1999;**14**:536–541.

Stirparo GG, Boroviak T, Guo G, Nichols J, Smith A, Bertone P. Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast. *Development* 2018;**145**:dev158501.

Straszewski-Chavez SL, Abrahams VM, Alvero AB, Aldo PB, Ma Y, Guller S, Romero R, Mor G. The isolation and characterization of a novel telomerase immortalized first trimester trophoblast cell line, Swan 71. *Placenta* 2009;**30**:939–948.

Su Y, Shi Q, Wei W. Single cell proteomics in biomedicine: High-dimensional data acquisition, visualization, and analysis. *Proteomics* 2017;**17**: 10.1002/pmic.201600267.

Sudheer S, Bhushan R, Fauler B, Lehrach H, Adjaye J. FGF inhibition directs BMP4-mediated differentiation of human embryonic stem cells to syncytiotrophoblast. *Stem Cells Dev* 2012:21;2987–3000.

Takebe T, Wells JM. Organoids by design. *Science* 2019;**364**: 956–959.

Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. Promotion of trophoblast stem cell proliferation by FGF4. *Science* 1998;**282**:2072–2075.

Tarrade A, Kuen RL, Malassiné A, Tricottet V, Blain P, Vidaud M, Evain-Brion D. Characterization of human villous and extravillous trophoblasts isolated from first trimester placenta. *Lab Invest* 2001; **81**:1199.

Tarui H, Tomigahara Y, Nagahori H, Sugimoto K, Mogi M, Kawamura S, Isobe N, Kaneko H. Species differences in the developmental toxicity of procymidone—placental transfer of procymidone in pregnant rats, rabbits, and monkeys. *J Pest Sci* 2018;**43**: 79–87.

Teasdale F, Jean-Jacques G. Morphometric evaluation of the microvillous surface enlargement factor in the human placenta from mid-gestation to term. *Placenta* 1985:6;375–381.

Theiler K. *The House Mouse: Atlas of Embryonic Development*. New York, NY: Springer, 1972.

Tilburgs T, Crespo AC, van der Zwan A, Rybalov B, Raj T, Stranger B, Gardner L, Moffett A, Strominger JL. Human HLA-G+ extravillous trophoblasts: Immune-activating cells that interact with decidual leukocytes. *Proc Natl Acad Sci USA* 2015;**112**:7219–7224.

Treissman J, Yuan V, Baltayeva J, Le HT, Castellana B, Robinson WP, Beristain AG. Low oxygen enhances trophoblast column growth by potentiating differentiation of the extravillous lineage and promoting LOX activity. *Development* 2020;**147**:dev181263.

Turco MY, Gardner L, Hughes J, Cindrova-Davies T, Gomez MJ, Farrell L, Hollinshead M, Marsh SGE, Brosens JJ, Critchley HO *et al*. Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium. *Nat Cell Biol* 2017;**19**:568–577.

Turco MY, Gardner L, Kay RG, Hamilton RS, Prater M, Hollinshead MS, McWhinnie A, Esposito L, Fernando R, Skelton H *et al*. Trophoblast organoids as a model for maternal-fetal interactions during human placentation. *Nature* 2018;**564**:263–267.

Tuuli MG, Longtine MS, Nelson DM. Review: oxygen and trophoblast biology—a source of controversy. *Placenta* 2011;**32** Suppl 2: S109–S118.

Valentin L, Sladkevicius P, Laurini R, Soderberg H, Marsal K. Uteroplacental and luteal circulation in normal first-trimester pregnancies: Doppler ultrasonographic and morphologic study. *Am J Obstet Gynecol* 1996;**174**:768–775.

Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, Park J-E, Stephenson E, Polański K, Goncalves A *et al*. Single-cell reconstruction of the early maternal–fetal interface in humans. *Nature* 2018;**563**:347–353.

Wagner DE, Weinreb C, Collins ZM, Briggs JA, Megason SG, Klein AM. Single-cell mapping of gene expression landscapes and lineage in the zebrafish embryo. *Science* 2018;**360**:981–987.

Wagner GP, Kin K, Muglia L, Pavlicev M. Evolution of mammalian pregnancy and the origin of the decidual stromal cell. *Int J Dev Biol* 2014;**58**:117–126.

Wakeland AK, Soncin F, Moretto-Zita M, Chang CW, Horii M, Pizzo D, Nelson KK, Laurent LC, Parast MM. Hypoxia directs human extravillous trophoblast differentiation in a hypoxia-inducible factor-dependent manner. *Am J Pathol* 2017;**187**: 767–780.

Wang X, Chen C, Wang L, Chen D, Guang W, French J. Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. *Fertil Steril* 2003;**79**:577–584.

Wang Z, Yan X. CD146, a multi-functional molecule beyond adhesion. *Cancer Lett* 2013;**330**:150–162.

Wathes DC, Wooding FB. An electron microscopic study of implantation in the cow. *Am J Anat* 1980;**159**:285–306.

Weiss G, Sundl M, Glasner A, Huppertz B, Moser G. The trophoblast plug during early pregnancy: a deeper insight. *Histochem Cell Biol* 2016;**146**:749–756.

Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC. Incidence of early loss of pregnancy. *N Engl J Med* 1988;**319**:189–194.

Wimsatt WA. Some comparative aspects of implantation. *Biol Reprod* 1975;**12**:1–40.

Xiang L, Yin Y, Zheng Y, Ma Y, Li Y, Zhao Z, Guo J, Ai Z, Niu Y, Duan K, He J, Ren S, Wu D, Bai Y, Shang Z, Dai X, Ji W, Li T. A developmental landscape of 3D-cultured human pre-gastrulation embryos. *Nature* 2020;**577**:537–542.

Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 2002;**20**:1261–1264.

Yabe S, Alexenko AP, Amita M, Yang Y, Schust DJ, Sadovsky Y, Ezashi T, Roberts RM. Comparison of syncytiotrophoblast generated from human embryonic stem cells and from term placentas. *Proc Natl Acad Sci USA* 2016;**113**:E2598–E2607.

Yan L, Yang M, Guo H, Yang L, Wu J, Li R, Liu P, Lian Y, Zheng X, Yan J *et al*. Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. *Nat Struct Mol Biol* 2013;**20**:1131–1139.

Yang D-H, Smith ER, Roland IH, Sheng Z, He J, Martin WD, Hamilton TC, Lambeth JD, Xu X-X. Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. *Dev Biol* 2002;**251**:27–44.

Yang Y, Adachi K, Sheridan MA, Alexenko AP, Schust DJ, Schulz LC, Ezashi T, Roberts RM. Heightened potency of human pluripotent stem cell lines created by transient BMP4 exposure. *Proc Natl Acad Sci USA* 2015;**112**:E2337–E2346.

Yu C, Shen K, Lin M, Chen P, Lin C, Chang GD, Chen H. GCMa regulates the syncytin-mediated trophoblastic fusion. *J Biol Chem* 2002;**277**:50062–50068.

Zdravkovic T, Nazor KL, Larocque N, Gormley M, Donne M, Hunkapillar N, Giritharan G, Bernstein HS, Wei G, Hebrok M *et al*. Human stem cells from single blastomeres reveal pathways of embryonic or trophoblast fate specification. *Development* 2015; **142**:4010–4025.

Zhou F, Wang R, Yuan P, Ren Y, Mao Y, Li R, Lian Y, Li J, Wen L, Yan L *et al*. Reconstituting the transcriptome and DNA methylome landscapes of human implantation. *Nature* 2019;**572**: 660–664.