

REVIEW

## Intrauterine trophoblast migration: A comparative view of humans and rodents

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### ABSTRACT

Trophoblast migration and invasion through the decidua and maternal uterine spiral arteries are crucial events in placentation. During this process, invasive trophoblast replace vascular endothelial cells as the uterine arteries are remodeled to form more permissive vessels that facilitate adequate blood flow to the growing fetus. Placentation failures resulting from either extensive or shallow trophoblastic invasion can cause pregnancy complications such as preeclampsia, intrauterine growth restriction, placenta creta, gestational trophoblastic disease and even maternal or fetal death. Consequently, the use of experimental animal models such as rats and mice has led to great progress in recent years with regards to the identification of mechanisms and factors that control trophoblast migration kinetics. This review aims to perform a comparative analysis of placentation and the mechanisms and factors that coordinate intrauterine trophoblast migration in humans, rats and mice under physiological and pathological conditions.

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### Introduction

The migration of trophoblasts at the maternal-fetal interface is a key process in implantation and placentation and is therefore essential for successful pregnancy outcomes in women and rodents.<sup>1,2</sup> Placentation failures resulting from either extensive or shallow trophoblastic invasion can cause pregnancy complications such as preeclampsia, intrauterine growth restriction, placenta creta, prematurity, gestational trophoblastic disease and even maternal or fetal death.<sup>3–6</sup> Consequently, some reports have attempted to evaluate the molecular mechanisms controlling trophoblastic invasion and migration under physiological conditions,<sup>7–9</sup> and under pathological conditions, e.g., preeclampsia, intrauterine growth restriction, gestational diabetes and maternal hypothyroidism.<sup>6,10–12,164</sup>

The trophoblasts that form the placenta originate from the embryonic trophoblast and are the first cell lineage in mammalian development.<sup>13</sup> In this moment, after its differentiation, whether the trophoblast is apposed to uterine epithelium, the endothelium of maternal vessels, or directly to maternal blood, placentas are classified as, respectively, epitheliochorial, endotheliochorial or hemochorial. In this last type of placenta, observed in humans, rats, mice, guinea pigs, armadillos,

rabbits and apes, specialized populations of trophoblasts are able to leave the placenta and move toward the decidua to directly contact maternal blood.<sup>13,14</sup> During this process, trophoblast stem cells proliferate and can differentiate into various trophoblast lineages. Between them, interstitial and endovascular trophoblasts exhibit migratory and invasive properties and have the capacity to recognize, modify and stimulate the behavior of other cell types at the maternal-fetal interface. This cellular communication is precisely controlled by maternal factors and factors released and/or expressed by trophoblastic cells themselves such as integrins, E-cadherin, proteases, cytokines, interleukins and growth factors. That allow the trophoblast cell to degrade extracellular matrix (ECM) proteins such as collagen IV, laminin, vitronectin and fibronectin to promote cell migration, while the decidua expresses a variety of inhibitory proteins that controls trophoblastic cell invasion.<sup>14</sup> Consequently, invasive trophoblasts replace vascular endothelial cells as the uterine arteries are remodeled to form more permissive vessels that facilitate adequate blood flow to the growing fetus.<sup>15</sup>

Changes or inadequate responses within the regulatory pathways that control trophoblast invasion and migration compromise placental development and can negatively affect maternal and fetal health, as well as

postnatal development.<sup>3,5,6,12</sup> During the 1970s, Brosens et al.<sup>10,11</sup> observed that failures in human trophoblast invasion and the absence of adequate vascular remodeling of the utero-placental arteries in the placental bed were associated with intrauterine growth restriction and/or preeclampsia. Since then, intrauterine trophoblast migration and invasion has been a major focus of placentation research. As there are some morphological and functional similarities among species that have hemochorial placenta, rat and mice animal models have been useful in the study of many aspects of human placentation.<sup>16,17</sup> This review aims to perform a comparative analysis of placentation and the mechanisms and factors involved in the cellular interactions that coordinate intrauterine trophoblast migration in humans, rats and mice under physiological and pathological conditions.

## Placental organization

Hemochorial placental development is characterized by close contact between maternal and fetal tissues and occurs in humans and rodents such as the rat and mouse. During this process, trophoblast stem cells originate from the embryonic trophoblast and can differentiate into various trophoblast lineages. One of the key activities of differentiated trophoblast cells is remodeling uterine spiral arteries. Vascular remodeling transforms tightly coiled uterine spiral arteries into dilated vessels that are no longer under maternal control. Restructuring maternal vasculature is essential for the optimal delivery of nutrients to the fetus.<sup>1-3,16,17,26</sup> However, despite the hemochorial placentation and especially trophoblast-directed vascular remodeling in humans, rats and mice are highly similar, there are differences in structure, placental development and some types of trophoblast cells between the human and rodent placentas.

### Human

One of the initial processes in human pregnancy is characterized by the adhesion of the blastocyst to the uterine decidua. This apposition is the first step in implantation and occurs approximately 6 to 7 d following conception (Fig. 1). At this stage, the endometrium has already been decidualized once; in contrast with mice, human decidualization is not dependent on blastocyst implantation and instead begins on day 14 of the menstrual cycle due to the effects of progesterone.<sup>46</sup>

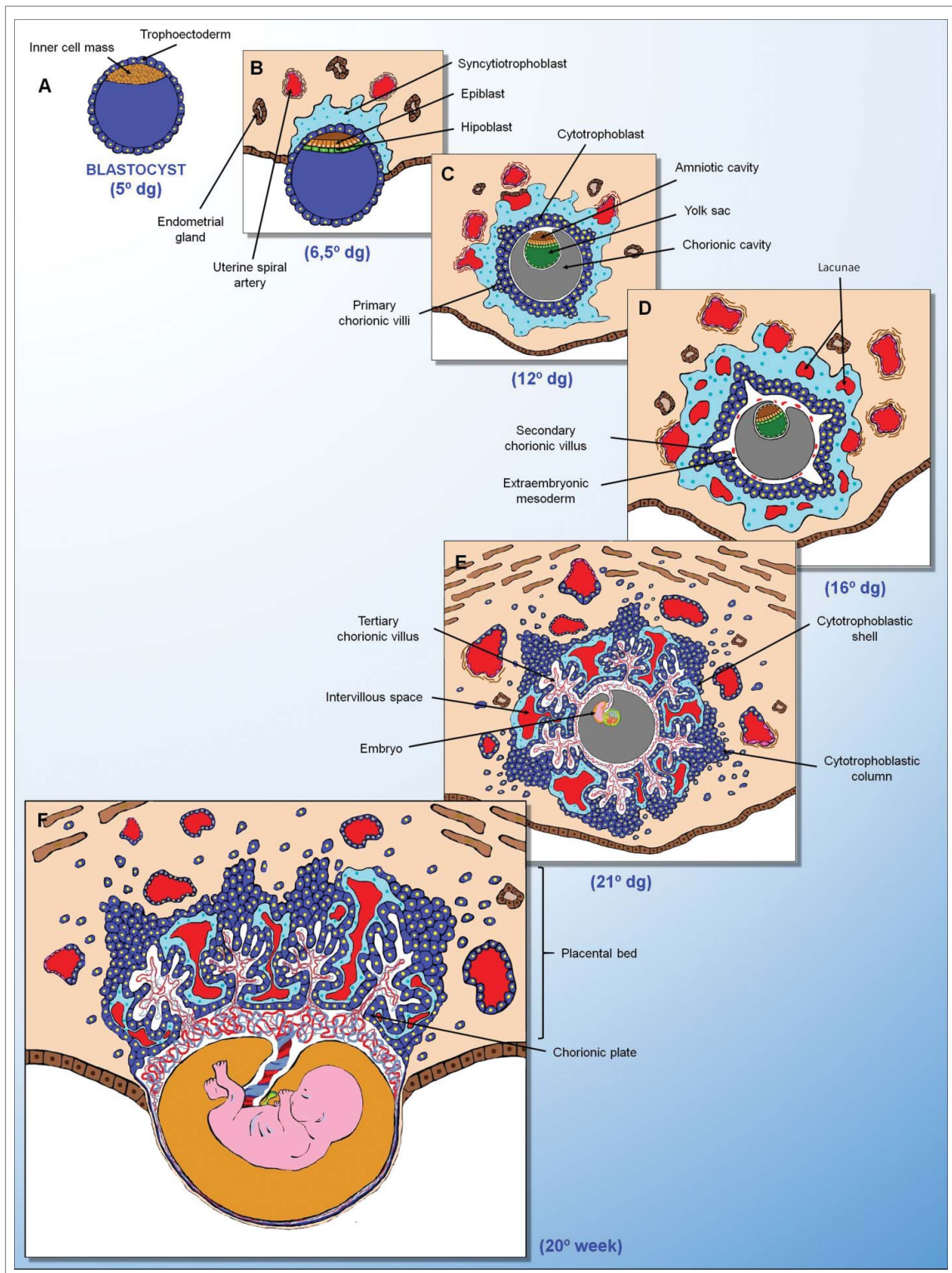
The outer layer of the blastocyst is formed by trophoblast. Close contact between the fetal trophoblasts and maternal uterine tissue leads to the formation of the maternal-fetal junctional zone. The junctional zone will form the basal plate, which is the maternal surface of the

placenta, and the fetal surface is called the chorionic plate.<sup>47,48</sup>

After the formation of the junctional zone, implantation continues through trophoblast proliferation and differentiation in cytotrophoblast (innermost) and syncytiotrophoblast (outermost).<sup>13,15,16,21</sup> The syncytiotrophoblast expands and erodes maternal capillaries and endometrial glands in the decidua to release blood and the contents of the glands inside the lacunae. The cytotrophoblast sequentially proliferates to form the primary, secondary and tertiary villi (villous trophoblast). The trophoblasts will then form columns, proliferate and extend beyond the syncytiotrophoblast, joining one another and forming the cytotrophoblastic shell. By the eighth week of pregnancy, the continuity of the cytotrophoblastic shell is lost and trophoblasts at the top of the anchoring villi will give rise to the extravillous trophoblast (EVT) that will migrate through the basal plate, decidualized endometrium and underlying myometrium to form the placental bed.<sup>47,48</sup>

The extravillous trophoblast, corresponding to all of the trophoblasts residing outside of placental villi, acquires the capacity to degrade extracellular matrix proteins to permit these cells to mobilize and invade the decidua (interstitial trophoblast) and the wall of maternal uterine spiral arteries (endovascular trophoblast). Endovascular trophoblasts invade the lumen of the spiral arteries and incorporate into the arterial wall (intramural trophoblast). The endothelium, the muscular layer and the elastic material of these arteries are destroyed and replaced with an extracellular matrix called the fibrinoid and by the EVT cells themselves. This process, known as trophoblastic arterial remodeling transforms the uterine spiral arteries into easily distensible, limp, thin-walled vessels that allow continuous and adequate blood supply in the placenta and thereby ensure the gestational success.<sup>6,7,16,50-52</sup>

The density of extravillous trophoblast and the depth of invasion of the utero-placental arteries are the most prominent in the central placental bed, and both the density and depth of invasion decrease with proximity to the placental margins. The control of this intrauterine migration allows a peak of trophoblastic invasion around the 12<sup>th</sup> week of pregnancy that is shortly followed by a decline. The space control restricts the depth of endovascular and interstitial trophoblast invasion to the decidua and proximal third of the myometrium.<sup>53,54</sup> Deregulation of these processes results in a wide variety of pregnancy complications such as intrauterine growth restriction, preeclampsia, placenta creta, gestational trophoblastic disease and abortion.<sup>3,55-57</sup> While preeclampsia and intrauterine growth restriction are characterized by a failure of trophoblastic invasion, placenta creta and



**Figure 1.** Human placental development. (A) Blastocyst (5° dg). (B) Implantation phase (6.5° dg). Trophectoderm differentiation and syncytiotrophoblast infiltration of the endometrium. (C) Post-implantation phase (12° dg). Syncytiotrophoblast-mediated erosion of the maternal blood vessels and endometrial glands, and formation of the primary chorionic villi from cytotrophoblasts. (D) Post-implantation phase (16° dg). Differentiation of the extraembryonic mesoderm and formation of the secondary chorionic villi. (E) Pre-placental phase (21° dg). Formation of the tertiary chorionic villi and cytotrophoblastic shell. (F) Definitive placenta (20° weeks). Evidence of the placental bed formed by the chorionic villi, decidua and adjacent myometrium. dg = days of gestation.

gestational trophoblastic disease are characterized by excessive trophoblastic invasion by either “normal” trophoblasts, or by trophoblastic tumor or pre-tumor cells, respectively.<sup>58,59</sup>

The villous trophoblast, which gives rise to extravillous trophoblast, covers the connective tissue shaft, extending from the chorionic plate until the basal plate and is where the greater diameter fetal vessels are located. The chorionic villi originate from these fibrous septa (also called villous septa). The space between the villous septa is called the intervillous space and is filled with maternal blood from the uterine arteries. The chorionic villi are formed by a central axis of connective tissue that contains a vast fetal capillary network surrounded by a layer of cytotrophoblasts and the multinucleate syncytiotrophoblast.<sup>6,49,50</sup>

The syncytiotrophoblast is more abundant and forms the outer surface of the villi, making direct contact with maternal blood and is formed from the fusion of cytotrophoblasts. The syncytiotrophoblast undergoes a constant and controlled process of renewal and apoptosis, and its primary functions are nutrient absorption, waste product removal, and synthesis of hormones such as hCG. To facilitate the transport of nutrients to the fetus and the removal of waste and harmful toxins from fetal circulation, a wide variety of transporters are expressed in the syncytiotrophoblast, including ion channels, aquaporins and ATP-binding cassette transporters.<sup>6,49,50</sup>

The cytotrophoblasts are located below the syncytiotrophoblast and are mononuclear and globular. The fetal blood makes exchanges with the maternal blood circulating in the intervillous space and bathing the villi. As the pregnancy progresses, the number of blood vessels in the chorionic villi increases while the amount of connective tissue decreases, facilitating the exchange of metabolites between the mother and the fetus.<sup>6,49,50</sup> In humans, the final structure of the placenta is discoid and becomes apparent at approximately 21 d of gestation.<sup>16,49</sup>

## Rodent

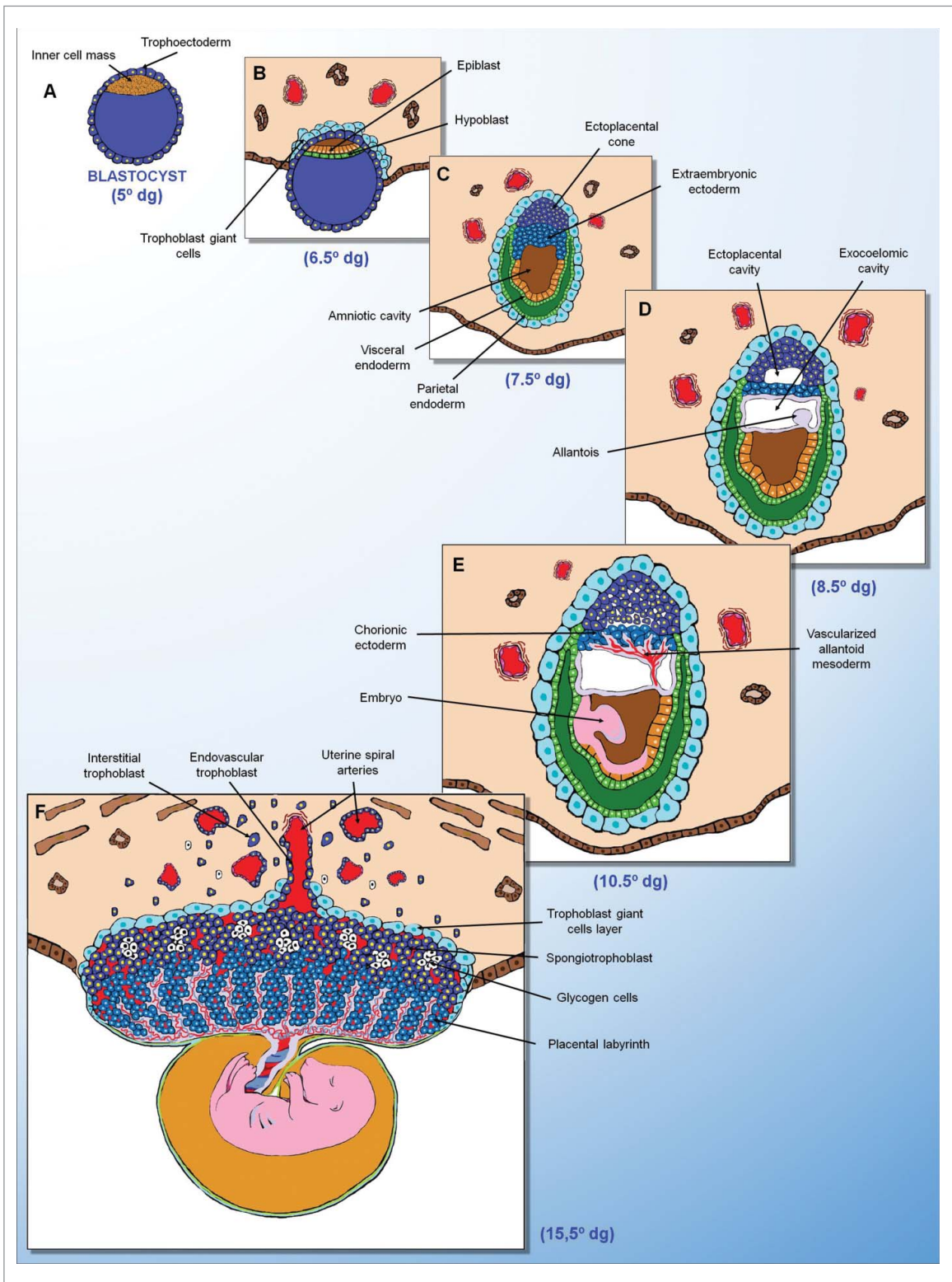
Following implantation, particularly in rats and mice, the embryo is completely covered by various trophoblast cell types with distinct spatial locations and gene expression patterns. In contrast with humans, the extraembryonic ectoderm, particularly the polar trophoblast, differentiates into chorionic ectoderm and the ectoplacental cone that will later give rise to the placental labyrinth and junctional zone, respectively (Fig. 2).<sup>60,61</sup> Mossman<sup>62</sup> suggested that the ectoplacental cone in rats and mice is analogous to cytotrophoblastic shell formed in the human placenta; however, in rodents, the ectoplacental cone proliferates in an orderly manner to form the

trophospongium, while the trophoblasts of the human cytotrophoblastic shell proliferate in a disorderly manner through the basal plate, decidua and underlying myometrium.<sup>16</sup>

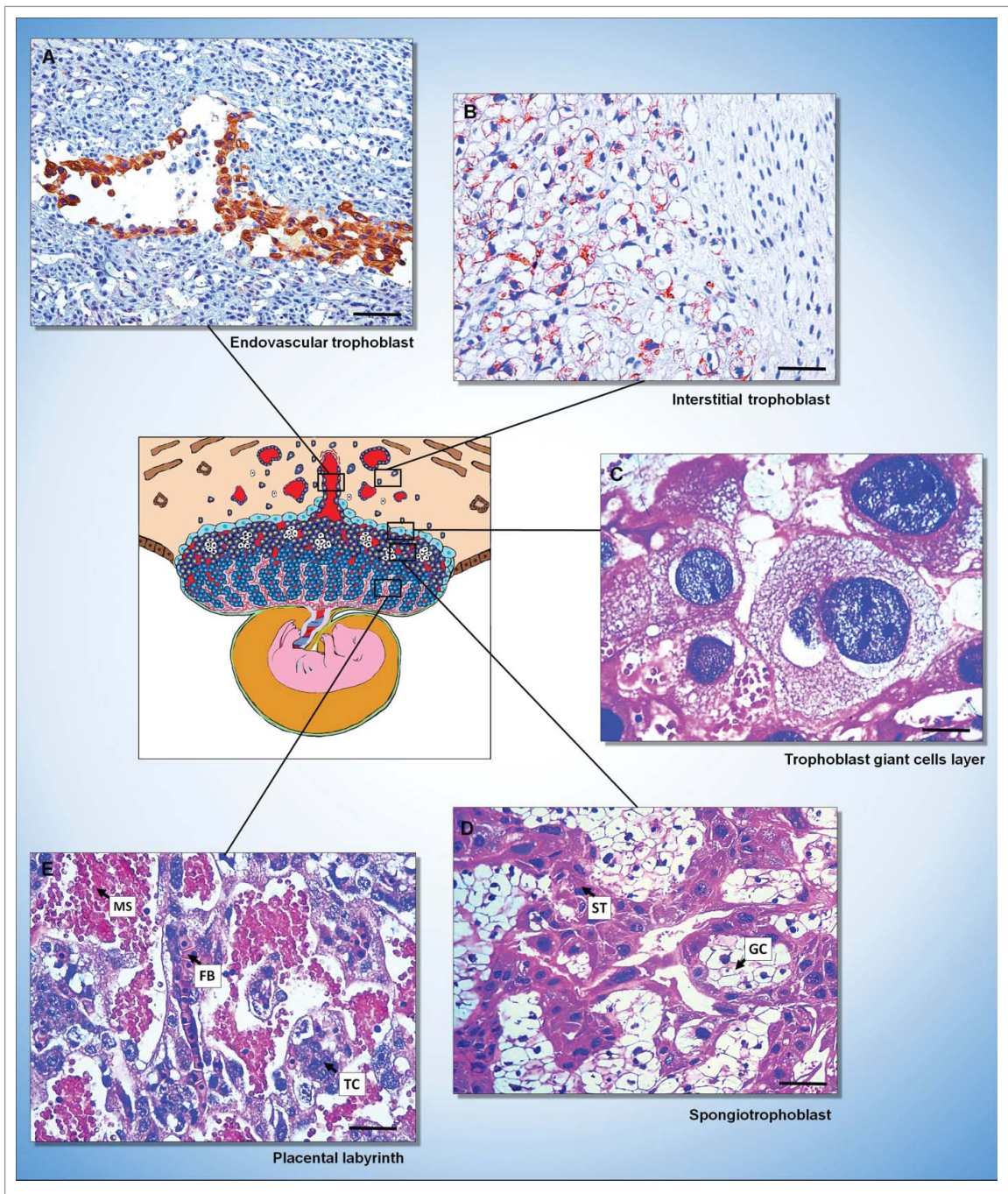
The definitive placenta of mice and rats is discoid and becomes established at approximately the 11th and 12th days of gestation, respectively, ie, in the middle of gestation. It has 3 anatomically and physiologically distinct regions (Fig. 3): the placental labyrinth located more internally on the fetal-placental unit; the junctional zone, which is composed of the spongiotrophoblast and glycogen cells and is formed from the original wall of the blastocyst and remains unicellular; and the trophoblast giant cell layer formed by the penetration of trophoblasts into the endometrium.<sup>18,63</sup>

The trophoblast giant cell layer (Fig. 3C) is located more externally on the fetal-placental unit and are the first cells that mediate fetal implantation and uterine invasion. These are known as secondary trophoblast giant cells and are derived from the polar trophoblast, whereas the primary trophoblast giant cells do not contribute to the definitive placenta and are derived from the mural trophoblast.<sup>18</sup> The trophoblast giant cells arise from trophoblasts that leave the cell cycle, stop dividing, and become polyploid through endoreplication, undergoing cycles of DNA replication without mitosis. They are one of the major endocrine cells of the placenta because they produce many cytokines and hormones that regulate the flow of maternal blood to the implantation site, ovarian progesterone synthesis and lactogenesis.<sup>67,68</sup> Most trophoblast giant cells migrate to the decidua; however, a subtype of trophoblastic giant cells that invades the spiral arteries that bring the maternal blood to the implantation site was identified in rat placenta.<sup>69</sup> Based on this evidence, the trophoblastic giant cells are also known to participate in the uterine vascular remodeling, at least in rats.<sup>68</sup>

The junctional zone, the middle layer of the placenta, is bordered by trophoblast giant cell layer and, more externally, by mesometrial decidua. This region is also referred to as the “trophospongium,” “spongiotrophoblast” and “spongy region.” Four trophoblast lineages differentiate from the progenitor cells present in the junctional zone: secondary trophoblastic giant cells, spongiotrophoblasts, glycogen cells, and invasive trophoblasts. In response to close contact with the inner cell mass, all of these cells undergo fibroblast growth factor 4 (FGF4)-mediated proliferation.<sup>18,67,69,70</sup> The spongiotrophoblasts are ovoid and mononuclear, while the glycogen cells, which appear in the placenta from the 12<sup>th</sup> day of gestation, possess more condensed nuclei and have glycogen granules in their cytoplasm.<sup>68,71</sup>



**Figure 2.** Rat placental development. (A) Blastocyst (5° dg). (B) Implantation phase (6.5° dg). Trophoectoderm differentiation and primary trophoblast giant cell infiltration of the endometrium. (C) Post-implantation phase (7.5° dg). Trophoectoderm differentiation with the formation of giant cells around the embryo and ectoplacental cone. (D) Post-implantation phase (8.5° dg). Allantois formation from the posterior epiblast. (E) Pre-placental phase (10.5° dg). Placental labyrinth formation from the fusion of the allantois mesoderm with the chorionic ectoderm. (F) Definite placenta (15° dg). Evidence of the trophoblast giant cell layer, spongiotrophoblast and placental labyrinth. dg = days of gestation.



**Figure 3.** Rat placenta. (A and B) Immunohistochemical evidence of the endovascular (A) and interstitial (B) trophoblast in the decidua stained with AE1/AE3 cytokeratin antibody. [Streptavidin-biotin-peroxidase method, Harris' hematoxylin counterstain, bar = 150  $\mu\text{m}$  (A); 64  $\mu\text{m}$  (B)]. (C-E) Histological evidence of the 3 layers of the placenta [trophoblast giant cells layer (C); spongiotrophoblast (D) formed by spongiotrophoblasts (ST) and glycogen cells (GC); placental labyrinth (E) formed by fetal blood vessels (FB), trophoblast cells (TC) and maternal sinusoids (MS)]. [Hematoxylin and eosin, bar = 64  $\mu\text{m}$ ].

The spongiotrophoblast (Fig. 3D) acts as an endocrine glandular compartment that maintains corpus luteum secretion of progesterone and performs other functions. The spongiotrophoblast, and secondarily, the syncytiotrophoblast, produce luteotropic and lactogenic hormones during pregnancy.<sup>8,13</sup> Another supposed spongiotrophoblast function is the limiting

of fetal endothelium growth in the maternal placenta by secreting anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and proliferin-related protein (rPlf).<sup>69,72</sup> In the event of changes in placental development, qualitative changes occur in the balance between spongiotrophoblasts and glycogen cells.<sup>70,73</sup>

Glycogen cells are trophoblasts of an uncertain origin that arise in the spongiotrophoblast. They are believed to originate from spongiotrophoblasts that express a specific gene, *Tpbp* (4311).<sup>69</sup> Alternatively, there is evidence that glycogen cells may be distinct from the spongiotrophoblast once they express protocadherin 12 (*PCDH12*), a specific marker of a cell group that is distinct from that which gives rise to spongiotrophoblasts in the ectoplacental cone.<sup>74</sup>

Glycogen cells are also similar to the human extravillous trophoblast in that they invade the uterine spiral arteries that carry blood to the implantation site and remodel the arterial muscle wall. This process results in increased blood flow, carrying oxygen and nutrients to the implantation site. In rats, glycogen cells invade the maternal decidua and can interact with the uterine natural killer cells (uNKs), stimulating them to modify the uterine spiral arteries.<sup>69</sup> Once in the decidua, the glycogen cells undergo a cytolytic process, releasing their intracellular components, glycogen and hormones into the interstitial space. These components are likely to be important as energy sources and in hormonal control of pregnancy and/or fetal development.<sup>74</sup>

Invasive trophoblasts, which originate from the junctional zone, also participate in uterine vascular remodeling along with the glycogen cells and uNKs, being that the exit of these cells from the placenta and their movements into the uterine stroma and/or myometrium is temporally and spatially well defined. These cells surround and penetrate the uterine spiral arteries and exhibit a retrograde migration similar to that of the human extravillous trophoblast.<sup>16</sup> The invasive endovascular trophoblasts (Fig. 3A) replace the endothelium while the interstitial trophoblasts are localized between the blood vessels.<sup>8,9</sup> Prior to 13.5 d of gestation, trophoblast invasion in rats and mice is limited to the endovascular environment and is confined to the mesometrial decidua. Following 14.5 d of gestation, endovascular and interstitial trophoblast (Fig. 3B) in rats can be identified in the metrial gland.<sup>8,32</sup> Research has demonstrated that arteries present at the maternal-fetal interface begin to dilate when they are near interstitial trophoblasts that are expressing nitric oxide synthase (NOS), endothelial nitric oxide synthase (eNOS) and possibly inducible nitric oxide synthase (iNOS).<sup>66</sup> Furthermore, Cartwright et al.<sup>75</sup> demonstrated that *in vitro* cell motility and trophoblast invasion is highly dependent upon trophoblast-produced NOS. The precise coordination of the entire uterine vascular remodeling process is essential to ensure proper delivery of nutrients to the fetus and prevent inadequate fetal exposure to the deleterious effects of reactive oxygen species (ROS).<sup>76</sup>

Based on these observations, the interstitial and endovascular trophoblast invasion and vascular changes in

rats and mice are very similar to those processes in humans.<sup>7,53</sup> In contrast, the incorporation of human invasive trophoblasts into the blood vessel wall is associated with a greater deposition of PAS-positive fibrinoid matrix than in rats. The incorporation of intramural trophoblasts in humans is also associated with the elimination of vascular smooth muscle and elastic fibers as observed in rats and mice, followed by a process of re-endothelialization.<sup>66</sup> One difference between these species is that interstitial invasion in humans precedes endovascular invasion, which can be related to the fact that in humans the interstitial trophoblast also plays a role in early vascular remodeling.<sup>53</sup> In contrast, rat and mice endovascular invasion precedes interstitial invasion.<sup>9</sup> Similarly to humans, rat trophoblasts deeply invade the decidua, extending to the mesometrial triangle. In mice, on the contrary, interstitial trophoblastic invasion does not extend into the myometrium and endovascular invasion is very limited and confined to the mesometrial decidua. Moreover, mice invasive trophoblasts follow a perivascular pathway and are not intraluminal as in rats. Therefore, the rat may represent a more promising animal model in relation to mouse for the study of experimental conditions limiting trophoblast invasion.<sup>69,77</sup>

The placental labyrinth (Fig. 3E), which is the largest portion of the placental disk, originates from the interaction of the allantois with the chorionic ectoderm, syncytium formation by trophoblasts and establishment of maternal-fetal barrier.<sup>64</sup> The chorionic ectoderm overlying the exocoelomic cavity and the allantois originates from the posterior primitive trunk of the epiblast within the exocoelomic cavity. The allantois develops toward the ectoplacental cone and fuses with the chorionic ectoderm at 8.5 and 9.5 d of gestation in mice and rats, respectively; this period coincides with the initiation of the heartbeat and circulation within the embryo.<sup>65</sup> It is known that chorioallantoic fusion is dependent on the cell adhesion molecule VCAM1, which is expressed on the allantois, and its ligand  $\alpha$ 4-integrin, which is expressed by the chorionic mesothelium. However, not all VCAM1- or  $\alpha$ 4-integrin- deficient mice fail in chorioallantoic fusion, suggesting that other redundant adhesion mechanisms are involved.<sup>64</sup>

During the chorioallantoic fusion, the trophoblast begins to differentiate into 3 layers, 2 syncytiotrophoblast layers in contact with the fetal endothelium, and one cytotrophoblast layer in contact with maternal blood. This type of placental development is known as haemotrichorial placentation, different from human placenta which is haemomonochorial.<sup>16,18</sup> Syncytiotrophoblast is formed by the fusion of trophoblast cells expressing *Gcm1* gene and its differentiation does not begin until chorioallantoic fusion in the endometrium. The placental

labyrinth is also comprised of stem cells capable of differentiating into trophoblast giant cells. However, that giant cells possess a restricted capacity for hormone production.<sup>8</sup> The placental labyrinth is the main area of maternal-fetal exchange, since syncytial trophoblast cells mediate the transfer of nutrients and wastes between maternal and fetal compartments. Maternal and fetal blood flows in a countercurrent manner within the labyrinth to maximize nutrient transport.<sup>8,64</sup> This region corresponds to the human placental villi that are surrounded by intervillous space through which maternal blood flows.<sup>66</sup>

## Trophoblast differentiation

Early in mammalian development, the conceptus differentiates into an inner cell mass and an outer sphere of cells, the trophoblast. During this process, to form the hemochorial placenta of humans, rats and mice, stem cells present in the trophoblast infiltrate the endometrial epithelium and differentiate into a variety of cell subtypes, each with specific functions.<sup>18,19</sup> The trophoblast does not contribute to the formation of any fetal tissues but does contribute to the formation of the placenta and other embryonic annexes. The proliferation and differentiation of trophoblast cells makes it the largest component of the placenta.<sup>19</sup> The tissues of the embryo are all derived from the inner cell mass as are the amnion and major components of the yolk sac and allantois.<sup>18,19</sup>

The molecular events involved in the differentiation of the trophoblast are not fully known.<sup>20</sup> In rats and mice, critical transcription factors such as *Eomes*, *Cdx2* and *Mash2* control the development of various trophoblast cell subtypes<sup>21</sup>; however, it is unclear what factors dictate invasive trophoblast differentiation in humans.<sup>22</sup> For example, although transcription factors that are important during invasive trophoblast giant cell differentiation, a subtype of trophoblast cell, have been described in mice, the expression and distribution of these factors in human invasive trophoblasts do not necessarily suggest the same roles.<sup>23</sup> These discrepancies may be explained not only by differences in observed placental morphology and trophoblast cell subtypes in human and rodent placenta but also by the difficulty of examining human invasive trophoblasts during various gestational periods. As an example, homozygous mutant embryos from *Hand1*-null mutant mice by gene targeting arrested by embryonic day 7.5 of gestation with defects in trophoblast giant cell differentiation, i.e. mice *Hand-1* promotes trophoblast stem cell differentiation into invasive giant cells during early pregnancy.<sup>24</sup> In human, on the contrary, *Hand-1* expression can only be

detected in blastocysts and during *in vitro* trophoblast differentiation.<sup>25</sup>

Regardless, some transcription factors, at least in rats and mice, are known to have critical roles in promoting and directing the initial differentiation of trophoblasts. The generation of mouse trophoblast stem cell lines from early-stage mouse embryos has been useful for studying this process as well as the mechanisms that promote self-renewal of the trophoblast stem cell population. Trophoblast stem cells (TSCs) can be derived and maintained *in vitro* in the presence of medium conditioned by mouse embryonic fibroblasts (MEF) supplemented with fibroblast growth factor 4 (FGF4). Without MEF-conditioned medium and FGF4 occurs the differentiation of trophoblast stem cells into various cell types of the placenta including spongiotrophoblast, syncytiotrophoblast and giant cells. MEF-conditioned medium provides TGF $\beta$  and/or Activin, that enable proliferation and maintenance of the undifferentiated state as inhibition of the TGF $\beta$  pathway results in TSCs differentiation.<sup>156,157</sup> However, attempts to use an FGF4-based strategy to derive human trophoblast stem cells have failed, which suggests that others factors are also required for self-renewal of human trophoblast stem cells.<sup>158</sup>

The first transcription factors to be expressed, *Eomes* and *Cdx-2*, influence trophoblast stem cell differentiation during the preimplantation period and are up-regulated in response to FGF4 treatment of mouse trophoblast stem cells.<sup>20</sup> Others transcription factors such as *Tead4*, *Elf5*, *GATA3*, *Tcfap2c*, *Esrrb* and *Sox2* are also critical for specification and/or maintenance of the multipotent state of mouse trophoblast stem cells, since knockdown mouse embryos to these genes do not express or have reduced expression of trophoblast specific genes, including *Eomes* and *Cdx2*, and died at pre-implantation stages.<sup>159,160</sup>

During the implantation and post-implantation periods, sequential expression of transcription factors *Hand1*, *AP-2y*, *ETs-2*, *Mash2*, *Gcm1*, *Ascl2*, *GATA2* and *FosL1* induce the differentiation of various trophoblast subpopulations. *Gcm1*-null mouse, for example, has defects in the formation of syncytiotrophoblasts of the labyrinth layer. However, *Gcm1* alone is not sufficient to promote syncytiotrophoblasts formation *in vitro* from mouse trophoblast stem cell, although expression of an antisense *Gcm1* transcript blocks syncytiotrophoblasts differentiation. *Hand1*, on the contrary, is important for mouse trophoblast giant cells formation, while *Mash2* is required for the production of the precursor population that gives rise to the spongiotrophoblast layer.<sup>20,27-29</sup> Human trophoblast progenitors also express *Mash2*, which is downregulated *in vitro* as the cells differentiate along the invasive pathway.<sup>158</sup>



Research in both humans and rodents has demonstrated that low oxygen tension directs trophoblast stem cell differentiation toward a phenotype that is associated with the junctional zone,<sup>30,31</sup> i.e., an invasive phenotype. This developmental process depends upon hypoxia-dependent signaling pathways, and the invasive trophoblast lineage in rats depends upon the hypoxia stimulus primarily between 8.5 and 9.5 d of gestation.<sup>30,32</sup> Similar to rats, *in vitro* exposure of human cytotrophoblastic cells to low oxygen tension also stimulates their differentiation along the extravillous trophoblast phenotype, which has invasive potential.<sup>33</sup>

Additionally, multiple homeobox genes controlling trophoblast differentiation potential such as HB24 and DLX4 have been identified in invasive trophoblasts from first-trimester human placenta.<sup>34,35</sup> Jia et al.<sup>36</sup> showed that Cdx2 promotes HTR-8/SVneo extravillous trophoblast cell invasiveness and migration to stimulate MMP9 expression and reduce metalloproteinase inhibitor 1 (TIMP1) expression. Unlike Cdx2, HTR-8/SVneo extravillous trophoblast cells transfected with Cdx1 reduces *in vitro* invasion and migration by reducing MMP9 expression and increasing TIMP1 expression.<sup>37</sup>

Another homeobox gene, the signal transducer and activator of transcription 3, also known as STAT3, was identified in JEG-3 human trophoblasts and in first trimester trophoblasts, but not in late gestation trophoblasts, suggesting a role in trophoblast invasion.<sup>38</sup> Placental leptin, which is known to inhibit trophoblastic invasion, increases STAT3 expression and activity in JEG-3 trophoblasts and human cytotrophoblasts.<sup>39</sup>

Activation of another transcription regulatory factor, peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ), also reduces the *in vitro* invasion of human cytotrophoblasts and HIPEC 65 trophoblastic cells, while its inhibition through of antagonists increases the invasiveness of these cells, suggesting an inhibitory role for this factor in trophoblast motility.<sup>40</sup> Interestingly, PPAR- $\gamma$  activation affects extravillous trophoblast expression and secretion of human chorionic gonadotropin (hCG), suggesting that the negative effects of PPAR- $\gamma$  on trophoblast invasion could be mediated by reductions in the levels of hCG during pregnancy.<sup>41</sup>

Yu et al.<sup>42</sup> also demonstrated that Notch-1 expression in the JEG3 human trophoblast lineage is important for trophoblast migration and invasion such that for these cells, the *in vitro* inhibition of this factor through RNA interference-mediated knockdown results in the reduced expression of MMPs 2 and 9, reduced NF- $\kappa$ B signaling and increased expression of E-cadherin, a marker for the epithelial-mesenchymal transition.<sup>43</sup> Notch-1 expression is reduced in placentas with preeclampsia.<sup>44</sup> Similar to the role of Notch-1 in human trophoblasts, inactivation

of Notch-2 in human trophoblast lineages also affects intrauterine trophoblastic migration, reducing trophoblastic invasion of the maternal uterine spiral arteries.<sup>45</sup>

### Intrauterine trophoblast migration

Trophoblast cells invade the decidua and possess a variety of functions such as communication with maternal immune cells, hormone and cytokine production, substitution of endothelial cells of maternal arterioles and angiogenesis. However, the regulation of these features depends upon plentiful extra- and intracellular signals.

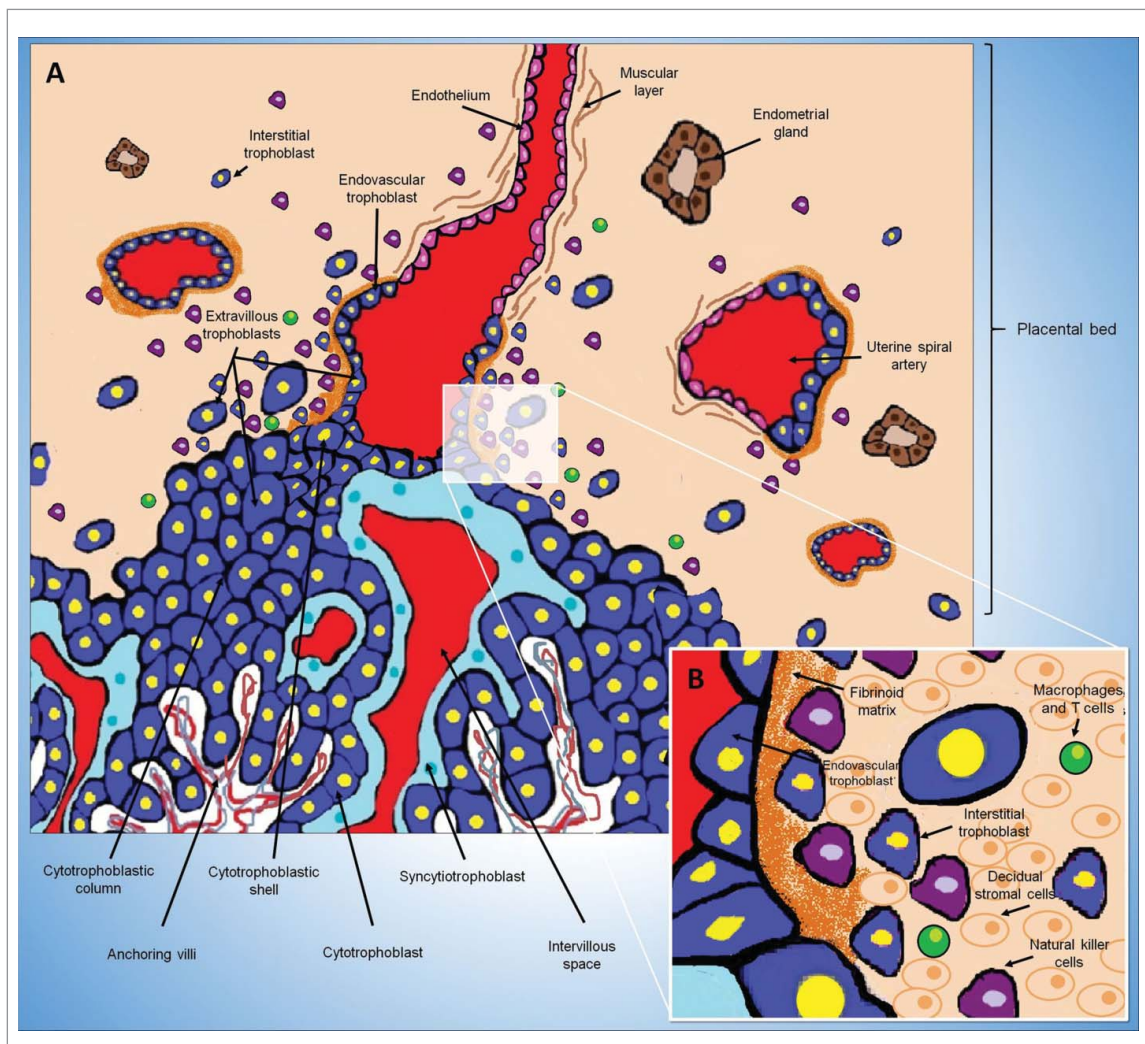
### Invasive trophoblasts

Placenta trophoblast invasion into the uterine tissue and maternal blood vessels is an essential process during rodent and human gestation and fetal development. Due to their remarkable plasticity, invasive trophoblasts fulfill numerous functions such as the anchoring of the placenta in maternal tissue, secreting hormones, modulating decidual angiogenesis/lymphangiogenesis and remodeling maternal uterine spiral arteries. This latter function is critical to increase blood flow to the placenta and thus ensure adequate transfer of nutrients and oxygen to the developing fetus.<sup>30,78</sup>

Failures in uterine and placental vascular remodeling are associated with important obstetric and neonatal complications such as preeclampsia, intrauterine growth restriction, early miscarriage, premature birth and even maternal or fetal death.<sup>1,2,6</sup> Consequently, basic research in this specific area has sought to identify the molecular mechanisms controlling trophoblast invasion under physiological and pathological conditions.

An increasing number of growth factors, angiogenic factors, cytokines and proteases that control trophoblastic cell migration kinetics have been identified, primarily through the use of first-trimester and full-term human chorionic villus explants and/or human trophoblast cell lines such as HIPEC 65, JEG3, BeWo and HTR8/SVneo.<sup>40,78,79</sup> However, the significance of most of these factors during *in vivo* placentation in humans, rats and mice is unknown.

Much of the *in vitro* control of trophoblastic invasion is exerted by growth factors and cytokines through activation of the phosphatidylinositol-3-kinase/serine-threonine (PI3K/Akt) intracellular signaling pathway, which regulates the trophoblast invasive phenotype in rats and humans.<sup>78</sup> Disruption of PI3K or Akt inhibits the expression of genes associated with the invasive phenotype and trophoblast cell invasion through an extracellular matrix. These actions are mediated, at least in part, through promoting the nuclear accumulation of FosL1 protein. FosL1



**Figure 4.** Intrauterine trophoblast invasion in humans. (A) The placental villi are covered by villous trophoblast cells (an inner cytotrophoblast layer covered by syncytiotrophoblast). The core of villus contains fetal blood vessels, fibroblasts and fetal macrophages. Maternal blood in the intervillous space reaches the placenta through the uterine spiral arteries. Cytotrophoblasts leave the top of the anchoring villi and become extravillous trophoblasts. Initially, the extravillous trophoblasts form cell columns and then invade and migrate toward the decidua. The extravillous trophoblast residing outside of placental villi acquires the capacity to degrade extracellular matrix proteins to invade the decidua (interstitial trophoblast) and the wall of maternal uterine spiral arteries (endovascular trophoblast). Endovascular trophoblasts invade the lumen of the spiral arteries and incorporate into the arterial wall. The endothelium, the muscular layer and the elastic material of these arteries are destroyed and replaced with an extracellular matrix called the fibrinoid and by the EVT cells themselves. (B) Representation of the placental bed showing interstitial trophoblast cells between decidual stromal cells, uterine natural killer cells and others maternal leukocytes (macrophages and T cells).

transcription factor is important in the regulation of the invasive trophoblast cell phenotype in humans and rats, but does not have the same function in mice. Knock-down of FosL1 in human trophoblast cells in vitro and in rat models disrupted the trophoblast migration and invasion and the expression of a subset of genes associated with the invasive-vascular remodeling trophoblast phenotype, including the MMPs.<sup>26,27</sup> In FosL1 null mouse, on the contrary, mutation in the FosL1 gene disrupts vascularization of the labyrinth zone with prenatal lethality.<sup>165</sup>

However, most of the PI3K/Akt signaling activators are secreted not only by the trophoblast itself but also by the decidua, uNKs cells and uterine macrophages, suggesting that a complex network of various cell types, mediators and signaling pathways regulates trophoblast invasiveness.<sup>2,78</sup>

During implantation and placentation in rodents, intrauterine trophoblast invasion is a prominent feature. Rat and mouse invasive trophoblast cells have been characterized by their polyploidy, epithelial nature, accumulation of glycogen and expression of a unique subset of

prolactin family cytokines. The first trophoblast cells penetrating into the uterine mesometrial compartment take an endovascular route and the depth of their invasion is generally limited to the mesometrial decidua compartment. In the rat, endovascular trophoblast cells can be identified at midgestation and expand into blood vessels situated in the uterine decidua and subsequently into the myometrium as gestation advances. During this process occurs an intraluminal invasion pathway, in contrast to the suggested invasion pathway in decidual spiral arteries of the mouse that is perivascularly followed by replacement of the endothelium from the outside.<sup>8,9,13,161-163</sup>

A second wave of trophoblast cell invasion begins after gestation day 13.5 and includes both interstitial and endovascular trophoblast cells. Invading interstitial trophoblast cells similarly penetrate the uterine decidua and in rats invade into the myometrium; however, their movements are restricted to the last third of gestation. It is not clear whether this invasion involves cell migration or proliferation-mediated extension toward the junctional zone, but it is believed that the invasion involves cell migration because there are no mitotic cells in the junctional zone at and after 14<sup>o</sup> day of gestation. Besides, invasive cells, unlike spongiotrophoblasts, are the only trophoblasts after 13<sup>o</sup> day of gestation expressing a gene coding for urokinase type plasminogen activator (uPA), a secreted proteolytic enzyme thought to be involved in cell migration.<sup>8,9,13,161-163</sup>

It is known that the trophoblast invasion in rats and mice involves degradation and remodeling of uterine extracellular matrix (ECM), partly regulated by MMPs and their tissue inhibitors (TIMPs). MMP-9 and MMP-14 have been reported to be highly expressed in the invading cells, and embryo-derived MMP-9 and uterus-derived TIMP-3 appear to be key regulators of ECM degradation by mouse trophoblast. However, the factors and regulatory mechanisms that controls the propulsion of endovascular and interstitial trophoblast cells along the decidua and myometrium of rats and mice have not been elucidated as in vitro research using human trophoblast cells. Some factors such as differential expression of adhesion molecules, colony stimulating factor-I, epidermal growth factor, hepatocyte growth factor, and insulin-like growth factor-I have been implicated as stimulators of human trophoblast cell invasion.<sup>8,9,13,161-163</sup> Whether these and others factors also participate in the regulation of trophoblast cell invasion in the rat or mouse remains to be elucidated.

During implantation in humans, cytotrophoblasts leave the top of the anchoring villi and become extravillous trophoblasts (Fig. 4). Initially, the extravillous trophoblasts form cell columns and then invade and

migrate toward the decidua. The extravillous trophoblasts at the top of the villi express  $\alpha6\beta4$  integrin, while extravillous trophoblasts from the column are positive for  $\alpha5\beta1$  integrin and the extravillous trophoblasts that invade the decidua express  $\alpha1\beta1$  integrin. All of these observations show that at least in humans, trophoblast differentiation toward an invasive phenotype depends on a change in the expression of integrins on the cell surface.<sup>80,81</sup>

### Adhesion molecules

Change in the integrin profile during human placental development is involved in the epithelial-mesenchymal transition exhibited by invasive trophoblasts.<sup>43</sup> The  $\alpha1\beta1$  integrin promotes an interaction with different collagens and laminins, such as laminin 2, which is abundantly expressed in the decidua.<sup>82</sup> Wang et al.<sup>83,84</sup> showed that RNA interference-mediated silencing of laminin receptor 1 (LR1) suppresses human trophoblast-like cell (JEG3) migration and invasion by reducing the expression of MMPs 2 and 9, and increasing TIMP expression. LR1 also contributes to hypoxia-induced trophoblastic migration via MMP9 expression.<sup>85</sup> The placentas of women with preeclampsia exhibit reduced LR1 expression in cytotrophoblasts and syncytiotrophoblasts.<sup>86</sup> Hypoxia also activates an epithelial-mesenchymal-like transformation in vitro in rat trophoblast stem cells that is associated with a decrease in the expression of E-cadherin and increases in the expression of MMPs 9 and 12 and movement through an extracellular matrix. These responses are dependent on activation of the HIF signaling pathway.<sup>2,17</sup>

Zhou et al.<sup>50,87</sup> suggest that failures in trophoblastic invasion of the spiral arteries result from a defect in the epithelial-mesenchymal transition and in the expression profile of vascular adhesion molecules. During normal pregnancy, extravillous trophoblast downregulate the expression of adhesion receptors that are characteristic of their epithelial origin, and upregulate the expression of adhesion receptors that are expressed by vascular cells. E-cadherin expression is reduced, while expression of VE-cadherin, vascular cell adhesion molecule 1 (VECAM-1), Platelet endothelial cell adhesion molecule 1 (PECAM-1) and  $\alpha4$  integrins increase. Endovascular trophoblasts continue to express these receptors and begin to express  $\alpha V\beta3$  integrins, similarly to activated endothelial cells. The extravillous trophoblasts of patients with preeclampsia fail to express most of these endothelial markers, suggesting that trophoblast expression of a vascular phenotype is necessary for endovascular invasion success.<sup>50,87</sup> In contrast, other studies yielded conflicting results, showing no extravillous trophoblast expression of PECAM-1, or that expression of

PECAM-1 and E-cadherin did not vary between normal pregnancies and those with preeclampsia and IUGR.<sup>88-90</sup>

Integrin glycosylation at the maternal-fetal interface is also of great importance in the control of trophoblastic migration and invasion during pregnancy as it mediates the interactions between trophoblasts and the extracellular matrix.<sup>91</sup> Liao et al.<sup>92</sup> demonstrated in vitro that  $\beta$ -1,4-galactosyltransferase III (B4GALT3)-transfected trophoblast cell have reduced invasion, increasing their adhesion to laminin and trophoblast degradation of  $\beta$ 1 integrin. As B4GALT3 expression in humans primarily occurs during the last trimester of pregnancy in the extravillous trophoblast, it is believed to be a critical regulator of trophoblast invasion in late pregnancy.<sup>92</sup> Similar roles have been suggested for mucin 1 and 15 (MUC1/MUC15), as their expression in human placenta increases during pregnancy and in vitro studies have demonstrated that MUC1 and MUC15-transfected trophoblast-like cells (JAR and JEG-3 cells) have reduced invasion and migration through reduced MMP9 expression, increased TIMP1 expression, and/or inhibition of  $\beta$ 1 integrin activity.<sup>93-95</sup> Shyu et al.<sup>93</sup> showed that MUC1 expression is increased in human placentas with preeclampsia.

### Growth factors

Numerous growth factors have been identified as acting at the maternal-fetal interface to stimulate human trophoblast cell proliferation, adhesion and/or invasion. Among these factors are epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), placental growth factor (PIGF), colony stimulating factor 1 (CSF-1) and insulin-like growth factors 1 and 2 (IGF1 and IGF2).<sup>96-98</sup> In contrast, addition in culture medium of inhibitory proteins such as transforming growth factor  $\beta$  (TGF $\beta$ ) family members, interferon gamma (INF $\gamma$ ), endostatin, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and fetuin-A limit human trophoblastic lineage migration and invasion in vitro.<sup>99-102</sup>

### Cytokines

Cytokines, e.g., CX3CL1, CCL14, CCL4, CXCL16 and CCL21 are also capable of stimulating human trophoblast migration and invasion,<sup>103,104</sup> unlike CXCL6 and CXCL14 cytokines that inhibit human trophoblast cell migration and invasion in vitro by inhibiting gelatinase activity. Human CXCL14 expression occurs primarily during early pregnancy and predominantly in villous cytotrophoblast, in contrast with CXCL6, which exhibits increased expression during pregnancy, particularly in the extravillous trophoblast.<sup>105,106</sup>

### Interleukins

Interleukins, such as IL1, IL6, IL8, and IL11, that are secreted by trophoblastic or decidual cells also promote increased human trophoblast migration and invasion, with increased expression of MMPs 2 to 9 and/or integrins  $\alpha$ 1,  $\alpha$ 5,  $\alpha$ 6 and  $\beta$ 1.<sup>107-109</sup> The leukemia inhibitory factor (LIF) is another key cytokine in development and embryo implantation, and increases the expression of protein O-fucosyltransferase 1 (poFUT1) in human trophoblasts in vitro, promoting their migration and invasion at the maternal-fetal interface by activating the PI3K/Akt signaling pathway and stimulating MMP2 expression.<sup>110</sup>

It is notable that the importance of the growth factors and interleukins may vary during pregnancy depending upon the patterns of temporal expression, abundance of soluble inhibitors and receptor expression.<sup>78</sup>

### Placental hormones

The hormones produced by the placenta itself also influence trophoblast migration and vascular remodeling at the maternal-fetal interface. Estradiol modulates the release in vitro of macrophage migration inhibitory factor (MIF) in first-trimester chorionic villous explants in a dose-dependent manner.<sup>112</sup> Krivokuca et al.<sup>113</sup> showed that MIF stimulates the in vitro migration and invasion of extravillous trophoblasts concomitant with increased levels of MMPs 2 and 9, and  $\alpha$ 1 integrin. Additionally, estrogen regulates the expression of placental leptin in a dose-dependent manner.<sup>114,115</sup> Placental leptin also increases the expression of MMPs 2 and 9 and trophoblastic migration in vitro.<sup>114,115</sup>

Progesterone is another hormone produced by the placenta and is one of the factors that controls trophoblastic cell invasiveness by reducing trophoblast secretion of gelatinases, although this effect is dependent upon the gestational period.<sup>116</sup> The progesterone-induced blocking factor (PIBF), identified in both humans and mice, was originally described as a progesterone-induced molecule that mediates the effects of progesterone during pregnancy. The distribution of PIBF within the human decidua during the first trimester of pregnancy coincides with sites of trophoblastic invasion, with strong PIBF immunostaining in the extravillous trophoblast.<sup>117</sup> Furthermore, PIBF has been shown to reduce the expression of placental leptin and its receptor, revealing another way in which progesterone affects trophoblastic invasion.<sup>118</sup>

Prolactin and placental growth hormone (pGH) also stimulate in vitro migration of human trophoblasts by increasing expression of the  $\alpha$ 1 and  $\alpha$ 5 integrins and galectin-1.<sup>119,120</sup> Galectin 1, expressed by the trophoblast column during cytotrophoblast differentiation to form

the extravillous trophoblast, also stimulates in vitro trophoblastic migration.<sup>121</sup> pGH is believed to potentially act as an autocrine stimulator of trophoblastic invasion, particularly during the early stages of cytotrophoblast differentiation into extravillous trophoblast.<sup>119</sup>

### **Others factors**

Recently, human and rodent kisspeptins were implicated not only in the inhibition of tumor metastasis but also in the inhibition of trophoblastic invasion via the G protein-coupled receptor Kiss-1R. In human placenta, the expression levels of kisspeptin and its receptor are highest during the first trimester of pregnancy, while for rats, they are most highly expressed at 12.5° days of pregnancy. This period coincides with the peak of trophoblastic invasion and with when regulation of trophoblastic invasion is critical. In rats, as in humans, the levels of Kiss-1 and its receptor decline during placental maturation, reaching low and/or undetectable levels at the end of gestation.<sup>48</sup> Human kisspeptins are primarily produced by the syncytiotrophoblast, while its receptor is expressed in the syncytiotrophoblast, cytotrophoblast and extravillous trophoblast, indicating paracrine regulation of trophoblastic invasion by the syncytiotrophoblast. In rat placenta, which is structurally different from human placenta, both Kiss-1 and its receptor are predominantly expressed by the invasive trophoblast giant cells, suggesting autocrine control over the invasion of these cells.<sup>48</sup> Research has shown that kisspeptins reduce trophoblastic migration by reducing MMP expression, and increasing TIMP expression and invasive trophoblast adhesion through type 1 collagen in the decidual stroma.<sup>48,111</sup>

Recent research has also shown that microRNAs (miRNAs) can influence trophoblastic invasion and migration. miRNAs are a class of endogenous small RNAs that act as post-transcriptional negative regulators of gene expression, and increased expression of certain miRNAs (miR-29b, miR-16, miR-222 and miR-519d-3p) in the plasma and/or placenta has been observed in women with preeclampsia.<sup>122,123</sup> Recently, Ding et al.<sup>122</sup> demonstrated that in vitro overexpression of miR-519d-3p significantly inhibit trophoblast cell migration and invasion, whereas transfection of a miR-519d-3p inhibitor enhance trophoblast cell migration and invasion. Besides, miR-519d-3p overexpression reduce MMP-2 mRNA and protein expression, while MMP-2 knock-down using a siRNA attenuates the increased trophoblast migration and invasion promoted by the miR-519d-3p inhibitor. miR-519d-3p is exclusively expressed in placenta and regulates fetal-maternal signaling.<sup>122</sup>

Interestingly, in vitro work by Liu et al.<sup>110</sup> also demonstrated that Gadd45 $\alpha$ , a downstream target of p53,

negatively regulates trophoblastic migration and invasion in early human pregnancy by reducing the expression of MMPs 2 and 9 and increasing the expression of TIMP1 and 2. Additionally, Gadd45 $\alpha$  expression is elevated in preeclamptic placenta.<sup>110</sup> Gadd45 $\alpha$  is an important factor in cell cycle regulation, apoptosis, signal transduction and genome stability, and its expression inhibits tumor cell invasion and metastasis by maintaining cell adhesion and reducing MMP expression.<sup>124</sup>

### **Interactions between invasive trophoblasts, extracellular matrix and uterine natural killer cells (uNK cells) during uterine vascular remodeling**

During gestation in humans and rats, the endovascular and interstitial trophoblast invasion is restricted to the decidua and the initial third of the myometrium, as the extent of trophoblast invasion is precisely controlled by maternal factors and factors released by trophoblastic cells themselves.<sup>96,97</sup> Tantbirojn et al.<sup>3</sup> showed that in humans, deep trophoblastic invasion occurs in the absence of decidua resulting from either scarce uterine tissue or ectopic pregnancy, revealing the importance of decidua-released factors in the control of trophoblast invasion.

In both humans and rodents, the invasive trophoblast is equipped with various proteases systems that allow it to degrade extracellular matrix (ECM) proteins such as collagen IV, laminin, vitronectin and fibronectin to promote cell migration. Acting in opposition, the decidua expresses a variety of inhibitory proteins that restrict trophoblastic cell invasion. Invasive trophoblasts express various members of the MMP family, such as cathepsins and urokinase plasminogen activator (uPA),<sup>125</sup> while decidual cells produce TIMPs and plasminogen activator inhibitor (PAI).<sup>78</sup> Extravillous trophoblasts also express CCN1 and CCN3, which are extracellular matrix proteins that are involved in cell migration, and their levels are decreased during conditions of preeclampsia; unglycosylated CCN3 protein increases the invasion and migration potential of JEG3 human trophoblast cells in vitro.<sup>126,127</sup> Both CCN1 and CCN3 also promote the migration of human trophoblasts under hypoxic conditions in vitro, and their expression levels are upregulated by hypoxia-inducible factor 1- $\alpha$  (HIF1 $\alpha$ ) and TGF $\beta$ 3.<sup>126,127</sup>

uNK cells are the principal maternal immune system cells that are present in the decidualized endometrium before and during placentation in humans, rats and mice. These cells are members of the lymphoid lineage, are similar to T and B lymphocytes, and can be divided into several subpopulations. Uterine NK cells are phenotypically and functionally distinct from those present in

the systemic circulation.<sup>17,128</sup> Their exact role in pregnancy is unknown, but they are believed to be involved in the regulation of placentation; however, research has shown that they function with trophoblasts to coordinate uterine spiral artery remodeling.<sup>128</sup> Mice lacking uNK cells (TgE26 mice) are fertile, but exhibit inappropriate uterine vascular remodeling, poor decidualization and low fetal weight, highlighting the importance of uNK cells in placentation.<sup>129</sup> These cells function by secreting a variety of cytokines and growth factors such as VEGF,  $\text{INF}\gamma$ , nitric oxide (NO), and interleukin 15 (IL-15), etc.<sup>1,32</sup> In humans, rats and mice, NK cells primarily participate in uterine vascular remodeling during the early stages of pregnancy as uNK cells are absent from the maternal-fetal interface during late pregnancy.<sup>1,17</sup>

Research using human placenta has suggested that extravillous trophoblasts are primarily responsible for the vascular remodeling of the uterine spiral arteries<sup>53</sup>; however, new evidence indicates that the early vascular remodeling process is primarily performed by uNK cells, meaning that the extravillous trophoblasts have a secondary role. The vascular remodeling of the maternal-fetal interface in humans and mice begins before intrauterine trophoblastic migration, when the decidual uNK cell population has already been established.<sup>69,130</sup>

In vitro experiments co-culturing human trophoblasts with uNK cells have shown that uNK cells inhibit invasive trophoblast migration to reduce cytotrophoblastic expression of MMP2 and 9, and PAI-1. These effects are believed to be mediated at least in part by  $\text{INF}\gamma$ ; however, uNK cells affect neither extravillous trophoblast cell proliferation and apoptosis nor the formation of trophoblastic villi.<sup>131</sup> uNK cells also produce other soluble molecules, e.g.,  $\text{TGF}\beta 1$ ,  $\text{TGF}\beta 2$ , IL-10, TIMP-1 and  $\text{TNF}\alpha$ , which also limit human trophoblast cell migration and invasion in vitro.<sup>1,2,132,133</sup> However, other factors that are also produced by uNK cells promote the migration of extravillous trophoblast, e.g., IL-8 and interferon gamma-induced protein 10 (IP-10).<sup>134</sup>

Recently, Hu et al.<sup>135</sup> showed that uNK cell-mediated inhibition of human trophoblast cell migration in vitro is mediated by DNA hypermethylation, causing a reduction in the expression of claudin-4 (CLDN4) and fucosyltransferase IV (FUTIV), which are important for trophoblastic migration. DNA methylation is an important epigenetic modification in programmed gene regulation during cell differentiation and adaptation to environmental conditions, and has been associated with changes in gene expression. Research has shown that DNA methylation is involved in pregnancy complications such as preeclampsia.<sup>136,137</sup>

### **Trophoblast migration, placental stress and diseases associated with failure of intrauterine trophoblast migration**

Blood flow at the maternal-fetal interface increases during pregnancy, and abnormal pregnancies can be predicted by changes in the flow as measured by Doppler. Normally, the utero-placental arteries are invaded by the endovascular trophoblast and remodeled into blood vessels that are dilated, inelastic and lack maternal vasomotor control. When intrauterine trophoblastic migration is deficient, vascular remodeling is impaired, and this impairment has been implicated as a cause of IUGR and preeclampsia.<sup>6,138-140</sup>

Failures in extravillous trophoblast invasion lead to the absence of arterial remodeling, resulting in vessels that permit highly pressured pulses to reach the delicate structures of the chorionic villi and intermittent blood flow in the placental site. During pregnancy, this condition leads to a hypoxic environment and/or ischemia reoxygenation, and the continual production of reactive oxygen species (ROS) and depletion of intracellular ATP concentrations. Consequently, inflammatory mediators are released into maternal circulation and cause preeclampsia syndrome that is characterized by hypertension and proteinuria.<sup>140</sup> Other clinical signs and changes may also be observed during preeclampsia, including oliguria, epigastric pain, thrombocytopenia, hyperuricemia, elevated liver enzymes and cyanosis.<sup>141</sup> Preeclampsia is a major cause of morbidity and mortality in pregnant women and neonates, affecting between 2 and 8% of pregnancies worldwide. Most cases begin late in the third trimester of pregnancy, but approximately 10% of cases start before 34 weeks of gestation; in these cases, which are also known as early onset preeclampsia, the disease is more severe and is often associated with IUGR.<sup>142</sup> Notably, even when IUGR is not accompanied by preeclampsia, failure of intrauterine vascular remodeling has also been observed; in these cases, interstitial trophoblastic invasion is increased, unlike that observed in preeclampsia.<sup>143</sup>

The placental stress associated with clinical changes observed in preeclampsia results from oxidative stress,<sup>139</sup> nitrosative stress<sup>144</sup> and as described more recently, endoplasmic reticulum (ER) stress at the maternal-fetal interface.<sup>138,145</sup> During this process, ischemia-reperfusion injury and hypoxia favor the occurrence of ER stress through changes in calcium homeostasis and increased ROS production via the mitochondrial pathway or proteolytic cleavage of xanthine dehydrogenase to form xanthine oxidase. Furthermore, the protein folding that occurs during ER stress generates ROS, and ER stress stimulates some inflammatory pathways.<sup>138,140</sup>

In preeclampsia, the NF- $\kappa$ B inflammatory pathway is activated and levels of plasma inflammatory cytokines such as TNF $\alpha$  and IL-6 increase.<sup>140</sup> Experimental evidence using Eif2s1(tm1RjK) mutants mice has shown that preeclampsia ER stress-induced apoptosis occurs particularly in the syncytiotrophoblast and decidua.<sup>146</sup> This process causes the release of placental microparticles and cell debris into the maternal bloodstream and activates several inflammatory pathways that trigger the inflammatory response and systemic endothelial dysfunction, the main pathophysiological events of preeclampsia.<sup>140</sup> Explants of late gestation human placenta under conditions of in vitro hypoxia-reoxygenation also exhibit increased secretion of pro-inflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$  in addition to increased levels of anti-angiogenic factors such as sFlt-1.<sup>147,148</sup>

Certain factors and metabolic disorders such as obesity, malnutrition, smoking, uterine infection and diabetes may also predispose mothers to preeclampsia. With regards to diabetes, although no research has shown changes in uterine vascular remodeling in response to hyperglycemia, maternal hyperinsulinemia in rats decreases the number and depth of endovascular invasive trophoblast cells.<sup>164</sup> Han et al.<sup>149</sup> recently showed that excess glucose reduces the in vitro migratory capacity of extravillous trophoblasts and promotes trophoblast synthesis of inflammatory cytokines such as IL-1 $\beta$ , IL-6, IL-8 and G-CSF. This pro-inflammatory status and the failure of trophoblastic migration in vitro suggests that diabetic hyperglycemia can result in the changes in uterine vascular remodeling that are observed in preeclampsia.<sup>149</sup> Interestingly, obesity in humans also favors a pro-inflammatory environment at the maternal-fetal interface, increasing the macrophage population and TNF $\alpha$  expression. This environment has been suggested to compromise trophoblastic migration and consequently affect uterine vascular remodeling.<sup>150</sup> Hayes et al.<sup>151</sup> demonstrated that obese rats have reduced trophoblast migration during late pregnancy, as well as failure of intrauterine vascular remodeling, corroborating the hypothesis of Challier et al.<sup>150</sup>

Recent studies in rats have also shown that other metabolic disorders, such as thyroid dysfunction, can affect intrauterine trophoblastic migration, and an environment of placental stress at the maternal-fetal interface is suspected in these animals.<sup>12,152</sup>

Silva et al.<sup>152</sup> observed that the placentas from hypothyroid rats have increased populations of glycogen cells in the spongiotrophoblast and of trophoblastic giant cells, suggesting a failure in the migration kinetics of these cells toward the decidua. In vitro work by Silva et al.<sup>153</sup> showed that trophoblasts of rats treated with triiodothyronine exhibit increased Tpbp and Prl3b1 gene

expression, which are only expressed in the spongiotrophoblast and invasive trophoblast, respectively, during the differentiation of trophoblasts from the ectoplacental cone.<sup>67</sup> In 2014,<sup>12</sup> the same group of researchers demonstrated that hypothyroid rats exhibited reduced endovascular and interstitial trophoblast invasion that could be associated with low placental and fetal weight. These animals also exhibited reduced iNOS expression during pregnancy,<sup>12</sup> and Cartwright et al.<sup>75</sup> demonstrated that in vitro, trophoblastic migration and invasion are heavily dependent on trophoblast-produced NOS.

Hypothyroid rats also exhibit reduced expression of MMPs 2 and 9 and placental leptin, in contrast with hyperthyroid rats that exhibit increased trophoblast MMP2 expression.<sup>12</sup> These results corroborate the work of Oki et al.<sup>154</sup> who observed that thyroid hormone deficiency in vitro reduced human extravillous trophoblast expression of MMPs 2 and 3, fetal fibronectin and integrins. Expression of MMPs 2 and 9 is also stimulated by placental leptin,<sup>114</sup> corroborating the findings of Silva et al.<sup>12</sup>

Hyperthyroid rats exhibit reduced endovascular trophoblast invasion during late gestation,<sup>12</sup> and this is believed to be related to the premature birth presented by these animals. For adequate delivery and afterbirth, invasive trophoblasts die and are removed.<sup>2,17</sup> Contrary to that observed in hyperthyroid rats,<sup>12</sup> increased trophoblastic invasion in late pregnancy, accompanied by failure to remove these cells from the decidua, is a major cause of retained placenta, dystocia and postpartum hemorrhage in women and domestic animal species, and can be fatal.<sup>3,55</sup>

Another example of excessive trophoblastic invasion occurs in placenta creta (accreta, increta and percreta), wherein the anchoring villi and extravillous trophoblasts penetrate beyond the initial third of the myometrium, reaching the final third of the myometrium, the perimetrium or even extra-uterine tissues and organs. Depending upon the extent of invasion, the placenta is considered to be accreta, increta or percreta, with placenta percreta being the most severe. In all these situations, failure of decidua formation is also observed, such that the placental villi and extravillous trophoblasts more easily reach the myometrium. Interestingly, although trophoblasts are more invasive in these situations, incomplete vascular remodeling of maternal uterine spiral arteries still occurs in placenta cretas. All of these placental abnormalities may result in heavy bleeding during pregnancy and following childbirth, as well as hinder the release of the placenta following the birth of the fetus.<sup>3,58</sup>

Unlike placenta creta, in which excessive trophoblastic invasion of 'normal' trophoblasts occurs, gestational trophoblastic disease (GTD) exhibits an excess of

trophoblastic invasion by tumor cells or pre-tumor cells derived from the placental villi, presenting 5 major clinical and pathological forms: hydatiform mole (complete or partial), invasive mole, choriocarcinoma, placental site trophoblastic tumor and epithelioid trophoblastic tumor. As observed in placenta creta, severe bleeding can occur in all GTD cases, in addition to the possibility of fatality if untreated. Despite its great invasive and sometimes even metastatic potential, little is known about the regulation of GTD development and growth.<sup>4,59</sup>

Regarding recurrent spontaneous abortion, another gestational condition that can occur at the beginning and end of pregnancy, failures in the uterine vascular remodeling have also been observed in humans, with an increase in the population of uNK cells and reduction of intramural and endovascular trophoblast populations.<sup>155</sup> The causes underlying these changes and the consequent abortion are still unknown; therefore, more research is needed in this area.

## Conclusions

Trophoblast migration and invasion through the decidua and maternal uterine spiral arteries are crucial events in placentation. This review shows that despite the differences in structure and some types of trophoblasts between the human and rodent placentas, some aspects and functions are highly similar, especially when considering intrauterine trophoblastic migration. Consequently, the use of experimental animal models such as rats and mice has led to great progress in recent years with regards to the identification of mechanisms and factors that control trophoblast migration kinetics. Some of this progress can also be attributed to the use of *in vitro* models of immortalized trophoblasts or placental explants in monoculture or co-culture system that are either bi or tri-dimensional, *in vivo* and *in vitro* gene transfection of trophoblast cells, and the use of genetically modified rats and mice. These tools have facilitated a better understanding of trophoblastic migration under physiological conditions and especially during some pathologies, such as preeclampsia and intrauterine growth restriction. In this context, the current perspectives in reproductive medicine allow the development of *in vitro* experimental techniques that can more accurately simulate the placental environment to promote a better understanding of all of the biological events occurring at the maternal-fetal interface during pregnancy, especially those concerning the human placenta, as there are limitations on *in vivo* research. Additionally, the rat should receive greater use an experimental model to determine whether the *in vivo* roles of various studied factors correspond to their functions in trophoblast culture. The greater our understanding of

placentation, the more disease prevention and maintenance of maternal-fetal and postnatal health will be improved.

## Abbreviations

B4GALT3	$\beta$ -1,4-galactosyltransferase III
CSF-1	colony stimulating factor 1
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
EVT	extravillous trophoblast
FGF4	fibroblast growth factor 4
GTD	gestational trophoblastic disease
hCG	human chorionic gonadotropin
HIF1 $\alpha$	hypoxia-inducible factor 1- $\alpha$
IGF1/ IGF2	insulin-like growth factors 1/2
IL	interleukin
INF $\gamma$	interferon gamma
iNOS	inducible nitric oxide synthase
IP-10	interferon gamma-induced protein 10
IUGR	intrauterine growth restriction
LIF	leukemia inhibitory factor
MIF	macrophage migration inhibitory factor
MMP-9	metalloproteinase 9
NOS	nitric oxide synthase
PAI	plasminogen activator inhibitor
PCDH12	protocadherin 12
PDGF	platelet-derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule 1
PI3K/Akt	phosphatidylinositol-3-kinase/serine-threonine
PIBF	progesterone-induced blocking factor, pGH, placental growth hormone
PIGF	placental growth factor
PPAR- $\gamma$	peroxisome proliferator-activated receptor gamma
ROS	reactive oxygen species
rPlf	proliferin-related protein
sFlt-1	soluble fms-like tyrosine kinase-1
STAT3	signal transducer and activator of transcription 3
TGF $\beta$	transforming growth factor $\beta$
TIMP1	metalloproteinase inhibitor 1
TNF $\alpha$	tumor necrosis factor $\alpha$
uNKs	uterine natural killer cells
uPA	urokinase plasminogen activator
VECAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

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