REPRODUCTION
REVIEW

Effects of extracellular vesicles on placentation and pregnancy disorders

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Abstract

In humans, pregnancy maintenance depends on normal placental formation following trophoblast invasion into the endometrium and vascular remodeling. In the early stages of pregnancy, immune tolerance, inflammatory response and adaptation to hypoxia need to be precisely regulated in the placental microenvironment. Various types of cells, such as trophoblasts, endothelial cells, immune cells, mesenchymal stem cells (MSCs) and adipocytes, induce normal placental development via intercellular interactions through soluble factors. Extracellular vesicles (EVs) are used to diagnose various diseases because their constituents vary depending on the type of cell of origin and pathological characteristics. EV-derived microRNAs (miRNAs) and proteins in the placenta regulate inflammatory responses and the invasion of trophoblasts through intercellular delivery in the placental microenvironment. If the placenta does not adapt to the changed environment during early pregnancy, pregnancy disorders such as pre-eclampsia, preterm birth and gestational diabetes mellitus can occur. Thus, the important roles of EVs during pregnancy and development is fast emerging. This review describes the physiological role of EVs during placentation and their composition in the human placenta. It also suggests the possibility of finding EV markers that can diagnose pregnancy disorders. Furthermore, it describes the properties of EVs that affect pregnancy in livestock.

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Introduction

In humans, a successful pregnancy depends on the normal implantation and development of the placenta, which is a temporary organ responsible for fetal growth and development during the pregnancy period. In the fetomaternal interface during early pregnancy, an inner layer called the cytotrophoblast (CT) is attached to the basement membrane and an outer layer called the syncytiotrophoblast (ST) originates from the fusion of CT. ST is in direct contact with maternal blood and is responsible for the exchange of gas, nutrients and waste products between the mother and fetus. Several evidences show that the release of apoptotic debris from the ST causes a systemic inflammatory response. The villous trophoblasts (VTs) avoid immune system attacks by forming an immune privilege at the fetomaternal interface (Sargent *et al.* 2003). The extravillous trophoblasts (EVTs) penetrate the endometrium and the underlying myometrium to induce intimate interactions between the placenta and uterine wall. Another group of EVTs enter the uterine spiral arteries and induce remodeling of the blood vessels. During the first trimester, EVT invasion is controlled by cytokines and chemokines secreted by the decidual and immune cells. In the early stages of fetal implantation, the oxygen tension is ~3% in the uterus and 8~12% in the decidua and myometrium (Jauniaux *et al.* 2003). In the fetomaternal interface, oxygen tension regulates EVT invasion and vascular remodeling in the myometrium.

Extracellular vesicles (EVs) are found in most body fluids, including blood, urine, saliva and breast milk and contain numerous types of RNAs, lipids and proteins. miRNA expression in trophoblast cells is reportedly altered in hypoxic and inflammatory placental environments. Not only are miRNAs abundant in EVs implicated in the pathological changes in diseases, but they can also stably exist in the circulatory system, which makes them potential biomarkers for diagnosing pregnancy disorders. Recent studies have shown that maternal obesity also contributes to the formation of pro-inflammatory environments and endothelial cell dysfunction in the placenta, which is related to the amount of maternal circulating EVs (Elfeky *et al.* 2017). Since EVs contain the metabolic products of cells, they make it possible to predict the physiological and pathological conditions of the cell of origin. Numerous trophoblast cell lines, such as JEG-3 choriocarcinoma and HTR8/SVneo EVT cell lines, have been used to elucidate the physiological effects of EVs. Furthermore,

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the placental explant culture model or perfusion methods have also helped to analyze the endothelial dysfunction mechanism using placenta-derived EVs (Gupta *et al.* 2008).

This review describes the effect of placenta-derived EVs on placental immune response regulation and trophoblast cell characteristics in humans. The contribution of EVs to pregnancy disorders such as pre-eclampsia (PE), preterm birth (PB) and gestational diabetes mellitus (GDM) is also analyzed. Finally, the role of EVs in interactions between the conceptus and endometrium in livestock is described.

Identification of placenta-derived EVs

The characterization of EVs is not yet fully established. The components of the recovered EVs can vary depending on the extraction methods (Lotvall *et al.* 2014). In addition, extracellular RNA can be delivered through non-EV carriers, which further makes research on EVs challenging (Vickers *et al.* 2011). The exosome is the most widely studied EV, but a method to accurately distinguish between exosomes has not been established. Numerous proteomic analyses of EVs have been conducted, but specific protein markers for each type of EV remain elusive.

Many studies have identified the composition of placental EVs derived from maternal circulation. Furthermore, the effect of EVs on placentation has been elucidated via *in vitro* studies by analyzing the changes in expression of miRNAs and proteins in EVs depending on the types and invasiveness of the trophoblast cells (Tong *et al.* 2016). The chromosome 19 miRNA cluster (C19MC), including miR-520c, is a highly expressed miRNA in placenta-derived EVs that changes its expression under hypoxic conditions and is likely to function in placental–maternal signaling (Donker *et al.* 2012).

Flow cytometry and nanoparticle tracking analysis (NTA) have not only enabled the detection of EVs in maternal circulation, but have also helped identify differences in the concentration and size of EVs between women with pregnancy disease and normal healthy women. Additionally, quantitative analysis of CD63, a typical EV marker, and placental alkaline phosphatase (ALPP), a placenta-specific marker, revealed that the concentration of placental EVs increased with the progress of pregnancy (Sarker *et al.* 2014). By injecting EVs labeled with fluorescent dyes into a rodent model, it was possible to image the delivery pattern of the EVs within the fetomaternal interface and other organs such as the lungs, liver and kidneys (Sheller-Miller *et al.* 2016, Tong *et al.* 2017). Engineered materials that can help analyze placenta-derived EVs more directly and sensitively have also been developed recently (Boriachek *et al.* 2019).

Physiological functions of EVs in the placenta

The functional activity of isolated EVs has been the focus of recent *in vitro* studies. However, investigation of physiological changes following delivery of EVs has been hindered by some experimental limitations (Lotvall *et al.* 2014). It is not easy to secure a treatment group capable of acting as a negative control in which EVs are depleted. It is also difficult to confirm the function of each EV subpopulation because various extraction methods are used. Since most of the studies that report the functional role of exosomes in the placenta have included these limitations, we have used the more general term 'EVs' rather than 'exosomes'.

Pregnancy is a condition characterized by mild immunosuppression and induced inflammation (Redman & Sargent 2007). The activation of T cells along with the expression of inflammatory cytokines during pregnancy has been associated with the onset of pregnancy disorders such as PE and intrauterine growth retardation (IUGR). Moreover, during early pregnancy, the placenta is hypoxic, which increases endothelial cell proliferation and ultimately increases the surface area of the blood vessels, maximizing oxygen and nutrient transfer. By determining the number of ALPP-positive EVs, it is possible to investigate the effects of placental EVs in comparison to those of the total EV population in maternal blood (Table 1).

Trophoblasts express a number of immunoregulatory proteins that regulate maternal immune cell function (Petroff *et al.* 2005). EVs exhibit either immunostimulatory or immunosuppressive properties depending on their origin and composition. For instance, placental Fas ligand (FASLG) is released from the ST via EVs at the fetomaternal interface in order to promote an immune privilege status (Stenqvist *et al.* 2013). EVs carrying FASLG and CD274 inhibit T-cell activation signals such as those from janus kinase 3 (JAK3) and have immunomodulatory effects (Sabapatha *et al.* 2006). In addition to FASLG, TNF superfamily member 10 (TNFSF10) is also released from the ST via EVs, leading to apoptosis of T cells and/or peripheral blood mononuclear cells (PBMCs) (Stenqvist *et al.* 2013). Furthermore, EVs bearing soluble MHC class I chain-related molecules (MIC) and UL16-binding proteins (ULBP) inhibit the killer cell lectin like receptor K1 (KLRK1)-dependent cytotoxic responses of PBMCs and induce fetal immune escape during early pregnancy (Mincheva-Nilsson *et al.* 2006, Hedlund *et al.* 2009). Moreover, endogenous retrovirus group W member 1, envelope (ERVW-1) transfers to PBMCs through EVs and inhibits the response of lipopolysaccharide (LPS)/phytohemagglutinin (PHA)-induced cytokines. C19MC in the EVs of trophoblast cells also induces viral resistance mediated by autophagy in nonplacental recipient cells (Delorme-Axford *et al.* 2013). Notably, miR-517a-3p is abundantly contained in EVs derived from trophoblast cells, is delivered to T cells and NK

Table 1 Biological function of EVs in the placenta.

Active substance in EVs	Recipient cells	Biological function	Target molecules/pathways	Reference
MIC	PBMCs	Fetal immune escape	KLRK1	Mincheva-Nilsson *et al.* 2006
FASLG, CD274	T cells	T-cell suppression	-	Sabapatha *et al.* 2006
ULBP	NK cells, T cells	Fetal immune escape	KLRK1	Hedlund *et al.* 2009
–	Monocytes, B cells, PBMCs	Th2 cytokine release	–	Southcombe *et al.* 2011
ERVW-1	PBMCs	Inhibition of Th1 cytokines	–	Tolosa *et al.* 2012
C19MC	Nonplacental cells	Viral resistance	Autophagy	Delorme-Axford *et al.* 2013
FASLG, TRAIL	T cells, PBMCs	Fetal immune escape	Apoptosis	Stenqvist *et al.* 2013
Fibronectin	Macrophages	Pro-inflammatory cytokine release	–	Atay *et al.* 2011*b*
–	Monocytes	Monocyte recruitment	–	Atay *et al.* 2011*a*
miR-146a-3p	Trophoblast	IL-8 secretion	TLR8	Gysler *et al.* 2016
–	Endothelial cells	Cytokine release	–	Elfeky *et al.* 2017
MiR-548c-5p	Macrophages	Inhibition of inflammatory cytokines	PTPRO	Wang *et al.* 2019
miR-520c-3p	EVTs	Increased invasion	CD44	Takahashi *et al.* 2017
miR-155	HUVECs	Inhibition of eNOS	–	Shen *et al.* 2018

cells and is known to target the *PRKG1* gene in recipient cells (Kambe *et al.* 2014).

Excessive pro-inflammatory effects in the placenta are associated with the onset of pregnancy disorders, which are characterized by systemic inflammation such as PE and PB. During pregnancy, macrophages are abundant in the maternal decidua and they regulate the inflammatory response at the fetomaternal interface by secreting various cytokines and chemokines. Fibronectin is transferred to macrophages via EVs derived from the trophoblast cells to promote the production of interleukin -1β (IL1B) (Atay *et al.* 2011*b*). In addition, EVs derived from trophoblasts increase the migration of monocytes, thus generating an inflammatory environment through the production of IL1B, IL6, SERPINE1, colony stimulating factor 2 (CSF2) (Atay *et al.* 2011*a*). The expression of miR-146a-3p in trophoblasts is increased in cell lysates and EVs via antiphospholipid antibodies, which promotes IL8 secretion through Toll-like receptor 8 (TLR8) activation (Gysler *et al.* 2016). On the contrary, miR-548c-5p, which is highly expressed in EVs derived from PE patients, inhibits the proliferation of macrophages and the expression of inflammatory cytokines such as IL12 and TNF (Wang *et al.* 2019). Maternal plasma EVs were also shown to cause inflammatory responses and PB in pregnant mice, which clarified the role of paracrine signaling in the progression of pregnancy inflammatory diseases (Sheller-Miller *et al.* 2019).

During the first trimester of pregnancy, placenta-derived EVs also induce vasculogenesis and angiogenesis through an oxygen-sensing mechanism under hypoxic conditions. Proteomic analysis suggests that trophoblast EVs induce the activation of matrix metalloproteinases (MMPs) and mitogen-activated protein kinase (MAPK) signaling pathways. miR-520c-3p of EVs promotes the invasiveness of EVTs, which occurs by targeting CD44 in EVTs (Takahashi *et al.* 2017). Other C19MC miRNAs as well as miR-520c-3p are also speculated to regulate EVT migration. In addition, ST-derived EVs containing endothelial nitric oxide synthase (eNOS) facilitate the prediction of low nitric oxide (NO) biological activity in PE patients (Motta-Mejia *et al.* 2017). miR-155, which is highly expressed in the plasma and placenta of PE patients, is delivered to endothelial cells via EVs and inhibits the expression of eNOS (Shen *et al.* 2018). Thus, many miRNAs and proteins in placental EVs regulate the placental immune response and invasiveness of trophoblast cells. However, more research is needed regarding the genetic and epigenetic networks involved in the development of pregnancy disorders (Fig. 1).

The microenvironment around placental trophoblast cells has important physiological functions with respect to placental development. In the placental microenvironment, soluble factors secreted by various types of cells such as endometrial cells, mesenchymal stem cells (MSCs), adipocytes and macrophages play various roles during intercellular interactions in the fetomaternal interface. Several tracking experiments have shown that miR-30d and let-7a secreted from endometrial cells can be transferred to the trophoblast through EVs (Vilella *et al.* 2015, Niu *et al.* 2017). Moreover, endometrial EVs are internalized by trophoblast cells, which mediate focal adhesion kinase (FAK) signaling, thereby increasing their ability to attach to the endometrium (Greening *et al.* 2016). MSCs, which can be easily isolated in the placenta, contribute to the formation of the vascular system in the placenta by producing numerous pro-angiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor2 (FGF2) (Kong *et al.* 2013). EVs derived from placental MSCs (pMSCs) promote migration and tube formation via placental microvascular endothelial cells in order to adapt to low oxygen tension. Moreover, pMSCs increase VEGF and miR-126 levels in the EVs and promote angiogenic processes in response to NO stimulation (Du *et al.* 2017).

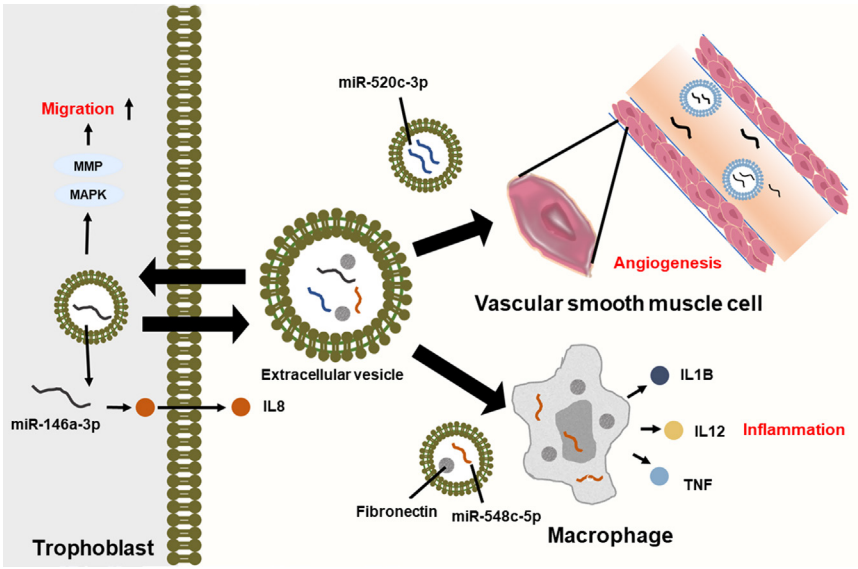

Figure 1 Effect of trophoblast-derived EVs on angiogenesis and inflammation. miRNAs and proteins in EVs control the migration of the trophoblast. An increase in the concentration of trophoblast EVs increases cell migration via the matrix metalloproteinase (MMP) and mitogen-activated protein kinase (MAPK) signaling pathways. Moreover, miR-146a-3p in EVs promotes the secretion of interleukin (IL)-8 from the trophoblast cells. EVs containing miR-520c-3p are also transferred to vascular smooth muscle cells (VSMCs) to increase cell migration and exhibit pro-angiogenic effects. Finally, trophoblast EVs internalized by macrophages promote cytokine secretion, including IL1B, IL12 and TNF and induce inflammatory responses.

The placenta also induces the secretion of pro-inflammatory cytokines by internalizing macrophage-derived EVs. Adipose EVs regulate glucose and glycogen metabolism in the placenta explant culture, which is associated with the onset of GDM. The endothelial cell EVs released due to high glucose levels associated with GDM may induce the disruption of the fetoplacental vascular system. The onset of GDM via endothelial cell-derived EVs has been reported to activate ROS production and the MAPK1 and AKT signaling proteins (Saez *et al.* 2018). Thus, pregnancy disorders such as GDM can be caused by interactions of various types of cells, including trophoblast cells, in the placental microenvironment (Fig. 2).

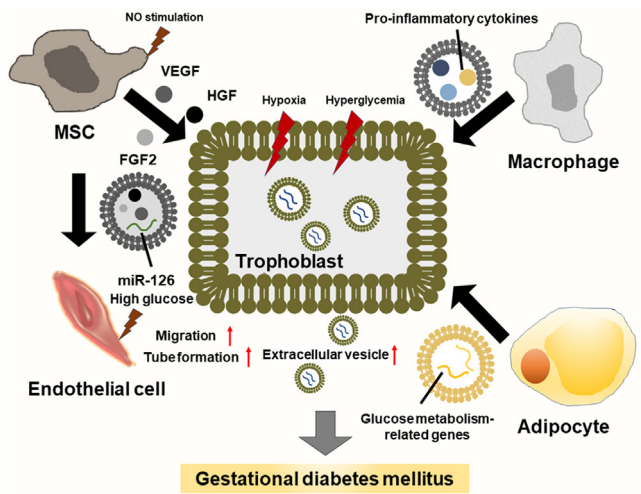

Figure 2 Hypothesis on the pathogenesis of gestational diabetes mellitus (GDM) following changes in the placental microenvironment. Under hypoxic and hyperglycemic conditions, the concentration of trophoblast EVs increases. MSCs increase endothelial cell migration through the delivery of miR-126 under high glucose conditions. Moreover, adipocytes release EVs containing transcripts related to glucose and glycogen metabolism. Pro-inflammatory cytokines in macrophage-derived EVs also induce the onset of GDM in the placental microenvironment.

Diagnosis of pregnancy disease using EVs

The ratio of placenta-specific EVs to total circulating EVs was reported to be positively correlated with fetal growth. Several studies have suggested that placental miRNAs may be involved in fetal growth restriction. Cohort studies show that miRNAs in the serum EVs of pregnant women in their second trimester can also help predict fetal growth (Rodosthenous *et al.* 2017). In addition to predicting fetal growth, much evidence suggests that placental EVs can be used to diagnose pregnancy disorders.

Pre-eclampsia

PE, characterized by hypertension and proteinuria after 20 weeks of gestation, adversely affects both the mother and fetus, exhibiting symptoms such as kidney and liver dysfunction and prematurity. PE is caused by abnormal placental development and is difficult to diagnose early due to insufficient understanding of its molecular pathogenesis mechanism. In a previous study, concentrations of placenta-specific EVs were clinically correlated to distinguish between normal pregnancy, early onset of PE and late onset of PE (Pillay *et al.* 2016). The expression of circulating miRNAs was also reported to be altered in the body fluids of PE patients when compared to controls. Notably, miR-885-5p exhibited higher expression than apoptotic bodies or microvesicles in the plasma-derived EV-rich fractions of PE patients. Moreover, miR-136, -494 and -495 in the EVs secreted by umbilical cord mesenchymal stem cells (UCMSCs) were also highly expressed in PE patients

when compared to healthy controls. The expression of miR-210 was found to be higher in PE placenta when compared to normal placenta; coincidentally, hypoxic conditions induce a high level of miR-210 release via EVs (Biro *et al.* 2019).

Proteins present in placental EVs can also be diagnostic markers due to their altered expression in pregnant women with PE when compared to that in normal pregnant women. The FLT1 binds to VEGF and interferes with vascular homeostasis. Levels of sFlt-1 in placenta-derived EVs are higher in patients with severe PE. Moreover, plasma-derived EVs of PE patients contain a large amount of FLT1 and endoglin (ENG), which cause vascular dysfunction when administered to pregnant mice (Chang *et al.* 2018). Proteomic analysis using umbilical cord blood-derived EVs revealed 29 instances of significantly different protein expression between PE patients and the control group (Jia *et al.* 2015). It is also likely that EVs isolated from urine can be used in the diagnosis of PE (Nielsen *et al.* 2017). Syncytin-2 expression, which was found to be reduced in the serum EVs of PE patients, is important for the cell uptake of EVs (Vargas *et al.* 2014). Galectin 13 (LGALS13) is one of the galectins that is specifically expressed in the placenta. The reduction of LGALS13 level in placental tissue and placenta-derived EVs is also associated with PE progression (Than *et al.* 2014).

Preterm birth

The precise cause of PB is still unclear; however, the action of biochemicals released by mature fetal organs is suspected to cause an inflammatory response that interferes with the maintenance of pregnancy. EVs derived from UCMSCs in term or preterm infants have been shown to have different oxidative metabolism (Panfoli *et al.* 2016). This suggests that the different effects of EVs on aerobic respiration by gestational age may affect the onset of preterm delivery. EVs derived from the milk of women who underwent PB survived even after gastric and pancreatic digestion, and miRNAs in EVs displayed different expression patterns when compared to same from mothers who underwent term birth (TB) (Kahn *et al.* 2018). Recent studies have shown that there are 173 different miRNAs in EVs between PB and TB, and bioinformatic analysis suggests that these miRNAs target TGFB1 and TP53 signaling (Menon *et al.* 2019). Various circulating microparticle proteins extracted from the plasma of 10- to 12-week pregnant women show correlation with PB. Moreover, evidence suggests that miRNA in the EVs of plasma can act as a biomarker to predict PB (Fallen *et al.* 2018). Complex genetic networks, including PI3K/AKT and VEGF signaling, regulated by miRNAs in EVs are assumed to be involved in the onset of PB. Furthermore, cytoscape analysis of the UCMSCs of PB and TB infants revealed a difference between inflammation and metabolic cluster between the two groups (Bruschi *et al.* 2018).

Gestational diabetes mellitus

GDM affects 9~15% of all pregnancies and is screened according to oral glucose tolerance tests at 24–28 weeks of gestation (Chu *et al.* 2007). An earlier diagnosis of GDM would allow for faster response to the damage accumulated by glucose intolerance. Hyperglycemia increases the release of EVs from primary trophoblast cells, which is further enhanced under hypoxic conditions (Rice *et al.* 2015). According to a cohort study, both normal and GDM pregnancies show a greater increase in the concentration of placenta-derived EVs as pregnancy progresses; however, a larger increase can be observed in GDM pregnancies (Salomon *et al.* 2016). The plasma EVs of pregnant women with GDM further promote the release of inflammatory cytokines from endothelial cells. Placental EVs from GDM pregnancy also show different miRNA expression patterns when compared to normal EVs and are associated with skeletal muscle insulin sensitivity. Notably, placental EVs in normal pregnant women increase glucose uptake in the diabetic skeletal muscle (Nair *et al.* 2018).

The effect of EVs on livestock pregnancy

In livestock, the endometrium secretes numerous soluble substances that are responsible for the growth and elongation of the conceptus, which is abundant in the EVs. Pregnancy loss occurs frequently during blastocyst hatching and conceptus implantation onto the endometrium; however, the factors responsible for intercellular interactions are still not completely understood. Even in livestock, placental EVs likely play an important role in maintaining pregnancy (Table 2). Several evidences suggest that the endometrium and placenta release microvesicles, including exosomes, to induce conceptus-endometrial interactions via the uterine luminal fluid (ULF). EVs isolated from the ULF of pregnant ewes show many differences in protein and miRNA profiles in EVs when compared to cyclic ULFs. Furthermore, EVs identified in the ULF of pregnant ewes contain endogenous beta retrovirus (enJSRV) RNA, which can be delivered to heterologous cells (Burns *et al.* 2014). Exosomes containing enJSRV protein also promote the proliferation and the secretion of interferon tau (IFNT) on ovine trophectoderm cells (Ruiz-Gonzalez *et al.* 2015). Moreover, EVs labeled with PKH67 dye were observed in both the trophectoderm and uterine epithelium (Burns *et al.* 2016). This suggests that the cell-to-cell interactions via EVs play an important role in maintaining normal pregnancy in sheep. Additionally, EVs isolated from the uterine flushings of pregnant ewes had proteins such as IFNT, macrophage-capping protein

Table 2 Effect of EVs on livestock pregnancy.

Species/EV source	Biological functions	Bioactive substances in EVs	Reference
Sheep			
Uterine luminal fluid	Conceptus–endometrial interactions	enJSRVs RNA	Burns *et al.* 2014
Uterine flushing	IFNT secretion	enJSRVs RNA	Ruiz-Gonzalez *et al.* 2015
Pig			
Trophectoderm cells	Angiogenesis	Angiogenic miRNAs	Bidarimath *et al.* 2017
Umbilical cord blood	Angiogenic regulation	miR-15	Luo *et al.* 2018
Cow			
Uterus	Development of SCNT embryos	–	Qiao *et al.* 2018
Uterine flushing	Genetic regulation	–	Kusama *et al.* 2018
Peripheral blood	Inflammatory regulation	miR-499	Zhao *et al.* 2018

(CAPG), and aldo-keto reductase family 1, member B1 protein (AKR1B1), which are not found in the EVs of the cyclic ewes.

In porcine pregnancies, placentation begins with the expression of adherent molecules in the trophectoderm and uterine luminal epithelium over days 15-20 during the gestation period. The expression of EV-related genes in the porcine uterus shows a specific difference depending on the cell type. The EVs derived from porcine trophectoderm cells induce the proliferation of the maternal endothelial cells and promote angiogenic processes (Bidarimath *et al.* 2017). The expression of porcine miR-150 is reduced in the umbilical cord blood-derived EVs of pigs undergoing IUGR (Luo *et al.* 2018). The increase in miR-150 in the EVs increases the proliferation and migration of endothelial cells, thereby exhibiting a pro-angiogenic effect.

When bovine EVs isolated from a luteal phase uterus were supplemented with bovine somatic cell nuclear transfer (SCNT) embryos, the blastocyst formation rate and inner cell mass/trophectoderm cell ratio were observed to increase (Qiao *et al.* 2018). This suggests that EVs released from bovine uteri may play an important role in embryo development. Treatment of EVs from the flushed fluids of a bovine uterus during pregnancy (at days 17, 20, and 22) leads to differences in the expression of apoptosis-related genes and adhesion molecules in endometrial epithelial cells depending on the gestational period (Kusama *et al.* 2018). Bovine placenta-specific EVs inhibit the activation of NF-κB via miR-499 transfer to endometrial epithelial cells and also inhibit the inflammatory response induced by LPS (Zhao *et al.* 2018).

Conclusion

The placenta during early pregnancy is in an environment of mild inflammation and hypoxia, and normal proliferation and invasion of trophoblasts is necessary for the maintenance of pregnancy. Various miRNAs and proteins in placenta-derived EVs are known to play a role in pregnancy maintenance in the trophoblast and placental microenvironment. Therefore, understanding the physiological activity of EVs during early pregnancy will present a new approach to overcome abnormal placentation and pregnancy disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

G S and W L designed and directed the study. C Y, G S and W L wrote and prepared the manuscript. C Y designed the figures. All authors provided critical feedback and helped to shape the manuscript.

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