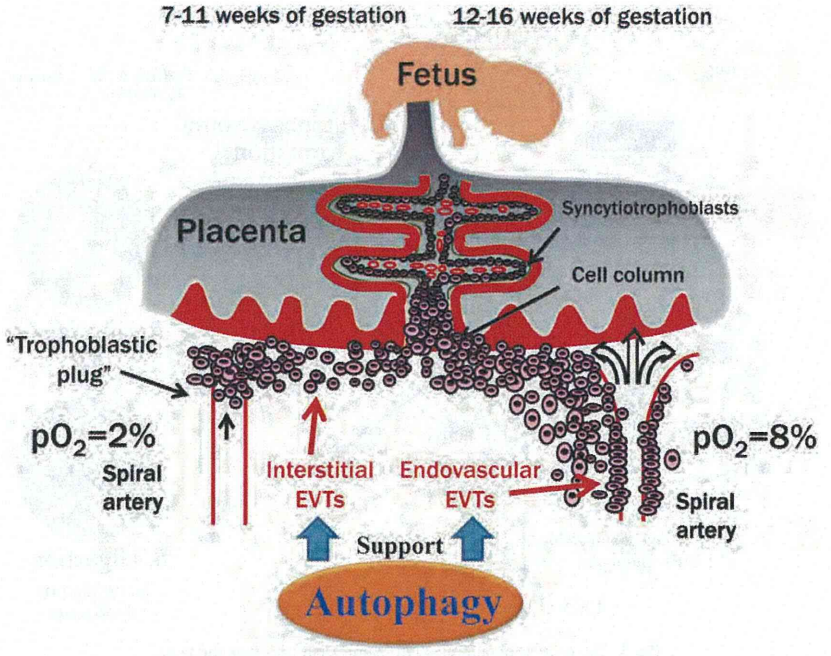

Fig. 2. Autophagy supports EVT functions under physiological hypoxia. Interstitial EVT invasion starts during 7–11 weeks of gestation, and vascular remodeling by EVTs starts during 12–16 weeks of gestation [24].

increased. The placenta generates ROS which may contribute to the oxidative stress seen even in normal pregnancy, but this is increased in pregnancies complicated by preeclampsia or IUGR, suggesting that such stress can lead to altered placental function via the covalent modification of protein structure and function [9].

4. Autophagy in embryogenesis and implantation

During embryogenesis, oocytes accumulate maternal proteins necessary for oogenesis. Although many of these proteins are provided to zygotes (fertilized embryos), the stockpile is largely degraded after fertilization, and newly synthesized proteins encoded by the zygotic genome are translated. In mice, zygotic transcripts are detected at the late one-cell stage, and most of the maternal RNAs are eliminated by the two-cell stage. The pattern of protein synthesis changes dramatically at the four- to eight-cell stages. The basal level of autophagy is low in unfertilized oocytes; however, autophagy is activated shortly after fertilization [10]. Using oocyte-specific Atg5 (autophagy-related 5) knockout mice, it was shown that Atg5-null oocytes can develop if they are fertilized with wild-type sperm, but cannot develop beyond the four- and eight-cell stages if they are fertilized with Atg5-null sperm [10]. Protein synthesis rates are reduced in autophagy-deficient embryos, suggesting that the degradation of maternal factors by autophagy is essential for pre-implantation development in mammals [10].

A mouse model of experimentally delayed implantation, established by ovariectomy before blastocyst implantation, showed that Atg7 and LC3 expression was up-regulated in blastocysts, made dormant by the elimination of 17β-estradiol (E2), in comparison with E2-activated blastocysts, suggesting that autophagy is sustained during the prolonged survival of dormant blastocysts [11]. The activation of autophagy was also observed in the inner cell mass. On the other hand, E2 or progesterone activated autophagy simultaneously with a decrease in the activation/phosphorylation of mTOR (mammalian target of rapamycin) in bovine mammary epithelial cells [12]. Progesterone is also known to play a crucial role in peripheral blood NK cell accumulation in the uterus. We have reported that hormonal regulation, using progesterone, prolactin and human chorionic gonadotropin, contributed to fetal tolerance by inducing the production of immunosuppressive NK subsets [13]. Hormonal regulation of maternal immune cells plays an important role in implantation; however, it is still unknown whether hormones affect autophagy in the maternal immune cells or stromal cells at the implantation site.

Mice lacking beclin 1, which is involved in both autophagy and endocytosis [14], experience early embryonic death (E7.5 or earlier) with defects in proamniotic canal closure [15]. Targeted deletion of FIP200 (a potential mammalian counterpart of the yeast autophagy protein Atg17p) in mice also leads to embryonic death at mid/late gestation associated with heart failure and liver degeneration [16]. A functional defect of Ambra1 (activating molecule in beclin1-regulated autophagy) causes embryonic death (E10-E14) with defects in neural tube development in midbrain/hindbrain exencephaly and/or spina bifida [17]. Therefore, autophagy has an important role in embryogenesis, survival of the embryo and embryonal development, though it is still unclear what affect autophagy has externally during embryogenesis externally.

5. Autophagy in extravillous trophoblasts and villous trophoblasts

In the placenta, LC3B expression is detected in villous cytotrophoblasts and syncytiotrophoblasts and LC3-II/actin levels are higher in the marginal than central portion of the placenta obtained by cesarean section [18]. LC3-II/actin levels are also more pronounced in placentas from cesarean sections than from vaginal deliveries [18]. Autophagic cell death in amniotic epithelium might be involved in the rupture of membranes in term placentas [19]. Atg9L2, a mammalian homolog of the autophagy-protein (Atg9p) first identified in yeast, is specifically expressed in the placenta and pituitary gland, while another homolog Atg9L1 (Atg9A) is

ubiquitously expressed in adult human tissues [20]. Atg9L1 functions as a regulator of innate immunity following double-stranded DNA stimulation as well as an essential autophagy protein [21]. All Atg9L1-knockout mice died within 1 day of delivery and mouse Atg9L2 was found to be more widely expressed at embryonic stages than in adulthood. Atg9L2 is markedly expressed in human primary cytotrophoblasts, but its level is significantly reduced in syncytiotrophoblasts (a 4.3-fold decrease). The role of Atg9L2 in the placenta and fetus is still unclear, but Atg9L2 may be involved in an early embryonic stage like Atg5.

As EVTs migrate away from the villi and invade into the maternal decidua, they progressively develop an invasive phenotype and are no longer able to proliferate [7]. Hypoxia reduces the invasive capacity of primary trophoblasts and the expression of molecules associated with an invasive trophoblast phenotype such as α1 integrin and matrix metalloprotease-2 (MMP-2). On the other hand, in cultures of HTR-8/SVneo cells, an EVT cell line, 1% oxygen increased cell invasion by more than 20% oxygen, by up-regulating the expression of urokinase-type plasminogen activator receptors. Thus, there are two opposing schools of thought on the effect of hypoxia on trophoblast invasion in the first trimester of human pregnancy. On using HTR-8/SVneo cells, GeneChip analysis showed that HTR8/SVneo cells were quite different from primary EVTs in EVT-specific gene expressions [22]. Regarding EVT invasion assays in our experiments, we used another EVT cell line, HChEpC1b, immortalized by infection with retroviral expression vectors containing the type 16 human papillomaviruses E6 and E7 in combination with a human telomerase reverse transcriptase, with a normal chromosomal number and no tumorigenic activity [23]. This cell line also shows invasive capacity under hypoxia [24]. To address the problem, it is necessary to use primary EVTs. In addition, EVTs start to invade the decidua when they encounter higher oxygen concentrations (5% O_2) than in the placenta [25]. It is worthwhile assessing the activation of autophagy and EVT invasion in primary EVTs under not only 2% but also 5% oxygen concentrations.

Hypoxia induces autophagy in primary trophoblasts [24,26]. To clarify the specific role of autophagy in trophoblast functions, we constructed autophagy-deficient cells by stably transfecting $ATG4B^{C74A}$, an inactive mutant of ATG4B, which inhibits the autophagic degradation and lipidation of MAP1LC3B paralogs [27]. The autophagy induced by hypoxia enhanced the invasive capacity of EVT cell lines, HTR-8/SVneo and HchEpC1b [24]. The invasion under hypoxia was significantly reduced in autophagy-deficient EVT cell lines, compared with wild-type EVT cell lines. The EVT functions, invasion and vascular remodeling, which precisely develop placentation, are sustained by hypoxia-induced autophagy at least in EVT cell lines. In these cell lines, no difference in HIF1α expression was observed between control and autophagy-suppressed EVT cell lines [24]. There are a few papers related to the HIF1 pathway for EVT invasion. A decrease in HIF1α expression caused by siRNA dramatically reduced the invasiveness of HTR8/SVneo cells under hypoxia and normoxia [28]. Hypoxia-induced autophagy is modulated by the inactivation of mTOR via AMPK (5′-AMP-activated protein kinase) [29]. Rapamycin or siRNA-mediated mTOR knockdown, activator of autophagy, reduced the invasiveness of HTR8/SVneo cells under normoxia [30]. Additionally, Atg5 knockout, but not wild-type, MEFs showed no activation of autophagy under hypoxia [31]. Thus, Atg5 plays a pivotal role in hypoxia-induced autophagy. In primary human trophoblasts, silencing of Atg7 also reduces autophagy [26]. Taken together, autophagy accelerates EVT-invasion under hypoxia, and a decrease in HIF1α or mTOR seems to substantially inhibit EVT-invasion. In addition, vascular remodeling by EVT cell lines is also inhibited in autophagy-suppressed EVT cells, compared with that in control cells, suggesting the importance of autophagy in EVT function (Fig. 2). The activation of autophagy by hypoxia is mainly dependent on the HIF1 pathway, but an independent pathway seems to exist; instead, the AMPK-mTOR and PKCδ (protein kinase Cδ)-JNK1 cascades are responsible for the signaling that triggers autophagy. HIF1α is indispensable to EVT-invasion under normoxia and hypoxia. Additionally, autophagy induced by hypoxia may be partially mTOR-dependent, though hypoxia-stimulated ER stress also plays a role.

6. Autophagy in preeclampsia and intrauterine growth restriction

We recently reported that autophagy was enhanced in EVTs in early placental tissues, which are under physiological hypoxia [24]. As mentioned before, invasion and vascular remodeling under hypoxia are significantly reduced in autophagy-deficient EVT cell lines. Furthermore, sENG, whose levels increase in sera in preeclamptic cases, suppresses invasion in EVT cell lines by inhibiting autophagy. The sENG-inhibited EVT invasion is recovered by TGF-β treatment in a dose-dependent manner. A low dose of sENG also inhibits the replacement of human umbilical vein endothelial cells (HUVECs) by EVT cell lines. This is the first report to show the role of autophagy in EVT functions under hypoxia. Concerning EVT invasion, there have been several studies linking TGF-β to the inhibition of EVT invasion [32–34]. On the other hand, there is a study to augment EVT invasion using TGF-β [35]. Our data showed that TGF-β neutralized the effect of sENG, resulting in the recovery of HTR-8/SVneo cell invasion under 2% oxygen tension, mimicking the physiological hypoxia in early pregnancy, but TGF-β showed no effect on HTR-8/SVneo cell invasion under 20% oxygen tension. Additionally, TGF-β has been shown to induce autophagosome formation and increase expression of beclin 1, Atg5, and Atg7 mRNA in human hepatoma cells, and increase autophagy in mammary carcinoma cells [36,37]. Hypoxia, which induces autophagy in EVTs, markedly altered gene expression in EVTs, compared with that under normoxia. Though it is still unknown how TGF-β affects the invasion in primary EVTs, TGF-β may recover EVT invasion under hypoxia by antagonizing sENG-inhibited autophagy but not enhancing EVT invasion. However, this must be confirmed using primary EVT cells.

A hypoplastic placenta with hypoxia, showing a complicated, damaged villous architecture with oxidative stress [38], is a feature of both preeclampsia and IUGR. The expression of LC3B mRNA or protein is significantly increased in placentas from patients with severe preeclampsia, compared with normal pregnancies [39,40]. Increased numbers of LC3B foci, a marker of autophagy, in villous trophoblasts were observed in cases of preeclampsia with IUGR or idiopathic IUGR placentas, compared with normal human pregnancy [39], indicating the activation of autophagy in villous trophoblasts in preeclampsia and IUGR. SQSTM1, a protein specifically digested by autophagy, accumulated in autophagy-defective human cell lines, suggesting inhibition of autophagy. The accumulation of SQSTM1 in syncytiotrophoblast is not observed in either preeclamptic or normal placentas, consistent with the activation of autophagy in syncytiotrophoblast [24]. On the other hand, expression of SQSTM1 is significantly higher in EVTs in preeclamptic placentas, showing the inhibition of autophagy in EVTs in preeclampsia. Taken together, there seems to be a difference in autophagous activity between syncytiotrophoblast and EVTs in preeclamptic placentas.

Beclin 1, a tumor suppressor protein, acts as an initiator of autophagy in mammals and up-regulation of beclin 1 expression represses cellular proliferation under hypoxia. The expression of beclin 1 mRNA or protein is significantly higher in IUGR without preeclampsia than in normal pregnancy [39], and another report

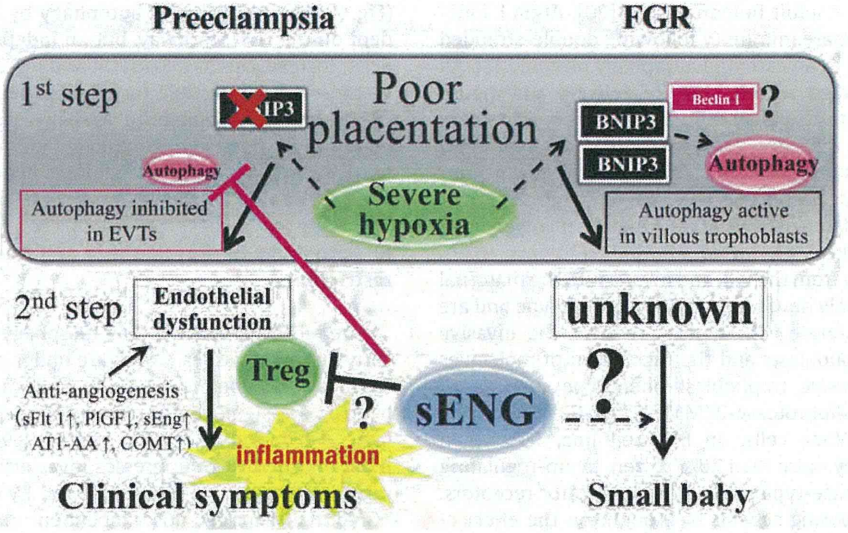

Fig. 3. Our proposed model of preeclampsia: The expression of beclin 1 between preeclampsia and IUGR was controversial. On the other hand, the expression of BNIP3 may contribute to activation of autophagy under severe hypoxia. Impairment of autophagy in EVTs in preeclampsia is induced by sENG. Additionally, sENG might inhibit Treg cells, resulting in inducing inflammation in placenta. There seems to be a difference in activation of autophagy between PE and IUGR.

shows that there is no significant difference in beclin 1 expression in syncytiotrophoblasts between preeclampsia and normal pregnancy [40]. Additionally, BNIP3 expression, induced by HIF1α, induced autophagy by disrupting Bcl-2-beclin 1 interaction and activating beclin 1 under hypoxia, and ablation of BNIP3 increased cell death under hypoxia. The expression of BNIP3 as well as HIF1α was transcriptionally up-regulated under hypoxia in HTR8/SVneo cells [41]. Although HIF1α is highly expressed in trophoblasts in preeclamptic cases, BNIP3 was not detected in syncytiotrophoblasts is cases of preeclampsia, and was only weakly expressed during normal pregnancy or IUGR [42]. For BNIP3L, BNIP3-like protein, weak staining was detectable only in preeclamptic placentas [42]. These reports suggest that a BNIP3/beclin 1-mediated autophagic pathway may contribute to hypoplastic placentation in idiopathic IUGR. On the other hand, the expression of beclin 1, BNIP3 and BNIP3L differs between preeclampsia with IUGR and idiopathic IUGR, suggesting disruption of this pathway under hypoxia. As one possible mechanism for preeclampsia, sENG, which is known to cooperate with sFLT1 in the induction of severe preeclampsia in humans as well as a rat model, inhibits autophagy in EVTs under hypoxia, resulting in disruption of invasion and vascular remodeling [24]. The sENG concentration in sera in preeclampsia is significantly higher than IUGR [43,44]. Thus, the disruption of autophagy in EVTs might contribute to the pathophysiology of preeclampsia, but not IUGR [24] (Fig. 3).

7. Future directions

A growing body of evidence is indicating key roles for autophagy in placentation, revealing differences among normal pregnancy, preeclampsia and IUGR. Not only the better molecular characterization of the autophagic pathways, but also the possibility of genetically manipulating these cellular processes, should further verify the close connection between autophagic malfunctioning and disease. Additionally, mice lacking autophagy-related genes, such as the Atg16L1 and LC3B genes, become susceptible to inflammation, suggesting that autophagy maintains cellular homeostasis. We believe that autophagy affects numerous functions, such as protection from stress, energy regulation, immune regulation, differentiation, proliferation and cell death, in the placenta.

Finally, Mizushima et al. [45] cautions that although mammalian LC3, yeast Atg8, and certain other autophagy-related genes may be transcriptionally upregulated in response to stress that induces autophagy, there is no clear evidence that autophagic activity per se is transcriptionally upregulated. Moreover, Atg proteins are constitutively expressed in sufficient amounts, and their post-translational modification and/or association with other members of the autophagic machinery, rather than regulation of their expression levels, seems to be critical for their activity in the autophagic pathway. Researchers need to keep all this in mind.

Conflict of interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2012.11.026.

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A review of the mechanism for poor placentation in early-onset preeclampsia: the role of autophagy in trophoblast invasion and vascular remodeling

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ABSTRACT

Shallow trophoblast invasion and impaired vascular remodeling of spiral arteries have been recognized in early-onset preeclampsia. Placentation and vascular remodeling are multistep processes, and hypoxia, placental oxidative stress, excessive or atypical maternal immune response to trophoblasts, exaggerated inflammation, and increased production of anti-angiogenic factors such as the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and soluble endoglin (sENG) may play a role in poor placentation in preeclampsia. Recent findings suggest that autophagy plays an important role in extravillous trophoblast (EVT) invasion and vascular remodeling under hypoxia, and sENG inhibits EVT invasion and vascular remodeling by the inhibition of autophagy under hypoxic conditions. In this review, we discuss the relationship between inadequate autophagy and poor placentation in preeclampsia.

1. Introduction

The pathogenesis of preeclampsia remains largely unknown. However, many researchers support the two-step theory (Steegers et al., 2010) or the three-step theory (Redman and Sargent, 2010). In normal pregnancy, extravillous trophoblasts (EVTs) deeply invade the uterine spiral arteries, disrupt the muscular coat and elastica, and replace the vascular endothelial cells (Pijnenborg et al., 1980). This remodeling dilates the spiral arteries and triggers increased uteroplacental blood flow. The placental oxygen curve estimated by Jauniaux et al. shows that this vascular remodeling starts from a gestational age of 10–12 weeks (Jauniaux et al., 2001). Therefore, failed remodeling at this stage leads to reduced uteroplacental blood flow and hypoxic stress to the fetus and placenta. In stage 1 of early-onset preeclampsia, impaired EVT invasion into maternal spiral arteries causes poor vascular remodeling and induces placental and endothelial damage (Khong et al., 1986).In stage 2, these damaged tissues release anti-angiogenic factors such as the soluble form of the vascular endothelial growth factor (VEGF) receptor (sFlt-1) and soluble endoglin (sENG), a co-receptor for transforming growth factor (TGF)-β_1 and -β_3, which induces maternal intravascular systemic inflammatory responses and endothelial dysfunction, resulting in hypertension and proteinuria after 20 weeks' gestation, especially in early-onset preeclampsia (Venkatesha et al., 2006; Levine et al., 2006).

During early pregnancy, the placental oxygen concentration is only 2%, while the decidual oxygen concentration is around 8% (Jauniaux et al., 2001). Furthermore, glucose concentration in the intervillous space at 5–12 weeks' gestation is only one quarter to one fifth of that in maternal serum, coelomic fluid, and amniotic fluid (Jauniaux et al., 2005). Nevertheless, EVTs invade the maternal decidua and myometrium, and induce vascular

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remodeling under harsh conditions. Indeed, the hypoxia-inducible factor (HIF) 1 system plays a critical role in EVT functions. Therefore, we should understand the mechanism by which EVTs can invade decidua or myometrium and induce vascular remodeling of spiral arteries under physiological conditions of low oxygen in normal pregnancy. In addition, we should understand why shallow EVT invasion and inadequate vascular remodeling occur in early-onset preeclampsia. Burton et al. have shown that total blood flow and hence oxygenation of the placenta was only slightly changed in patients with failure to transform the spiral arteries (Burton et al., 2009); thus, not only hypoxia, but also other unknown factors, may play important roles in the pathogenesis of early-onset preeclampsia.

Recent studies have demonstrated that autophagy is a process of self-degradation of cellular components in which double-membrane autophagosomes sequester organelles and fuse with lysosomes so that the contents can be digested by lysosomal enzymes (Mizushima et al., 2010, 2011; Ichimura and Komatsu, 2011). By using this system, cells can survive under starvation or stress conditions such as hypoxia or oxidative stress.

The expression of autophagy-related proteins in the placenta (Oh et al., 2008; Signorelli et al., 2011) and activated autophagy in the placenta of intrauterine growth restriction (IUGR) pregnancies (Hung et al., 2012) have been reported. Recently, we reported that autophagy is recognized in deeply invaded EVTs in the uterine myometrium and perivascular region. We have found that autophagy is essential for EVT functions, invasion, and vascular remodeling under physiological conditions of low oxygen (Nakashima et al., 2013; Saito and Nakashima, 2013). We also reported that anti-angiogenic factor sENG inhibits autophagy in EVTs under hypoxia, resulting in poor EVT invasion and vascular remodeling (Nakashima et al., 2013).

In this review, we discuss the mechanisms of poor placentation in preeclampsia from the perspective of autophagy.

1.1. Previous reports on the mechanisms of poor endovascular trophoblast invasion in preeclampsia

In normal pregnancy, invasive extravillous trophoblasts (EVTs) express integrin $\alpha_1\beta_1$, a receptor for collagen 1, collagen IV, and laminin. However, in preeclampsia, the expression of integrin $\alpha_1\beta_1$ is downregulated, and this failure to acquire the vascular repertoire of adhesion molecules may explain the impaired invasion of EVTs (Zhou et al., 1993) (Fig. 1). Early in the first trimester (<10 weeks), placental oxygen tension is very low (~2%; 25.6 mmHG O_2) (Jauniaux et al., 2001), and this low-oxygen environment maintains trophoblasts in an immature, proliferative state mediated by TGF-β_3 through HIF-1α (Caniggia et al., 1999, 2000). After the gestational age of 10 weeks, increased placental oxygen tension increases, and may reduce the pool of proliferating trophoblasts and increase the number of invasive trophoblasts. Caniggia et al. (1999) speculated that increased placental oxygen tension reduces the expression of HIF-1α and TGF-β_3 and the failure of TGF-β_3 production at around 9 weeks' gestation results in shallow trophoblast invasion. However, Lyall et al. (2001) reported that TGF-β_1 and -β_2, and to a much lesser extent, TGF-β_3, were present within the placental bed, and no change in the expression of either isoform of TGF-β was found in the placenta and placental bed in preeclampsia and fetal growth restriction (FGR) compared with those in normal pregnancy.

Smith et al. (2009) reported that NK cells and macrophages were present in the vascular wall at the stage of remodeling (gestational age of 9~10 weeks). These NK cells produce matrix metalloproteinase −7 and −9, and urokinase plasminogen activator (uPAR) (Fig. 1) (Smith et al., 2009; Naruse et al., 2009a,b). These enzymes can break down the extracellular matrix and induce the separation of vascular smooth vessel cells. Cell culture supernatant of uterine NK cells at a gestational age of 12–14 weeks stimulated EVT invasion (Lash et al., 2010; Lash and Bulmer, 2011), and this effect was partially abrogated in the presence of neutralizing antibodies to IL-8 and IP-10 (Fig. 1) (Hanna et al., 2006). Adequate NK cell stimulation might be necessary for EVT invasion and vascular remodeling of uterine spiral arteries. In this regard, Fraser et al. (2012) reported some very interesting findings. They studied the resistance indices using uterine artery Doppler ultrasound. Uterine NK cells isolated from pregnant women with higher resistance indices, i.e., impaired vascular remodeling, were less able to promote invasive behavior of trophoblasts. Furthermore, uterine NK cells isolated from high-resistance-index pregnancies failed to induce vascular apoptosis (Fraser et al., 2012). These findings suggest that dysregulation of uterine NK cells may contribute to the impaired vascular remodeling (Fig. 1). Indeed, Hiby et al. (2004) reported that the combination of maternal killer-cell immunoglobulin-like receptor (KIR) AA and fetal HLA-C2 was a risk factor for preeclampsia (Fig. 1). KIR AA lacks the activation receptor for HLA-C2; therefore, inadequate NK cell activation might induce poor EVT invasion and vascular remodeling, and adequate NK cell activation might be necessary for the placentation. Kaufmann et al. (2003) reported that activated macrophages induce trophoblast apoptosis by the secretion of TNFα and by the expression of indoleamine 2,3-dioxygenase (IDO), which depletes the local level of tryptophan. They speculated that activated macrophages reduce EVT invasion by the induction of apoptosis of EVTs in preeclampsia (Fig. 1). Indeed, the serum level of TNFα is elevated (Meekins et al., 1994) and peripheral blood mononuclear cells produce a lot of TNFα in such cases (Saito et al., 1999).

2. The mechanisms of autophagy

Extravillous trophoblast s invade the myometrium and maternal spiral arteries and replace the endothelial cells at low oxygen concentration and under stressful conditions. Autophagy may explain this mechanism, because autophagy is a cellular bulk degradation system to maintain cellular homeostasis under stress (Mizushima et al., 2010, 2011; Ichimura and Komatsu, 2011). By the degradation of cellular components, autophagy supplies energy so that cells can survive under starvation conditions (Fig. 2). In nutrient-sufficient conditions, amino acids, glucose, and insulin activate mTORC1, resulting in the regulation of autophagy (Fig. 3). However, under starvation

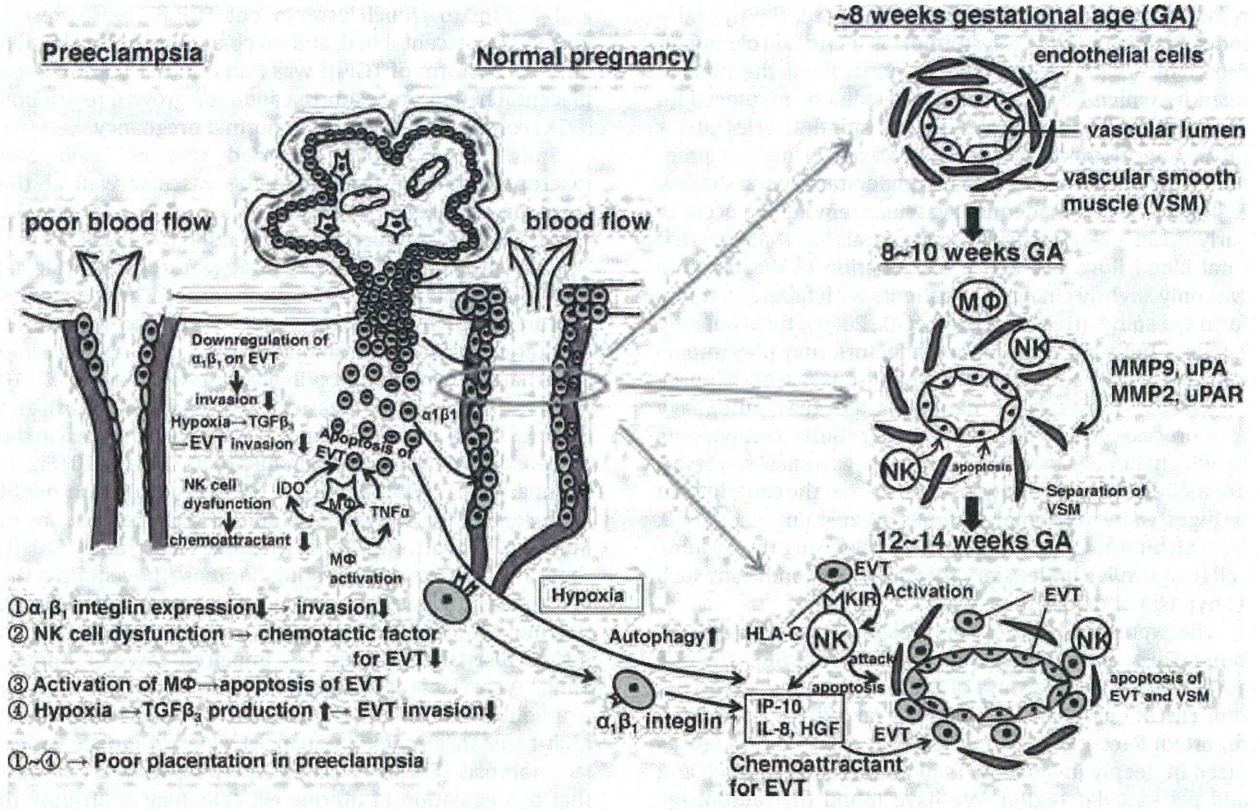

Fig. 1. The mechanisms of vascular remodeling in normal pregnancy and inadequate vascular remodeling in preeclampsia that have already been reported. At a gestational age of 8–10 weeks, uterine NK cells produce MMP-2, MMP-9, and uPA, and these enzymes induce the separation of vascular smooth muscle. NK cells also induce the apoptosis of endothelial epithelial cells and vascular smooth muscle, partly through Fas–Fas ligand signaling. These initial morphological changes have been called "trophoblast-independent remodeling." At a gestational age of 12–14 weeks, extravillous trophoblasts (EVTs) accumulate in spiral arteries owing to chemoattractant factors such as IL-10, IL-8, and HGF, and they invade the wall of the myometrial spiral arteries and replace endothelial cells. These changes have been called "trophoblast-dependent remodeling". In preeclampsia, poor EVT invasiveness and inadequate vascular remodeling have been considered to result from poor α1β1 integrin expression on EVTs, NK cell dysfunction, and the activation of macrophages, resulting in the induction of apoptosis of EVTs, and TGF-β production by hypoxic stress.

conditions, mTORC1 are suppressed, resulting in release of the mTORC1-ULK complex. Free ULK complex induces autophagy (Fig. 3). Hypoxia also induces autophagy, and HIF-1α plays an important role in its induction (Fig. 3). Hypoxia stabilizes HIF1α protein and activates BNIP3. BNIP3 releases the Baclin 1 and Bcl-2 complex. Then, free Bcl-2 inhibits PI3 kinase, resulting in the activation of autophagy (Fig. 3). Autophagy also plays an important role in the removal of old or misfolded proteins and damaged organelles before they cause damage (Fig. 2). This is a cellular homeostasis system, and basal autophagy acts as quality control machinery for cytoplasmic components (Mizushima et al., 2010, 2011). Autophagy also plays a role in the cell defense mechanism to clear intracellular bacteria or viruses. However, excessive autophagy can promote cell death, which is called autophagic cell death.

When cells recognize stress, an isolation double-membrane arises from the endoplasmic reticulum or the Golgi apparatus. Next, this isolation membrane elongates to engulf cytoplasmic components, including mitochondria and endoplasmic reticulum (Fig. 2). Association with the Atg5–Atg12 complex forms the isolation membrane. LC3-II derived from LC3-I localizes to the elongated isolation membrane during the latter step of autophagosome formation. Finally, the isolation membrane is enclosed to form an autophagosome. The diameter of the autophagosome is approximately 1 μm. Lastly, the lysozome fuses with the autophagosome to form an autolysosome, and lysosomal enzymes degrade the cell components in the autolysosome. They obtain energy by eating themselves (autophagy; Fig. 2).

2.1. Autophagy in trophoblasts

Hypoxia induces autophagy in choriocarcinoma cell lines JEG-3 and BeWo (Oh et al., 2008; Curtis et al., 2013), and in EVT cell lines HTR/SV40-neo and HchEpc1b (Nakashima et al., 2013). Autophagy was also observed in primary cultured trophoblasts under hypoxic conditions (Chen et al., 2012) or hypoxia–reoxygenation (Hung et al., 2010). Hypoxia was also shown to promote autophagy in placental chorionic plate-derived mesenchymal stem cells (Lee et al., 2013). Increased autophagy in the placenta was also found in fetal growth restriction (FGR) (Hung et al., 2012; Curtis et al., 2013; Chang et al., 2013) and preeclampsia (Oh et al., 2008).

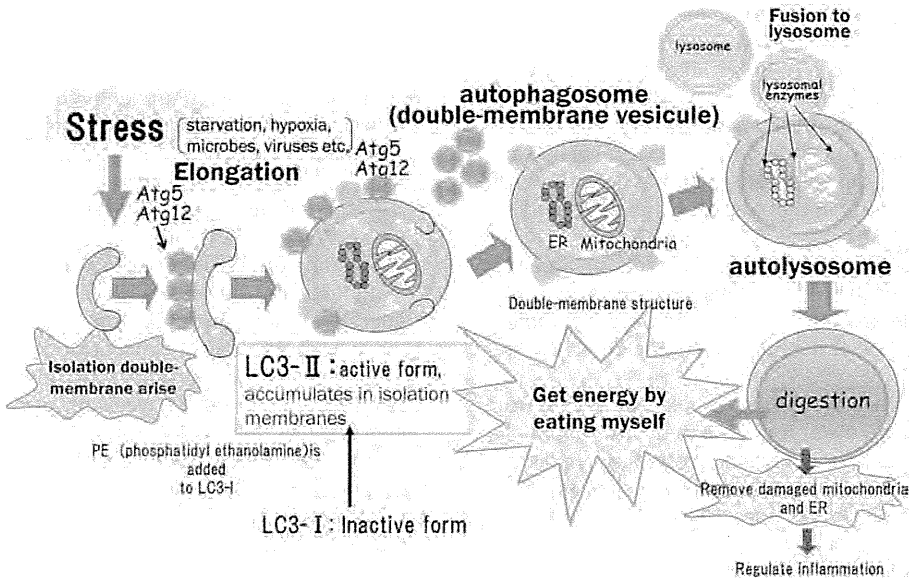

Fig. 2. Schematic diagram of autophagy. When cells experience stress, the isolation membrane arises and elongates, finally forming an autophagosome. Lastly, the autophagosome fuses with lysosomes, resulting in degradation of the cytoplasmic components by lysosomal hydrolases. Then, the cells obtain energy. At the same time, damaged mitochondria and ER are removed by autophagy, resulting in the regulation of inflammation.

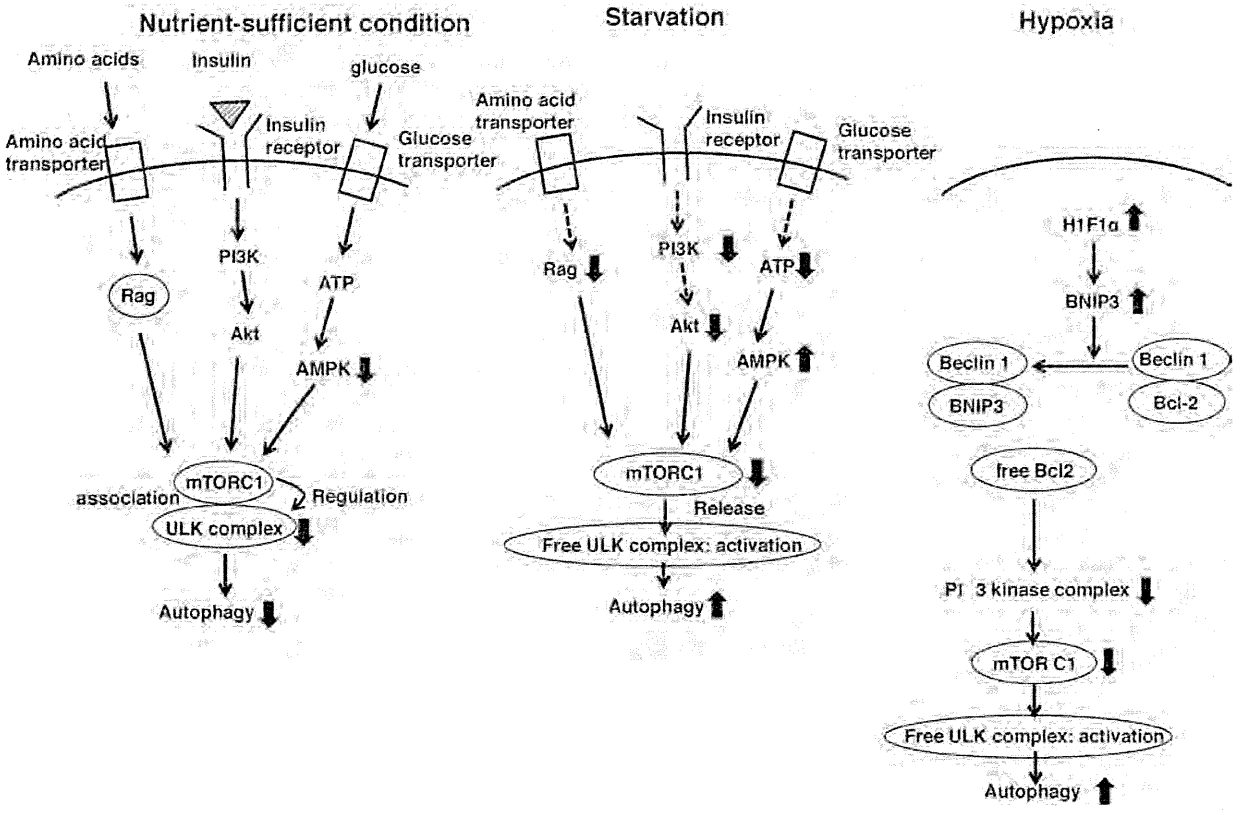

Fig. 3. The molecular mechanisms that induce autophagy. Under nutrient-sufficient conditions, amino acids, glucose, and insulin induce mTORC1 activation. mTORC1 associates with the UCL complex, resulting in the regulation of autophagy. Under starvation conditions, mTOR activity is reduced, resulting in autophagy. Hypoxia induces HIF-1α expression. HIF-1 induces BNIP3 expression, resulting in the release of Baclin 1 and Bcl2 complex. Free Bcl2 reduces mTORC1 activity, resulting in autophagy.

When primary cultured trophoblasts were cultured under hypoxic conditions, autophagy was mainly observed in syncytiotrophoblasts. Tuuli et al. (2011) proposed that hypoxia results in marked increases in p53 activity and yields apoptosis in cytotrophoblasts. In syncytiotrophoblasts, hypoxia results in a diminished level of p53 and promotes autophagy. Indeed, autophagy has been shown to localize predominantly in the syncytiotrophoblasts in FGR cases (Curtis et al., 2013).

Autophagic cell death is also observed in amniotic epithelial cells near the ruptured parts (Shen et al., 2008). Autophagic cell death may be involved in the development of membrane rupture of the human amnion in term gestation.

A higher level of autophagy in placental villi from cesarean section was observed compared with that in placentas from spontaneous vaginal delivery (Signorelli et al., 2011). Maternal fasting prior to the operation is commonly performed, so that the lower glucose level of the cord blood of infants may increase the autophagy in the placenta from cesarean section. Indeed, maternal starvation in a mouse model results in enhanced placental autophagy (Broad and Keverne, 2011). These findings suggest that autophagy is activated as a survival mechanism during nutrient deprivation (Bildirici et al., 2012).

2.2. The role of autophagy in EVT invasion and vascular remodeling by EVT

We have studied autophagy in EVTs obtained from induced abortion cases before 10 weeks' gestation (Nakashima et al., 2013). Interestingly, LC3B puncta, which exhibited autophagosomes, were present in cytokeratin 7-positive EVT cells in the decidua basalis at a deeper site than the fetal site (interstitial EVTs) and perivascular site (perivascular EVTs). We could not obtain the decidual samples after 10 weeks' gestation; thus further analysis is necessary whether EVTs experiencing higher oxygen during invasion (e.g., at 14–16 weeks' gestation) show a different autophagy pattern or not. These findings suggest that autophagy might play a role in EVT invasion and vascular remodeling by them under hypoxic conditions. To clarify the specific roles of autophagy in EVT functions, we constructed two autophagy-deficient EVT cells such as SV40 large T transformed-HTR8/SV neo and human papilloma virus (HPV) E6- and hTERT-transfected EchEpc 1b cells by stable transfection of $ATG4B^{C74A}$, an inactive mutant of ATG4B, which inhibits autophagic degradation and lipidation of LC3B paralogs.

Under 20% O_2, EVT invasion was similar in wild-type EVT cell lines and autophagy-deficient EVT cell lines. However, importantly, the depth of invasion of autophagy-deficient cell lines was significantly shallower than that of wild-type EVT cell lines in three-dimensional culture assays under hypoxic conditions (O_2: 2%), although levels of cell viability and proliferation were similar in wild-type EVT cells and autophagy-deficient EVT cells (Nakashima et al., 2013). The oxygen level in the decidua is 6–8%; thus, a 2% oxygen level in this study is considered a pathological hypoxic condition. Thus, it may be different from the chronically mild hypoxic condition. Kadyrov et al. reported that there was a much deeper invasion of more EVTs (Kadyrov et al., 2003). Under pathological hypoxic conditions, autophagy may play a role in EVT invasion. Supplementation of ATP under hypoxic conditions rescued the impairment of cell invasion in our recent study (unpublished data); thus, obtaining energy by autophagy plays a role in EVT invasiveness under pathological hypoxia.

We can evaluate vascular remodeling by tube formation assays with EVT cells and human umbilical vascular endothelial cells (HUVECs) (Kalkunte et al., 2010). Under a pathologically low oxygen level, 2% O_2, HUVEC did not undergo vascular formation on a Matrigel-coated plate; thus, we set the oxygen concentration at 8%. This concentration reflects the physiologically low oxygen level at a gestational age of 10–18 weeks in placenta and decidua, when vascular remodeling occurs. In the culture with wild-type EVT cells and HUVECs, tube areas were formed by EVT cells and HUVEC at 6 h, but the tubes were mostly occupied by EVT cells at 12 h or later. Meanwhile, tubes were still occupied by HUVEC when autophagy-deficient EVT cells were co-cultured with HUVECs, suggesting that replacement of endothelial cells by EVT under typical hypoxic conditions might require autophagy.

2.3. Impaired autophagy by soluble endoglin might be involved in poor placentation in preeclampsia

We have studied whether preeclampsia-related substances such as soluble endoglin (sENG), sFlt-1, TGF-β, and TNF-α affect autophagy in EVTs under hypoxia (2% O_2) (Fig. 4). Only sENG inhibited the number of autophagosomes in cytoplasm using confocal microscopy and reduced the LC3B-II conversion in western blotting under hypoxia. Importantly, sENG inhibited the cell invasion of EVT under 2% O_2 and reduced the vascular remodeling under 8% O_2, although sENG did not affect the EVT invasion and vascular remodeling under 20% O_2 (Nakashima et al., 2013). These findings were from an in vitro study; thus, we studied the evidence showing impaired autophagy in EVTs in preeclampsia using placental bed biopsy samples. It has been reported that p62/SQSTM1 is selectively digested by autolysosome; therefore, p62/SQSTM1 levels were suppressed under hypoxia in hepatic carcinoma cells (Pursiheimo et al., 2009) and EVT cells (Nakashima et al., 2013), but not in autophagy-deficient EVT cells. p62/SQSTM1 expression in interstitial EVTs and endovascular EVTs was markedly enhanced compared with that in normal pregnancy. The ratio of p62/SQSTM1 to CK7 expression was significantly higher in preeclampsia than in normal pregnancy in both interstitial EVTs and endovascular EVTs (Nakashima et al., 2013). These findings suggest that impaired autophagy in EVTs might be present in preeclampsia. It is unknown whether the sENG level is elevated at a gestational age of 10–18 weeks in that EVTs invade the myometrium and replace the endovascular cells of spiral arteries. Farina et al. (2008) examined the mRNA expression in chorionic villi at 11 weeks' gestation obtained by chorionic villous sampling. Five patients subsequently developed preeclampsia, while 25 did not. They showed that sENG mRNA expression was significantly higher in patients who subsequently developed preeclampsia than

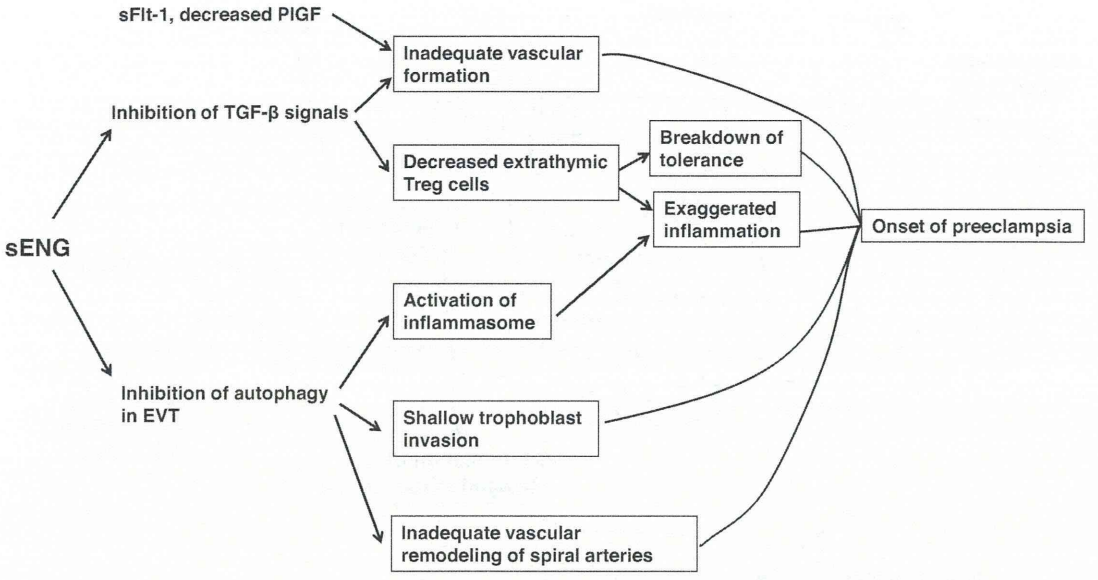

Fig. 4. The mechanisms for the pathophysiology of preeclampsia from the perspective of autophagy. The high level of sENG inhibits the autophagy in EVTs under hypoxic conditions, resulting in shallow trophoblast invasion and inadequate vascular remodeling of spiral arteries. Inhibition of autophagy reduces the recycling of damaged mitochondria and endoplasmic reticulum, resulting in the activation of inflammasomes. Inhibition of TGF-β signals, in association with increased sFlt-1 and decreased PlGF, induces inadequate vascular formation. Inhibition of TGF-β signals also decreases the induction of extrathymic Treg cells, resulting in the breakdown of tolerance and exaggerated inflammation.

in the controls. This report may show that sENG level at the feto-maternal interface is already upregulated before the onset of clinical symptoms of preeclampsia, although serum sENG during early pregnancy did not increase in a longitudinal study.

Poor early placentation is associated with early onset of preeclampsia and FGR patients without hypertension and proteinuria (Steegers et al., 2010; Redman and Sargent, 2010). Thus, the next question that arises is whether impaired autophagy is present in the EVTs of FGR patients. Our preliminary data showed that the ratio of p62/SQSTM1 to CK7 expression in FGR cases without preeclampsia was similar to that in normal pregnancy. The serum sENG level of FGR patients was reported to be lower than that of preeclampsia (Stepan et al., 2007; Jeyabalan et al., 2008); therefore, elevated sENG might play some role in the impairment of autophagy in EVTs, resulting in poor EVT invasion and inadequate vascular remodeling. This impaired autophagy reduces the 'trophoblast-dependent vascular remodeling' in preeclampsia (Fig. 1). Meanwhile, dysfunction of uterine NK cells may disturb the 'trophoblast-independent vascular remodeling' in preeclampsia (Fig. 1).

2.4. Correlation between impaired autophagy and systemic inflammation and inadequate tolerance in preeclampsia

Redman et al. (1999) proposed that an excessive maternal inflammatory response to pregnancy induces endothelial dysfunction, resulting in hypertension, proteinuria, edema, and clotting dysfunction. Oxidative stress (Stark, 1993) and system inflammation play an important role in the pathophysiology of preeclampsia. Recent studies have revealed that impaired autophagy in macrophages and adipocytes induce inflammation (Saitoh et al., 2008; Yoshizaki et al., 2012). Autophagy plays an important role in the elimination of damaged mitochondria or endoplasmic reticulum (ER) (Fig. 5). Signals from damaged mitochondria induced by uric acid or toll-like receptors induce oxidative stress and activate inflammasome components such as NALP-3 and caspase-1, resulting in overproduction of IL-1β and IL-18 (Saitoh et al., 2008; Yoshizaki et al., 2012) (Fig. 5). Uric acid also induces trophoblast IL-1β production via the inflammasomes (Mulla et al., 2011) (Fig. 5). Impaired autophagy additionally leads to ER stress, resulting in excessive inflammation. These findings suggest that autophagy impairment by sENG might play a part in the systemic inflammation of preeclampsia (Fig. 5).

Regulatory T cells (Treg) play essential roles in implantation and allogeneic pregnancy maintenance (Aluvihare et al., 2004; Sasaki et al., 2004). Treg cells are classified into thymic (naturally occurring) Treg cells that differentiate in the thymus and extrathymic (peripheral or inducible) Treg cells that differentiate in the periphery upon stimulation of naïve $CD4^+$T cells in the presence of TGF-β. Thymic Treg cells account for 70% of Treg cells, while extrathymic Treg cells account for the rest. Samstein et al. (2012) reported that extrathymic Treg cells play an essential role in the maintenance of allogeneic pregnancy. Interestingly, sENG inhibits TGF-β activity by competition with the binding of TGF-β receptor. Therefore, it can be readily speculated that increased sENG disturbs the differentiation of extrathymic Treg cells, resulting in the breakdown of maternal tolerance to the fetus (Fig. 4). Indeed, decreased Treg cells in preeclampsia have been reported (Sasaki et al., 2007; Saito et al., 2007). One of the important roles of Treg