transferred into Jurkat cells. Expression of *PRKG1* in Jurkat cells cultured with vehicle, Wt-Ex, or 517a-Ex for 48 h was evaluated by real-time PCR. Data were normalized to *GAPDH*. Expression in Jurkat cells incubated without exosomes (Vehicle) was defined as 1. D) *PRKG1* 3′-UTR luciferase reporter assay in Jurkat cells treated with BeWo exosomes. Jurkat cells transfected with pMIR-cont or pMIR-PRKG1 were incubated with Wt-Ex or 517a-Ex (each 50 µg/ml) for 48 h at 37°C. Luciferase activity in cells transfected with pMIR-cont was defined as 1. E) TrypLE digestion of Jurkat cells after incubation with BeWo exosomes. Jurkat cells were incubated with BeWo exosomes (Wt-Ex) for 24 h and then treated with (+) or without (−) TrypLE. Expression of *miR-517a-3p* in Jurkat cells was evaluated by real-time PCR. Data were normalized to *SNORD44*. Expression in cells without TrypLE treatment was defined as 1. F) Real-time PCR analysis of *miR-1* in Jurkat cells incubated with BeWo exosomes. Jurkat cells were treated with Pre-miR-NC-loaded exosomes (NC-Ex) or Pre-miR-1-loaded exosomes (1-Ex), each 50 µg/ml, for 24 h at 37°C. Data were normalized to *SNORD44*. Expression in Jurkat cells incubated with NC-Ex was defined as 1. G) *TWF1* silencing by exosomal *miR-1* transferred into Jurkat cells. Expression of *TWF1* in Jurkat cells cultured with NC-Ex or 1-Ex for 24 h was evaluated by real-time PCR. Data were normalized to *GAPDH*. Expression in Jurkat cells incubated with NC-Ex was defined as 1. Data are means ± SD from three (A–D, F, and G) and six (E) independent examinations. Student *t*-test (A, D–G) or Tukey test (B and C); **P < 0.01, ***P < 0.001.

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were detected in maternal MNCs before delivery (data not shown). Furthermore, we evaluated the expression of *miR-517a-3p* and *miR-518b* in maternal NK and Treg cells (Fig. 5, A and B) because these cell types play important roles in the maintenance of pregnancy [34, 35]. In NK cells, abundant *miR-517a-3p* and *miR-518b* were detected before delivery; the levels of these miRNAs were significantly reduced at 4 days after delivery (Fig. 5, A and B). In contrast, negligible levels of *miR-517a-3p* and *miR-518b* were detected in Treg cells even before delivery; no differences were found in miRNA levels between before and after delivery (Fig. 5, A and B). In addition, *miR-517a-3p* and *miR-518b* were undetectable in NK and Treg cells from women with no history of pregnancy (Fig. 5, A and B). We also investigated whether expression of *PRKG1* mRNA in maternal NK and Treg cells changes after delivery. As shown in Figure 5C, expression of *PRKG1* in maternal NK cells was significantly upregulated at 4 days after delivery. In maternal Treg cells, the change of *PRKG1* levels was not significant (Fig. 5C). Expression levels of transferred exosomal *miR-517a-3p* and its target *PRKG1*mRNA were inversely correlated and were dependent on pregnancy status, supporting the above-mentioned hypothesis that placental exosomal miRNAs modulate the expression of target genes in maternal immune cells.

To determine whether the uptake of placenta-associated miRNA *miR-517a-3p* into maternal immune cells is dependent on exosomes, we employed an in vitro cell-cell communication model, this time employing male NK and Treg cells instead of Jurkat cells. A significant increase in *miR-517a-3p* levels was detected in NK cells treated with BeWo wild-type exosomes compared with NK cells incubated without BeWo exosomes (Fig. 5D), providing direct evidence that BeWo exosomes can deliver *miR-517a-3p* to NK cells. Unlike maternal Treg cells, exosome-mediated uptake of *miR-517a-3p* into Treg cells was observed in this in vitro model system; however, lower levels of *miR-517a-3p* were transferred into Treg cells than into NK cells (Fig. 5D).

DISCUSSION

Until recently, intercellular communication had been thought to be take place only through cell-to-cell adhesion conduits or secreted signals, such as hormones, cytokines, and neurotransmitters. In addition to these, exosomal RNAs and proteins have been shown to play an important role in intercellular communication [10, 36, 37]. During pregnancy, exosomes are involved in cell-to-cell communication between the placenta and peripheral blood immune cells. For example, Taylor et al. [38], Sabapatha et al. [39], and Atay et al. [40] reported that placenta-derived exosomes exist in the maternal circulation and suppress T-cell signaling components. However, these functional studies measured placental exosome-associated proteins, such as Fas ligand, MHC class I chain-related proteins, and fibronectin. Although it is likely that exosomal C19MC miRNAs contribute to placental-maternal communication, few studies have examined whether exosomal placenta-associated miRNAs modulate the expression of their targets in recipient maternal immune cells.

In the present study, we investigated the possible involvement of exosomal placenta-associated miRNAs in communication between trophoblasts and immune cells using in vitro and in vivo approaches. First, we employed an in vitro model system. We attempted to search target genes of placenta-associated miRNA *miR-517a-3p* using an in vitro trophoblast (BeWo)-immune cell (Jurkat) model system because little has been reported on experimentally validated targets of *miR-517a-*

3p. In the present study, we identified *PRKG1* as a target gene of the placenta-associated *miR-517a-3p* (Fig. 3) and demonstrated that BeWo exosomal *miR-517a-3p* was transferable to Jurkat cells and suppressed the expression of *PRKG1* mRNA within the recipient cells (Fig. 4). Next, using maternal peripheral blood cells as an in vivo approach, we confirmed that *miR-517a-3p* in the maternal circulation was delivered into NK cells in peripheral blood as it was into Jurkat cells in vitro. We revealed a negative correlation between the expression levels of transferred exosomal *miR-517a-3p* and its target mRNA *PRKG1* in maternal NK cells before and after delivery (Fig. 5, A and C). In addition, we presented that *miR-517a-3p* was transferred into NK cells via BeWo exosomes (Fig. 5D). These in vivo and in vitro results suggest that exosome-mediated transfer of placenta-associated miRNAs and subsequent modulation of their target genes occur in maternal NK cells during pregnancy.

PRKG1 is a serine/threonine kinase that acts as a key mediator of the nitric oxide (NO)/cGMP signaling pathway [41, 42]. PRKG1 plays a central role in regulating cardiovascular and neuronal functions in addition to relaxing smooth muscle tone, preventing platelet aggregation, and modulating cell growth. In mammals, PRKG1 is strongly expressed in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, the end plates of neuromuscular junctions, and kidney [43, 44]. In terms of PRKG1 in immune cells, Fischer et al. [45] reported that the NO/cGMP/PRKG1 signaling system negatively regulates T-cell activation and proliferation. Although the biological function of PRKG1 in NK cells is unclear, our present data together with previous findings suggest that *miR-517a-3p* is involved in regulating the activation and proliferation of maternal immune cells by inhibiting NO/cGMP/PRKG1 signaling.

Placenta-associated miRNAs were reduced in maternal NK cells rapidly after delivery (Fig. 5, A and B). What mechanism is involved in this rapid clearance of placenta-associated miRNAs in NK cells? There are some possible explanations for rapid turnover dynamics of placenta-associated miRNAs in NK cells. First, it is possible that the supply of placenta exosomes, including placenta-associated miRNAs, is stopped after a termination of pregnancy. Another explanation may be that rapid degradation of miRNAs occurs in NK cells. Because placenta-associated miRNA levels in maternal plasma decreased dramatically after delivery [20], it seems likely that the halt of the supply of placenta exosomes affects rapid clearance of placenta-associated miRNAs rather than miRNA turnover in NK cells.

Natural killer cells are well known to play important roles in the maintenance of human pregnancy [34]. Although we identified one target gene of *miR-517a-3p* using the in vitro model system used in this study, we could not find other target genes that are associated with immune tolerance in pregnancy. A single miRNA targets multiple mRNAs, and a single mRNA is regulated by multiple miRNAs. This relationship between miRNAs and target genes makes it difficult to study the functional roles of miRNAs. Recently, Olarerin-George et al. [46] have shown *miR-517a-3p* to be a potent activator of nuclear factor-kappa B signaling. Nuclear factor-kappa B plays pivotal roles in immune response [47]. Further identification of targets of these miRNAs and studies of their functions are warranted to comprehensively understand the precise mechanisms by which placental exosomal miRNA-mediated modulation of maternal NK cells contributes to fetomaternal immunotolerance during pregnancy.

The specificity of the exosome-mediated communication between trophoblasts and immune cells remains to be
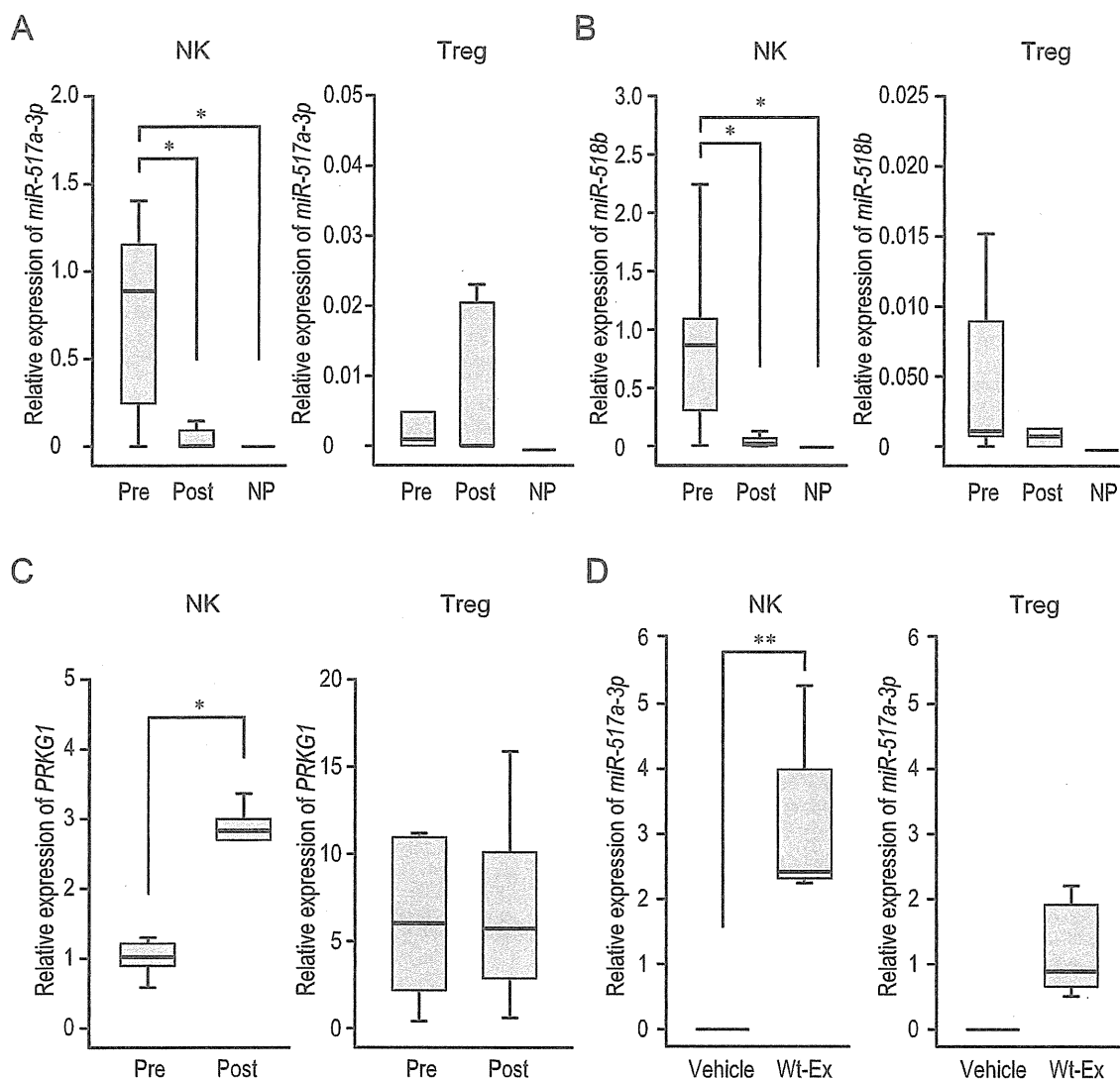
Article 129

FIG. 5. Transferred placenta-associated miRNAs in the peripheral blood immune cells of pregnant women. A and B) Quantification of transferred placenta-associated miRNAs in maternal immune cells. Transferred miR-517a-3p (A) and miR-518b (B) were measured by real-time PCR in NK and Treg cells isolated from maternal peripheral blood samples at 1 day before delivery (Pre) and 4 days after delivery (Post). NK, n = 15; Treg, n = 9. The NK and Treg cells were also obtained from nonpregnant women with no history of pregnancy (NP; n = 5). Data were normalized to SNORD44. Expression in NK before delivery was defined as 1. Lines inside boxes denote medians; boxes represent the interquartile range; and whiskers extend to the most extreme values within 1.5 times the interquartile range. C) Differential expression of PRKG1 mRNA in maternal peripheral blood immune cells before and after delivery. PRKG1 expression in maternal NK cells (n = 5) and Treg cells (n = 6) obtained from pregnant women both 1 day before delivery (Pre) and 4 days after delivery (Post) was investigated by real-time PCR. Data were normalized to GAPDH. Expression in NK before delivery was defined as 1. D) In vitro transfer of BeWo miR-517a-3p into male NK and Treg cells. After incubation of each cell type in the presence (Wt-Ex) or absence (Vehicle) of BeWo wild-type exosomes (50 µg/ml) for 48 h at 37°C, levels of transferred miR-517a-3p were analyzed by real-time PCR (n = 5 each). Data were normalized to SNORD44. Expression in Treg cells incubated with exosomes was defined as 1. Data are means ± SD. Kruskal-Wallis test; *P < 0.05, **P < 0.01.

elucidated. Exosomes differ in their cellular origin [48]. Thus, cell type-specific molecules on the cell surface of exosomes may promote specific delivery to target cells. In addition to the exosome-mediated communication between BeWo and Jurkat cells, we investigated the possible involvement of miR-517a-3p in exosome-mediated communication between BeWo and human umbilical vein endothelial cells. Although BeWo exosomal miR-517a-3p was transferable to human umbilical vein endothelial cells as well as Jurkat cells, it suppressed the expression of different target genes within the nonimmune recipient cells (our unpublished data). The mechanism by which placental exosomes preferentially target and modulate specific cells has not yet been determined. In the present study,

cell lines were primarily employed. Differential ultracentrifugation employed in this study could not separate relatively large vesicles (e.g., 150- to 200-nm vesicles). As reported by Bobrie et al. [49], improved techniques will be needed to accurately purify the different populations of extracellular vesicles. To further characterize trophoblast-derived exosomes (e.g., the cell surface properties and target cell-specific delivery) and reveal the functional roles of exosome-mediated placenta-associated miRNAs in maternal NK cells, future work should be performed using a combination of primary immune cells and exosomes isolated from primary human trophoblasts.

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Original Article

Intrauterine growth restriction modifies gene expression profiling in cord blood

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Abstract   Background: Small-for-gestational-age (SGA) newborns are at an increased risk for perinatal morbidity and mortality and development of metabolic syndromes such as cardiovascular disease and type 2 diabetes mellitus (T2DM) in adulthood. The mechanism underlying this increased risk remains unclear. In this study, genetic modifications of cord blood were investigated to characterize fetal change in SGA newborns.

Methods: Gene expression in cord blood cells was compared between 10 SGA newborns and 10 appropriate-for-gestational-age (AGA) newborns using microarray analysis. Pathway analysis was conducted using the Ingenuity Pathways Knowledge Base. To confirm the microarray analysis results, quantitative real-time polymerase chain reaction (RT-PCR) was performed for upregulated genes in SGA newborns.

Results: In total, 775 upregulated and 936 downregulated probes were identified in SGA newborns and compared with those in AGA newborns. Of these probes, 1149 were annotated. Most of these genes have been implicated in the development of cardiovascular disease and T2DM. There was good agreement between the RT-PCR and microarray analyses results.

Conclusions: Expression of certain genes was modified in SGA newborns in the fetal period. These genes have been associated with metabolic syndrome. To clarify the association between modified gene expression in cord blood and individual vulnerability to metabolic syndrome in adulthood, these SGA newborns will be have long-term follow up for examination of genetic and postnatal environmental factors. Gene expression of cord blood can be a useful and non-invasive method of investigation of genetic alterations in the fetal period.

Key words   cord blood, gene expression, intrauterine growth restriction, metabolic syndrome, microarray analysis.

Intrauterine growth restriction (IUGR) is used to designate a fetus that has not reached its growth potential; it can be caused by fetal, placental, or maternal factors.[1] IUGR results in the birth of an infant who is small for gestational age (SGA).[1] SGA newborns are at increased risk for perinatal morbidity, mortality, and adverse developmental outcome.[2,3] After Barker et al. reported an association between restricted fetal growth and increased risk of mortality from cardiovascular disease in adulthood,[4] several studies noted the same or similar results in a variety of ethnicities.[5–7] Using several animal models of IUGR, other studies have confirmed the association between restricted fetal growth and increased risk of developing metabolic and cardiovascular disease in the future.[8–10] Despite the evidence of the relationship between fetal undernutrition and adult diseases,[5–10] the specific mechanism underlying this increased risk remains

unclear. In the recent studies, they hypothesized that fetal and neonatal undernutrition may lead to epigenetic alterations, resulting in an increased susceptibility to development of metabolic syndrome and other chronic diseases in adulthood.[11–13] The high rate of DNA synthesis of the fetal and neonatal genome is particularly vulnerable to environmental factors, and the elaborate DNA methylation pattern required for normal tissue development is established during the early stages of fetal development.[14]

The microarray method provides a means to examine the expression profiles of several hundred genes. In combination with this method, pathway analysis technologies allow the mapping of gene expression data into the relevant pathway maps on the basis of their functional annotation and known molecular interactions.[15] Previous studies have shown that the total RNA derived from circulating blood can be used to distinguish control subjects and patients with certain diseases.[16,17] Cord blood is a non-invasive and readily available source of fetal cells. In this study, the global gene expression profiling of SGA newborns was characterized in cord blood cells using Affymetrix Gene Chip technology by comparing appropriate-for-gestational-age (AGA) with SGA newborns.

Methods

Subjects

In this study, SGA was defined as birthweight and height below the 10th percentile of the local sex-specific distribution for gestational age, and AGA as these parameters between the 10th and 90th percentiles for gestational age. Singleton newborns were selected, and those with congenital malformations or exposure to intrauterine infection were excluded. After application of these criteria, 10 SGA newborns and 10 AGA newborns were included in the study. The AGA newborns were randomly selected from among infants whose birthweight and height matched these criteria. This study was approved by the Research Ethics Committee of the University of Toyama. Written informed consent was obtained from the families of all participants before the study.

RNA extraction from whole blood

Whole blood samples (0.5 mL) were obtained from umbilical cords, conserved in RNA later® (Ambion, Austin, TX, USA) following the manufacturer's instructions and stored at −20°C. Following complete thawing, RNA later® was eliminated by centrifugation. Total RNA was extracted from cord blood cells using a RiboPure-Blood Kit (Ambion) and treated with DNase I (Ambion) for 30 min at 37°C to remove residual genomic DNA. Whole blood messenger (m)RNA consists of a relatively large population of globin mRNA transcripts. Because globin mRNA interferes with the expression profiling of whole blood samples, α and β globin mRNA were depleted from the total RNA preparations using a GLOBIN clear Kit (Ambion).

Microarray hybridization and pathway analysis

Gene expression profiling of cord blood cells from AGA and SGA newborns was performed on an Affymetrix HG-U133A plus 2.0 array that was spotted with 54 674 probe sets (Affymetrix, Santa Clara, CA, USA). In this study, a total of 20 arrays were used: 10 for the AGA newborns and 10 for the SGA newborns. Samples were prepared for array hybridization following the manufacturer's instructions. In short, biotin-labeled complementary (c)RNA was prepared from 500 ng of globin-depleted total RNA using a 3′-IVT Express Kit (Affymetrix) and hybridized to the array. The arrays were scanned with a probe array scanner. The data files (CEL files) resulting from the analysis using the Affymetrix GCOS software were imported into GeneSpring GX 11.0 software (Agilent Technologies, Santa Clara, CA, USA) to extract the differentially expressed genes in the cord blood of SGA and AGA newborns.

Quantitative real-time polymerase chain reaction

To confirm the microarray analysis results, quantitative real-time polymerase chain reaction (RT-PCR) was performed to examine mRNA expression in four genes: fibronectin 1 (*FN1*), collagen type 1alpha2 (*COL1A2*), farnesyl diphosphate synthase (*FDPS*) and mitochondrial tumor suppressor 1 (*MTUS1*). Complementary DNA (cDNA) was synthesized from total RNA (1 μg) using

an oligo (dT) 16 primer and Omniscript reverse transcriptase (Qiagen, Hilden, Germany). RT-PCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Tokyo, Japan) and a Mx3000P QPCR system (Agilent Technologies). The following primer pairs were used: *FN1*: forward, 5′-gagagaagtgggaccgtcag-3′; reverse, 5′-tggcacc gagatattccttc-3′; *COL1A2*: forward, 5′-gcccaacctgaaaacatccc-3′; reverse, 5′-aggttgccagtctcctcatc-3′; *FDPS*: forward, 5′-aggacaaca aatgcagctgg-3′; reverse, 5′-ggtgctgcgtactgttcaat-3′; *MTUS1*: forward, 5′-gaaagctcggatggacaagc-3′; reverse, 5′-gtccccattgtg cagtttcc-3′. RT-PCR was performed under the following conditions: 95°C for 10 s and 60°C for 30 s. All mRNA expression was normalized with respect to the mRNA of glyceraldehyde phosphate dehydrogenase (*GAPDH*).
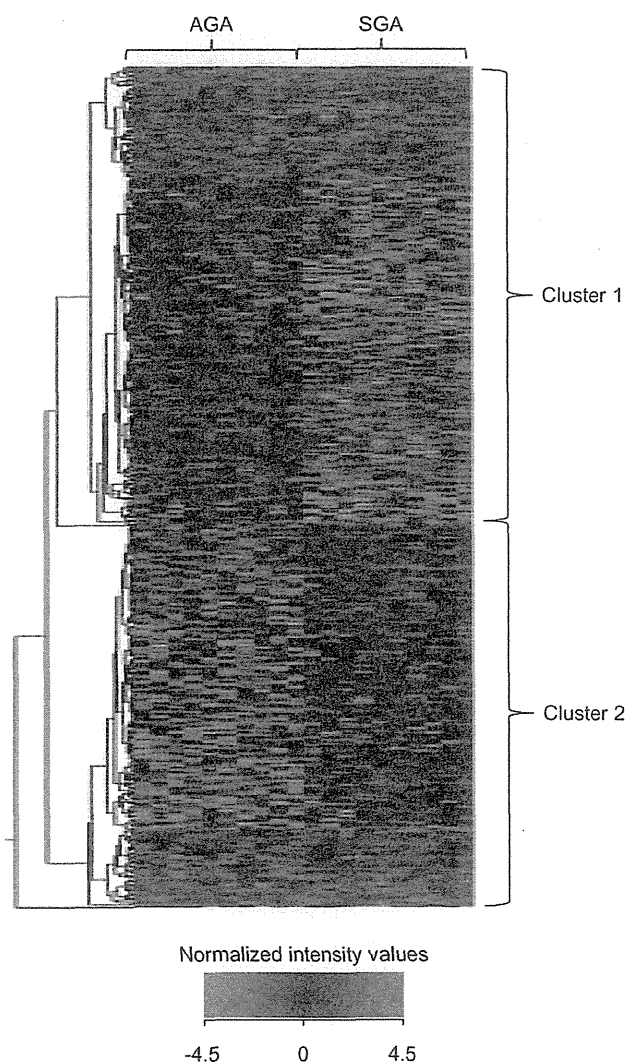

Normalized intensity values

-4.5   0   4.5

Fig. 1   Hierarchical clustergram. Each probe set is represented by a single row of colored bars. Red, upregulated; and green, downregulated; black, no change. Each line represents a cord blood sample from among the appropriate-for-gestational-age (AGA; *n* = 10) and small-for-gestational-age (SGA; *n* = 10) newborns.

Table 1 Neonatal and maternal characteristics

	GA	Sex	Delivery	Birthweight (g)	Birth height (cm)	Cord blood pH	Placental weight (g)	Cord insertion	Age (years)	Maternal Height (cm)	Weight (g)	BMI	Pregnancy weight gain (kg)
SGA 1	39 weeks 5 days	M	V	2400	46	7.31	440	Central	30	157	55	22.3	8.9
2	39 weeks 4 days	M	V	2430	46	7.25	430	Marginal	31	154	46.2	19.5	7.8
3	39 weeks 5 days	F	V	2066	43	7.30	330	Velamentous	28	159	48.	19	8
4	40 weeks 0 days	F	V	2194	45	7.36	340	Marginal	26	155	48	20	12.5
5	37 weeks 2 days	F	C	1960	43.5	7.23	345	Eccentric	31	160	64	25	7.8
6	37 weeks 6 days	M	C	2214	43	7.40	270	Central	21	151	52	21.9	3.5
7	37 weeks 0 days	M	V	2070	40	7.35	440	Marginal	32	153	40	17.1	8
8	38 weeks 1 days	F	V	2398	43.5	7.33	510	Marginal	25	148	40	18.3	10
9	39 weeks 4 days	F	C	2054	44	7.33	430	Marginal	33	154	48	20.2	8
10	38 weeks 3 days	M	C	2355	44.2	7.22	375	Central	34	153	47	20.1	8
Mean	38 weeks 4 days			2214	43.8	7.31	391		29.1	154.4	48.8	20.3	8.3
AGA 1	40 weeks 3 days	F	V	3224	50.5	7.31	605	Central	35	155	58	24.1	10.8
2	38 weeks 2 days	F	V	2814	50	7.35	584	Central	26	157	52	21.1	9
3	38 weeks 0 days	M	V	2684	46.5	7.30	580	Marginal	31	149	44	19.8	5.9
4	38 weeks 1 days	F	C	2840	48.5	7.31	400	Central	39	168	61	21.6	6
5	39 weeks 2 days	F	V	2904	49.5	7.31	640	Marginal	37	149	42	18.9	6.5
6	37 weeks 2 days	M	C	3220	47	7.25	622	Central	35	167	54.5	19.5	9
7	39 weeks 2 days	M	V	3334	51	7.30	800	Central	25	168.5	56	19.7	11.3
8	40 weeks 3 days	M	V	3392	50.5	7.33	600	Central	25	162	65	24.8	14.8
9	39 weeks 5 days	M	V	3014	49.5	7.39	520	Marginal	37	154	50	21.1	9.3
10	37 weeks 0 days	F	C	2530	47.5	7.30	590	Central	39	149	44	19.9	9
Mean	38 weeks 5 days			2996	49.1	7.31	595		33	157.9	52.7	21.1	9.2

AGA, appropriate for gestational age; BMI, body mass index; GA, gestational age; SGA, small for gestational age.

Statistical analysis

Statistical analysis was done using unpaired *t*-test. Statistically significant differences were identified at $P < 0.05$. A fold change >2 (upregulated) or <2 (downregulated) was considered to be biologically important.[18] We used Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA, USA) to investigate the following characteristics of upregulated and downregulated genes from cord blood of SGA newborns: biological processes, cellular components, molecular functions, genetic networks, and toxicity functions. IPA allowed the estimation of the likelihood of SGA newborns developing disease. Differences in demographic characteristics and clinical measures between SGA and AGA newborns were also examined using unpaired *t*-test.

Results

Cord blood samples were collected from 10 SGA and 10 AGA newborns. The characteristics of the newborns and their mothers are given in Table 1. Mothers were free of diseases such as gestational diabetes mellitus and hypertension. Significantly lower birthweight, height, and placental weight were recorded for SGA newborns than for AGA newborns (Table 1). Abnormal cord insertion was identified in 60% of placentas from SGA infants, suggesting that the placenta tended to be light or cord insertion tended to be abnormal in SGA newborns. There was no significant difference in maternal height, weight, body mass index before pregnancy, alcohol consumption or pregnancy weight gain between the two groups (Table 1). One mother had a smoking history in each group.

Gene expression was compared between cord blood samples from SGA and AGA newborns to characterize fetal changes in SGA newborns. Hierarchical clustering based on similarities in gene expression using differentially expressed genes (>twofold increase or decrease in RNA) demonstrated a clear distinction between SGA and AGA newborns (Fig. 1). In SGA newborns, 936 downregulated (Fig. 1, cluster 1) and 775 upregulated probes (Fig. 1, cluster 2) were identified for a total of 1711 changed probes. Of these 1711 probes, 1149 were annotated and labeled as SGA-specific genes.

To identify the biologically relevant networks and pathways of these genes, pathway analysis was conducted on the SGA-specific genes using the Ingenuity Pathways Knowledge Base. SGA-specific genes were classified into functional groups related to diseases and disorders, and the top 10 diseases are identified in Table 2 and the top five genes in Table 3. Most of these listed genes overlapped with biological processes and were related to metabolic syndromes, including cardiovascular disease and diabetes mellitus.

Four genes (*FN1*, *COL1A2*, *FDPS* and *MTUS1*) from Table 3 were selected for confirmation of the microarray analysis results. Overall, there was good agreement between the RT-PCR and microarray analyses results (Fig. 2).

Discussion

To characterize gene expression of the IUGR fetus, we compared cord blood between SGA and AGA newborns. IUGR was found to modify the expression patterns of certain genes during the fetal period. These genes are associated with development of metabolic syndromes including cardiovascular disease and type 2 diabetes mellitus (T2DM; Table 2). In the top five upregulated SGA-specific genes (Table 3), *FN1* is outstanding. Fibronectin regulates a wide variety of cellular interactions with the extracellular matrix (ECM) and plays crucial roles in cell adhesion, migration, growth, differentiation and embryogenesis.[19,20]

Table 2 Functional analysis of the genes specific for SGA newborns

Category	Function	P	Associated genes
Immunological disease	Autoimmune disease	1.55×10^{-9}	198
	Immunological disorder	1.25×10^{-8}	219
	Insulin-dependent diabetes mellitus	2.74×10^{-7}	101
Genetic disorder	Genetic disorder	1.64×10^{-9}	483
	Alzheimer's disease	5.96×10^{-7}	86
	Coronary artery disease	3.14×10^{-6}	104
Metabolic disease	Metabolic disorder	1.22×10^{-7}	220
	Diabetes mellitus	3.72×10^{-7}	186
Endocrine system disorder	Endocrine system disorder	3.35×10^{-7}	198
	Glucose intolerance	1.10×10^{-2}	7
Cardiovascular disease	Arteriosclerosis	3.51×10^{-7}	115
	Atherosclerosis	4.49×10^{-7}	114
	Cardiovascular disorder	1.69×10^{-6}	190
Neurological disease	Neurodegenerative disorder	5.41×10^{-7}	90
	Neurological disorder	7.45×10^{-6}	286
Inflammatory disease	Inflammatory disorder	1.47×10^{-6}	231
	Arthritis	4.09×10^{-6}	146
Cardiovascular system development	Development of blood vessel	1.67×10^{-6}	50
Organismal development	Angiogenesis	3.88×10^{-5}	39
Cancer	Tumorigenesis	2.82×10^{-6}	245

SGA, small for gestational age.

Table 3 Most strongly upregulated genes specific for SGA newborns

Category	AffyID	Gene symbol	Description	Log ratio
Immunological disease	211719_x_at	FN1	Fibronectin 1	2.235
	206683_at	ZNF165	Zinc finger protein 165	1.164
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	211151_x_at	Gh	Growth hormone 1	0.991
	202404_s_at	COL1A2	Collagen, type I, alpha 2	0.956
Genetic disorder	211719_x_at	FN1	Fibronectin 1	2.235
	236517_at	MEGF10	Multiple EGF-like-domains 10	1.641
	217344_at	FDPS	Farnesyl diphosphate synthase	1.308
	1569701_at	PER3	Period homolog 3 (Drosophila)	1.267
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123
Metabolic disease	211719_x_at	FN1	Fibronectin 1	2.235
	217344_at	FDPS	Farnesyl diphosphate synthase	1.308
	206683_at	ZNF165	Zinc finger protein 165	1.164
	211151_x_at	Gh	Growth hormone 1	0.991
	202404_s_at	COL1A2	Collagen, type I, alpha 2	0.956
Endocrine system disorder	211719_x_at	FN1	Fibronectin 1	2.235
	217344_at	FDPS	Farnesyl diphosphate synthase	1.308
	212093_s_at	MTUS1	Mitochondrial tumor suppressor 1	1.204
	206683_at	ZNF165	Zinc finger protein 165	1.164
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123
Cardiovascular disorder	211719_x_at	FN1	Fibronectin 1	2.235
	1562301_at	C8orf34		1.079
	208190_s_at	LSR	Lipolysis-stimulated lipoprotein receptor	0.762
	211506_s_at	IL8	Interleukin 8	0.678
	202988_s_at	RGS1	Regulator of G-protein signaling 1	0.663
Neurological disorder	236517_at	MEGF10	Multiple EGF-like-domains 10	1.641
	243483_at	TRPM8	Transient receptor potential cation channel, subfamily M, member 8	1.417
	217344_at	FDPS	Farnesyl diphosphate synthase	1.308
	1569701_at	PER3	Period homolog 3 (Drosophila)	1.267
	206084_at	PTPRR	Protein tyrosine phosphatase, receptor type, R	1.207
Inflammatory disorder	211719_x_at	FN1	Fibronectin 1	2.235
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	209278_s_at	TFPI2	Tissue factor pathway inhibitor 2	0.936
	220406_at	TGFB2	Transforming growth factor, beta 2	0.847
	226189_at	ITGB8	Integrin, beta 8	0.718
Cardiovascular system development and function	211719_x_at	FN1	Fibronectin 1	2.235
	208357_x_at	CSH1	Chorionic somatomammotropin hormone 1 (placental lactogen)	1.417
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	211151_x_at	GH1	Growth hormone	0.991
	202404_s_at	COL1A2	Collagen, type I, alpha 2	0.956
Organismal development	211719_x_at	FN1	Fibronectin 1	2.235
	208357_x_at	CSH1	Chorionic somatomammotropin hormone 1 (placental lactogen)	1.417
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123
	211151_x_at	GH1	Growth hormone	0.991
	202404_s_at	COL1A2	Collagen, type I, alpha 2	0.956
Cancer	211719_x_at	FN1	Fibronectin 1	2.235
	243483_at	TRPM8	Transient receptor potential cation channel, subfamily M, member 8	1.417
	217344_at	FDPS	Farnesyl diphosphate synthase	1.308
	1555471_a_at	Fmn2	Formin 2	1.160
	221796_at	NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	1.123

SGA, small for gestational age.

Interestingly, ECM such as integrin and collagen were also upregulated in SGA cord blood (Table 3). Although these activated interactions could affect the fetal development of SGA newborns, we need further research to account for this pathological meaning.

Many studies have reported an association between low birthweight and the development of cardiovascular diseases in the future.[4–7] In the present study, 190 SGA-specific genes were associated with cardiovascular disease and 115 were related to arteriosclerosis (Table 2). Hence, we speculated that cardiovascular disease could develop during the fetal period. Several reports noted that the aortic wall in SGA newborns was thicker and stiffer than that in AGA newborns.[21–24] Fetal IUGR has also been associated with significant thickening of the aortic intima–media, suggesting that atherosclerosis may occur during the fetal period.[24] Interestingly, systolic blood pressure in these SGA infants significantly increased at a mean postnatal age of 18 months compared with AGA infants,[24] suggesting that fetal
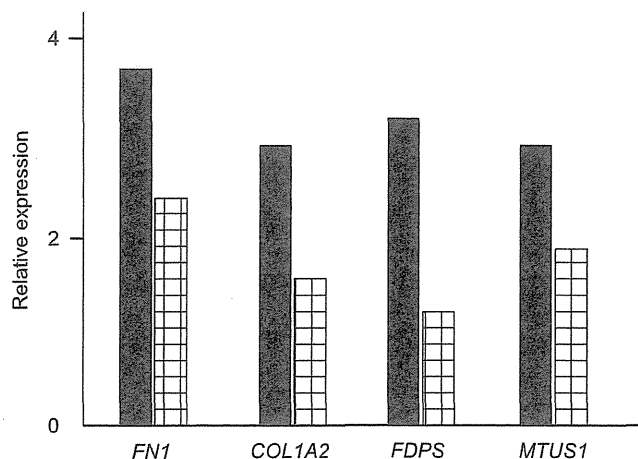
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Fig. 2 (▦) Real-time polymerase chain reaction (RT-PCR) confirmation of (▨) microarray analysis results. Complete agreement was found between RT-PCR and microarray results. GAPDH expression was used for normalization of RT-PCR. *COL1A2*, collagen type 1alpha2; *FDPS*, farnesyl diphosphate synthase; *FN1*, fibronectin 1; *MTUS1*, mitochondrial tumor suppressor 1.

atherosclerosis may have an effect on blood pressure in the future. Thus, the risk for cardiovascular disease may originate from as early as the fetal period.

Many reports of the relationship between low birthweight and increased incidence of T2DM have been reviewed.[13,25] The precise mechanisms of this relationship, however, remain unknown. Genome-wide association studies have identified T2DM susceptibility variants in various ethnic groups.[26–29] In addition, several reports have proposed an association between variants of T2DM risk genes and reduced birthweight because insulin is a key fetal growth factor.[29,30] This fetal insulin hypothesis proposes that genetic variants reduce insulin secretion or action, which may predispose toward the development of T2DM and reduce birthweight. Among the SGA-specific genes identified here, 186 have been associated with T2DM (Table 2). Some of these may be consistent with the fetal insulin hypothesis.

Maternal undernutrition exposes the fetus to hormonal, metabolic, and vascular alterations.[31] Maternal stress or undernutrition during gestation also disturbs the hypothalamic-pituitary-adrenal (HPA) axis in SGA newborns.[32,33] This disturbance may provide a mechanism for susceptibility to cardiovascular and metabolic diseases.[32] In this study, 220 genes related to metabolic disorders and 198 genes related to endocrine system disorders were found among the SGA-specific genes (Table 2). These data suggest that fetal undernutrition induces dramatic changes in the fetal HPA axis, which may induce metabolic diseases in the future.

Although many animal models of fetal undernutrition have been reported, biopsy analysis of tissues of healthy infants for research is unethical. Therefore, gene expression in cord blood was investigated in this study. Genes in cord blood have had direct contact with many organs such as the pancreas, liver, and heart. Hayashi *et al.* showed that the transcriptional levels of genes in leukocytes could be a diagnostic marker for T2DM.[34]

The present analysis has demonstrated that cord blood can be utilized as a good resource for non-invasive investigation of fetal genetic alterations.

This study has some limitations that must be considered in the interpretation of the results. First, family history of metabolic syndrome was not included in the analysis; hence, the effect of heredity on the results cannot be excluded. Second, SGA newborns may be a mixture of IUGR and constitutionally small newborn. Although maternal height, weight and parity are risk factors for SGA, we did not recognize any difference in risk factors between the AGA and SGA group.[35] Moreover, the SGA group had significantly lower placenta weight and a high percentage of abnormal cord insertion. This implies that most of the present SGA newborns were IUGR. Third, the association between modified gene expression in cord blood and development of metabolic disease in adulthood was not clarified. In addition, the relationship between low birthweight and future risk of disease may be mediated by epigenetic mechanisms.[14,36] Modified gene expression in SGA newborns has been associated with metabolic disease, but epigenetic factors were not investigated in this study. Previous studies have found an association between rapid weight gain during infancy and higher blood pressure and risk of coronary heart disease in the future.[37,38] Moreover, not all SGA newborns are at risk of insulin resistance and diabetes.[25] The results of these reports indicate that the association between IUGR and future development of diseases may be due to genetic and postnatal environmental interactions. In the future, long-term follow up of these SGA newborns will be conducted for the analysis of genetic and postnatal environmental factors to determine the precise pathological consequences of the genetic findings of this study.

Conclusion

A total of 1149 genes were differentially expressed in SGA cord blood in comparison with AGA cord blood. Interestingly, most of these genes have been implicated in the development of cardiovascular disease and T2DM. To clarify the association between modified gene expression in cord blood and individual vulnerability to metabolic syndrome in future, these SGA newborns will undergo long-term follow up for examination of genetic and postnatal environmental factors. Gene expression of cord blood can be a useful and non-invasive method of investigation of genetic alterations in the fetal period.

Acknowledgments

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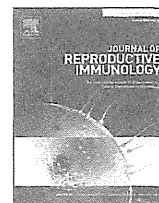
A review of the mechanism for poor placentation in early-onset preeclampsia: the role of autophagy in trophoblast invasion and vascular remodeling

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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

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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

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
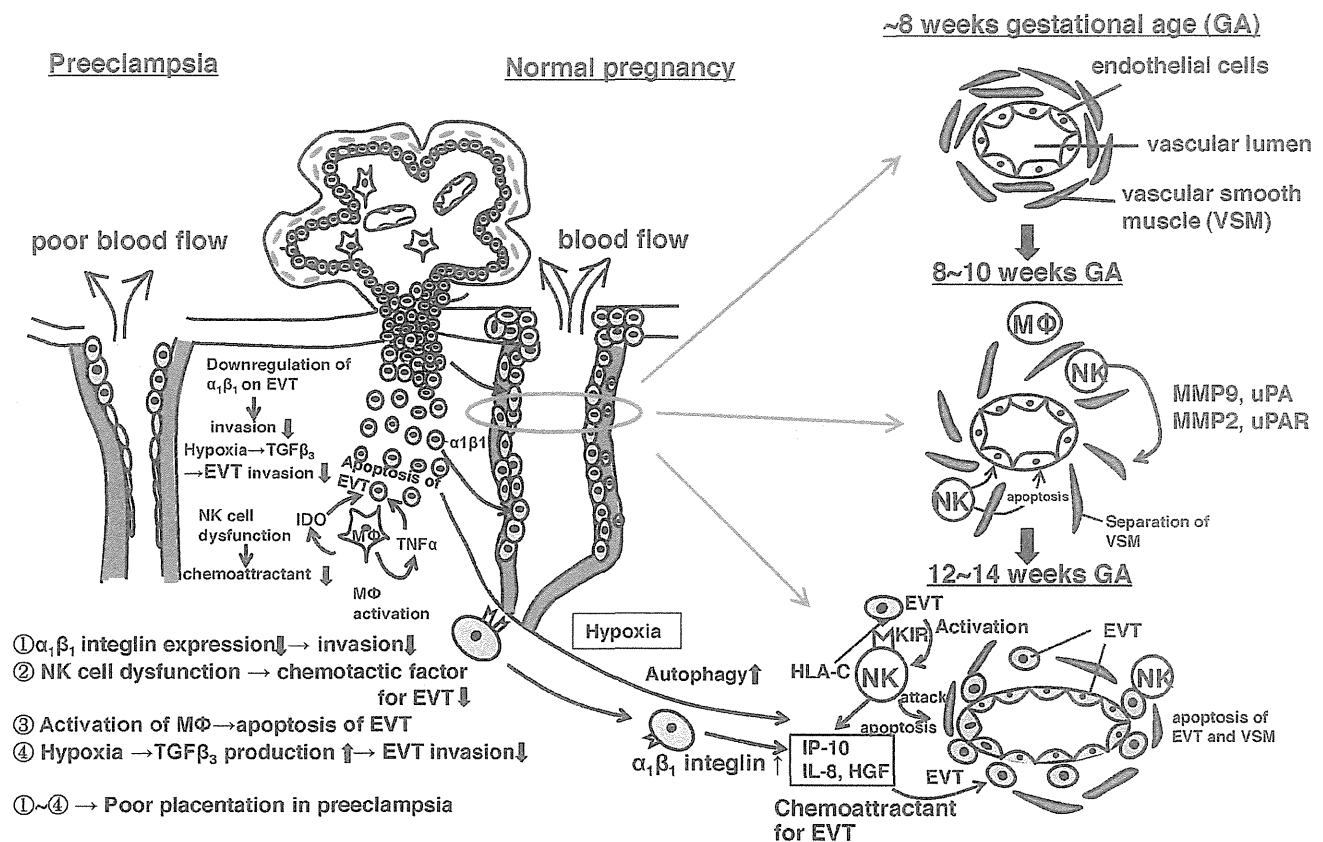
Fig. 1. The mechanisms of vascular remodeling in normal pregnancy and inadequate vascular remodeling in preeclampsia that have already been reported. At a gestational age of 8–10 weeks, uterine NK cells produce MMP-2, MMP-9, and uPA, and these enzymes induce the separation of vascular smooth muscle. 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 $\alpha1\beta1$ 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).
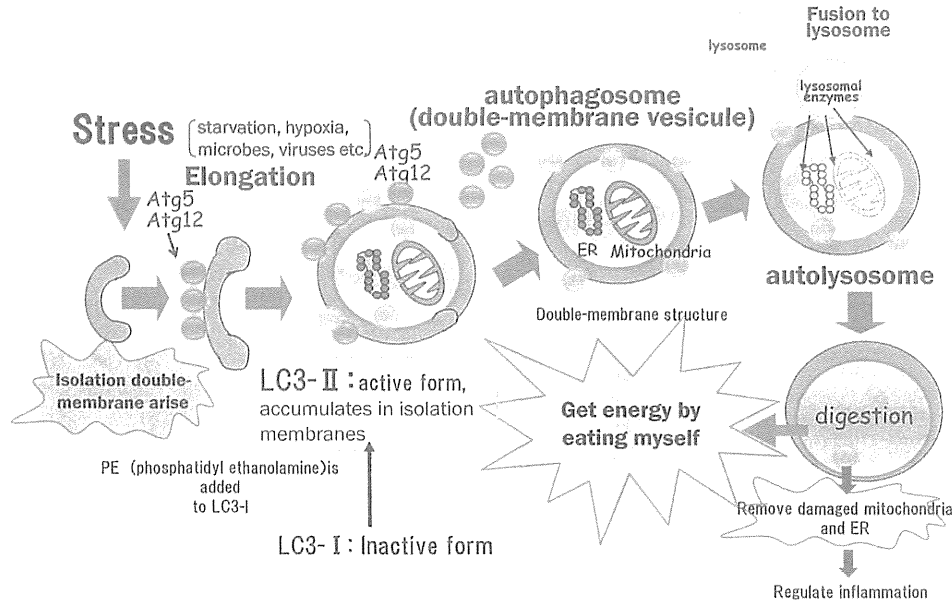
Fig. 2. Schematic diagram of autophagy. When cells experience stress, the isolation membrane arises and elongates, finally forming an autophagosome. Lastly, the autophagosome fuses with lysosomes, resulting in degradation of the cytoplasmic components by lysosomal hydrolases. Then, the cells obtain energy. At the same time, damaged mitochondria and ER are removed by autophagy, resulting in the regulation of inflammation.
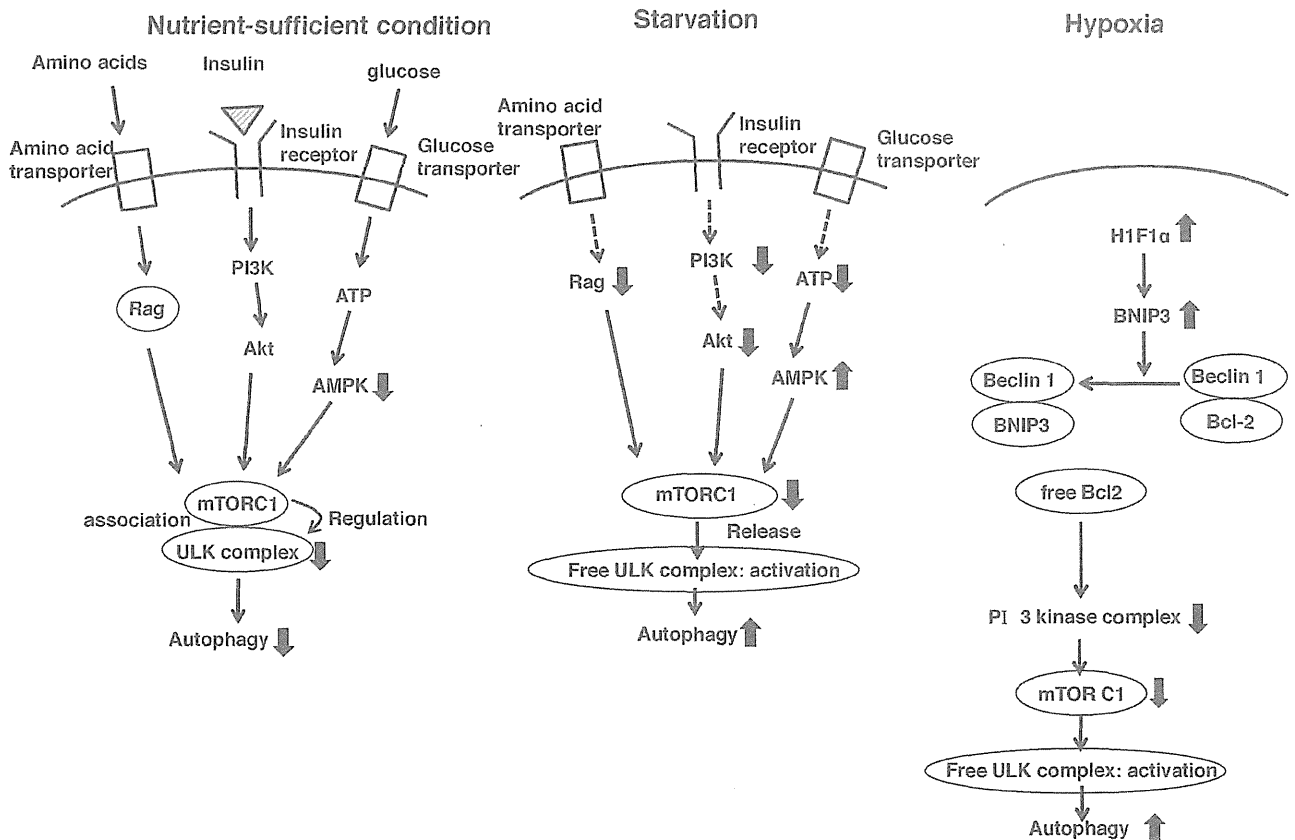

Fig. 3. The molecular mechanisms that induce autophagy. Under nutrient-sufficient conditions, amino acids, glucose, and insulin induce mTORC1 activation. mTORC1 associates with the UCL complex, resulting in the regulation of autophagy. Under starvation conditions, mTOR activity is reduced, resulting 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
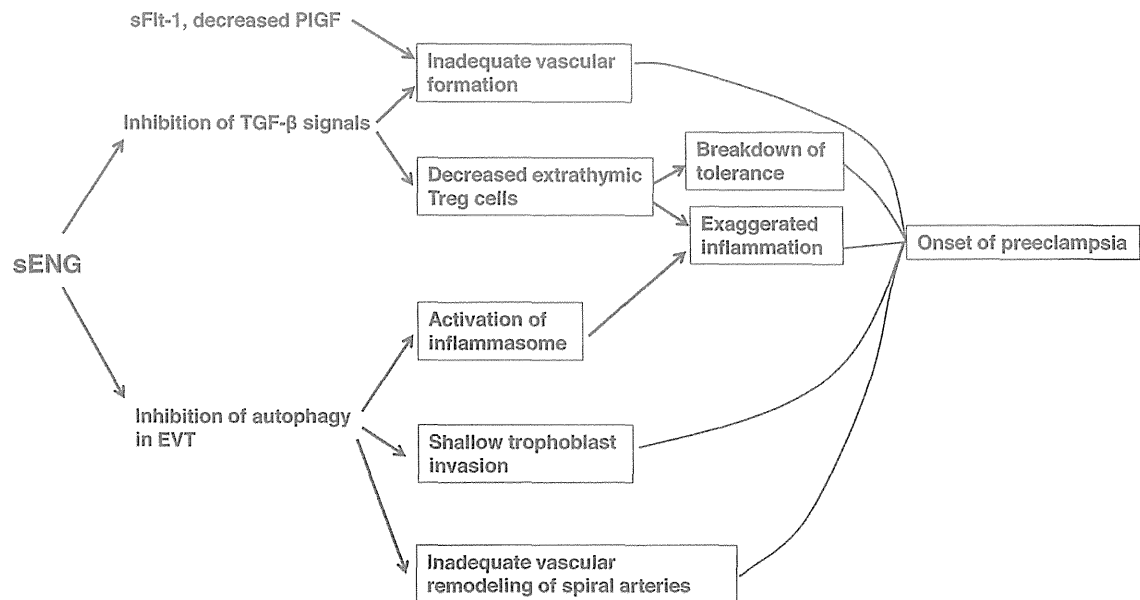
Fig. 4. The mechanisms for the pathophysiology of preeclampsia from the perspective of autophagy. The high level of sENG inhibits the autophagy in EVTs under hypoxic conditions, resulting in shallow trophoblast invasion and inadequate vascular remodeling of spiral arteries. Inhibition of autophagy reduces 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
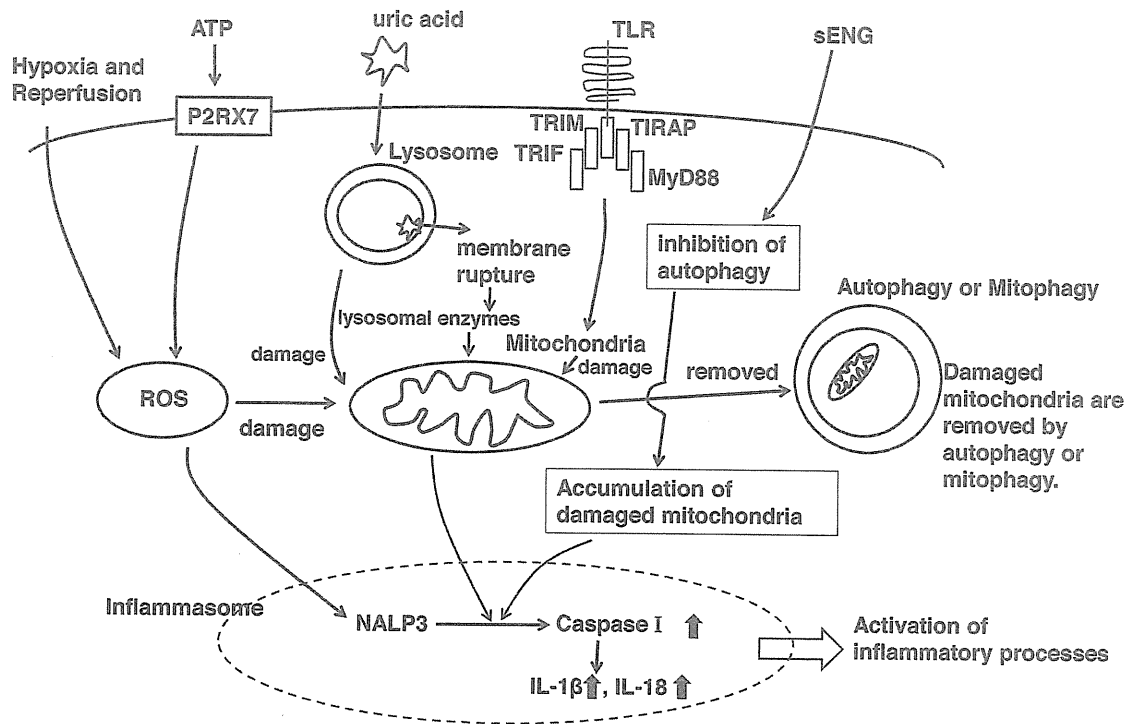
Fig. 5. The role of autophagy in NALP3 inflammasome activation. Quality control of mitochondria is essential for cellular homeostasis, and this process is achieved through autophagy. When autophagy is inhibited, damaged mitochondria activate inflammasomes, resulting in exaggerated inflammation.

cells consists of the regulation of inflammation; therefore, decreased Treg cells induce exaggerated inflammation (Fig. 4). This inflammation induces Th1-type immunity, which subsequently induces rejection (Saito et al., 2007), inhibits the function of Treg cells (Yang et al., 2004), and induces endothelial dysfunction (Redman et al., 1999). Redman and Sargent (2010) proposed that the inflammatory drive of preeclampsia results from the three-way interactions among oxidative stress, ER stress and inflammatory responses. Inadequate autophagy of EVTs may accelerate oxidative stress, ER stress and inflammation. Further studies are needed to clarify this hypothesis.

3. Conclusion

Autophagy plays an important role in trophoblast function under physiologically low oxygen conditions in normal pregnancy. Autophagy plays an important role in EVT invasion and vascular remodeling under a physiologically low oxygen concentration. Autophagy impaired by sENG may be involved in poor placentation due to impaired EVT invasion and poor vascular remodeling, systemic inflammation by activating inflammasome and induction of ER stress, and inadequate tolerance by reducing the pool of Treg cells in preeclampsia.

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