intervillous space.[7] EVT invasion in humans is also much deeper than that in other mammalian species. Trophoblasts can invade into the maternal decidua under harsh conditions, such as low oxygen (2–5% O_2) and low glucose concentrations (1 mM) until 11 weeks of gestation (**Fig. S1a**).[8-11] As trophoblast invasion of the uterus proceeds, the hypoxia inducible factor (HIF) system plays a critical role in EVT functions.[7] After 12 weeks, endovascular EVTs invade the uterine spiral arteries, replace the endothelial cells, and participate in the degradation of tunica media smooth muscle cells. This remodeling of the spiral arteries is essential for proper placental perfusion to sustain fetal growth (**Fig. S1b**). Preeclampsia is considered to be caused by poor placentation, and a two-step process has been proposed.[2] The first preclinical stage, hypoplastic placentation, can be explained by the shallow invasion by trophoblast until 20 weeks of gestation. This impairment is closely related with a failure of vascular remodeling and decreased circulation in the early placenta, resulting in continuous placental ischemia after 20 weeks of gestation. The initial events leading to these changes remain little understood. At the second stage, a growing body of evidence is accumulating to show that releasing and genetic factors, soluble fms-related tyrosine kinase 1 (sFLT1), soluble endoglin (sENG), angiotensin II receptor, type 1 receptor agonistic autoantibody (AGTR1-AA) and catechol-O-methyltransferase (COMT) among others, are responsible for maternal endothelial dysfunction, causing the maternal signs of hypertension and proteinuria.[12-17]

Autophagy is a process for degrading cellular components under stress to maintain cellular homeostasis. In addition to its well-understood physiological role in recycling intracellular materials under starvation, there is growing evidence for the participation of autophagy in other cellular processes including cellular differentiation, tissue remodeling, growth control, adaptation to adverse environments and cellular immunity.[18-21] Additionally, autophagy is essential for preimplantation development beyond the four- and eight-cell stages and blastocyst survival in mammals.[22,23] Apart from the placental energy supply, autophagy helps neonates with the production of amino acids by autophagic degradation of 'self ' proteins.[24] Some papers reported the expression of autophagy-related proteins in placentas, there is, however, no direct evidence that autophagy is involved in human placentation.[25,26]

We now report that autophagy was essential for EVT functions, invasion and vascular remodeling, under physiological hypoxia, which is necessary for the first stage of placentation, in vitro and in vivo. Furthermore, we first report that sENG, an exacerbation factor in the second stage of preeclampsia, is a physiologically active substance that inhibited autophagy in EVTs under hypoxia, resulting in a disruption of EVT functions. Additionally, the expression of SQSTM1, a hallmark of autophagic inhibition, was observed in EVTs in preeclampsia cases. These results suggested that autophagy may be essential in elucidating the pathophysiology of preeclampsia.

Results

Detection of autophagy induced in primary extravillous trophoblasts (EVTs) or EVT cell lines under hypoxia. To evaluate autophagy in EVTs, microtubule-associated protein 1 light chain 3 β (MAP1LC3B) staining was performed under hypoxia or normoxia. MAP1LC3B puncta were observed in the cytoplasm of primary EVT cells under hypoxia (2% O_2; physiological O_2 concentration in early pregnancy period); on the other hand, few MAP1LC3B puncta were observed in EVT under normoxia (**Fig. 1a and b**). MAP1LC3B puncta were not seen in EVTs in early placental tissues at a shallower site from the fetus at the implantation site (**Fig. 1f**), but were clearly seen at a deeper site from the fetus side (**Fig. 1g**) and around the spiral arteries (**Fig. 1h**, arrows) in the implantation site.

No such cells were seen at the villous trophoblast region (**Fig. 1h**, arrowheads). To confirm the occurrence of autophagy in the deeper invading EVT cells, double immunohistochemical staining was performed. MAP1LC3B puncta (**Fig. 1j**) were present in cytokeratin 7 (KRT7)-positive EVT cells (**Fig. 1k**) in the maternal decidua basalis at a deeper site from the fetus side at the implantation site (**Fig. 1l**, arrows). An enlarged image of KRT7-positive EVT cells with MAP1LC3B puncta is shown in **Figure 1m**.

An electron microscopic analysis showed the accumulation of autophagosomes 0.5–1.5 μm in size, which are double-membrane structures containing undigested cytoplasmic materials including organelles (**Fig. 1n and o**), in HTR8/SVneo cells under hypoxia for 4 h. The number of MAP1LC3B puncta was significantly increased under hypoxia as well as starvation in HTR8/SVneo cells at 24 h, compared with that under normoxia (**Fig. 2A**, $p = 0.005$). The proportion of autophagic cells was increased over 50% from 4–24 h under hypoxia, and then gradually decreased (**Fig. 2B**). In the western blot analysis, the amount of MAP1LC3B-II was increased under hypoxia, which induced HIF1A expression, and the band of MAP1LC3B-II was further augmented in the presence of E64d and pepstatin, protease inhibitors, under hypoxia (**Fig. 2C**). Additionally, SQSTM1 expression, a biomarker for autophagy flux, was decreased in HTR8/SVneo cells under hypoxia, suggesting the activation of autophagy. Flow cytometry showed that the total amount of MAP1LC3B decreased in a time-dependent manner under hypoxia (**Fig. 2D**). These results directly showed the enhancement of autophagy in EVT cells under hypoxia.

The role of autophagy in EVT cell functions under hypoxia. 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 autophagic degradation and lipidation of MAP1LC3B paralogs (see *Materials and Methods*).[27] As shown in **Figure 3A** and **Figure S2a**, no MAP1LC3B puncta were observed in the HTR8-$ATG4B^{C74A}$ cells and HchEpC1b-$ATG4B^{C74A}$ cells under hypoxia. Western blot analysis also showed a complete MAP1LC3B-II deficiency in the HTR8-$ATG4B^{C74A}$ cells (**Fig. 3B**). The expression of SQSTM1, a protein selectively degraded by autophagy, in HTR8-mStrawberry, a control cell line that expressed only monomeric red fluorescent protein, showed a large decline under hypoxia, whereas no clear decline in SQSTM1 expression in HTR8-$ATG4B^{C74A}$ cells was observed (**Fig. 3B**). Enzyme-linked immunosorbent assays showed a significant decrease of SQSTM1

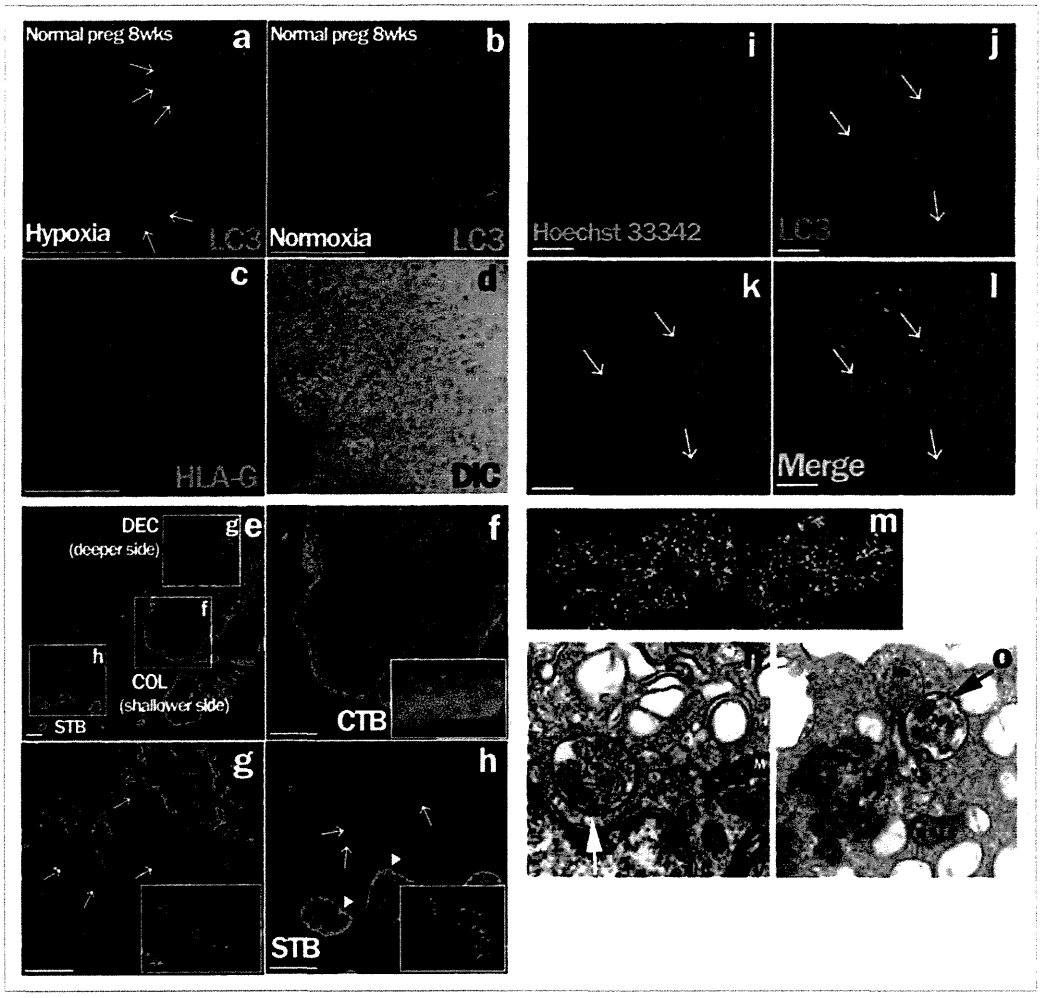

Figure 1. Detection of autophagy in EVTs from human subjects in early pregnancy. (**a–d**) Early chorionic and decidual tissues were obtained from patients who had undergone elective termination at 8 weeks of gestation, and villous explant cultures were performed on Matrigel under hypoxia, 2% oxygen tension (**a**), and normoxia, 20% oxygen tension (**b**). HLA-G, one of the markers for EVTs, was expressed on primary cultured trophoblasts in (**c**). DIC is shown in (**d**). Arrows indicate MAP1LC3B (LC3) dot formation, a marker of autophagic activation. (**e–l**) MAP1LC3B staining in normal pregnant subjects at 8 weeks of gestation (**e**). MAP1LC3B dot formation (**g and h**, arrows) was detected in the maternal decidua basalis, the implantation site, at a deeper site from the fetus side in square DEC (**g**), but not at a shallower site from the fetus in the implantation site (**f**) or in the villous trophoblast region (**h**, arrowheads). (**f–h**) Enlarged images of the white squares. (**i–l**) Double immunohistochemical staining for cytokeratin 7 (KRT7), an EVT marker, (**k**) and MAP1LC3B (**j**) revealed that cells with punctated MAP1LC3B were KRT7-positive EVTs (**l**). (**m**) Enlarged image of the KRT7-positive EVTs with MAP1LC3B puncta in (**l**). Hoechst33342 staining, nuclei staining, is shown in (**i**). Scale bar: 50 μm. STB, syncytiotrophoblasts; CTB, cytotrophoblasts; COL, cell column; DEC, decidua basalis. Electron microscopic images of autophagic structures in HTR8/SVneo cells under hypoxia (**n and o**). HTR8/SVneo cells, which were cultured under hypoxia for 4 h, were fixed and observed by electron microscopy (×20,000). The smooth endoplasmic reticulum, some destroyed organelles and autophagosomes (Ap) were present (**n**, arrow). The autophagosome (Ap) had come into contact with multiple vesicle bodies (Mb) (**o**, arrow). AL, autolysosome; M, mitochondria.

expression under hypoxia in the HTR8-mStrawberry cells, but not in the HTR8-ATG4B^{C74A} cells (**Fig. 3C**). Flow cytometric analysis showed that the total amount of cytoplasmic MAP1LC3B did not decrease under hypoxia in the HchEpC1b-ATG4B^{C74A} cells (**Fig. S2b**). In regard to cell growth and cell death, there were no differences between HchEpC1b-mStrawberry cells and HchEpC1b-ATG4B^{C74A} cells (**Fig. S2C and S2D**).

EVT invades the maternal decidua under 2% O_2 for intact placentation before 12 weeks of gestation. To clarify the role of autophagy in trophoblast invasion, conventional invasion assays were performed under 2% O_2, mimicking the physiological hypoxia in the early stages of pregnancy before 12 weeks of gestation (**Fig. S1a**). Under hypoxia the number of invading HchEpC1b-ATG4B^{C74A} cells, an autophagy-deficient EVT cell line, was significantly decreased ($p = 0.0001$), compared with that of HchEpC1b-mStrawberry-expressing cells (**Fig. 3D**). Similar results were obtained in HTR8-ATG4B^{C74A} cells under hypoxia (**Fig. S2f**). In three-dimensional culture assays, the depth of HchEpC1b-ATG4B^{C74A} cells was significantly shallower than that of HchEpC1b-mStrawberry cells under normoxia (**Fig. 3E**, $p = 0.001$). In the next stage, endovascular EVTs invade the uterine spiral arteries and replace spiral artery endothelial cells after 12 weeks of gestation. Tube formation assays with EVT cells and human umbilical vascular endothelial cells (HUVECs),

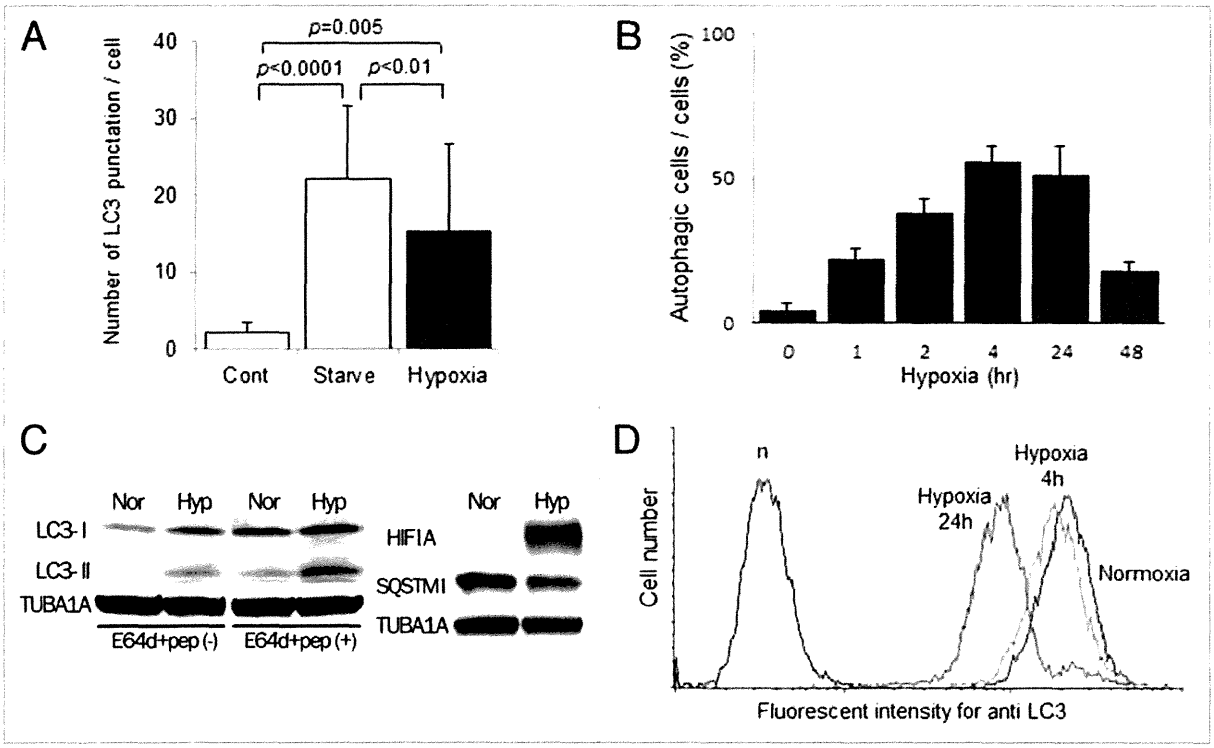

Figure 2. Activation of autophagy in EVT cells under hypoxia. (**A**) The number of MAP1LC3B (LC3) puncta in HTR8/SVneo cells, infected with the GFP-MAP1LC3B adenovirus vector, in the presence of E64d and pepstatin under 20% (Cont), starvation (Starved) or 2% oxygen tension (hypoxia) for 24 h. (**B**) The proportion of autophagic cells, identified as cells containing more than 5 MAP1LC3B puncta, among HTR8/SVneo cells in the presence of E64d and pepstatin under 2% oxygen tension for 48 h. (**C**) Western blots in HTR8/SVneo cells under normoxia and hypoxia (2% oxygen tension) are shown as follows: MAP1LC3B (LC3), HIF1A, SQSTM1 and TUBA1A. Expression of MAP1LC3B-II was examined in the presence or absence of E64d and pepstatin under normoxia and hypoxia (2% oxygen tension). Nor, normoxia; Hyp, hypoxia. (**D**) Intracellular MAP1LC3B levels determined by flow cytometry. The amount of MAP1LC3B was estimated at 4 and 24 h under 2% oxygen tension (hypoxia) in HTR8/SVneo cells.

an in vitro model of vascular remodeling by EVT cells after 12 weeks of gestation, were performed under 8% oxygen tension (**Fig. S1b**).[2,28,29] This level of oxygen tension is thought to match the placental conditions after 12 weeks of gestation, the so-called "second wave," in which vascular remodeling takes place.[11] Both HTR8-mStrawberry cells and HTR8-ATG4B^{C74A} cells formed a tube structure with HUVECs at 12 h (**Fig. 4f and l**), but they did not form a tube structure without HUVECs (**Fig. 4m and n**). The tube structure was predominantly formed by HUVECs (green) in both settings at 6 h (**Fig. 4b and h**), and there was no significant difference in the area occupied by HTR8 cells (red) in the total tube area formed by dual cells at 6 h (**Fig. 4p**). In the culture with HTR8-mStrawberry cells and HUVECs, the tubes were mostly occupied by HTR8-mStrawberry cells (red) at 12 h or later (**Fig. 4d, f and p**, blue line); meanwhile, the tubes were occupied by HUVECs (green) when HTR8-ATG4B^{C74A} cells were cocultured with HUVECs (**Fig. 4j, l and p**, red line). The proportion of HTR8 (red) in the total tube area was significantly higher in HTR8-mStrawberry cells than in HTR8-ATG4B^{C74A} cells (**Fig. 4p**, blue and red lines), suggesting that replacement of endothelial cells by EVT cells required autophagy. These results indicated that autophagy plays an important role in the endovascular interaction between EVT and endothelial cells.

Soluble endoglin inhibits hypoxia-induced autophagy in EVT cells. Soluble endoglin (sENG) is known to cooperate with sFLT1 in the induction of severe preeclampsia in humans as well as a rat model.[16,30] We examined whether preeclampsia-related substances such as sENG, sFLT1, transforming growth factor, β 1

Figure 3 (See opposite page). Confirmation of autophagy-deficient cells. (**A**) Representative panels show mStrawberry (red), anti-MAP1LC3B (LC3) (green) and merged images (lower panels) in HTR8-ATG4B^{C74A} (ATG4B^{C74A}: left panels), an autophagy-deficient EVT cell line, and HTR8-mStrawberry cells (mStrawberry: right panels) under hypoxia for 24 h. (**B**) Western blots in HTR8-ATG4B^{C74A} and -mStrawberry cells under normoxia and hypoxia (2% oxygen tension) are shown as follows: MAP1LC3B (LC3), SQSTM1, HIF1A and TUBA1A. (**C**) SQSTM1 expression in HTR8-ATG4B^{C74A} and -mStrawberry cells under normoxia (white bars) and hypoxia (black bars) estimated by ELISA. Results were normalized per milligram of protein from cell lysate. (**D**) An in vitro invasion assay model before 12 weeks of gestation. Hypoxia (2% oxygen tension) was similar to the placental pO_2 level before 12 weeks of gestation. Invasion assays were performed with HchEpC1b-ATG4B^{C74A} or -mStrawberry cells under normoxia (gray bars) or hypoxia (black bars) for 48 h. The *Y*-axis indicates the number of invading cells. Data were normalized to 1 for normoxia at 24 h. (**E**) Three-dimensional invasion assays were performed with HchEpC1b-ATG4B^{C74A} or -mStrawberry cells under normoxia (gray bars) and hypoxia (black bars) for 48 h. Photographs show the invading cells (mStrawberry) at the incision face of the gel, in which cells were seeded. The *Y*-axis indicates the depth of invasion from the surface of the gel. Scale bar: 300 μm.

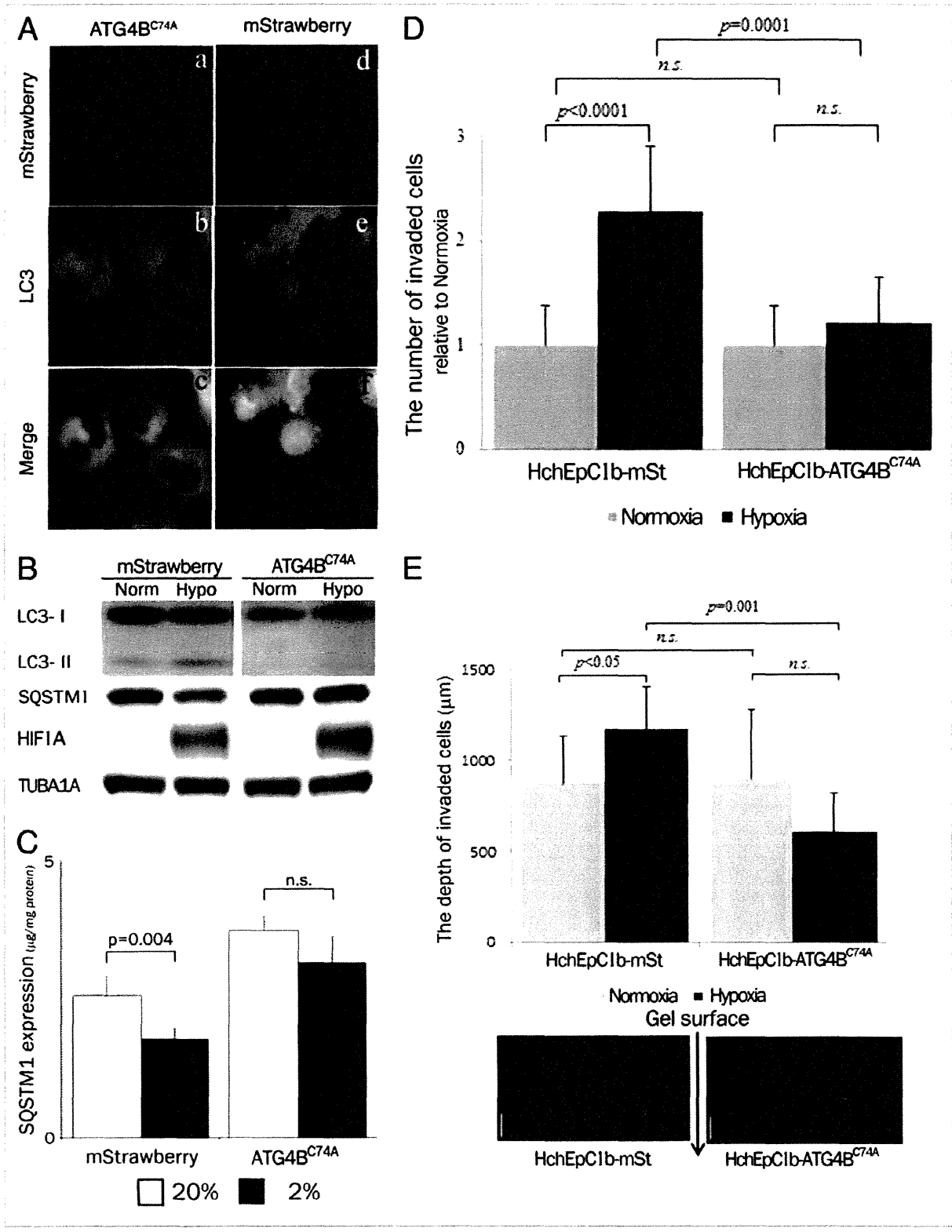

Figure 3. For figure legend, see page 4.

(TGFB1) and tumor necrosis factor-α (TNF) affect autophagy in EVT under hypoxia (2% O_2) (**Fig. 5A**). Only sENG significantly inhibited the number of MAP1LC3B puncta in HchEpC1b-mStrawberry cells, the control cell line, under hypoxia at 24 h in the presence of E64d and pepstatin (**Fig. 5A**, p = 0.007) or even in the absence of E64d and pepstatin (**Fig. 5B**, p < 0.0001). sENG repressed the MAP1LC3B-II conversion under hypoxia in western blotting (**Fig. 5C**), and flow cytometric analysis showed

that hypoxia-induced MAP1LC3B degradation was diminished by sENG (**Fig. 5D**). These results indicated that sENG inhibited the induction of autophagy in EVTs under hypoxia.

Disruption of EVT by soluble endoglin. To assess the effects of sENG-induced inhibition of autophagy on EVT functions, invasion and vascular remodeling assays were performed. sENG significantly decreased the number of invading HchEpC1b-mStrawberry cells, a control cell line, but not HchEpC1b-ATG4B^{C74A} cells, an autophagy-deficient EVT cell line, under hypoxia (2% O_2) at 48 h (**Fig. 6A**, $p = 0.0006$). On the other hand, the number of HchEpC1b-mStrawberry cells under normoxia was not affected by sENG (**Fig. S3a**). The inhibition of HchEpC1b-mStrawberry cells by 100 ng/ml sENG under hypoxia was recovered by TGFB1 in a dose-dependent manner and recovered to the control level under hypoxia by an additional 20 ng/ml of TGFB1 (**Fig. 6B**). In regard to vascular remodeling, a dose of more than 250 ng/ml of sENG significantly inhibited the formation of tubes compared with that in the control under 8% O_2 tension, but there were no significant differences in the total tube area formed by HchEpC1b-mStrawberry cells with HUVECs in the presence of 125 ng/ml sENG (**Fig. 6C**; **Fig. S3c**). HTR8-mStrawberry cells (red) replaced HUVECs (green) in the absence of sENG at 12 h (**Fig. 6De and Di**, blue solid line). On the other hand, sENG (100 ng/ml) impaired the replacement of HUVECs (green) by HTR8-mStrawberry cells (red) at 12 h (**Fig. 6Df and Di**, blue dotted line); however, 100 ng/ml sENG did not affect the proportion of HTR8-ATG4B^{C74A} cells (red) in the total tube area at 12 h (**Fig. 6Dg, Dh and Dj**, red solid and dotted lines), suggesting that replacement of endothelial cells by EVT cells (a model for vascular remodeling) was impaired by a low dose (100 ng/ml) of sENG in the HTR8-mStrawberry cells. These results suggested that sENG inhibited the EVT functions, such as invasion and tube formation, through inhibition of autophagy.

Accumulation of SQSTM1 in EVTs in preeclampsia. Finally, to evaluate the autophagy in EVTs of preeclamptic cases in vivo, the expression of SQSTM1 was assessed as a marker of autophagy inhibition in placental bed biopsy samples. It has been reported that hypoxic activation of autophagy induces clearance of the SQSTM1 protein in carcinoma cells.[31] Our results showed that SQSTM1 expression was significantly decreased under hypoxia in HTR8-mStrawberry or HchEpC1b-mStrawberry cells, control cell lines, although it was not significantly decreased under hypoxia in HTR8-ATG4B^{C74A} or HchEpC1b-ATG4B^{C74A} cells, autophagy-deficient EVT cell lines (**Fig. 3B and C**; **Fig. S2e**). SQSTM1 expression in interstitial EVT cells was not found in normal pregnancy (**Fig. 7b**), but was markedly found in preeclampsia (**Fig. 7d**). Similar results were obtained in endovascular EVT cells (**Fig. 7 f and h**). Little expression of SQSTM1 was, however, seen in villous trophoblasts in preeclampsia (**Fig. 7j**). Additionally, the distribution of SQSTM1 was similar to that of KRT7, which indicates EVTs, in the serial sections of preeclampsia placental bed biopsy samples. The ratio of SQSTM1 to KRT7 expression was significantly higher in preeclampsia than in normal pregnancy in both interstitial and endovascular EVTs (**Fig. 7K and L**, $p < 0.0001$, $p = 0.0008$). Patients with placenta previa, who all delivered preterm, did not show significantly high expression of SQSTM1 in EVTs (**Fig. 7K and L**, triangle marks). On the other hand, there were no significant differences in SQSTM1 expression in villous trophoblasts between the preeclampsia and normal pregnancy samples (**Fig. 7M**). These results suggested that disruption of autophagy occurred in EVTs, but not villous trophoblasts, in preeclampsia.

Discussion

We first showed the enhancement of autophagy even under physiological hypoxia in EVTs, suggesting that autophagy has physiological functions during early placentation in vivo. As shown in **Figure S1**, the oxygen concentrations, at which EVT functions, were known to be different before and after 12 weeks of gestation. As for EVT functions, autophagy was an indispensable mechanism to promote EVT-invasion as well as crosstalk between EVTs and the endothelium at the feto-maternal interface under physiological hypoxia during early pregnancy. On the other hand, many reports have shown that a disruption of EVT functions contributes to the pathophysiology of preeclampsia during early pregnancy.[2,6,28,29] Our results showed that the failure of EVT invasion and vascular remodeling by EVTs were induced by autophagic inhibition. Paradoxically, there might be a correlation between the inhibition of autophagy and onset of preeclampsia. Therefore, we hypothesized that impairment of autophagy may be involved in poor placentation. Maternal endothelial dysfunction induced by sENG in concert with sFLT1 is recognized as one of the major causes of preeclampsia.[16,30] Thus, this study first showed that sENG inhibited the autophagy in EVTs under hypoxia, resulting in a failure of vascular remodeling by EVTs as well as failure of EVT invasion. Our data also showed the accumulation of SQSTM1 in EVTs in preeclampsia cases, suggesting that autophagy in EVTs is impaired in preeclampsia. Our results are the first to show the importance of autophagy to the pathophysiology of preeclampsia.

In regard to the pathophysiology of preeclampsia, a placental hypoxic model in rats clearly showed that placental hypoxia itself led to increased plasma and placental expression of sENG, resulting in decreased fetal weight and increased blood pressure in dams.[32] This animal model mimicked the two stages of preeclampsia, hypoplastic placentation and endothelial dysfunction. The release of sENG under 2% O_2 tension was also increased in primary human trophoblasts obtained from placentas of preeclampsia patients, compared with those from normal pregnant women.[33] Thus, hypoxia initiates the induction of preeclampsia and then augments sENG production from syncytiotrophoblasts or endothelial cells in the placenta. Our results suggested that inhibition of autophagy in EVTs, which induces shallow trophoblast invasion, could cause preeclampsia, by emphasizing hypoxia at the feto-maternal interface.

Recent data show that mRNAs for *sENG* and *TGFB1* were already elevated in chorionic villous samples at 11 weeks of gestation from women who later developed preeclampsia.[34] The sENG concentration in sera was already elevated at 17–20 weeks of gestation before the onset of early-onset preeclampsia.[30,35] The sENG

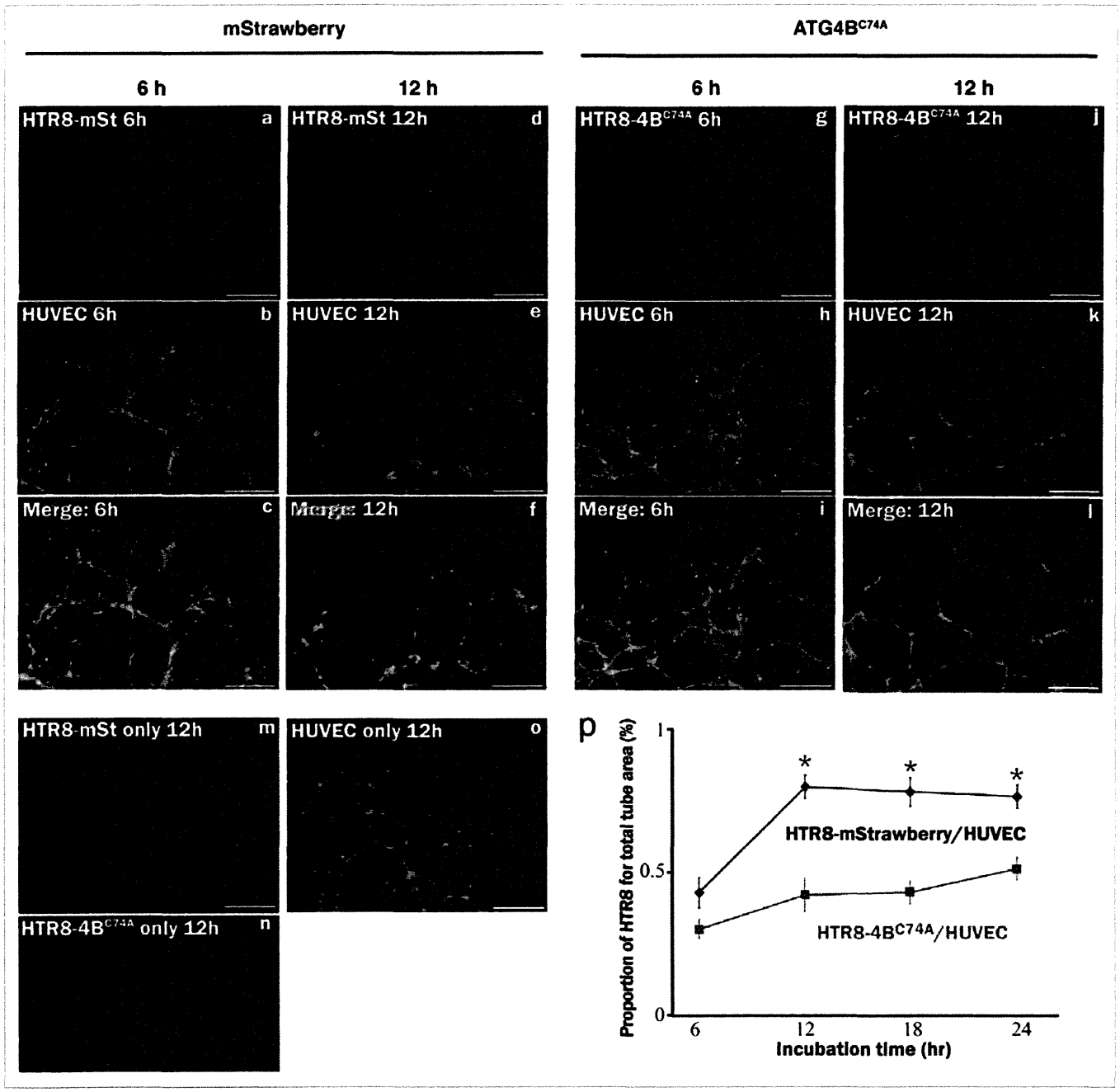

Figure 4. Tube formation assay in the HUVEC and EVT coculture system. The tube formation assay was used as an in vitro model of vascular remodeling after 12 weeks of gestation. Hypoxia (8% oxygen tension) was similar to the placental pO_2 level after 12 weeks of gestation. Tube formation assays with human umbilical vein endothelial cells (HUVECs, **b, e, h, k**, labeled with green) with HTR8-ATG4B^{C74A} (**g and j**, labeled with red), an autophagy-deficient EVT cell line, or HTR8-mStrawberry cells (**a and d**, labeled with red) were performed under 8% oxygen tension for 6 or 12 h. Representative merged images are shown from cultures for 6 h (**c and i**) or 12 h (**f and l**). Representative figures in the lower panels show single cell cultures, HTR8-mStrawberry cells (**m**), HTR8-ATG4B^{C74A} cells (**n**) or HUVECs (**o**). (**p**) The graph shows the HTR8 (red) area as a proportion of the total area (red and green). *$p < 0.001$: the proportion in HTR8-mStrawberry and HUVECs (blue line) was significantly higher than that in HTR8-ATG4B^{C74A} and HUVECs (red line). Data are shown as the mean ± S. E. for three independent experiments. Scale bar: 300 μm.

concentration in the intervillous space could be higher than that in maternal sera; therefore, vascular remodeling by EVTs might be disrupted by sENG in the first or second trimester of pregnancy, resulting in shallow trophoblast invasion and inadequate vascular remodeling in preeclampsia. Mano et al. report that membrane-bound ENG negatively regulates the invasion of normal HTR8/SVneo cells under normoxia, but sENG does not affect invasion in HTR8/SVneo cells under normoxia.[36] In our study, sENG diminished EVT functions by inhibiting autophagy in hypoxia, but not normoxia. Additionally, the suppressive effect by sENG on EVT invasion under hypoxia was removed by the addition of TGFB1 (**Fig. 6b**). Hypoxia also enhances the release

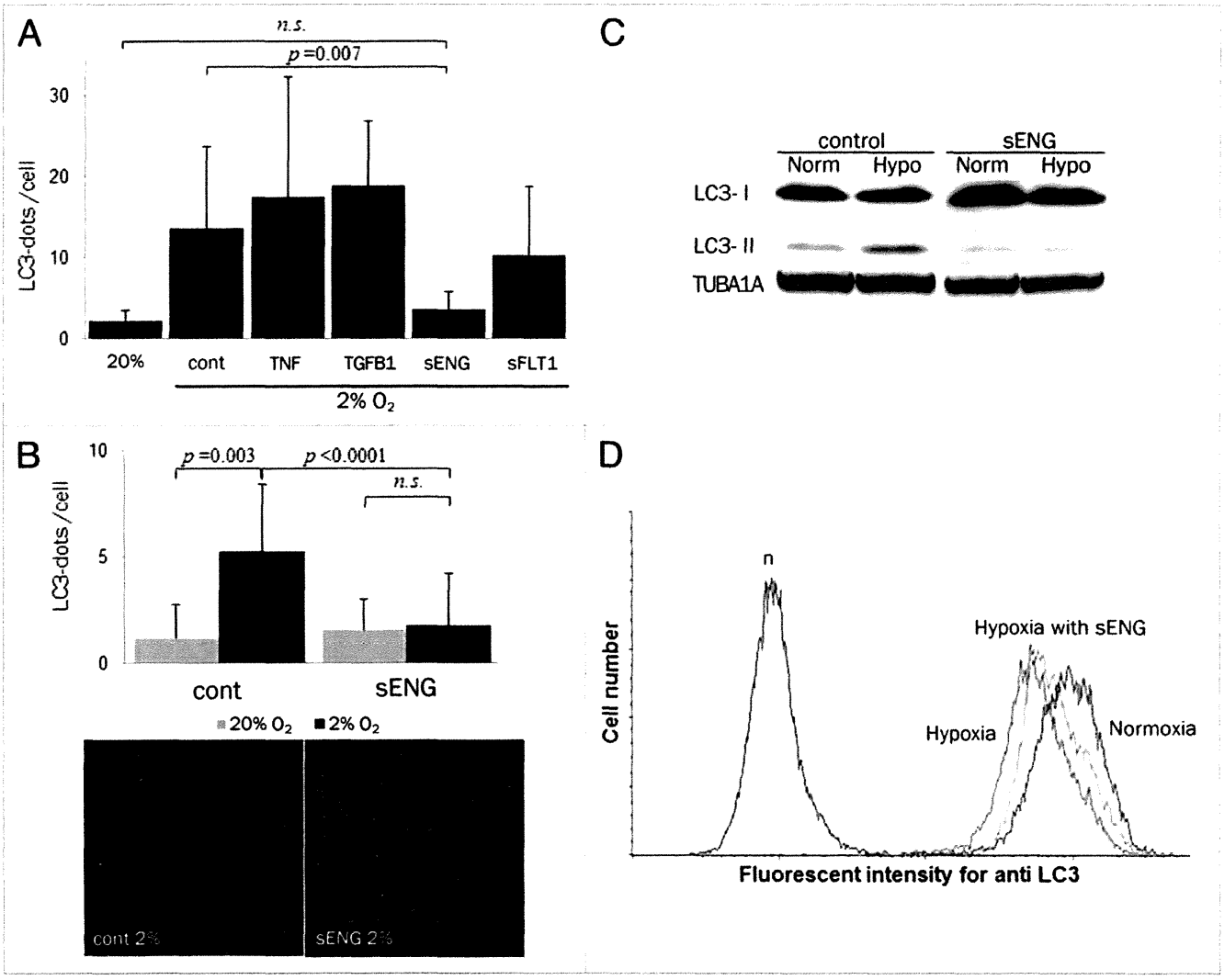

Figure 5. sENG inhibited autophagy in EVT cells under hypoxia. (**A**) The numbers of MAP1LC3B (LC3) puncta in HchEpC1b-mStrawberry cells in the presence of 5 ng/ml TNF, 5 ng/ml TGFB1, 100 ng/ml sENG or 5 μg/ml sFLT1 with E64d and pepstatin under 2% oxygen tension for 24 h. (**B**) The numbers of MAP1LC3B puncta in HchEpC1b-mStrawberry cells in the presence of 100 ng/ml sENG without E64d and pepstatin under 20% or 2% oxygen tension for 24 h. Representative figures show MAP1LC3B staining in HchEpC1b-mStrawberry cells in the presence or absence of 100 ng/ml sENG under 2% oxygen tension. (**C**) Western blot analysis showed that MAP1LC3B-II expression was not enhanced in hypoxic conditions in the presence of 100 ng/ml sENG. (**D**) The amount of MAP1LC3B in HchEpC1b-mStrawberry cells under 20% oxygen tension (normoxia), 2% oxygen tension (hypoxia) or 2% oxygen tension in the presence of 100 ng/ml sENG (hypoxia with sENG). n, negative control. Data are represented as means ± S.E. n.s., not significant. Statistical significance was calculated using the Mann-Whitney *U* test.

of sENG in syncytiotrophoblasts.[33] If autophagy, which is indispensable for EVT invasion under hypoxia in the first trimester, is impaired in EVTs, hypoxia in the placenta caused by shallow invasion might enhance sENG production in the second trimester, resulting in insufficient vascular remodeling.

Not only EVT invasion but also vascular formation between EVTs and HUVECs was impaired by a disruption of autophagy in this study. High concentrations of sENG (250 and 500 ng/ml) inhibited the tube formation by HUVECs and HchEpC1b-mStrawberry cells (**Fig. 6C**; **Fig. S3c**); on the other hand, a low concentration of sENG, 100 ng/ml, which is close to the serum level in preeclampsia patients, suppressed the vascular remodeling by EVT cells like autophagy-deficient cells. Although this is indirect evidence, inadequate vascular remodeling of EVTs might be, in part, related to the disruption of autophagy in EVTs during the second stage of preeclampsia.

Impaired selective turnover of SQSTM1 by autophagy corresponds to the pathophysiological conditions seen in human hepatocellular carcinoma.[37] A recent study shows that a failure of autophagy enhances oxidative stress, accompanied by SQSTM1 accumulation, in a mouse kidney cell line under metabolic stress, low glucose and 1% O_2 tension.[38] Mice lacking ATG16L1 in hematopoietic cells, which accumulate SQSTM1, are also highly susceptible to endotoxin-induced inflammatory immune responses, resulting in Crohn disease like colitis.[39] In our study, a decline of SQSTM1 expression under hypoxia was observed in autophagy-normal EVT cells but not in autophagy-deficient EVT cells (**Fig. 3B and C**; **Fig. S2e**).

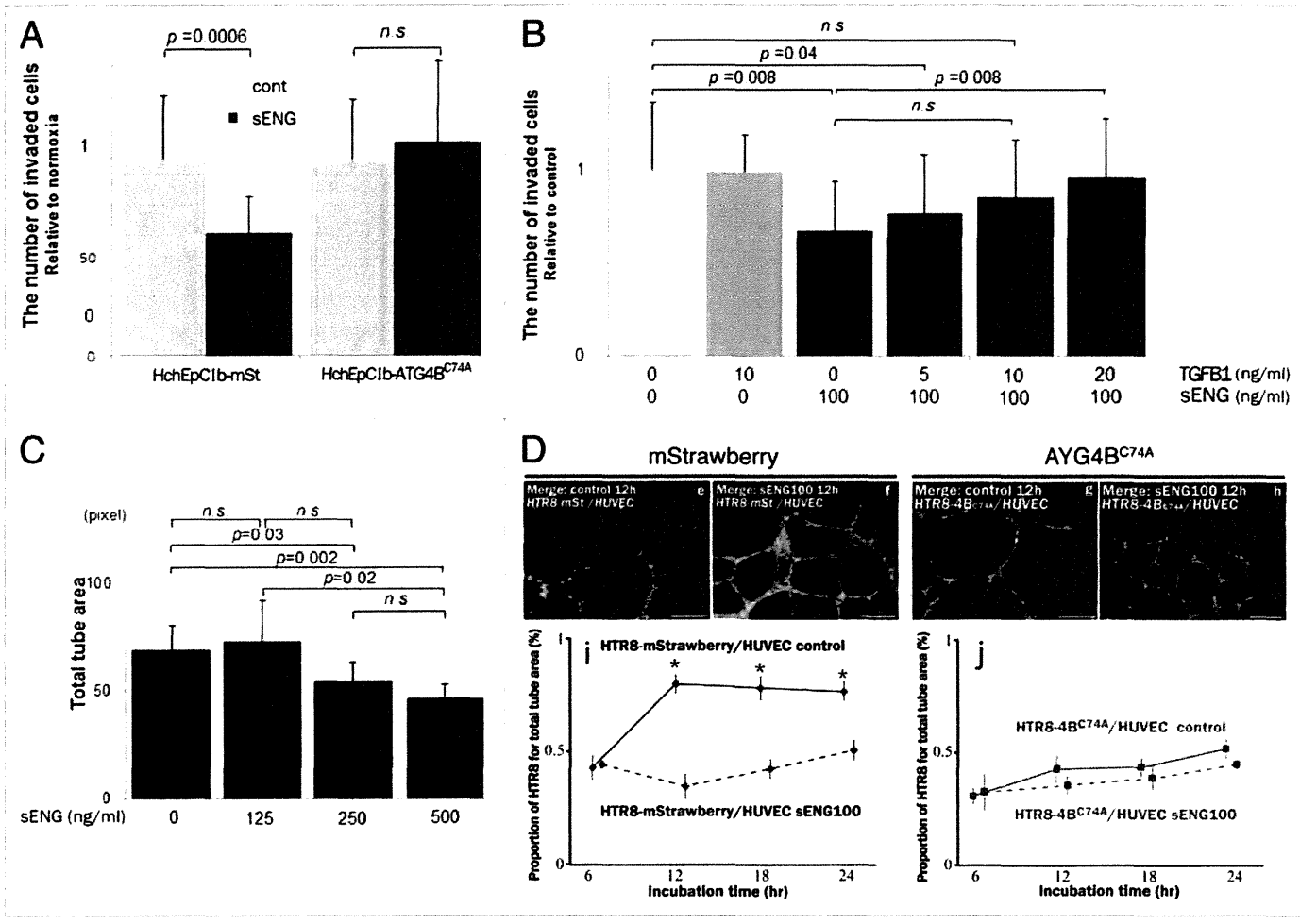

Figure 6. sENG inhibited EVT invasion and vascular remodeling. (**A**) Invasion assays were performed with HchEpC1b-ATG4B^{C74A}, an autophagy-deficient EVT cell line, or HchEpC1b-mStrawberry cells in the presence or absence of 100 ng/ml sENG under 2% oxygen tension for 48 h. The Y-axis indicates the number of invading cells. Data were normalized to 1 for the control at 48 h. (**B**) HchEpC1b-mStrawberry cells were treated with 100 ng/ml sENG and several doses of TGFB1 under 2% oxygen tension for 48 h. Data were normalized to 1 for the control at 48 h. (**C**) Tube formation assays by HUVECs with HchEpC1b-mStrawberry cells were performed under 8% oxygen tension for 24 h in the presence of 0, 125, 250 or 500 ng/ml sENG. The quantification of total tube area formed with dual cell lines was evaluated at 24 h. (**D**) Representative figures of tube formation by HUVECs (green) with HTR8-ATG4B^{C74A} (red) or HTR8-mStrawberry cells (red) in the presence (**f and h**) or absence (**e and g**) of 100 ng/ml sENG under 8% oxygen tension for 12 h. (**i and j**) The graphs show the HTR8 (red) area as a proportion of the total area in the HTR8-mStrawberry cells and HUVECs (**i**) or HTR8-ATG4B^{C74A} cells and HUVECs (**j**). *p < 0.001: the proportion in HTR8-mStrawberry and HUVECs control (blue solid line) is significantly higher than that in HTR8-mStrawberry and HUVECs with 100 ng/ml sENG (blue dotted line). There was no significant difference between the HTR8-ATG4B^{C74A} and HUVECs control (red solid line) and the HTR8-ATG4B^{C74A} and HUVECs with 100 ng/ml sENG (red dotted line). Data are shown as the mean ± S.E. for three independent experiments. Scale bar: 300 μm. n.s., not significant.

Marked SQSTM1 expression in EVTs in placental bed biopsies in preeclampsia was found in vivo, suggesting the disruption of autophagy by sENG in EVT cells in preeclamptic cases. Preeclampsia is an inflammatory disease in pregnant women, and the level of TNF is increased in the plasma of preeclampsia women.[40-42] EVT cells which express SQSTM1 at high levels in preeclampsia women might be susceptible to inflammatory cytokines.

In regard to the correlation between autophagy and placenta, a few papers reported the expression of autophagy-related proteins in placentas following vaginal delivery and caesarean section, or normal placenta and preeclampsia.[25,26] Oh et al. showed that the expression of *MAP1LC3B* mRNA was significantly increased in placentas from patients with preeclampsia, compared with normal pregnancies. Immunostaining for MAP1LC3B in placental villi was significantly increased in preeclamptic placentas, suggesting that autophagy is activated in syncytiotrophoblasts.[25] Additionally, Hung T et al. reported that autophagy is activated in the villous trophoblasts in preeclampsia with intrauterine growth restriction (PE+IUGR) or IUGR placentas, compared with that in normal human pregnancy.[43] In our study, we did not detect the accumulation of SQSTM1, a marker of autophagic inhibition, in placental villi in either preeclamptic placentas or normal placentas, suggesting that autophagy was not inhibited in syncytiotrophoblasts. Taken together, autophagy was activated in syncytiotrophoblasts, and inhibited in EVTs in preeclamptic

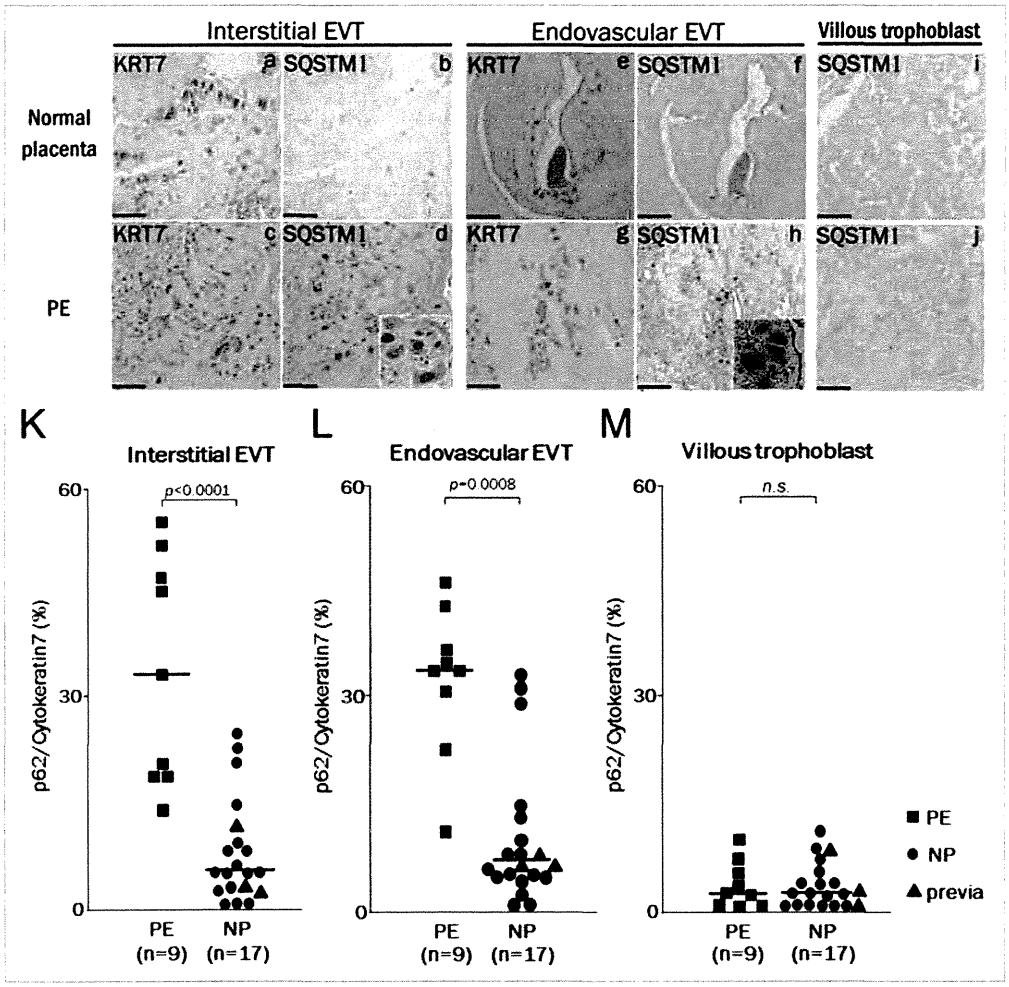

Figure 7. The expression of SQSTM1 in EVTs in preeclamptic placentas. Representative panels show the expression of cytokeratin 7 (KRT7) and SQSTM1 in placental bed biopsies in normal placenta (NP) and preeclamptic placenta (PE) by immunohistochemistry. Four panels on the left show the interstitial EVTs (**a–d**), four panels in the center show the endovascular EVT (**e–h**) and two panels on the right show the villous region (**I and j**). (**d and h**) Enlarged images of the SQSTM1-positive cells. KRT7 staining, indicating EVTs, was performed in (**a, c, e and g**). SQSTM1 staining, a marker of autophagic inhibition, was performed in (**b, d, f, and h–j**). KRT7-positive cells in the myometrium around the vessels were counted as endovascular EVTs, and those in the myometrium without vessels, as interstitial EVTs. (**K and L**) The graphs show ratios of SQSTM1-positive cells to KRT7-positive cells in the interstitial EVTs (**K**), in the endovascular EVTs (**L**) and in the villous trophoblast (**M**) between preeclampsia (PE, n = 9) and normal pregnancy (NP, n = 17). Dots indicate the ratios of individuals, and bars indicate the median values. Square, PE; Circles, NP and triangles, placenta previa. Scale bar: 50 μm.

placentas. Preeclamptic placentas are well known to suffer from severe hypoxia. The differences in SQSTM1 expression between EVTs and syncytiotrophoblasts in preeclamptic placentas may indicate a difference of susceptibility to hypoxia dependent on autophagic activation. In other words, these findings suggested that hypoxia induced-autophagy activation maintains cellular homeostasis in syncytiotrophoblasts in preeclamptic placentas, and the disruption of autophagy in EVTs might contribute to hypoplastic placentation.

As for trophoblast invasion, there are differences between mouse and human placentation. Human EVTs invade into the uterine myometrium up to one third of its depth. In mice, the invasion is shallower. Additionally, *ATG5*$^{-/-}$ mice, an autophagy-deficient model, are born with minimal abnormalities at birth and do not show IUGR.[24] Furthermore, Kumasawa et al. reported that the physiological excessive expression of human sFLT1 in placenta induced symptoms of preeclampsia in pregnant mice.[13] But mouse sFLT1 in placenta did not induce preeclampsia (personal communication). There might be quite a difference in function between humans and mice in regard to anti-angiogenic factors as well as EVT-invasion. As autophagy is well conserved from yeast to humans, it may be developmentally involved in the pathophysiology of preeclampsia, a feature of human pregnancy.

In conclusion, we showed for the first time that autophagy played an important role in trophoblast functions under physiologically hypoxic conditions, suggesting a contribution of autophagy to normal placentation. On the other hand, the activation of autophagy in EVTs under hypoxia was inhibited by sENG, and autophagic inhibition in EVTs was observed in preeclampsia cases. Therefore, identification of the disruption of EVT due to inhibition of autophagy by sENG will break new ground for studying the pathophysiology of preeclampsia.

Materials and Methods

Reagents. Recombinant human sENG (R&D, 1097-EN), human sFLT1 (R&D, 321-FL/CF), human TNF (R&D, 210-TA/CF) and human TGFB1 (R&D, 240-B) were purchased from R&D Systems. The following antibodies (Ab) were used: rabbit polyclonal Ab for MAP1LC3B (MBL, PM036), rabbit polyclonal Ab for MAP1LC3B (ABGENT, AP1802a), mouse monoclonal Ab for SQSTM1 (MBL, 5F2), mouse monoclonal Ab for HIF1A (BD PharMingen, H72320), mouse monoclonal Ab for TUBA1A (Sigma-Aldrich, T8203), mouse monoclonal Ab for KRT7 (NOVUS Biological, NB120-17069) and mouse monoclonal Ab for HLA-G (Abcam, Ab7759). Protease inhibitors, E64d (Peptide institute, 4321-v) and pepstatin A (Peptide institute, 4397), were purchased from Peptide Institute, Inc.

Subjects. All clinical studies were approved by the University of Toyama Ethics Committee, and informed consent was obtained from all patients. Clinical samples were obtained in early or late pregnancy. Normal early placental tissues in the early pregnant period were obtained from patients (four cases, median maternal age 27 y, range 23–32 y; median gestational age 8 weeks, range 7–10 weeks) who had undergone elective termination by vaginal curettage. The termination was done legally at the patient's wish. The women had received no medication, or had infectious, autoimmune, or other systemic diseases. Karyotype was not analyzed in the abortive specimens. These specimens were treated as normal early pregnant specimens. All placental bed biopsy samples, late placental tissue samples, were obtained when caesarean section was performed. Preeclampsia was defined by the American College of Obstetricians and Gynecologists criteria. Clinical information is described in **Table S1**. The normal pregnancy group (NP) contained healthy pregnant women who received a caesarean section due to breech presentation or previous caesarean section. Additionally, three patients with placenta previa, which were all preterm deliveries, are shown in **Figure 7K–M**. They are clearly shown in the normal pregnancy groups in **Figure 7**.

Construction of an autophagy-deficient cell line. To establish autophagy-deficient HTR8/SVneo cells or autophagy-deficient HchEpC1b cells, we used pMRX-IRES-puro-mStrawberry-ATG4B^{C74A}, an ATG4B^{C74A} mutant expression vector that inhibits MAP1LC3B-II formation, and pMRX-IRES-puro-mStrawberry, a control vector encoding monomeric red fluorescent protein. The procedures for constructing the vectors were reported previously.[44] After infection with these vectors, the cells were incubated in DMEM (Sigma, D5671) supplemented with 10% FBS (Cell culture technologies, CC3008-504) containing 0.3 μg/ml puromycin (Sigma, P8833). Growing cells were collected, and the expression of mStrawberry was confirmed by fluorescence microscopy. The constructed autophagy-deficient cell lines and the control vector-infected cell lines were named HTR-ATG4B^{C74A} cells and HchEpC1b-ATG4B^{C74A} cells and HTR8-mStrawberry cells and HchEpC1b-mStrawberry cells, respectively.

Cell culture. EVT cell lines, HTR8/SVneo (a gift from Dr. Charles H. Graham, Department of Anatomy and Cell Biology, Queen's University) and HchEpC1b, were used in this study. HchEpC1b is an HPV E6 and hTERT-transfected immortalized EVT cell line.[45] HTR8/SVneo cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, 15140) at 37°C in a 5% CO_2 atmosphere, and HchEpC1b cells with RPMI1640 medium (Sigma, R8758) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. HUVECs (Lonza, CC-2517) were cultured in EGM-2 Bullet Kit (Lonza, CC-3162) according to the manufacturer's instructions. For hypoxic cultures, the cells were plated on a 35-mm dish at 2×10^5 cells/dish. After 24 h, the cells were transferred to an incubator under a 2% O_2 and 5% CO_2 atmosphere at 37°C. For starved cultures, the culture medium was exchanged for Hanks' solution (Nacalai Tesque Inc., 17460).

Small pieces of tissue (2–3 mm) from the placental villi, which were obtained from 1st trimester placental tissues, were dissected (usually 8–16 villus tips per sample). For each explant, 5 μg/cm^2 of Fibronectin^{TM} (BD Biosciences, 356008) was placed in the center of an 8-well culture slide (Thermo Fisher Scientific, Inc., 177402). After the formation of gels (30 min at 37°C), the dissected tissue pieces were carefully placed at the center of each chamber, covered with 200 μl of medium, and incubated for 24 h at 37°C with 5% CO_2 and 20% O_2. After an initial period of 24 h, villi with good attachment and outgrowth on the gel were selected for the hypoxic culture. Before the experiment, the outgrown cells were characterized by immunocytochemical analysis for KRT7 and HLA-G to confirm that they were EVTs.

Quantitative analysis of GFP-LC3 puncta. For the quantitative analysis of MAP1LC3B, the cells were stained with MAP1LC3B or transfected with a green fluorescent protein-microtubule-associated protein 1 light chain 3 protein (GFP-MAP1LC3B) adenovirus vector (5×10^9 pfu/ml) and incubated for 24 h. The cells were pretreated with lysosomal protease inhibitors, E64d (10 ng/ml) and pepstatin A (10 ng/ml), for 2 h to distinguish cytoplasmic MAP1LC3B puncta, and then fixed with 4% paraformaldehyde-PBS.[46] The incidence of autophagy was estimated by quantifying the number of MAP1LC3B puncta within MAP1LC3B-stained cells by manual counting in five independent visual fields using a fluorescence microscope (KEYENCE, BZ-8000) or a confocal microscope (Carl Zeiss, LSM700). Cells containing more than 5 GFP-MAP1LC3B punta in cytoplasm were counted as autophagic. These experiments were independently performed at least three times.

Invasion assays and three-dimensional invasion assay. A conventional invasion assay was performed using a BD BioCoat Growth Factor Reduced Matrigel Invasion Chamber (BD Biosciences, 354483) according to the manufacturer's instructions. Cells were plated in the upper insert at 5×10^4 /well and incubated under normoxia (20% O_2) or hypoxia (2% O_2). These cells were incubated for 48 h, and then the upper surface of the membrane in each insert was gently scrubbed with a cotton swab to remove all of the noninvading cells, and the cells on the under surface of the membranes were fixed in 100% methanol (Wako Pure Chemical Industries, Ltd., 131-01826), stained with 0.05%

Toluidine Blue Solution (Wako, 206-14555), and then the number of invading cells was counted by microscopy.

The three-dimensional invasion assay was performed as described previously.[47] Collagen matrix, Cellmatrix® (Kurabo Industries, Ltd., KP-2020), was prepared according to the manufacturer's instructions. Cells at 2×10^5 /ml were then placed on the top of the matrix and incubated under normoxia (20% O_2) or hypoxia (2% O_2) for 48 h. The collagen matrix was fixed with 4% paraformaldehyde (Wako, 163-20145) and cut for observation. The invasion distance was measured by fluorescence microscopy.

Tube formation assay using an EVT cell and endothelial cell coculture system. This assay was from a previous report.[48] This three-dimensional dual cell culture model is suitable for evaluating vascular remodeling by trophoblasts. Briefly, EVT cells (1×10^5 /ml) or HUVECs (1×10^5 /ml), labeled with cell tracker green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes, C-2925) or cell tracker red CMTMR [5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine, Molecular Probes, C-2927], respectively, were cocultured on thick Matrigel (BD, 356234)-coated plates in the presence or absence of sENG. The cells were then incubated in an 8% O_2 and 5% CO_2 atmosphere at 37°C for 24 h. The images were imported as TIFF files into the National Institutes of Health Image J software (http://rsbweb.nih.gov/ij/). The total area of the tube-like structures per image was quantified using Image J. The proportion, HTR8/HUVECs, was calculated by dividing the area of HTR8 by the area of HUVECs. The averages of these factors were calculated from the data for five independent visual fields. These experiments were independently performed at least three times. HUVECs, for which passages were performed 3 times, were used in this assay.

Immunohistochemistry. Immunohistochemistry was performed as described previously.[49] The sections were incubated overnight at 4°C, with the primary antibodies, anti-KRT7 (1:250) and anti-MAP1LC3B (1:100; ABGENT), diluted with 5% BSA/DW. After extensive washing with TBS-Tween solution, the sections were stained with the secondary antibodies, Alexa Fluor 594 donkey anti-mouse IgG (1:2000; Molecular Probes, A-21203) and Alexa Fluor 488 donkey anti-rabbit IgG (1:2000; Molecular Probes, A-21206), and with Hoechst33342 (1:500; Molecular Probes, H-3570) for nuclear staining overnight at 4°C and then observed by confocal microscopy. Confocal images of fluorescent materials were collected using a confocal microscope (Carl Zeiss).

Immunocytochemistry. Primary EVT cells, HTR8/SVneo or HchEpC1b cells, were fixed in 4% paraformaldehyde/PBS for 15 min and labeled with the primary antibody, anti-MAP1LC3B (MBL), at a dilution of 1:500. Negative controls were produced by replacing the primary antibody with normal rabbit serum and then were secondarily stained with Alexa Fluor 488 donkey anti-rabbit IgG. They were finally stained with Hoechst33342 for 10 min, washed with PBS and observed under a fluorescence microscope or a confocal microscope.

Electron microscopy. Electron microscopy was performed as described previously.[50] Cells were fixed with 0.1 M phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C for 1 h. The cells were washed with the same buffer three times and post-fixed with 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer for 1 h. After washing, cells were incubated with a 3% uranyl solution, further dehydrated with a graded series of ethanol and propylene oxide solutions, and embedded. Areas containing cells were block-mounted and cut into 50-nm sections. The sections were stained with uranyl acetate and lead citrate and observed under an electron microscope.

Flow cytometry. Cells were harvested, washed once and then fixed in 4% paraformaldehyde-PBS for 15 min at room temperature. After being permeabilized with 100 μg/ml digitonin (Sigma-Aldrich, D141) for 15min at room temperature, the cells were resuspended in PBS solution containing anti-MAP1LC3B antibody (1:200, MBL) for 30 min at room temperature. Subsequently, the cells were stained with FITC goat anti-rabbit IgG (BD PharMingen, 554020) and incubated for 30 min at room temperature. Samples were then analyzed with a FACS Calibur flow cytometer (BD Biosciences) using the Cell Quest software as described previously (BD Biosciences).[51] Negative controls were performed by replacing the primary antibody with normal rabbit serum. To estimate the proportion of dead cells, the harvested cells were suspended in PBS solution containing 5 μg/ml propidium iodide (Molecular Probes, P3566), and incubated for 30 min at room temperature. Samples were then analyzed with a FACS Calibur flow cytometer using the Cell Quest software (BD Bioscience).

Cell proliferation assay. To estimate cell proliferation, we used two methods: the first was manual counting and the second involved use of the cell proliferation reagent WST-1 (Roche, 5015944) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA). SQSTM1 expression was measured with a p62 ELISA kit (Enzo Life Sciences, ADI-900-212-0001) according to the manufacturer's instructions. The cells were cultured under 2% or 20% oxygen tension for 24 h. The cells were lysed with Radio-Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors. The range of the standard curve was 625–40,000 pg/ml. For each assay, all samples were assayed in duplicate in the same plate. Results were normalized per milligram of protein from cell lysate.

Statistical analysis. Results are presented as the mean ± S.E. and comparisons between multiple groups were made using ANOVA. Values of $p < 0.05$ were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank all the staff at the Department of Obstetrics, University of Toyama, for help with patient identification and recruitment. We thank Dr. E. Morita for providing advice and M. Suzuki, Y. Shimizu and A. Ushijima for technical assistance with some assays. This research was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan [Grant-in-Aid for Scientific Research (B)-23390386 and Grant-in-Aid for Young Scientists (B)-23791817].

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/22927

References

1. Duley L. The global impact of pre-eclampsia and eclampsia. Semin Perinatol 2009; 33:130-7; PMID:19464502; http://dx.doi.org/10.1053/j.semperi.2009.02.010
2. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R. Pre-eclampsia. Lancet 2010; 376:631-44; PMID:20598363; http://dx.doi.org/10.1016/S0140-6736(10)60279-6
3. World Health Organization. Policy and Coordination Comittee 16th meeting. Department of Reproductive Health and Research. Summary Medium-term Programme of Work 2004-2009 HRP/PCC(16)/2003/8.1
4. Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. Lancet 2001; 357:2002-6; PMID:11438131; http://dx.doi.org/10.1016/S0140-6736(00)05112-6
5. Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, et al. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? J Clin Invest 1997; 99:2139-51; PMID:9151786; http://dx.doi.org/10.1172/JCI119387
6. Huppertz B. The feto-maternal interface: setting the stage for potential immune interactions. Semin Immunopathol 2007; 29:83-94; PMID:17621696; http://dx.doi.org/10.1007/s00281-007-0070-7
7. Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, et al. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. J Clin Invest 2004; 114:744-54; PMID:15372095
8. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. Science 1997; 277:1669-72; PMID:9287221; http://dx.doi.org/10.1126/science.277.5332.1669
9. Jauniaux E, Hempstock J, Teng C, Battaglia FC, Burton GJ. Polyol concentrations in the fluid compartments of the human conceptus during the first trimester of pregnancy: maintenance of redox potential in a low oxygen environment. J Clin Endocrinol Metab 2005; 90:1171-5; PMID:15562012; http://dx.doi.org/10.1210/jc.2004-1513
10. Jauniaux E, Watson A, Burton G. Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation. Am J Obstet Gynecol 2001; 184:998-1003; PMID:11303211; http://dx.doi.org/10.1067/mob.2001.111935
11. Tuuli MG, Longtine MS, Nelson DM. Review: Oxygen and trophoblast biology--a source of controversy. Placenta 2011; 32(Suppl 2):S109-18; PMID:21216006; http://dx.doi.org/10.1016/j.placenta.2010.12.013
12. Kanasaki K, Palmsten K, Sugimoto H, Ahmad S, Hamano Y, Xie L, et al. Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. Nature 2008; 453:1117-21; PMID:18469803; http://dx.doi.org/10.1038/nature06951
13. Kumasawa K, Ikawa M, Kidoya H, Hasuwa H, Saito-Fujita T, Morioka Y, et al. Pravastatin induces placental growth factor (PGF) and ameliorates pre-eclampsia in a mouse model. Proc Natl Acad Sci U S A 2011; 108:1451-5; PMID:21187414; http://dx.doi.org/10.1073/pnas.1011293108
14. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004; 350:672-83; PMID:14764923; http://dx.doi.org/10.1056/NEJMoa031884
15. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003; 111:649-58; PMID:12618519
16. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med 2006; 12:642-9; PMID:16751767; http://dx.doi.org/10.1038/nm1429
17. Zhou CC, Zhang Y, Irani RA, Zhang H, Mi T, Popek EJ, et al. Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice. Nat Med 2008; 14:855-62; PMID:18660815; http://dx.doi.org/10.1038/nm.1856
18. Cuervo AM. Autophagy: in sickness and in health. Trends Cell Biol 2004; 14:70-7; PMID:15102438; http://dx.doi.org/10.1016/j.tcb.2003.12.002
19. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 2004; 6:463-77; PMID:15068787; http://dx.doi.org/10.1016/S1534-5807(04)00099-1
20. Mizushima N. Autophagy: process and function. Genes Dev 2007; 21:2861-73; PMID:18006683; http://dx.doi.org/10.1101/gad.1599207
21. Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. Biochem Biophys Res Commun 2004; 313:453-8; PMID:14684184; http://dx.doi.org/10.1016/j.bbrc.2003.07.023
22. Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N. Autophagy is essential for preimplantation development of mouse embryos. Science 2008; 321:117-20; PMID:18599786; http://dx.doi.org/10.1126/science.1154822
23. Lee JE, Oh HA, Song H, Jun JH, Roh CR, Xie H, et al. Autophagy regulates embryonic survival during delayed implantation. Endocrinology 2011; 152:2067-75; PMID:21363932; http://dx.doi.org/10.1210/en.2010-1456
24. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. Nature 2004; 432:1032-6; PMID:15525940; http://dx.doi.org/10.1038/nature03029
25. Oh SY, Choi SJ, Kim KH, Cho EY, Kim JH, Roh CR. Autophagy-related proteins, LC3 and Beclin-1, in placentas from pregnancies complicated by preeclampsia. Reprod Sci 2008; 15:912-20; PMID:19050324; http://dx.doi.org/10.1177/1933719108319159
26. Signorelli P, Avagliano L, Virgili E, Gagliostro V, Doi P, Braidotti P, et al. Autophagy in term normal human placentas. Placenta 2011; 32:482-5; PMID:21459442; http://dx.doi.org/10.1016/j.placenta.2011.03.005
27. Fujita N, Noda T, Yoshimori T. Atg4B(C74A) hampers autophagosome closure: a useful protein for inhibiting autophagy. Autophagy 2009; 5:88-9; PMID:19104152; http://dx.doi.org/10.4161/auto.5.1.7183
28. Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol 2000; 157:2111-22; PMID:11106583; http://dx.doi.org/10.1016/S0002-9440(10)64849-3
29. Kalkunte S, Boij R, Norris W, Friedman J, Lai Z, Kurtis J, et al. Sera from preeclampsia patients elicit symptoms of human disease in mice and provide a basis for an in vitro predictive assay. Am J Pathol 2010; 177:2387-98; PMID:20889559; http://dx.doi.org/10.2353/ajpath.2010.100475
30. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, et al.; CPEP Study Group. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006; 355:992-1005; PMID:16957146; http://dx.doi.org/10.1056/NEJMoa055352
31. Pursiheimo JP, Rantanen K, Heikkinen PT, Johansen T, Jaakkola PM. Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. Oncogene 2009; 28:334-44; PMID:18931699; http://dx.doi.org/10.1038/onc.2008.392
32. Gilbert JS, Gilbert SA, Arany M, Granger JP. Hypertension produced by placental ischemia in pregnant rats is associated with increased soluble endoglin expression. Hypertension 2009; 53:399-403; PMID:19075097; http://dx.doi.org/10.1161/HYPERTENSIONAHA.108.123513
33. Gu Y, Lewis DF, Wang Y. Placental productions and expressions of soluble endoglin, soluble fms-like tyrosine kinase receptor-1, and placental growth factor in normal and preeclamptic pregnancies. J Clin Endocrinol Metab 2008; 93:260-6; PMID:17956952; http://dx.doi.org/10.1210/jc.2007-1550
34. Farina A, Sekizawa A, De Sanctis P, Purwosunu Y, Okai T, Cha DH, et al. Gene expression in chorionic villous samples at 11 weeks' gestation from women destined to develop preeclampsia. Prenat Diagn 2008; 28:956-61; PMID:18792924; http://dx.doi.org/10.1002/pd.2109
35. Noori M, Donald AE, Angelakopoulou A, Hingorani AD, Williams DJ. Prospective study of placental angiogenic factors and maternal vascular function before and after preeclampsia and gestational hypertension. Circulation 2010; 122:478-87; PMID:20644016; http://dx.doi.org/10.1161/CIRCULATIONAHA.109.895458
36. Mano Y, Kotani T, Shibata K, Matsumura H, Tsuda H, Sumigama S, et al. The loss of endoglin promotes the invasion of extravillous trophoblasts. Endocrinology 2011; 152:4386-94; PMID:21914777; http://dx.doi.org/10.1210/en.2011-1088
37. Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O, et al. Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. J Cell Biol 2011; 193:275-84; PMID:21482715; http://dx.doi.org/10.1083/jcb.201102031
38. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, et al. Autophagy suppresses tumorigenesis through elimination of p62. Cell 2009; 137:1062-75; PMID:19524509; http://dx.doi.org/10.1016/j.cell.2009.03.048
39. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 2008; 456:264-8; PMID:18849965; http://dx.doi.org/10.1038/nature07383
40. Conrad KP, Miles TM, Benyo DF. Circulating levels of immunoreactive cytokines in women with preeclampsia. Am J Reprod Immunol 1998; 40:102-11; PMID:9764352; http://dx.doi.org/10.1111/j.1600-0897.1998.tb00398.x
41. Vince GS, Starkey PM, Austgulen R, Kwiatkowski D, Redman CW. Interleukin-6, tumour necrosis factor and soluble tumour necrosis factor receptors in women with pre-eclampsia. Br J Obstet Gynaecol 1995; 102:20-5; PMID:7833306; http://dx.doi.org/10.1111/j.1471-0528.1995.tb09020.x

42. Visser W, Beckmann I, Bremer HA, Lim HL, Wallenburg HC. Bioactive tumour necrosis factor alpha in pre-eclamptic patients with and without the HELLP syndrome. Br J Obstet Gynaecol 1994; 101:1081-2; PMID:7826964; http://dx.doi.org/10.1111/j.1471-0528.1994.tb13587.x
43. Hung TH, Chen SF, Lo LM, Li MJ, Yeh YL, Hsieh TT. Increased autophagy in placentas of intrauterine growth-restricted pregnancies. PLoS One 2012; 7:e40957; PMID:22815878; http://dx.doi.org/10.1371/journal.pone.0040957
44. Fujita N, Hayashi-Nishino M, Fukumoto H, Omori H, Yamamoto A, Noda T, et al. An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. Mol Biol Cell 2008; 19:4651-9; PMID:18768752; http://dx.doi.org/10.1091/mbc.E08-03-0312
45. Omi H, Okamoto A, Nikaido T, Urashima M, Kawaguchi R, Umehara N, et al. Establishment of an immortalized human extravillous trophoblast cell line by retroviral infection of E6/E7/hTERT and its transcriptional profile during hypoxia and reoxygenation. Int J Mol Med 2009; 23:229-36; PMID:19148547
46. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 2005; 1:84-91; PMID:16874052; http://dx.doi.org/10.4161/auto.1.2.1697
47. Bayless KJ, Kwak HI, Su SC. Investigating endothelial invasion and sprouting behavior in three-dimensional collagen matrices. Nat Protoc 2009; 4:1888-98; PMID:20010936; http://dx.doi.org/10.1038/nprot.2009.221
48. Kalkunte S, Lai Z, Tewari N, Chichester C, Romero R, Padbury J, et al. In vitro and in vivo evidence for lack of endovascular remodeling by third trimester trophoblasts. Placenta 2008; 29:871-8; PMID:18775564; http://dx.doi.org/10.1016/j.placenta.2008.07.009
49. Nakashima A, Shiozaki A, Myojo S, Ito M, Tatematsu M, Sakai M, et al. Granulysin produced by uterine natural killer cells induces apoptosis of extravillous trophoblasts in spontaneous abortion. Am J Pathol 2008; 173:653-64; PMID:18688023; http://dx.doi.org/10.2353/ajpath.2008.071169
50. Yoshimori T, Yamagata F, Yamamoto A, Mizushima N, Kabeya Y, Nara A, et al. The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. Mol Biol Cell 2000; 11:747-63; PMID:10679028.
51. Nakashima A, Ito M, Yoneda S, Shiozaki A, Hidaka T, Saito S. Circulating and decidual Th17 cell levels in healthy pregnancy. Am J Reprod Immunol 2010; 63:104-9; PMID:20015328; http://dx.doi.org/10.1111/j.1600-0897.2009.00771.x.

REVIEW ARTICLE

Which Types of Regulatory T cells Play Important Roles in Implantation and Pregnancy Maintenance?

Shigeru Saito, Tomoko Shima, Kumiko Inada, Akitoshi Nakashima

Department of Obstetrics and Gynecology, University of Toyama, Toyama, Japan

Keywords
Extrathymic Treg, heterogeneity of Treg, memory Treg, miscarriage, tolerance

Correspondence
Shigeru Saito, Department of Obstetrics and Gynecology, University of Toyama, 2630 Sugitani, Toyama-shi 930-0194, Japan.
E-mail: s30saito@med.u-toyama.ac.jp

Submission January 22, 2013;
accepted January 25, 2013.

Citation
Saito S, Shima T, Inada K, Nakashima A. Which types of regulatory T cells play important roles in implantation and pregnancy maintenance?. Am J Reprod Immunol 2013; 69: 340–345

doi:10.1111/aji.12101

Regulatory T cells (Treg) play essential roles in implantation and allogeneic pregnancy maintenance in mice and humans. Recent data have shown the heterogeneity of Treg, such as thymic (naturally occurring) Treg, extrathymic (inducible or peripheral) Treg, naïve Treg, effector Treg, resting (non-proliferating) Treg and activated (proliferating) Treg. Importantly, Foxp3, which was believed to be a specific marker for Treg, is transiently expressed in T cells when conventional T cells are activated and proliferating in humans showing that Foxp3 is not a specific marker for Treg. Therefore, we should evaluate the true Treg level and clarify which types of Treg cells play important roles in implantation and pregnancy maintenance in mice and humans.

Introduction

Regulatory T cells were first reported as $CD4^+$ $CD25^+$T cells[1] (Fig. 1). Nine years later, Aluvihare et al.[2] and Sasaki et al.[3] reported that Treg cells might mediate maternal tolerance to fetuses in mice and humans. Depletion of Treg cells could induce abortion in allogeneic pregnancy, but not in syngeneic pregnancy in mice,[2] and a decreased level of Treg cells in the decidua was observed in miscarriage in humans.[3] In 2005, Zenclussen et al. reported that adoptive transfer of Treg cells purified from normal pregnant mice prevented fetal loss in CBA/J mated with DBA/2J abortion-prone mice.[4] Transfer of Treg cells from non-pregnant CBA/J mice was ineffective, suggesting that pregnancy-specific or paternal antigen-specific Treg cells contribute to the maintenance of pregnancy. In 2007, Sasaki et al. reported that Treg cells were decreased in the peripheral blood and in the decidua of pre-eclamptic patients.[5] In 2008, very important findings were reported by Tilburgs et al.[6] They provided direct evidence for the selective migration of fetal HLA-C-specific Treg cells from the peripheral blood to the decidua in human pregnancy. The data of Zenclussen et al. and Tilburgs et al. show that paternal antigen-specific Treg cells play important roles in the maintenance of allogeneic pregnancy in mouse and human, so we should consider the mechanisms that explain why paternal antigen-specific Treg cells were expanded. Supportive data were also reported later.[7,8] Robertson et al. provided an answer to the above issue. They showed that seminal fluid induced the expansion of the total Treg cell pool and induced tolerance to paternal alloantigens in mice.[9] Schumacher et al. reported that some of the Treg cells expressed hCG/LH receptor on their surface, and hCG produced by trophoblasts attracted Treg cells into the feto-maternal interface in human pregnancy.[10] Interestingly, Treg cells also increase in syngeneic pregnant mice[2] because male antigen (H-Y)-specific Treg cells contribute to tolerance to male fetuses in syngeneic pregnancy.[11]

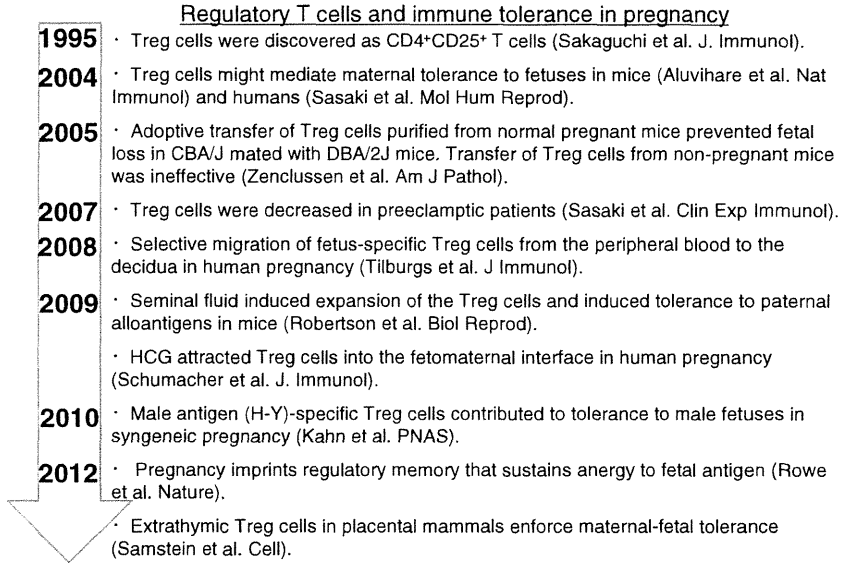

Fig. 1 *Progress of the studies of Treg cells for induction of tolerance during pregnancy.*

When Treg cells were depleted in the implantation period, implantation failure occurred in allogeneic pregnancy, but not in syngeneic pregnancy,[4,12,13] and Treg cell depletion in the early pregnancy period induces fetal resorption in allogeneic pregnancy, but not in syngeneic pregnancy.[13,14] Treatment with a small amount (0.2 mg) of anti-CD25 monoclonal antibody induced implantation failure,[4,12] but a moderate amount (0.5 mg) of anti-CD25 monoclonal antibody was required to induce abortion in allogeneic pregnancy.[13] In our study, when 0.25 mg and 0.5 mg of anti-CD25 monoclonal antibody was injected into C57BL/6 female mice mated with BALB/c male mice, the Treg cell population decreased by 10 and 80%, respectively.[13] These findings suggest that mild or moderate Treg cell depletion or suppression might cause implantation failure, and severe Treg cell depletion or suppression might induce immune-mediated pregnancy loss. However, depletion of Treg cells in the late pregnancy period does not induce fetal resorption, fetal growth restriction, maternal hypertension, and maternal proteinuria,[13] suggesting that Treg cells are essential for the implantation and for the success of allogeneic pregnancy during the early pregnancy period.

In 2012, very important findings were reported. Rowe et al. reported that memory-type fetal antigen-specific Treg cells were present in a second pregnancy, and these Treg cells induced fetal antigen-specific tolerance.[15] These findings explain the epidemiological findings that the first conception and first pregnancy with a new partner and cases when there has been a long interval between births are risk factors for pre-eclampsia.[16]

Treg cells are classified into thymic (naturally occurring) Treg that differentiate in the thymus and extrathymic (inducible or peripheral) Treg cells. Some thymocytes expressing T-cell receptor (TCR) with a heightened reactivity for 'self' antigen up-regulate Foxp3 and differentiate into thymic Treg cells in the thymus (Fig. 2). Another type of Treg cells called extrathymic (inducible or peripheral) Treg cells differentiate into Treg cells in the periphery upon stimulation of naïve $CD4^+$ T cells with high-affinity cognate TCR ligands in the presence of TGFβ and retinoic acid[17] (Fig. 2). Samstein et al. reported that extrathymic (inducible or peripheral) Treg plays an important role in the maintenance of pregnancy.[18]

A Foxp3 enhancer, conserved non-coding sequence 1 (CNS1), is essential for extrathymic Treg cell generation. CNS1-deficient female mice showed increased fetal resorption in allogeneic pregnancy. It was speculated that a CNS1-dependent mechanism of extrathymic differentiation of Treg cells emerged in placental animals to enforce maternal-fetal tolerance.[18] These recent findings suggest that we should reanalyze the populations of Treg in each subtype and find out which subtypes of Treg cells increase during normal pregnancy and decrease in complicated pregnancy.

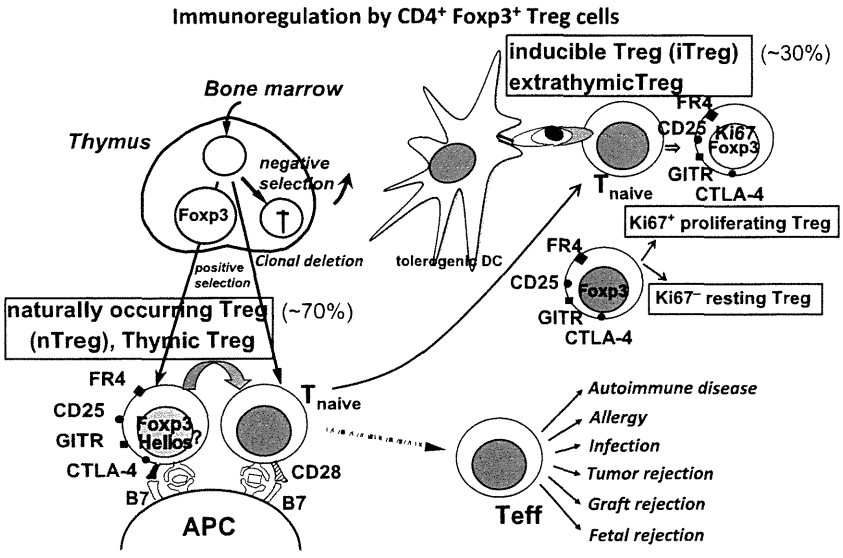

Fig. 2 The differentiation of thymus-derived Treg cells and extrathymic Treg cells. The major population of Treg cells is produced from the thymus and prevents autoimmune disease. However, some population of Treg cells is produced from the periphery in the presence of TGFβ and retinoic acid. Extrathymic Treg cells play an important role in the maintenance of pregnancy.

Naïve Treg cells and Effector Treg cells

In 2003, it was reported that Foxp3 is specifically expressed in Treg cells and is a master gene that induces Treg cells from naïve T cells;[19,20] in addition, mutation of human *FOXP3* was found to be a cause of immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX).[21,22] As a result, Treg cells were subsequently evaluated as $Foxp3^+$ T cells instead $CD4^+$ $CD25^+$ T cells. However, recent data showing that naïve $CD4^+$ T cells transiently express Foxp3 after activation confuse the definition of Treg cells in humans.[23] Miyara et al. reported that $Foxp3^+$ T cells were classified into $Foxp3^{low}$ $CD45RA^+$ $CD4^+$-naïve Treg cells, $Foxp3^{high}$ $CD45RA^-CD4^+$-effector Treg cells, and $Foxp3^{low}$ $CD45RA^+$ $CD4^+$- non-Treg cells (Fig. 3).[24] Naïve Treg cells proliferate and up-regulate Foxp3 and differentiate into effector Treg cells by *in vitro* and *in vivo* stimulation.

In contrast to naïve Treg cells, effector T cells do not proliferate and are susceptible to apoptotic cell death after stimulation.[24] The naïve Treg population was found to be decreased in aged donors and cases of sarcoidosis, but was increased in patients with active SLE.[23] The effector Treg population was found to be increased in aged donors and cases of sarcoidosis, and was decreased in patients with active SLE.[23] Non-proliferating-HLA-DR^+ Treg cells were shown to express a higher level of Foxp3 and to induce more vigorous rapid T-cell suppression than those of HLA-DR^- Treg cells.[25] Our recent data showed that the

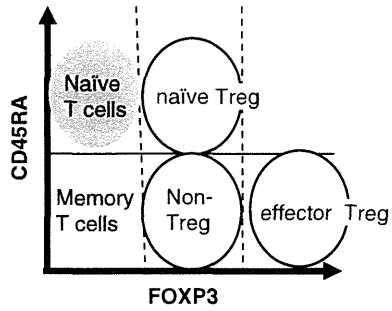

Fig. 3 CD4+Foxp3+ cells are classified into naïve Treg cells, effector Treg cells, and non-Treg cells by the expression of CD45RA and Foxp3. $CD4^+CD45RA^+Foxp3^{low}$ cells are naïve Treg cells, $CD4^+CD45RA^-Foxp3^{high}$ cells are effector Treg cells, and $CD4^+CD45RA^-Foxp3^{low}$ cells are cytokine-producing activated T cells.

population of decidual non-proliferating ($Ki67^-$) $Foxp3^+$ Treg was significantly smaller in cases of miscarriage with a normal embryo than in normal pregnancy, suggesting that non-proliferated Treg cells may play an important role in the induction of immune tolerance, although HLA-DR expression was not evaluated.[26] Recent data showed that HLA-DR^+ $CD45RA^-$-effector Treg cells decreased and HLA DR^- $CD45RA^+$ naïve Treg cells increased in the peripheral blood of healthy pregnant women.[27] In contrast, the naïve Treg cell population was reduced in pre-eclampsia and preterm labor.[27] At present, the population of naïve Treg cells and effector Treg cells in the decidua is unknown. Naïve Treg cells in the periphery might differentiate into memory Treg cells at feto-maternal interface.

Functional Treg Cell Markers for Induction of Maternal Tolerance

Regulatory T cells express cytotoxic T lymphocyte antigen 4 (CLTA4), and mice with CTLA4 conditional knockout in Treg cells showed autoimmune disease.[28] When Treg cells are activated, intracellular CTLA-4 moves to the cell surface. Surface CTLA-4 ligation by the B7 complex triggers a negative signaling pathway; thus, Treg cells regulate the immune system.[29] Decidual $CD4^+$ $CD25^{bright}$ Treg cells express surface CTLA-4 in normal pregnancy, but these levels were found to be decreased in miscarriage cases,[3] suggesting that the functional Treg cell population might be down-regulated in miscarriage.

Kallikourdis et al. reported the accumulation of $CCR5^+$ Treg cells at the feto-maternal interface.[30] In addition, $CCR5^+$ Treg cells functionally regulate the proliferation of maternal lymphocytes, suggesting that $CCR5^+$ Treg plays an important role in the induction of tolerance in murine pregnancy. We have studied the frequency of decidual $CCR5^+Foxp3^+CD4^+$ Treg cells. Unexpectedly, the frequencies of $CCR5^+$ Treg cells did not differ between normal pregnancy subjects, cases of miscarriage with a karyotypically normal embryo and cases of miscarriage with a karyotypically abnormal embryo.[26] Functional Treg marker might differ between mice and humans.

Another candidate for a functional Treg marker during pregnancy is CCR7. Guerin et al. reported that seminal fluid regulates the accumulation of Treg cells in the peri-implantation mouse uterus through CCD19-CCR7-mediated recruitment.[31] To date, the population of human CCR7-expressing Treg cells during pregnancy has not been reported. In human pregnancy, Schumacher et al. reported that human chorionic gonadotropin (hCG) attracted hCG/LH $receptor^+$ Treg cells into the feto-maternal interface.[10] If hCG/LH receptor is a functional marker for the induction of tolerance, hCG/LH R^+ Treg might be a useful tool for evaluating the immune environment in implantation failure and recurrent pregnancy loss.

Thymic (naturally occurring) Treg and Extrathymic (peripheral or inducible) Treg

The majority of Treg cells (~70%) are produced from the normal thymus, and they are capable of recognizing both self and non-self antigen.[30] Some population of Treg cells (~30%) is produced from peripheral T cells by ex vivo stimulation with antigens in the presence of TGFβ and retinoic acid[32] (Fig. 2). A recent study revealed that extrathymic Treg cells induce feto-maternal tolerance, resulting in the maintenance of allogeneic pregnancy.[18] Therefore, specific markers for extrathymic Treg cells are needed to evaluate feto-maternal tolerance during pregnancy. Thornton et al. reported that Helios, an Ikaros transcription factor family member, is a specific marker of the thymic Treg cells in mice and humans.[33] Around 70% of Treg cells expressed Helios, suggesting that a significant percentage of Treg cells is generated extrathymically.[33] Helios is a marker of proliferating Treg.[34,35] Our recent data[26] showed that non-proliferated Treg cells were decreased in the decidua of miscarriage cases with a normal embryo suggesting extra-thymic Treg cell population was decreased in these cases. However, a recent study showed that Helios was not a suitable marker to distinguish thymic Treg cells from extrathymic Treg cells,[34] and Helios is a marker for activation and proliferation,[34,35] or immunosuppressive capacity of Treg.[35] Therefore, we should identify a reliable marker for thymic or extrathymic Treg cells.

TGFβ is a very important cytokine for the differentiation of extrathymic Treg cells. Interestingly, decidual NK cells are the source of TGFβ at feto-maternal interface,[36,37] and TGFβ-producing NK cell population decreases in miscarriage. These findings suggest decidual NK cells play some roles for the differentiation of extrathymic Treg cells.

Conclusion

It is well known that Treg cells play an essential role in implantation and pregnancy maintenance, and extrathymic Treg cells seem to be important. As a next step, we should identify suitable markers for functional Treg cells during pregnancy. These markers might be used for evaluating implantation failure, unsuccess of IVF-ET or immune-mediated pregnancy loss.

References

1 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M: Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; 155:1151–1164.

2 Aluvihare VR, Kallikourdis M, Betz AG: Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol* 2004; 5:266–271.
3 Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S: Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Mol Hum Reprod* 2004; 10:347–353.
4 Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, Kotsch K, Leber J, Volk HD: Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. *Am J Pathol* 2005; 166:811–822.
5 Sasaki Y, Darmochwal-Kolarz D, Suzuki D, Sakai M, Ito M, Shima T, Shiozaki A, Rolinski J, Saito S: Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. *Clin Exp Immunol* 2007; 149:139–145.
6 Tilburgs T, Roelen DL, van der Mast BJ, De Groot-Swings GM, Kleijburg C, Scherjon SA, Claas FH: Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. *J Immunol* 2008; 180:5737–5745.
7 Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-V D Voort-Maarschalk M, Roelen DL, van Rood JJ, Claas FH: Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J Reprod Immunol* 2009; 82:148–57.
8 Yin Y, Han X, Shi Q, Zhao Y, He Y: Adoptive transfer of CD4+CD25+ regulatory T cells for prevention and treatment of spontaneous abortion. *Eur J Obstet Gynecol Reprod Biol* 2012; 161:177–81.
9 Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlström AC, Care AS: Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. *Biol Reprod* 2009; 80:1036–1045.
10 Schumacher A, Brachwitz N, Sohr S, Engeland K, Langwisch S, Dolaptchieva M, Alexander T, Taran A, Malfertheiner SF, Costa SD, Zimmermann G, Nitschke C, Volk HD, Alexander H, Gunzer M, Zenclussen AC: Human chorionic gonadotropin attracts regulatory T cells into the fetal-maternal interface during early human pregnancy. *J Immunol* 2009; 182:5488–5497.
11 Kahn DA, Baltimore D: Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. *Proc Natl Acad Sci USA* 2010; 107:9299–9304.
12 Darrasse-Jèze G, Klatzmann D, Charlotte F, Salomon BL, Cohen JL: CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. *Immunol Lett* 2006; 102:106–109.
13 Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, Saito S: Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. *J Reprod Immunol* 2010; 85:121–129.
14 Rowe JH, Ertelt JM, Aguilera MN, Farrar MA, Way SS: Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens. *Cell Host Microbe* 2011; 10:54–64.
15 Rowe JH, Ertelt JM, Xin L, Way SS: Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature* 2012; 490:102–106.
16 Saito S, Sakai M, Sasaki Y, Nakashima A, Shiozaki A: Inadequate tolerance induction may induce pre-eclampsia. *J Reprod Immunol* 2007; 76:30–39.
17 Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM: Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; 198: 1875–1886.
18 Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY: Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* 2012; 150:29–38.
19 Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; 4:330–336.
20 Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299:1057–1061.
21 Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME: X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001; 27:18–20.
22 Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD: The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001; 27:20–21.
23 Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD, Rudensky AY: Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA* 2006; 103:6659–6664.
24 Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S: Functional delineation and differentiation dynamics of human CD4 + T cells expressing the FoxP3 transcription factor. *Immunity* 2009; 30:899–911.
25 Baecher-Allan C, Wolf E, Hafler DA: MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol* 2006; 176:4622–4631.
26 Inada K, Shima T, Nakashima A, Aoki K, Ito M, Saito S: Characterization of regulatory T cells in decidua of miscarriage cases with abnormal or normal fetal chromosomal content. *J Reprod Immunol* 2013. in press. (http://dx.doi.org/10.1016/j.jri.2012.12.001)
27 Steinborn A, Schmitt E, Kisielewicz A, Rechenberg S, Seissler N, Mahnke K, Schaier M, Zeier M, Sohn C: Pregnancy-associated diseases are characterized by the composition of the systemic regulatory T cell (Treg) pool with distinct subsets of Tregs. *Clin Exp Immunol* 2012; 167:84–98.
28 Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S: CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008; 322:271–275.
29 Saito S, Sasaki Y, Sakai M: CD4(+)CD25high regulatory T cells in human pregnancy. *J Reprod Immunol* 2005; 65:111–120.
30 Kallikourdis M, Andersen KG, Welch KA, Betz AG: Alloantigen-enhanced accumulation of CCR5+ 'effector' regulatory T cells in the gravid uterus. *Proc Natl Acad Sci USA* 2007; 104: 594–599.
31 Guerin LR, Moldenhauer LM, Prins JR, Bromfield JJ, Hayball JD, Robertson SA: Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. *Biol Reprod* 2011; 85:397–408.

32 Sakaguchi S: Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; 22:531–562.
33 Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM: Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 2010; 184:3433–3441.
34 Akimova T, Beier UH, Wang L, Levine MH, Hancock WW: Helios expression is a marker of T cell activation and proliferation. *PLoS ONE* 2011; 6:e24226.
35 Zabransky DJ, Nirschl CJ, Durham NM, Park BV, Ceccato CM, Bruno TC, Tam AJ, Getnet D, Drake CG: Phenotypic and functional properties of Helios+ regulatory T cells. *PLoS ONE* 2012; 7:e34547.
36 Clark DA, Vince G, Flanders KC, Hirte H, Starkey P: CD56+ lymphoid cells in human first trimester pregnancy decidua as a source of novel transforming growth factor-beta 2-related immunosuppressive factors. *Hum Reprod* 1994; 9:2270–2277.
37 Higuma-Myojo S, Sasaki Y, Miyazaki S, Sakai M, Siozaki A, Miwa N, Saito S: Cytokine profile of natural killer cells in early human pregnancy. *Am J Reprod Immunol* 2005; 54:21–29.

NKG2D Blockade Inhibits Poly(I:C)-Triggered Fetal Loss in Wild Type but Not in IL-10$^{-/-}$ Mice

Jessica E. Thaxton,* Tania Nevers,* Eliana O. Lippe,* Sandra M. Blois,† Shigeru Saito,‡ and Surendra Sharma*

Infection and inflammation can disturb immune tolerance at the maternal-fetal interface, resulting in adverse pregnancy outcomes. However, the underlying mechanisms for detrimental immune responses remain ill defined. In this study, we provide evidence for immune programming of fetal loss in response to polyinosinic:polycytidylic acid (polyI:C), a viral mimic and an inducer of inflammatory milieu. IL-10 and uterine NK (uNK) cells expressing the activating receptor NKG2D play a critical role in poly(I:C)-induced fetal demise. In wild type (WT) mice, poly(I:C) treatment induced expansion of NKG2D$^+$ uNK cells and expression of Rae-1 (an NKG2D ligand) on uterine macrophages and led to fetal resorption. In IL-10$^{-/-}$ mice, NKG2D$^-$ T cells instead became the source of fetal resorption during the same gestation period. Interestingly, both uterine NK and T cells produced TNF-α as the key cytotoxic factor contributing to fetal loss. Treatment of WT mice with poly(I:C) resulted in excessive trophoblast migration into the decidua and increased TUNEL-positive signal. IL-10$^{-/-}$ mice supplemented with recombinant IL-10 induced fetal loss through NKG2D$^+$ uNK cells, similar to the response in WT mice. Blockade of NKG2D in poly(I:C)-treated WT mice led to normal pregnancy outcome. Thus, we demonstrate that pregnancy-disrupting inflammatory events mimicked by poly(I:C) are regulated by IL-10 and depend on the effector function of uterine NKG2D$^+$ NK cells in WT mice and NKG2D$^-$ T cells in IL-10 null mice. *The Journal of Immunology*, **2013, 190: 3639–3647.**

Maintenance of pregnancy is acutely prone to infections and inflammatory triggers. Viral and bacterial infections, clinical or subclinical, can disturb immune tolerance at the maternal-fetal interface and predispose to adverse pregnancy outcomes (1–4). However, the mechanisms underlying detrimental immune microenvironment and pregnancy loss remain poorly understood. The immune constitution of the pregnant uterus (decidua) is distinct from other organs, consisting of a large number of innate immune cells such as NK cells and macrophages. Uterine NK (uNK) cells compose 50–60% of the total mononuclear cell population during early to mid-gestation in both humans and mice (5, 6), whereas macrophages make up 20% (7, 8). uNK cells possess alternate phenotype and function when compared with peripheral blood NK cells, because they are empowered with angiogenic characteristics and lack killing activity (9–11). In humans, uNK cells are CD56^{bright}CD16^{dim} and play a distinctive role in placental vasculature and decidualization (12, 13). In mice, distinct populations of uNK cells have been identified that mirror the functions of human uNK cells (14, 15). Like uNK cells, macrophages in the decidua also contribute to the balance required for immune protection of the fetus (7, 8). Not surprisingly, conventional T cells are found in low proportions in human and murine uterine tissue to limit reactivity to paternally derived fetal-antigens (16). Moreover, regulatory T cells (Tregs), present at ~10% of the uterine immune population in early gestation, can further dampen cytotoxic T cells and guide embryo acceptance in the hormonally receptive uterus (17–19).

Inflammatory events including stress, commensal bacterial activation, and excessive local tissue necrosis can also propel an immune-tolerant microenvironment toward induction of a detrimental response (20–22). Although the placenta is a highly potent immune regulator, it is not known yet whether viral infections alone or in combination with other triggers will elicit fetal ablating immune responses. We recently demonstrated that pregnant IL-10$^{-/-}$ mice when challenged with low doses of LPS or CpG, ligands for TLR4 and TLR9, respectively, experienced fetal resorption or preterm birth depending on the gestational age–dependent exposure. These adverse pregnancy outcomes were directly associated with activation and amplification of TNF-α–producing uNK cells or macrophages (7, 23, 24). In these models, IL-10 had a critical role as a vascular and anti-inflammatory cytokine for maintaining pregnancy in response to mild or moderate levels of inflammation (4, 25). However, it is not clear which infectious or inflammatory events will overwhelm the IL-10 proficient uterine milieu and cause pregnancy complications. This scenario warrants further analysis of detrimental immune responses activated by diverse inflammatory triggers (26–28). In the TLR cascade, TLR3 can respond to double-stranded nucleic acids emanating from viral infections or necrotic cells (22). In this regard, the viral mimic polyinosinic:polycytidylic acid (polyI:C) has been shown to induce fetal loss (29, 30). However, what detrimental immune responses are elicited at the maternal-fetal interface by poly(I:C) and whether cytokine milieu affects the nature of the response to this viral mimic are poorly understood.

*Department of Pediatrics, Women and Infants Hospital of Rhode Island–Warren Alpert Medical School of Brown University, Providence, Rhode Island 02905; †Charité Center 12 Internal Medicine and Dermatology, Reproductive Medicine Research Group, Medical University of Berlin, 10117 Berlin, Germany; and ‡Department of Obstetrics and Gynecology, Faculty of Medicine, University of Toyama, Toyama 930-0194, Japan

Received for publication December 20, 2012. Accepted for publication January 30, 2013.

This work was supported in part by Superfund Research Program National Institute of Environmental Health Sciences Grant P42ES013660 and National Institutes of Health Grant P20RR018728. J.E.T. was supported by a Environmental Toxicology Training Grant awarded to Brown University. S.M.B. was supported by Deutsche Forschungsgemeinschaft Grant BL1115/1-1.

Address correspondence and reprint requests to Dr. Surendra Sharma, Department of Pediatrics, Women and Infants Hospital, 101 Dudley Street, Providence, RI 02905. E-mail address: ssharma@wihri.org

Abbreviations used in this article: DBA, *Dolichos biflorus*; GD, gestation day; polyI:C, polyinosinic:polycytidylic acid; PRF, perforin; Treg, regulatory T cell; uNK, uterine NK; WT, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1203488