Differential Expression and the Anti-apoptotic Effect of Human Placental Neurotrophins and Their Receptors

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ABSTRACT

Neurotrophin (NT) is important in the survival, maintenance and differentiation of neuronal tissue, and functions in follicle maturation, tumor growth, angiogenesis and immunomodulation; however, the expression of NT and its receptors (NTR) in human placenta and their influence on fetal growth are unclear. Here we investigated the correlation of NT and NTR in human placenta with uterine environment and fetal growth. *TrkB*, an NTR, mRNA was expressed on decidual and villous tissue and increased with gestational age, localizing in the trophoblast layer and endothelium by immunohistochemistry. Villous *TrkB* mRNA was significantly increased in preeclampsia (PE) than in controls and was higher in the normotensive small for gestational age (SGA) placenta, although it was not significant. It was also significantly increased in the small twin of discordant twin pregnancies. Brain-derived neurotrophic factor (BDNF), the main ligand of TrkB, was expressed in membranous chorion and villous tissue and was significantly higher in maternal plasma in normotensive SGA and PE than in controls. *TrkB* mRNA expression was up-regulated on cultured villous tissue explants and on JEG-3, a choriocarcinoma cell line, by H_2O_2 treatment. BDNF decreased apoptotic cells in H_2O_2-treated JEG-3, indicating that BDNF/TrkB signaling had anti-apoptotic effects against oxidative stress in JEG-3, suggesting a protective role of BDNF/TrkB in human villous tissue under unfavorable conditions in utero.

1. Introduction

Preeclampsia (PE) is a pregnancy-induced disease characterized by elevated blood pressure and proteinuria after 20 weeks of gestation. The disease is estimated to occur in 3-5 % of pregnancies. Especially in early onset or in severe type, PE is one of the major causes of maternal mortality because of its severe symptoms (e.g. HELLP syndrome, eclampsia, renal failure) in addition to causing fetal and neonatal mortality by preterm birth or intra-uterine growth restriction (IUGR) [1]. A number of studies have suggested possible mechanisms for the development of PE, including shallow trophoblast invasion and impaired spiral artery remodeling [2] with subsequent placental hypoperfusion and endothelial dysfunction. In addition, a variety of factors are thought to contribute to the pathogenesis of PE: inflammation [3], immune maladaptation [4] and metabolic disorders [5]. Increased placental apoptosis is reported to be observed in PE and IUGR by a variety of stimuli and damage, including hypoxia and oxidative stress [6-8]. Despite the progress of clinical and basic researches, the cause of PE has not been completely elucidated and there is no specific therapy except for placental delivery. It is known that some growth factors, including epidermal growth factor (EGF) and insulin-like growth factor (IGF), can rescue trophoblast apoptosis mediated by cytokine or oxidative stress in vitro [9, 10]. These are examples of the potent protective mechanisms against various stresses in the feto-maternal environment.

Neurotrophin (NT) is known to be an important factor in the survival, maintenance and differentiation of neuronal tissue [11-13]. The NT family is composed of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Although they share greater than 80 % identity in their amino acid structure, each NT interacts with a specific high-affinity tropomyosin-related kinase (Trk) receptor: NGF activates TrkA, BDNF and NT-4 activate TrkB, and NT-3 activates TrkC [14]. Recently, NT has also been reported to play an important role in follicle maturation [15], tumor growth [16], angiogenesis [17-19], immunomodulation [20, 21], inflammation [22, 23], energy metabolism [24], and so on. Moreover, signaling mediated by BDNF through its receptor TrkB has been reported to play an important role in embryo implantation, subsequent placental development and fetal growth by increasing trophoblast cell growth and survival in mice [25]; therefore, we hypothesized that the NT/NTR system can also play an important role in the human placenta, as reported in neural or some non-neural tissue.

There are a few reports about their expressions on human placenta and fetal membranes. Toti et al. reported that NGF was expressed in human placenta [26] and Casciaro et al. reported that NT-3 was expressed in human placenta [27]; however, the overall expression profile of NT and its receptors (NTR) on human placenta and their influences on fetal growth and pathological pregnancy, such as PE and IUGR, are not well elucidated. Here, we tested the hypothesis that NT and NTR might have an important role in fetal growth and an unfavorable environment, especially in the feto-maternal interface. The aim of this study was to investigate the expression profile of NT/NTR in human placenta and maternal plasma, especially in association with PE and/or fetal growth, and to assess their roles in the pathological environment.

2. Materials and methods

2.1. Patient characteristics and tissue collection

We collected normal villous tissues in the first (6 to 13 weeks of gestation, n = 11) and second trimester (16 to 25 weeks of gestation, n = 7), including 6 legal abortions and 1 preterm birth. Pathological placentas in the third trimester included 15 complicated with PE and 11 with normotensive SGA (small for gestational age). Sixteen uncomplicated normal controls were also collected. The clinical characteristics are shown in Table 1. PE was defined as maternal systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg in 2 consecutive measurements, with an interval of 6 h, and proteinuria ≥300 mg per 24 h after 20 weeks of gestation. SGA was defined as birth weight less than the 10th percentile. The numbers of PE with SGA and PE without SGA were 11 and 4, respectively. In addition, we collected villous tissues from dichorionic twins as a separate group as they share the same maternal and uterine environment. Seven discordant twins and 5 concordant twins were included in this study. The discordant twin was defined as having discordancy of more than 15 % difference in neonatal birth weight. Villous tissues were taken from the central part of the placenta and were free of visible infarction or calcification, and separated amnion and membranous chorion and decidua of the basal plate were collected within 20 min after Cesarean section without labor. After brief rinsing in saline, these tissues were quickly frozen in liquid nitrogen and stored at -80 ˚C until the experiment. Informed consent was obtained from each patient before sampling. The protocol was approved by the local ethics committee of Kyoto University Graduate School of Medicine.

2.2. Real-time quantitative PCR

Total RNA was extracted from the samples using the QIAGEN RNeasy Mini kit (QIAGEN, Germantown, MD) according to the manufacturer’s instructions. Five micrograms of total RNA were reversed into cDNA using a First-Strand cDNA Synthesis Kit (GE Healthcare, Little Chalfont, UK). Primers for the genes examined (Table 2) were designed using GeneFisher 2 software (Bielefeld University Bioinformatics Service, Bielefeld, Germany). The primers for human *GAPDH* were purchased from Applied Biosystems (Foster City, CA). Quantitative PCR (qPCR) amplification was performed with a final volume of 20 µl containing 33 ng template cDNA, 0.4 µM of each primer, and 10 µl SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan). The reaction was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the following PCR conditions: 95 ˚C for 10 sec, followed by 95 ˚C for 5 sec and 60 ˚C for 31 sec, repeated for 40 cycles. For dissociation after PCR amplification, the protocol included slow heating from 60 to 95 ˚C to ensure amplification specificity. The gene expression was estimated using the $2^{-\Delta Ct}$ method. Ct values were used to read off relative RNA amounts. The values of NT and NTR mRNA expression were obtained by the relative value for *GAPDH* mRNA. All samples were run in duplicate, and quantitative detection was averaged.

2.3. Immunohistochemistry

Immunohistochemical staining was conducted by the streptavidin-biotin-peroxidase method. Formalin-fixed, paraffin-embedded specimens of uncomplicated third trimester pregnancies were cut into 4 µm-thick sections. The tissue sections were deparaffinized in xylene (3×10 min) and dehydrated through graded alcohol (99 %, 80 % and 70 %) to water. Tissue samples were heated to retrieve antigens in Tris-EDTA buffer (pH 9.0) at 120 ˚C for 5 min. Endogenous peroxidase activity was blocked using 0.3 % H_2O_2. The sections were incubated with mouse monoclonal antibody against TrkB (diluted 1:100; R&D Systems, Minneapolis, MN, code MAB397) and rabbit polyclonal antibody against BDNF (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, code SC-546) overnight at 4 ˚C. Corresponding nonspecific IgG (Dako, Carpinteria, CA) was used as a negative control and processed in parallel. They were then incubated with biotinylated rabbit anti-mouse Ig secondary antibody for TrkB or with biotinylated goat anti-rabbit Ig for BDNF (Nichirei, Tokyo, Japan), followed by incubation with streptavidin-peroxidase complex solution for 30 min at room temperature (RT). Peroxidase activity was visualized by treatment with diaminobenzidine. Finally, the nuclei of sections were counterstained with Mayer's hematoxylin and observed under a microscope (Olympus, Tokyo, Japan).

2.4. Plasma assays

Maternal blood from uncomplicated pregnancies was obtained from the first (n = 9), second (n = 7) or third (n = 11) trimester. Samples from normotensive SGA (n = 6) and PE (n = 12) patients were taken in the third trimester after the onset of disease. Umbilical blood was obtained at the time of Cesarean section in the third trimester, including normal controls (n = 8), PE (n = 5) and normotensive IUGR (n = 2). The blood was sampled into heparinized tubes, plasma separated by centrifugation at 3000 rpm for 30 min, and stored at -20 ˚C until analysis. Plasma BDNF was measured using the human BDNF enzyme-linked immunosorbent assay

(ELISA) kit (R&D Systems) in duplicate each sample as instructed by the manufacturer. According to the manufacturer's protocol, coefficients of variation for BDNF ELISA were as follows: intra-assay precision and inter-assay precision were 3.8-6.2 % and 7.6-11.3 %, respectively.

2.5. Tissue and cell culture and oxidative stress

Placental villous tissues were collected from normal term pregnancies (n = 10) delivered by elective Cesarean section. Tissues were taken from midway between the chorionic and basal plates and were free of visible infraction or calcification. After brief rinsing in ice-cold phosphate-buffered saline (PBS), tissues were placed in ice-cold RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan). Samples were taken to the laboratory and processed within 30 min. They were further dissected into small pieces (about 2-3 mm in diameter), and 3 fragments were placed in 6-well plates with 3 ml culture medium (RPMI 1640 containing streptomycin, penicillin, and 10 % fetal calf serum) per well. Subsequently, tissues were incubated with or without H_2O_2 (100 μM) for 2 h in a culture incubator with 5 % CO_2/95 % air at 37 ˚C. Placental villous explants were then collected and stored at -80 ˚C. H_2O_2 concentration was determined according to a previous report [9] showing that 100 μM H_2O_2 was effective to increase apoptosis in the placental explant culture.

The JEG-3 (HTB-36) choriocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI medium containing streptomycin, penicillin, and 10 % fetal calf serum at 37 ˚C with 5 % CO_2 / 95 % air. JEG-3 were incubated for 2 h with or without H_2O_2 (5 µM) and harvested. A marked number of JEG-3 cells died in a concentration of 100 µM. After preliminary experiments of 1, 5 and 50 µM, we selected 5 µM as an appropriate concentration for the cell culture that showed increased apoptosis but less cell death.

TrkB mRNA in these tissues and cell samples were measured by qPCR. The amount of *TrkB* mRNA from 3 wells was averaged and the experiments were repeated 6 times.

2.6. Apoptosis analysis

Apoptosis analysis was assessed using the Annexin V-FITC Apoptosis Detection kit I (BD Biosciences, San Jose, CA) by a fluorescence-activated cell sorter (FACS). Annexin V identifies cells in early apoptosis by detecting externalized phosphatidylserine, and propidium iodide (PI) identifies necrotic or late apoptotic cells that have lost plasma membrane integrity. JEG-3 cells on 6 cm culture dishes were administered 50 ng/ml recombinant human (rh) BDNF (PeproTech, Rocky Hill, NJ) 24 h prior to H_2O_2 (5 µM) treatment. Vehicle only was used as a control. This was selected according to the manufacturer’s instructions depending on previous reports [28, 29]. We did not perform a dose response experiment but performed the experiment once using 10 ng/ml, which was less effective than 50 ng/ml.

For the blocking experiment, 100 nM k252a (inhibitor of pan Trk signaling; Calbiochem, Darmstadt, Germany) or k252b, which is almost equipotent to k252a but without inhibiting activity, was added just prior to rhBDNF (50 ng/ml) administration, followed by H_2O_2 treatment 24 h later. After incubation for 2 h with H_2O_2, the cells were harvested by trypsin-EDTA and centrifuged at 3000 rpm for 10 min. The cells were washed twice with PBS (containing 0.2 % fetal calf serum) at RT, resuspended in 100 µl of 1× binding buffer supplemented with 5 µl FITC conjugated Annexin V and 2 µl PI, and incubated at RT in the dark for 15 min according to the manufacturer’s instructions. Following the addition of 400 µl of 1× binding buffer, stained cells were kept on ice and subjected immediately to FACS analysis using a FACSCalibur flow cytometer with CellQuest software (BD, Franklin Lakes, NJ). The cell debris and small particles were excluded from analysis. When cells were double stained, 4 different groups of cells were observed: both negative cells (Annexin V (-) / PI (-)) were defined as viable cells, cells stainable with Annexin V but not with PI were early apoptotic cells, both positive cells were late apoptotic or necrotic cells, and PI (+) -only cells were debris of dead cells.

2.7. Cell proliferation assay (WST assay)

WST assay was performed using Cell Count Reagent SF (Nacalai, Kyoto, Japan) according to the manufacturer's protocol to examine the proliferation of JEG-3 cells. JEG-3 cells on 96-well plates were cultured with rhBDNF (50 ng/ml) for 24 h or 48 h. Vehicle-only was used as a control. The media were changed to 110 µl fresh medium containing 10 µl Reagent SF. After 4 h, the absorbance of the media at 450 nm was measured using an Emax microplate reader (Molecular Devices, Tokyo, Japan).

2.8. Statistical analysis

The results of normally distributed continuous variables are expressed as the mean ± SEM (range), while those with skewed distribution were expressed as the median value with [interquartile range]. Continuous variables were analyzed by the Wilcoxon *t* test, Mann-Whitney *U* test and Kruskal-Wallis H test, as appropriate. Pearson's correlation coefficient was used for evaluation of a possible association between neonatal birth weight and *TrkB* expression in villous tissue. A p value of < 0.05 denoted statistical significance. Statistical analyses were performed using Prism 3.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Patient characteristics

The features of the patients are shown in Table 1. Gestational age at delivery was lower in the PE group than in controls. Neonatal and placental weights were lighter in the normotensive SGA and PE group than in controls. Among 15 patients with PE, 11 were complicated with SGA and 4 were not; 5 were early onset type and 10 were late onset type.

3.2. NT and NTR mRNA expression in villous tissue and fetal membranes

The expression profile of NT and NTR mRNA was investigated in separate fetal membranes (amnion, membranous chorion, and decidua) and villous tissue samples in the 3rd trimester (Fig. 1A). Among 4 NTs, *NGF*, *BDNF*, and *NT-3* were detected in the amnion and membranous chorion, but there was no significant difference between these tissues. *NT-4* was less expressed in those tissues. The *BDNF* mRNA level was higher in the membranous chorion and villous tissue than amnion and decidua, although they did not reach statistical significance (Fig. 1A). Among 3 NTRs, the expression of *TrkB* in decidua and villous tissue was higher than in other tissues and other NTRs; however, they were not statistically significant (Fig. 1B). *TrkB* expression in villous tissue was significantly increased in the second and third trimesters compared to the first trimester (Fig. 1C).

3.3. Localization of BDNF and TrkB

In the third trimester placenta, BDNF immunostaining was observed in the membranous chorion (Fig. 2A), trophoblast layer and endothelium (Fig. 2B), whereas TrkB immunostaining was observed in the decidua (Fig. 2D), trophoblast layer and endothelium (Fig. 2E). In the trophoblast layer, TrkB was confirmed to localize in both cytotrophoblast cells and syncytiotrophoblast cells in the first and second trimesters (Fig. 2G and H). The localization of TrkB was not different throughout the gestational age (Fig. 2G, H and E) and appeared similar even in PE (Fig. 2I).

3.4. Placental *TrkB* mRNA expression according to pathological status

Among 42 samples, including normal term controls, normotensive SGA and PE, *TrkB* mRNA was significantly higher in PE placentas than in normal term controls (Fig. 3A). There was no significant difference between PE with SGA (0.85 [0.53-0.11] $\times 10^{-2}$, n = 11) and PE without SGA (0.47 [0.38-0.94] $\times 10^{-2}$, n = 4). The increase of *TrkB* in normotensive SGA was not significant. Pearson's correlation tests of 42 samples demonstrated that *TrkB* expression was reversely correlated with neonatal birth weight (Fig. 3B).

3.5. *TrkB* mRNA expression in discordant twin placentas

Theoretically, dichorionic twins share the same maternal environment and their genomes are different; therefore, the difference in *TrkB* between discordant dichorionic twin placentas was thought to be regulated by the placental environment, which causes fetal growth discordancy. *TrkB* was significantly higher in villous tissues of small twins than their co-twins (Fig. 3C), whereas it was not different between concordant twins (Fig. 3D). Conversely, *BDNF* was not different in discordant twins (data not shown).

3.6. Plasma BDNF level

Maternal plasma BDNF levels were significantly higher in the normotensive SGA and PE group than in non-complicated pregnant women in the third trimester (Fig. 3E). Gestational age did not affect plasma BDNF levels (first: 262.8 pg/ml [130.6-352.7], second: 236.4 pg/ml [155.5-1609.8] and third: 623.8 pg/ml [330.9-1024.0]). In umbilical plasma, BDNF levels were not influenced by the pathological status, such as in PE, normotensive SGA and discordant twins (data not shown).

3.7. Effect of BDNF/TrkB against oxidative stress in vitro

The expression of *TrkB* in villous explants was significantly increased when cultured with 100 µM H_2O_2 for 2 h (Fig. 4A). *TrkB* on JEG-3 cells was also significantly increased with 5 µM H_2O_2 for 2 h (Fig. 4B).

In the FACS experiment, JEG-3 cells were evaluated by double-staining with Annexin V and PI: both negative cells were defined as viable, Annexin V positive / PI negative were early apoptotic, and both positive cells were late apoptotic or necrotic. The cells were divided into these populations by the lines indicated. Representative FACS plots are shown in the presence or absence of H_2O_2 and rhBDNF (Fig. 5A-C). The level of late apoptotic or necrotic cells was significantly increased at 2 h with 5 µM of H_2O_2 (Fig. 5B and D). When cells were pretreated with rhBDNF (50 ng/ml) 24 h prior to H_2O_2 treatment, late apoptotic or necrotic cells were not increased even with H_2O_2 treatment (Fig. 5C and D). On the other hand, viable cells were significantly decreased by H_2O_2 treatment, whereas rhBDNF pre-treatment diminished the decrease of viable cells (Fig. 5B, C and F). The relative cell number in each group was evaluated as the ratio to those in the group without H_2O_2 and rhBDNF treatment.

In the culture model of JEG-3 with H_2O_2 and rhBDNF, k252a (inhibitor of pan Trk) treatment significantly induced late apoptosis or necrosis but k252b (equipotent to k252a without inhibiting activity) caused no marked change (Fig. 5G).

As for early apoptosis, a similar result was observed in that k252a treatment significantly induced apoptotic cells (Fig. 5H). On the other hand, viable cells were significantly decreased by k252a administration compared to k252b treatment (Fig. 5I). These results were evaluated as the relative cell number compared to those treated with H_2O_2 and rhBDNF without k252a and k252b.

3.8. Proliferation of JEG-3 by rhBDNF

Cell proliferation assay revealed no statistically significant difference in the growth of JEG-3 cultured with rhBDNF compared with vehicle only. The absorbance of the media at 450 nm was as follows: 0.086 [0.075-0.099] with rhBDNF vs. 0.118 [0.111-0.130] with vehicle only for 24 h, 0.263 [0.247-0.260] with rhBDNF vs. 0.349 [0.319-0.377] with vehicle only for 48 h (n = 4).

4. Discussion

In the present study, we investigated the expression profile of NT and NTR and their potential roles in human pregnancy. We present a detailed expression profile for the first time for NT and NTR in human placenta and the fetal membrane. We also investigated the regulation of these expressions in association with PE and/or fetal growth. We then assessed their effects in the pathological environment in vitro. The expressions of *NGF*, *BDNF* and *NT-3* mRNAs were detected in these tissues, although no significant difference was detected among the tissues examined. On the other hand, we demonstrated the expression of *TrkB* on villous tissue and decidua among 4 NTRs. *TrkB* expression increased with gestational age and was up-regulated in PE patients. These findings strongly suggest a relationship between TrkB and the pathological status in the placenta; thus, we focused on BDNF, the main ligand of TrkB among the NTs. Both BDNF and TrkB in villous tissue were localized in the trophoblast layer as well as the endothelium, which is consistent with previous reports [18, 19]. BDNF was detected in maternal plasma and its level was significantly higher in normotensive SGA and PE patients than normal controls in the third trimester. These findings suggest that the BDNF/TrkB system may be activated at the feto-maternal interface under unfavorable conditions during pregnancy.

We also demonstrated that the expression of *TrkB* was up-regulated in PE despite the presence or absence of SGA and there was no significant difference between these two groups. We did not detect significant up-regulation of TrkB in SGA placenta; however, Mayeur et al. reported that mRNA expression of *TrkB* was significantly increased in human IUGR placenta [30]. This discrepancy seems to be due to the severity of IUGR; the patients in Mayeur’s report were more severely affected than our patients. Villous *TrkB* expression correlated with neonatal birth weight and was significantly increased in the smaller twin of discordant twins; therefore, the increase of TrkB expression in human placenta may be related to fetal growth.

NTs are known to be an important factor in the survival, maintenance and differentiation of neuronal tissue [11-13]. Although TrkB$^{-/-}$ mice were not embryonically lethal but showed neuronal deficiencies in the central and peripheral nervous systems and neonatal death due to insufficient feeding activity [31], the effect of TrkB$^{-/-}$ on the placenta was unclear in this article, but we suppose that TrkB is not essential for placental development in normal pregnancy but functions under unfavorable conditions.

As maternal BDNF reaches the fetal brain through the utero-placental barrier, it contributes to development in mice [32]. Thus, previous studies on NTs during pregnancy mostly focused on the effect on the fetal nervous system; for example, Marx reported that NTs were detectable in human amniotic fluid and their decreased levels may reflect abnormalities in the fetal brain in utero [33]. Although the anti-apoptotic effect of NTs on neuronal and non-neuronal tissues, such as endothelial cells, has already been reported [34], we showed in the present study that BDNF administration decreased the rate of apoptotic or necrotic cells in H_2O_2- treated JEG-3 cells, which suggests that BDNF/TrkB signaling also has an anti-apoptotic effect on trophoblast cells. Placental apoptosis increased with placental growth according to gestational age even in normal development [35]; however, it was marked in complicated pregnancies, such as hydatidiform mole, PE and IUGR [6-8, 36, 37]. An increased number of syncytial knots were observed in PE and IUGR placentas and could be replicated in vitro by reactive oxygen species (ROS) or hypoxia [38]. Our in vitro findings suggest that BDNF/TrkB can play a protective role in the placenta against oxidative stress by reducing or slowing the increase of apoptotic cells in PE or SGA.

Although the precise mechanism of the anti-apoptotic effects of BDNF/TrkB has not been elucidated, some reports have demonstrated relationships between BDNF/TrkB and some important factors associated with PE. BDNF induces vascular endothelial growth factor (VEGF) expression via hypoxia-inducible factor-1 alpha in neuroblastoma cells [39] and may act on angiogenesis through VEGF. It is known that VEGF and placental growth factor (PlGF) and their receptors play important roles in PE. The increase of circulating soluble fms-like tyrosine kinase-1 (sFlt-1), which is a soluble receptor of VEGF, is associated with the symptoms of PE, partly by inhibiting the VEGF effect [40, 41]; therefore, we speculate that BDNF might supplement the impaired VEGF system.

Recent studies demonstrated a possible relationship between the stress-induced steroid hormone, glucocorticoid, and BDNF/TrkB, which has a neuroprotective effect [42]. It was also reported that stress induces BDNF in rat submandibular glands [43]; therefore, BDNF/TrkB may function to contribute to the maintenance of an impaired placenta in a stressful environment. Accordingly, it will be valuable to investigate the network or balance of various factors, including BDNF/TrkB as well as VEGF, sFlt-1 and so on in PE or IUGR patients.

We showed elevated levels of BDNF in the maternal plasma of PE patients. As the BDNF level in maternal blood is reported to be consistent during normal pregnancy [44], which is compatible with our results, we found that BDNF levels are up-regulated in the plasma of PE patients. BDNF is reported to be secreted from endothelial cells, macrophages and monocytes [45], and platelets contain a large amount of BDNF and release it when activated [46]. It will be interesting to investigate the source of increased BDNF and the regulation of its secretion in PE patients.

In conclusion, we have demonstrated that placental TrkB and maternal BDNF are up-regulated in the pathological environment, such as PE and small twins. BDNF/TrkB signaling may have an anti-apoptotic effect in response to oxidative stress on the choriocarcinoma cell line, and suggests a protective role of BDNF/TrkB in villous tissue under stress-induced unfavorable conditions. Further investigation of the physiology and relationship among the responsible factors, including BDNF/TrkB signaling, in maternal and fetal units may reveal the mechanism of placental maintenance in a stressful environment, which will give some insights into the management of PE and SGA patients.

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Figure Legends

Fig. 1. Expression of neurotrophins (NTs) and neurotrophin receptors (NTRs) in human placenta. Relative mRNA expression of NTs (A) and NTRs (B) in fetal membranes and villous tissues (n = 4) in normal term pregnancy. (C) *TrkB* mRNA expression on villous tissue in the first, second and third trimesters (n = 11, 7 and 16, respectively). * $p < 0.05$, **$p < 0.001$. Each mRNA expression was normalized by *GAPDH*. Lines within the boxes represent the median value; top and bottom lines of the boxes represent 25th and 75th percentiles, and upper and lower bars outside the box represent 90th and 10th percentiles, respectively.

Fig. 2. Localized expression of BDNF and TrkB in human placenta. Representative immunohistochemical staining of BDNF (A-C) and TrkB (D-I) in fetal membrane (A, D), villous tissue (B, C, E, F) in normal term placenta, first trimester placenta (G), second trimester placenta (H), and preeclamptic placenta (I). (C and F) Negative control for BDNF and TrkB, respectively. Arrowhead: trophoblast layer; arrow: endothelium.

Fig. 3. *TrkB* mRNA expressions and plasma *BDNF* levels in the pathological status. (A) *TrkB* mRNA expression in villous tissue in normal term control (n = 16), normotensive SGA (n = 11) and PE (n = 15). (B) Correlation between neonatal birth weight and *TrkB* expression in villous tissue of 42 patients. SD: standard deviation, *r*: correlation coefficient.

TrkB mRNA expression in villous tissue of discordant twins (C, n = 7) and concordant twins (D, n = 5). (E) Maternal plasma BDNF levels in third trimester in normal pregnancies (n = 11), normotensive SGA (n = 6) and PE (n = 12). The mRNA expressions were normalized by *GAPDH*. * $p < 0.05$, ** $p < 0.01$

Fig. 4. Expression of *TrkB* mRNA under oxidative stress in vitro.

TrkB mRNA in villous explants (A, n = 10) and JEG-3 cells (B, n = 6) cultured with or without H_2O_2 for 2 hours. The mRNA expressions were normalized by *GAPDH*. * $p < 0.05$, ** $p < 0.01$

Fig. 5. Effect of BDNF/TrkB signaling against oxidative stress on JEG-3 cells.

Induction of apoptosis by H_2O_2 and the effect of BDNF on JEG-3 cells were examined by FACS (n = 6). (A-C) Representative FACS plots by double-staining with Annexin V and PI. Non-treatment group (A) treated with H_2O_2 (B) and H_2O_2 plus rhBDNF (C). Positive cells for both were defined as late apoptosis or necrosis (D, G); Annexin V positive/PI negative as early apoptosis (E, H); both negative as viable (F, I). (D-F) Relative number of JEG-3 cells treated with H_2O_2 (lane 2) and H_2O_2 plus rhBDNF (lane 3) compared to non-treatment group (lane 1). (G-I) Inhibition of the anti-apoptotic effect of BDNF by k252a, an inhibitor of Trk. Relative number of JEG-3 cells pretreated with k252a (lane 2) or k252b (lane 3) followed by

H_2O_2 and rhBDNF compared to cells treated with H_2O_2 and rhBDNF alone (lane 1). $^*p < 0.05$, $^{**}p < 0.01$.

Table 1
Patient characteristics.

	Controls (n = 16)	Normotensive SGA (n = 11)	PE (n = 15)	*p* value		
				Controls vs normotensive SGA	Controls vs PE	Normotensive SGA vs PE
Patient’s age at delivery (years)	32.0±1.6 (21-41)	31.8±1.5 (24-40)	33.7±1.4 (27-41)	n.s.	n.s.	n.s.
Primipara (n)	3/16	6/11	13/15	-	-	-
Gestational age at delivery (weeks)	37 [37-38]	37 [34-38]	34 [30-35]	n.s.	$p < 0.01$	n.s.
Body mass index at delivery (kg/m^2)	23.9±0.5 (20.8-27.7)	23.9±0.9 (20.1-28.6)	25.2±0.8 (20.5-30.4)	n.s.	n.s.	n.s.
Systolic blood pressure (mmHg)	108±2 (98-120)	116±2 (107-130)	168±4 (140-190)	n.s.	$p < 0.001$	$p < 0.001$
Diastolic blood pressure (mmHg)	65±2 (50-77)	66±3 (48-80)	96±3 (70-110)	n.s.	$p < 0.001$	$p < 0.001$
Neonatal weight (g)	2954 [2715-3058]	1550 [1311-2273]	1657 [1096-2021]	$p < 0.001$	$p < 0.001$	n.s.
Placental weight (g)	497 [468-570]	360 [275-369]	288 [245-384]	$p < 0.01$	$p < 0.001$	n.s.
SGA (n)	0	11/11	11/15	-	-	-

SGA: small for gestational age, PE: preeclampsia, Values are the mean ± SEM and (range) or median value with [interquartile range].

Table 2

Summary of the primers analyzed in real-time quantitative PCR.

Gene	Forward	Reverse	accession number
TrkA	5’ - ACTGAGCTCTACATCGAGA	5’ - CTGCACAGTTTTCCAGGA	NM_002529
TrkB	5’ - AGAGGCTAAATCCAGTCCA	5’ - CAGGTTACCAACATCCCAA	NM_006180
TrkC	5’ - ATACTACCAAGAGGGAGAGA	5’ - TGGGTCACAGTGATAGGA	NM_001007156
NGF	5’ - ACTGAGGTGCATAGCGTA	5’ - GTGTCAAGGGAATGCTGA	NM_002506
BDNF	5’ - GTGAGAAGAGTGATGACCA	5’ - CTCTTCTATCACGTGTTCGA	NM_170735
NT-3	5’ - TGGCATCCAAGGTAACAACA	5’ - GGCAGGGTGCTCTGGTAAT	NM_002527
NT-4	5’ - CCCTCTCCTGAGATGTCA	5’ - GGAGGAGGAAAAGGAGGA	NM_006179

Fig. 1.

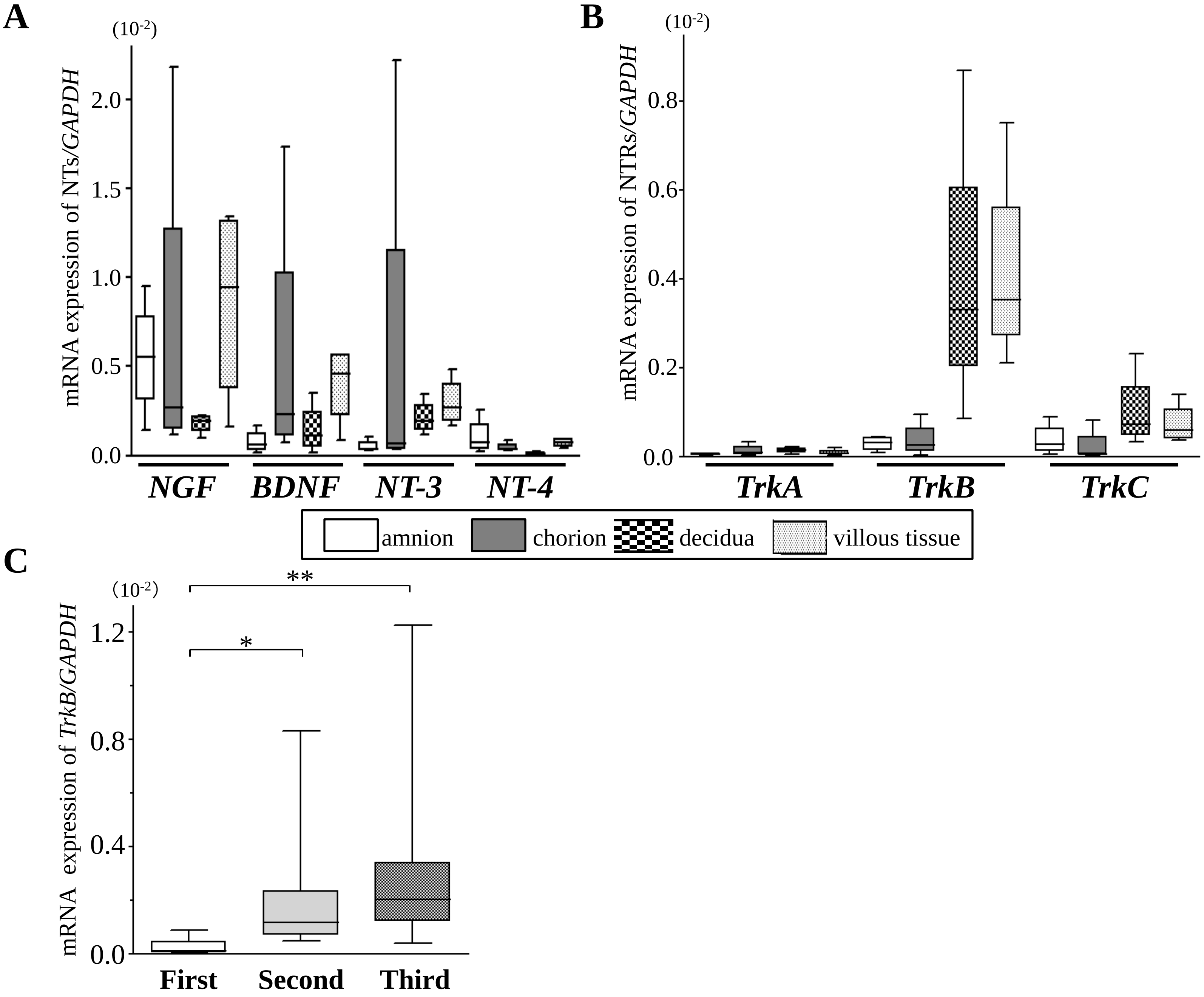

A
(10-2)
mRNA expression of NTs/GAPDH
2.0
1.5
1.0
0.5
0.0
NGF
BDNF
NT-3
NT-4
B
(10-2)
mRNA expression of NTRs/GAPDH
0.8
0.6
0.4
0.2
0.0
TrkA
TrkB
TrkC
amnion
chorion
decidua
villous tissue
C
(10-2)
mRNA expression of TrkB/GAPDH
**
*
1.2
0.8
0.4
0.0
First
Second
Third

Fig. 2.

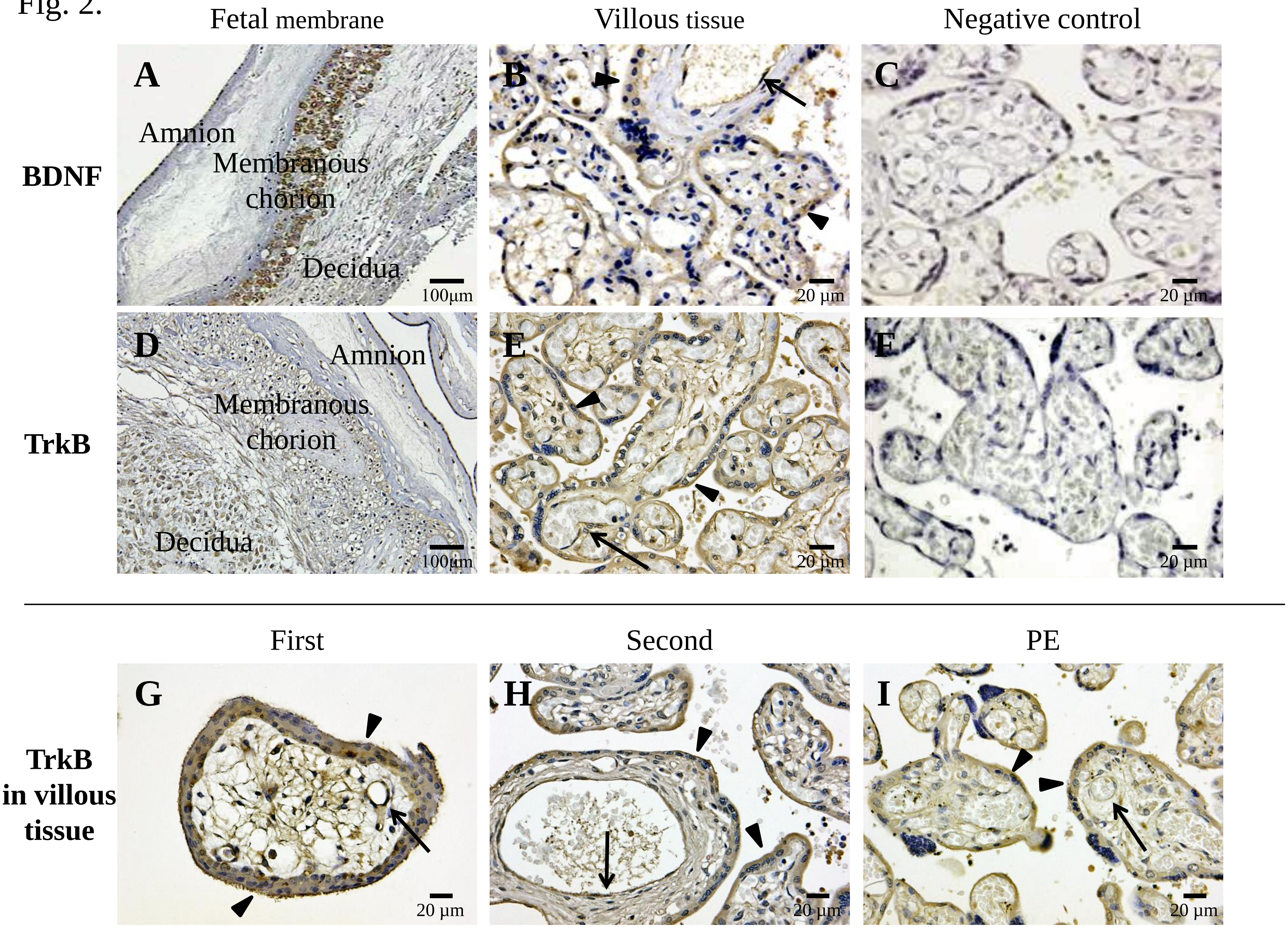

Fetal membrane
Villous tissue
Negative control
BDNF
TrkB
A
Amnion
Membranous chorion
Decidua
100µm
B
20 µm
C
20 µm
D
Amnion
Membranous chorion
Decidua
100µm
E
20 µm
F
20 µm
First
Second
PE
TrkB in villous tissue
G
20 µm
H
20 µm
I
20 µm

Fig. 3.

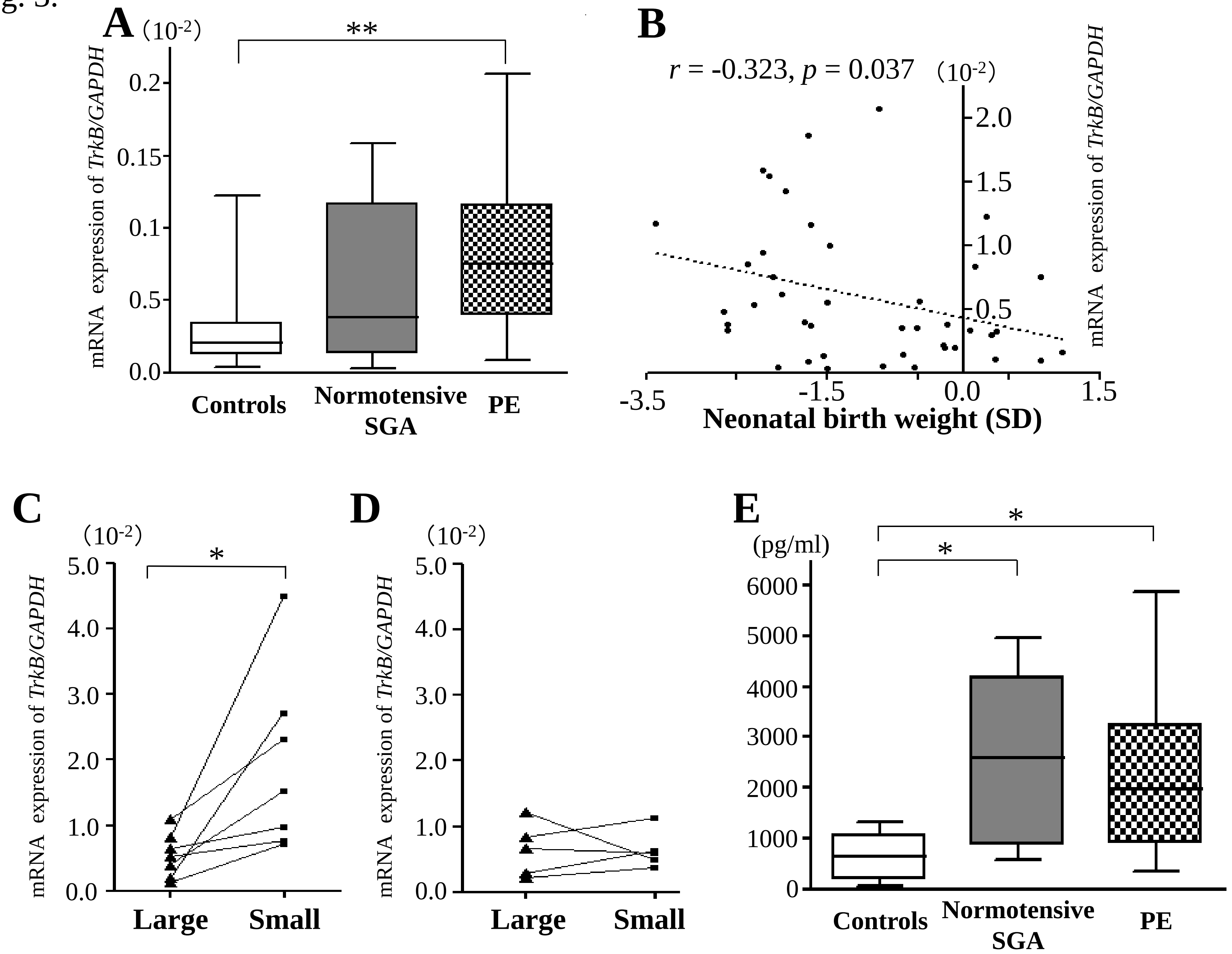

A
(10-2)
**
mRNA expression of TrkB/GAPDH
0.2
0.15
0.1
0.5
0.0
Controls
Normotensive SGA
PE
B
r = -0.323, p = 0.037
(10-2)
mRNA expression of TrkB/GAPDH
2.0
1.5
1.0
0.5
-3.5
-1.5
0.0
1.5
Neonatal birth weight (SD)
C
(10-2)
*
mRNA expression of TrkB/GAPDH
5.0
4.0
3.0
2.0
1.0
0.0
Large
Small
D
(10-2)
mRNA expression of TrkB/GAPDH
5.0
4.0
3.0
2.0
1.0
0.0
Large
Small
E
(pg/ml)
*
*
6000
5000
4000
3000
2000
1000
0
Controls
Normotensive SGA
PE

Fig. 4.

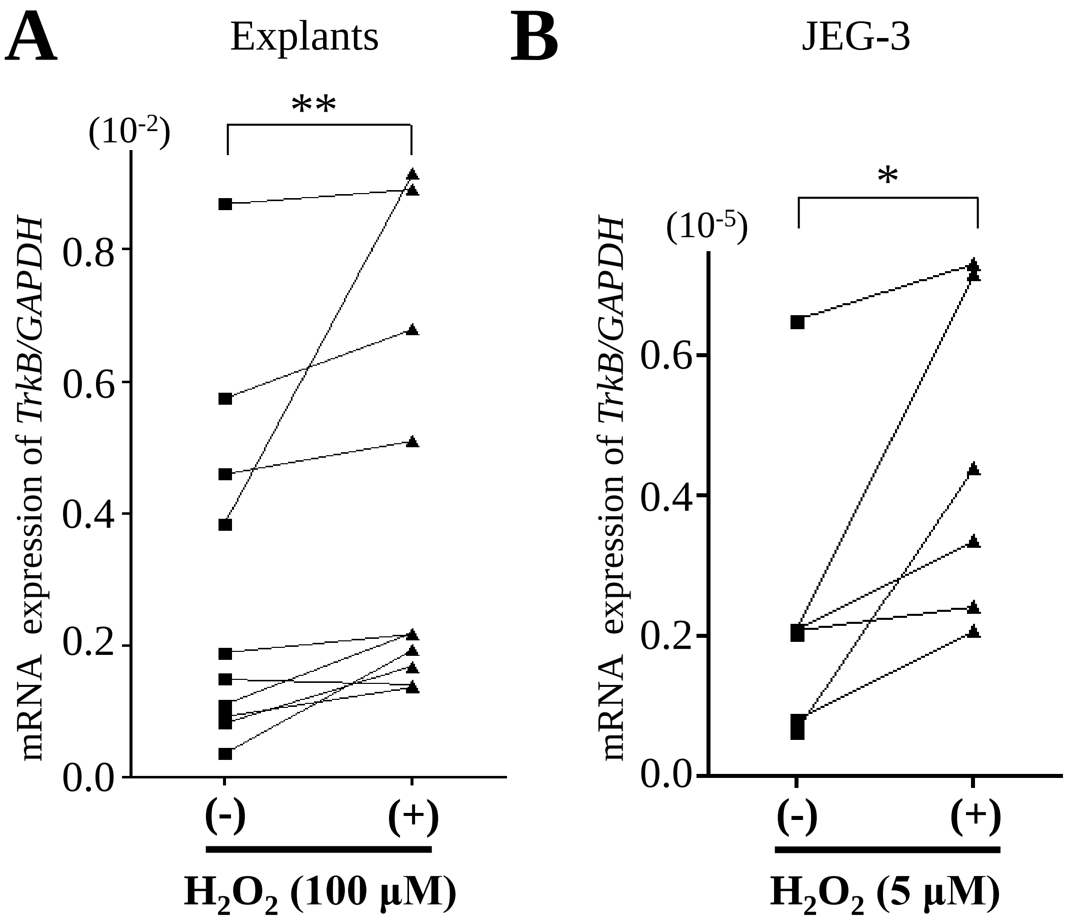
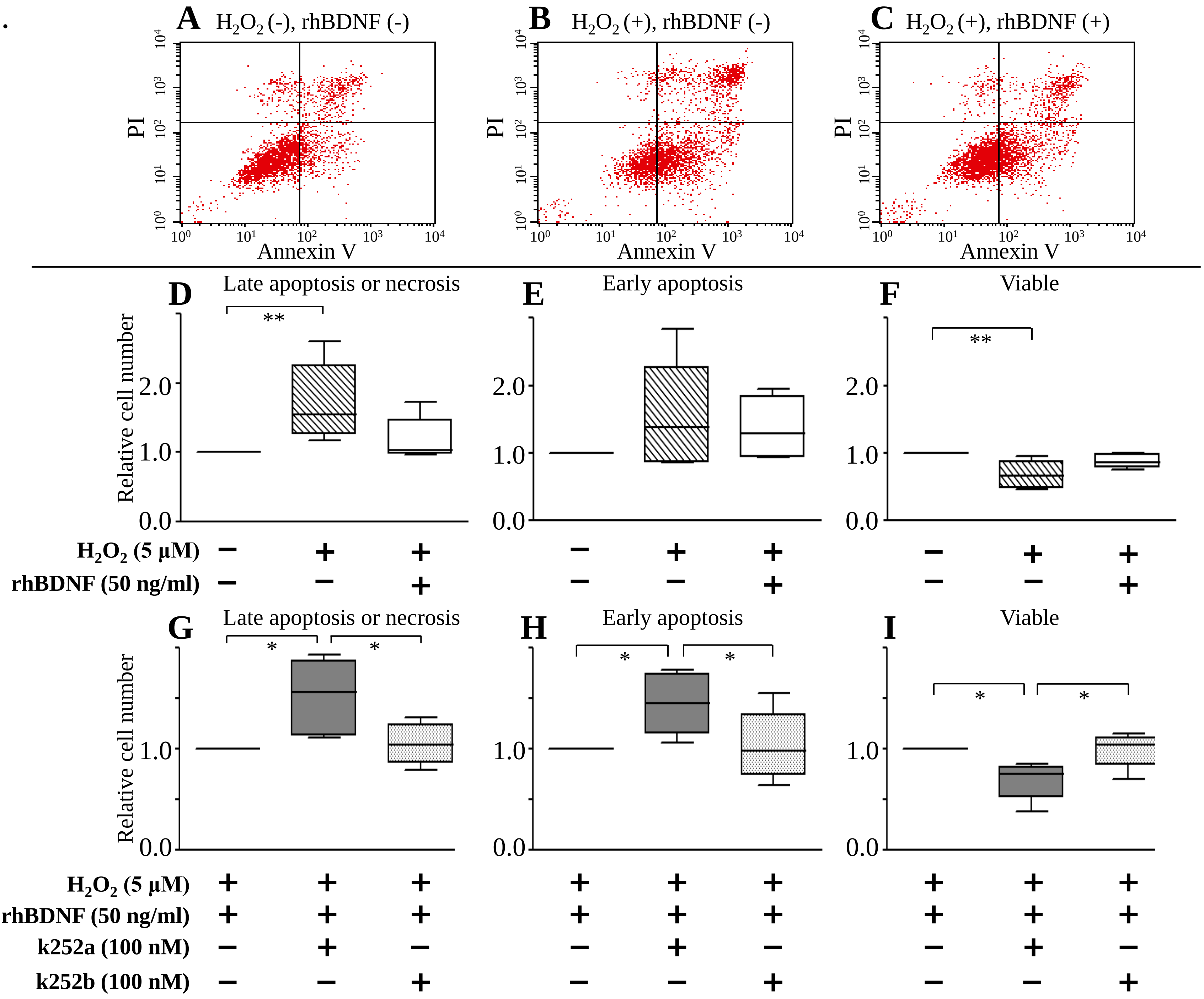

A
Explants
**
(10-2)
mRNA expression of TrkB/GAPDH
0.8
0.6
0.4
0.2
0.0
(-)
(+)
H2O2 (100 µM)
B
JEG-3
*
(10-5)
mRNA expression of TrkB/GAPDH
0.6
0.4
0.2
0.0
(-)
(+)
H2O2 (5 µM)

Fig. 5.


A H2O2 (-), rhBDNF (-)
B H2O2 (+), rhBDNF (-)
C H2O2 (+), rhBDNF (+)
PI
Annexin V
10^0
10^1
10^2
10^3
10^4
D Late apoptosis or necrosis
E Early apoptosis
F Viable
Relative cell number
0.0
1.0
2.0
**
H2O2 (5 μM) − + +
rhBDNF (50 ng/ml) − − +
G Late apoptosis or necrosis
H Early apoptosis
I Viable
*
H2O2 (5 μM) + + +
rhBDNF (50 ng/ml) + + +
k252a (100 nM) − + −
k252b (100 nM) − − +