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Author(s)	Fujita, K; Tatsumi, K; Kondoh, E; Chigusa, Y; Mogami, H; Fujii, T; Yura, S; Kakui, K; Konishi, I
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1 **Differential Expression and the Anti-apoptotic Effect of Human Placental**

2 **Neurotrophins and Their Receptors**

3

4 Kohei Fujita, Keiji Tatsumi, Eiji Kondoh, Yoshitsugu Chigusa, Haruta Mogami,

5 Tsuyoshi Fujii, Shigeo Yura, Kazuyo Kakui, Ikuo Konishi

6

7 Department of Gynecology and Obstetrics, Kyoto University Graduate School of Medicine,

8 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

9

10 Corresponding author: Keiji Tatsumi, M.D., Ph.D.

11 Department of Gynecology and Obstetrics

12 Kyoto University Graduate School of Medicine

13 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

14 Tel.: +81-75-751-3269

15 Fax: +81-75-761-3967

16 E-mail: ktat@kuhp.kyoto-u.ac.jp

17

18 **Key words:** neurotrophin, preeclampsia, fetal growth, apoptosis, oxidative stress, placenta

19

20 1

1 **ABSTRACT**

2

3 Neurotrophin (NT) is important in the survival, maintenance and differentiation of neuronal
4 tissue, and functions in follicle maturation, tumor growth, angiogenesis and
5 immunomodulation; however, the expression of NT and its receptors (NTR) in human
6 placenta and their influence on fetal growth are unclear. Here we investigated the correlation
7 of NT and NTR in human placenta with uterine environment and fetal growth. *TrkB*, an NTR,
8 mRNA was expressed on decidual and villous tissue and increased with gestational age,
9 localizing in the trophoblast layer and endothelium by immunohistochemistry. Villous *TrkB*
10 mRNA was significantly increased in preeclampsia (PE) than in controls and was higher in
11 the normotensive small for gestational age (SGA) placenta, although it was not significant. It
12 was also significantly increased in the small twin of discordant twin pregnancies.
13 Brain-derived neurotrophic factor (BDNF), the main ligand of TrkB, was expressed in
14 membranous chorion and villous tissue and was significantly higher in maternal plasma in
15 normotensive SGA and PE than in controls. *TrkB* mRNA expression was up-regulated on
16 cultured villous tissue explants and on JEG-3, a choriocarcinoma cell line, by H₂O₂ treatment.
17 BDNF decreased apoptotic cells in H₂O₂-treated JEG-3, indicating that BDNF/TrkB signaling
18 had anti-apoptotic effects against oxidative stress in JEG-3, suggesting a protective role of
19 BDNF/TrkB in human villous tissue under unfavorable conditions in utero.

1 **1. Introduction**

2

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

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

14 There are a few reports about their expressions on human placenta and fetal membranes.
15 Toti et al. reported that NGF was expressed in human placenta [26] and Casciaro et al.
16 reported that NT-3 was expressed in human placenta [27]; however, the overall expression
17 profile of NT and its receptors (NTR) on human placenta and their influences on fetal growth
18 and pathological pregnancy, such as PE and IUGR, are not well elucidated. Here, we tested
19 the hypothesis that NT and NTR might have an important role in fetal growth and an

1 unfavorable environment, especially in the feto-maternal interface. The aim of this study was
2 to investigate the expression profile of NT/NTR in human placenta and maternal plasma,
3 especially in association with PE and/or fetal growth, and to assess their roles in the
4 pathological environment.

5

6 **2. Materials and methods**

7

8 2.1. Patient characteristics and tissue collection

9

10 We collected normal villous tissues in the first (6 to 13 weeks of gestation, n = 11) and
11 second trimester (16 to 25 weeks of gestation, n = 7), including 6 legal abortions and 1 preterm
12 birth. Pathological placentas in the third trimester included 15 complicated with PE and 11
13 with normotensive SGA (small for gestational age). Sixteen uncomplicated normal controls
14 were also collected. The clinical characteristics are shown in Table 1. PE was defined as
15 maternal systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg in 2
16 consecutive measurements, with an interval of 6 h, and proteinuria ≥ 300 mg per 24 h after 20
17 weeks of gestation. SGA was defined as birth weight less than the 10th percentile. The numbers
18 of PE with SGA and PE without SGA were 11 and 4, respectively. In addition, we collected
19 villous tissues from dichorionic twins as a separate group as they share the same maternal and

1 uterine environment. Seven discordant twins and 5 concordant twins were included in this
2 study. The discordant twin was defined as having discordancy of more than 15 % difference
3 in neonatal birth weight. Villous tissues were taken from the central part of the placenta and
4 were free of visible infarction or calcification, and separated amnion and membranous chorion
5 and decidua of the basal plate were collected within 20 min after Cesarean section without labor.
6 After brief rinsing in saline, these tissues were quickly frozen in liquid nitrogen and stored at
7 -80 °C until the experiment. Informed consent was obtained from each patient before sampling.
8 The protocol was approved by the local ethics committee of Kyoto University Graduate School
9 of Medicine.

10

11 2.2. Real-time quantitative PCR

12

13 Total RNA was extracted from the samples using the QIAGEN RNeasy Mini kit
14 (QIAGEN, Germantown, MD) according to the manufacturer's instructions. Five micrograms
15 of total RNA were reversed into cDNA using a First-Strand cDNA Synthesis Kit (GE
16 Healthcare, Little Chalfont, UK). Primers for the genes examined (Table 2) were designed
17 using GeneFisher 2 software (Bielefeld University Bioinformatics Service, Bielefeld,
18 Germany). The primers for human *GAPDH* were purchased from Applied Biosystems (Foster
19 City, CA). Quantitative PCR (qPCR) amplification was performed with a final volume of 20

1 μl containing 33 ng template cDNA, 0.4 μM of each primer, and 10 μl SYBR Premix Ex Taq
2 II (Takara Bio, Otsu, Japan). The reaction was performed using the ABI PRISM 7000
3 Sequence Detection System (Applied Biosystems) with the following PCR conditions: 95 °C
4 for 10 sec, followed by 95 °C for 5 sec and 60 °C for 31 sec, repeated for 40 cycles. For
5 dissociation after PCR amplification, the protocol included slow heating from 60 to 95 °C to
6 ensure amplification specificity. The gene expression was estimated using the $2^{-\Delta Ct}$ method. Ct
7 values were used to read off relative RNA amounts. The values of NT and NTR mRNA
8 expression were obtained by the relative value for *GAPDH* mRNA. All samples were run in
9 duplicate, and quantitative detection was averaged.

10

11 2.3. Immunohistochemistry

12

13 Immunohistochemical staining was conducted by the streptavidin-biotin-peroxidase
14 method. Formalin-fixed, paraffin-embedded specimens of uncomplicated third trimester
15 pregnancies were cut into 4 μm-thick sections. The tissue sections were deparaffinized in
16 xylene (3×10 min) and dehydrated through graded alcohol (99 %, 80 % and 70 %) to water.
17 Tissue samples were heated to retrieve antigens in Tris-EDTA buffer (pH 9.0) at 120 °C for 5
18 min. Endogenous peroxidase activity was blocked using 0.3 % H₂O₂. The sections were
19 incubated with mouse monoclonal antibody against TrkB (diluted 1:100; R&D Systems,

1 Minneapolis, MN, code MAB397) and rabbit polyclonal antibody against BDNF (diluted
2 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, code SC-546) overnight at 4 °C.
3 Corresponding nonspecific IgG (Dako, Carpinteria, CA) was used as a negative control and
4 processed in parallel. They were then incubated with biotinylated rabbit anti-mouse Ig
5 secondary antibody for TrkB or with biotinylated goat anti-rabbit Ig for BDNF (Nichirei,
6 Tokyo, Japan), followed by incubation with streptavidin-peroxidase complex solution for 30
7 min at room temperature (RT). Peroxidase activity was visualized by treatment with
8 diaminobenzidine. Finally, the nuclei of sections were counterstained with Mayer's
9 hematoxylin and observed under a microscope (Olympus, Tokyo, Japan).

10

11 2.4. Plasma assays

12

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

1 (ELISA) kit (R&D Systems) in duplicate each sample as instructed by the manufacturer.

2 According to the manufacturer's protocol, coefficients of variation for BDNF ELISA were as
3 follows: intra-assay precision and inter-assay precision were 3.8-6.2 % and 7.6-11.3 %,
4 respectively.

5

6 2.5. Tissue and cell culture and oxidative stress

7

8 Placental villous tissues were collected from normal term pregnancies (n = 10) delivered
9 by elective Cesarean section. Tissues were taken from midway between the chorionic and basal
10 plates and were free of visible infarction or calcification. After brief rinsing in ice-cold
11 phosphate-buffered saline (PBS), tissues were placed in ice-cold RPMI 1640 medium (Nacalai
12 Tesque, Kyoto, Japan). Samples were taken to the laboratory and processed within 30 min.
13 They were further dissected into small pieces (about 2-3 mm in diameter), and 3 fragments
14 were placed in 6-well plates with 3 ml culture medium (RPMI 1640 containing streptomycin,
15 penicillin, and 10 % fetal calf serum) per well. Subsequently, tissues were incubated with or
16 without H₂O₂ (100 μM) for 2 h in a culture incubator with 5 % CO₂/95 % air at 37 °C. Placental
17 villous explants were then collected and stored at -80 °C. H₂O₂ concentration was determined
18 according to a previous report [9] showing that 100 μM H₂O₂ was effective to increase
19 apoptosis in the placental explant culture.

1 The JEG-3 (HTB-36) choriocarcinoma cell line was obtained from the American Type
2 Culture Collection (Manassas, VA). The cells were maintained in RPMI medium containing
3 streptomycin, penicillin, and 10 % fetal calf serum at 37 °C with 5 % CO₂ / 95 % air. JEG-3
4 were incubated for 2 h with or without H₂O₂ (5 μM) and harvested. A marked number of
5 JEG-3 cells died in a concentration of 100 μM. After preliminary experiments of 1, 5 and 50
6 μM, we selected 5 μM as an appropriate concentration for the cell culture that showed
7 increased apoptosis but less cell death.

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

10

11 2.6. Apoptosis analysis

12

13 Apoptosis analysis was assessed using the Annexin V-FITC Apoptosis Detection kit I (BD
14 Biosciences, San Jose, CA) by a fluorescence-activated cell sorter (FACS). Annexin V
15 identifies cells in early apoptosis by detecting externalized phosphatidylserine, and propidium
16 iodide (PI) identifies necrotic or late apoptotic cells that have lost plasma membrane integrity.

17 JEG-3 cells on 6 cm culture dishes were administered 50 ng/ml recombinant human (rh)

18 BDNF (PeproTech, Rocky Hill, NJ) 24 h prior to H₂O₂ (5 μM) treatment. Vehicle only was

19 used as a control. This was selected according to the manufacturer's instructions depending

1 on previous reports [28, 29]. We did not perform a dose response experiment but performed
2 the experiment once using 10 ng/ml, which was less effective than 50 ng/ml.

3 For the blocking experiment, 100 nM k252a (inhibitor of pan Trk signaling; Calbiochem,
4 Darmstadt, Germany) or k252b, which is almost equipotent to k252a but without inhibiting
5 activity, was added just prior to rhBDNF (50 ng/ml) administration, followed by H₂O₂
6 treatment 24 h later. After incubation for 2 h with H₂O₂, the cells were harvested by
7 trypsin-EDTA and centrifuged at 3000 rpm for 10 min. The cells were washed twice with PBS
8 (containing 0.2 % fetal calf serum) at RT, resuspended in 100 µl of 1× binding buffer
9 supplemented with 5 µl FITC conjugated Annexin V and 2 µl PI, and incubated at RT in the
10 dark for 15 min according to the manufacturer's instructions. Following the addition of 400 µl
11 of 1× binding buffer, stained cells were kept on ice and subjected immediately to FACS
12 analysis using a FACSCalibur flow cytometer with CellQuest software (BD, Franklin Lakes,
13 NJ). The cell debris and small particles were excluded from analysis. When cells were double
14 stained, 4 different groups of cells were observed: both negative cells (Annexin V (-) / PI (-))
15 were defined as viable cells, cells stainable with Annexin V but not with PI were early
16 apoptotic cells, both positive cells were late apoptotic or necrotic cells, and PI (+) -only cells
17 were debris of dead cells.

18

19 2.7. Cell proliferation assay (WST assay)

20

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

7

8 2.8. Statistical analysis

9

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

17

18 **3. Results**

19

1 3.1. Patient characteristics

2

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

7

8 3.2. NT and NTR mRNA expression in villous tissue and fetal membranes

9

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

1

2 3.3. Localization of BDNF and TrkB

3

4 In the third trimester placenta, BDNF immunostaining was observed in the membranous

5 chorion (Fig. 2A), trophoblast layer and endothelium (Fig. 2B), whereas TrkB

6 immunostaining was observed in the decidua (Fig. 2D), trophoblast layer and endothelium

7 (Fig. 2E). In the trophoblast layer, TrkB was confirmed to localize in both cytotrophoblast

8 cells and syncytiotrophoblast cells in the first and second trimesters (Fig. 2G and H). The

9 localization of TrkB was not different throughout the gestational age (Fig. 2G, H and E) and

10 appeared similar even in PE (Fig. 2I).

11

12 3.4. Placental *TrkB* mRNA expression according to pathological status

13

14 Among 42 samples, including normal term controls, normotensive SGA and PE, *TrkB*

15 mRNA was significantly higher in PE placentas than in normal term controls (Fig. 3A). There

16 was no significant difference between PE with SGA ($0.85 [0.53-0.11] \times 10^{-2}$, $n = 11$) and PE

17 without SGA ($0.47 [0.38-0.94] \times 10^{-2}$, $n = 4$). The increase of *TrkB* in normotensive SGA was

18 not significant. Pearson's correlation tests of 42 samples demonstrated that *TrkB* expression

19 was reversely correlated with neonatal birth weight (Fig. 3B).

1

2 3.5. *TrkB* mRNA expression in discordant twin placentas

3

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

10

11 3.6. Plasma BDNF level

12

13 Maternal plasma BDNF levels were significantly higher in the normotensive SGA and PE
14 group than in non-complicated pregnant women in the third trimester (Fig. 3E). Gestational
15 age did not affect plasma BDNF levels (first: 262.8 pg/ml [130.6-352.7], second: 236.4 pg/ml
16 [155.5-1609.8] and third: 623.8 pg/ml [330.9-1024.0]). In umbilical plasma, BDNF levels
17 were not influenced by the pathological status, such as in PE, normotensive SGA and
18 discordant twins (data not shown).

19

1 3.7. Effect of BDNF/TrkB against oxidative stress in vitro

2

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

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

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

1 As for early apoptosis, a similar result was observed in that k252a treatment significantly
2 induced apoptotic cells (Fig. 5H). On the other hand, viable cells were significantly decreased
3 by k252a administration compared to k252b treatment (Fig. 5I). These results were evaluated
4 as the relative cell number compared to those treated with H₂O₂ and rhBDNF without k252a
5 and k252b.

6

7 3.8. Proliferation of JEG-3 by rhBDNF

8

9 Cell proliferation assay revealed no statistically significant difference in the growth of
10 JEG-3 cultured with rhBDNF compared with vehicle only. The absorbance of the media at
11 450 nm was as follows: 0.086 [0.075-0.099] with rhBDNF vs. 0.118 [0.111-0.130] with
12 vehicle only for 24 h, 0.263 [0.247-0.260] with rhBDNF vs. 0.349 [0.319-0.377] with vehicle
13 only for 48 h (n = 4).

14

15 **4. Discussion**

16

17 In the present study, we investigated the expression profile of NT and NTR and their
18 potential roles in human pregnancy. We present a detailed expression profile for the first time
19 for NT and NTR in human placenta and the fetal membrane. We also investigated the

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

14 We also demonstrated that the expression of *TrkB* was up-regulated in PE despite the
15 presence or absence of SGA and there was no significant difference between these two groups.
16 We did not detect significant up-regulation of TrkB in SGA placenta; however, Mayeur et al.
17 reported that mRNA expression of *TrkB* was significantly increased in human IUGR placenta
18 [30]. This discrepancy seems to be due to the severity of IUGR; the patients in Mayeur's
19 report were more severely affected than our patients. Villous *TrkB* expression correlated with

1 neonatal birth weight and was significantly increased in the smaller twin of discordant twins;
2 therefore, the increase of TrkB expression in human placenta may be related to fetal growth.

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

9 As maternal BDNF reaches the fetal brain through the utero-placental barrier, it
10 contributes to development in mice [32]. Thus, previous studies on NTs during pregnancy
11 mostly focused on the effect on the fetal nervous system; for example, Marx reported that
12 NTs were detectable in human amniotic fluid and their decreased levels may reflect
13 abnormalities in the fetal brain in utero [33]. Although the anti-apoptotic effect of NTs on
14 neuronal and non-neuronal tissues, such as endothelial cells, has already been reported [34],
15 we showed in the present study that BDNF administration decreased the rate of apoptotic or
16 necrotic cells in H₂O₂-treated JEG-3 cells, which suggests that BDNF/TrkB signaling also has
17 an anti-apoptotic effect on trophoblast cells. Placental apoptosis increased with placental
18 growth according to gestational age even in normal development [35]; however, it was marked
19 in complicated pregnancies, such as hydatidiform mole, PE and IUGR [6-8, 36, 37]. An

1 increased number of syncytial knots were observed in PE and IUGR placentas and could be
2 replicated in vitro by reactive oxygen species (ROS) or hypoxia [38]. Our in vitro findings
3 suggest that BDNF/TrkB can play a protective role in the placenta against oxidative stress by
4 reducing or slowing the increase of apoptotic cells in PE or SGA.

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

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

1 patients.

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

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

17

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2

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6

7

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3

4

1 **Figure Legends**

2

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

10

11 **Fig. 2.** Localized expression of BDNF and TrkB in human placenta.

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

16

17 **Fig. 3.** *TrkB* mRNA expressions and plasma *BDNF* levels in the pathological status.

18 (A) *TrkB* mRNA expression in villous tissue in normal term control (n = 16), normotensive
19 SGA (n = 11) and PE (n = 15). (B) Correlation between neonatal birth weight and *TrkB*
20 expression in villous tissue of 42 patients. SD: standard deviation, r : correlation coefficient.

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

5

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

7 *TrkB* mRNA in villous explants (A, n = 10) and JEG-3 cells (B, n = 6) cultured with or
8 without H₂O₂ for 2 hours. The mRNA expressions were normalized by *GAPDH*. * $p < 0.05$,
9 ** $p < 0.01$

10

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

12 Induction of apoptosis by H₂O₂ and the effect of BDNF on JEG-3 cells were examined by
13 FACS (n = 6). (A-C) Representative FACS plots by double-staining with Annexin V and PI.
14 Non-treatment group (A) treated with H₂O₂ (B) and H₂O₂ plus rhBDNF (C). Positive cells for
15 both were defined as late apoptosis or necrosis (D, G); Annexin V positive/PI negative as
16 early apoptosis (E, H); both negative as viable (F, I). (D-F) Relative number of JEG-3 cells
17 treated with H₂O₂ (lane 2) and H₂O₂ plus rhBDNF (lane 3) compared to non-treatment group
18 (lane 1). (G-I) Inhibition of the anti-apoptotic effect of BDNF by k252a, an inhibitor of Trk.
19 Relative number of JEG-3 cells pretreated with k252a (lane 2) or k252b (lane 3) followed by

- 1 H₂O₂ and rhBDNF compared to cells treated with H₂O₂ and rhBDNF alone (lane 1). * $p < 0.05$,
- 2 ** $p < 0.01$.

Table 1
Patient characteristics.

	Controls (n = 16)	Normotensive SGA (n = 11)	PE (n = 15)	<i>p</i> 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.	<i>p</i> < 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.	<i>p</i> < 0.001	<i>p</i> < 0.001
Diastolic blood pressure (mmHg)	65±2 (50-77)	66±3 (48-80)	96±3 (70-110)	n.s.	<i>p</i> < 0.001	<i>p</i> < 0.001
Neonatal weight (g)	2954 [2715-3058]	1550 [1311-2273]	1657 [1096-2021]	<i>p</i> < 0.001	<i>p</i> < 0.001	n.s.
Placental weight (g)	497 [468-570]	360 [275-369]	288 [245-384]	<i>p</i> < 0.01	<i>p</i> < 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
<i>TrkA</i>	5' - ACTGAGCTCTACATCGAGA	5' - CTGCACAGTTTTCCAGGA	NM_002529
<i>TrkB</i>	5' - AGAGGCTAAATCCAGTCCA	5' - CAGGTTACCAACATCCCAA	NM_006180
<i>TrkC</i>	5' - ATACTACCAAGAGGGAGAGA	5' - TGGGTCACAGTGATAGGA	NM_001007156
<i>NGF</i>	5' - ACTGAGGTGCATAGCGTA	5' - GTGTCAAGGGAATGCTGA	NM_002506
<i>BDNF</i>	5' - GTGAGAAGAGTGATGACCA	5' - CTCTTCTATCACGTGTTCGA	NM_170735
<i>NT-3</i>	5' - TGGCATCCAAGGTAACAACA	5' - GGCAGGGTGCTCTGGTAAT	NM_002527
<i>NT-4</i>	5' - CCCTCTCCTGAGATGTCA	5' - GGAGGAGGAAAAGGAGGA	NM_006179

Fig. 1.

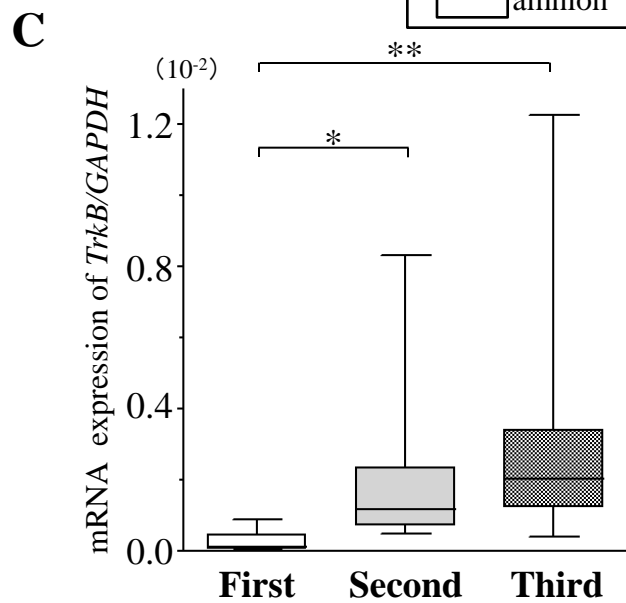
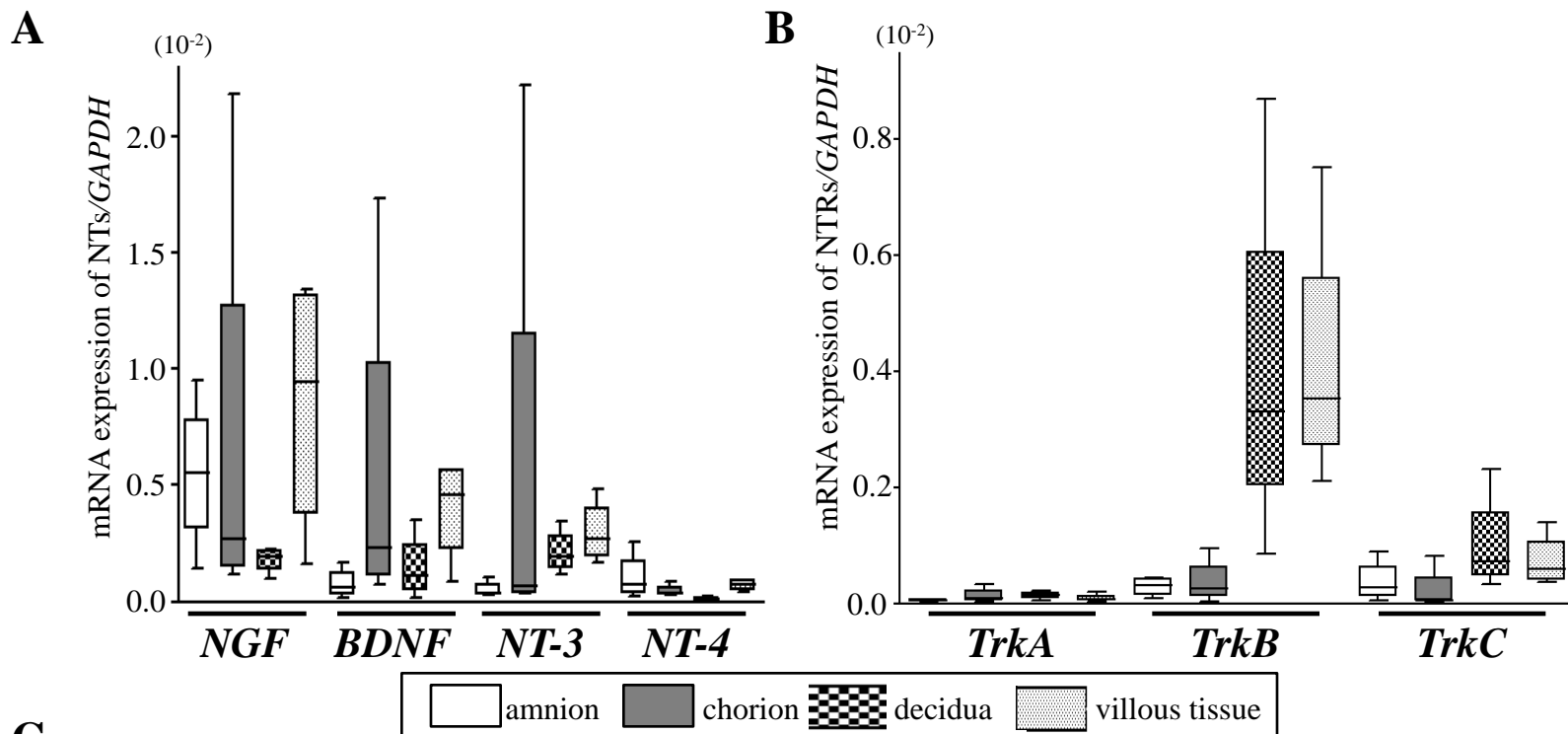


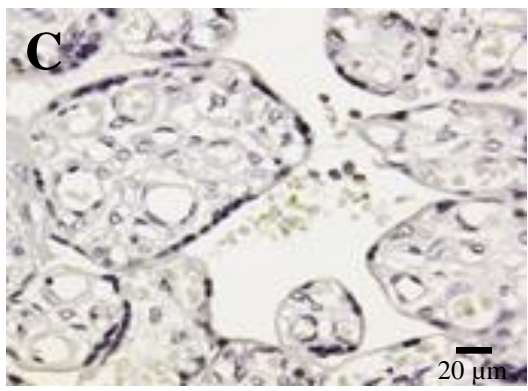
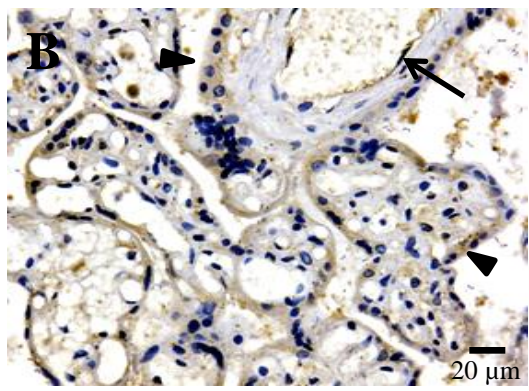
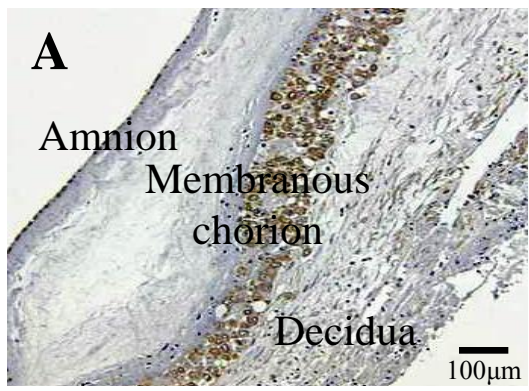
Fig. 2.

Fetal membrane

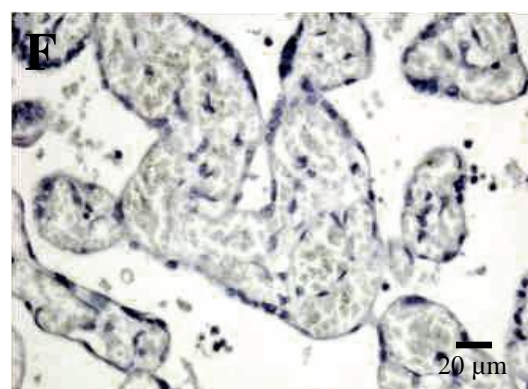
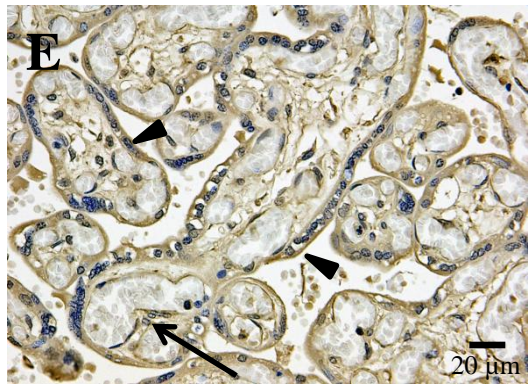
Villous tissue

Negative control

BDNF



TrkB



First

Second

PE

**TrkB
in villous
tissue**

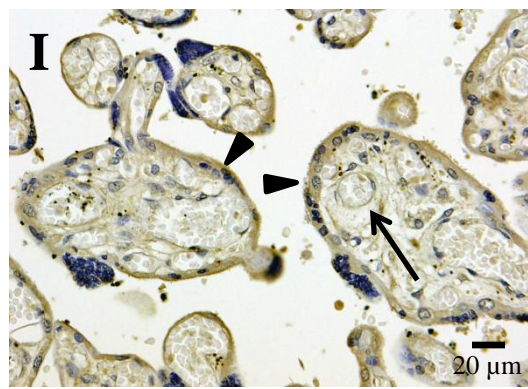
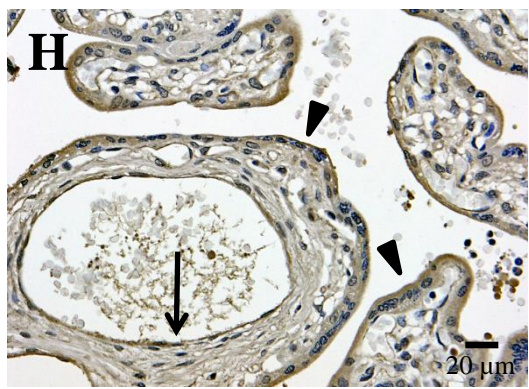
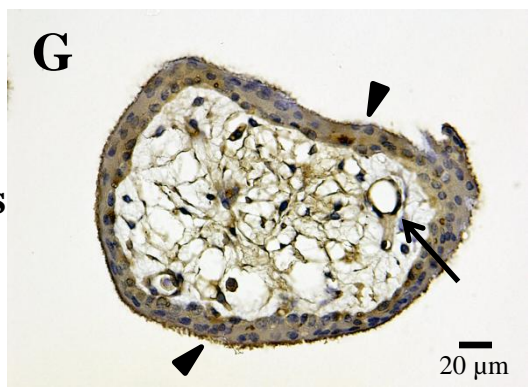


Fig. 3.

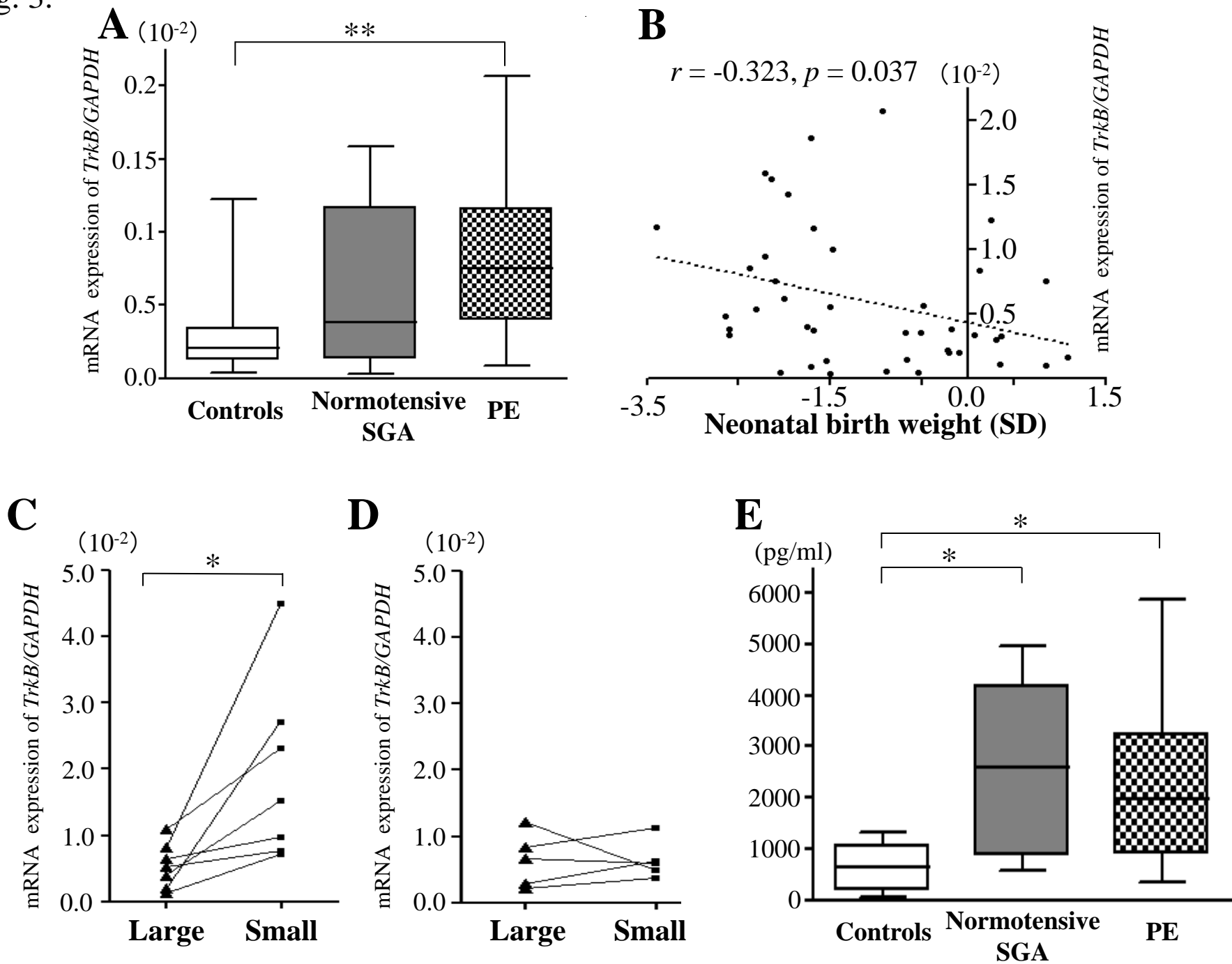


Fig. 4.

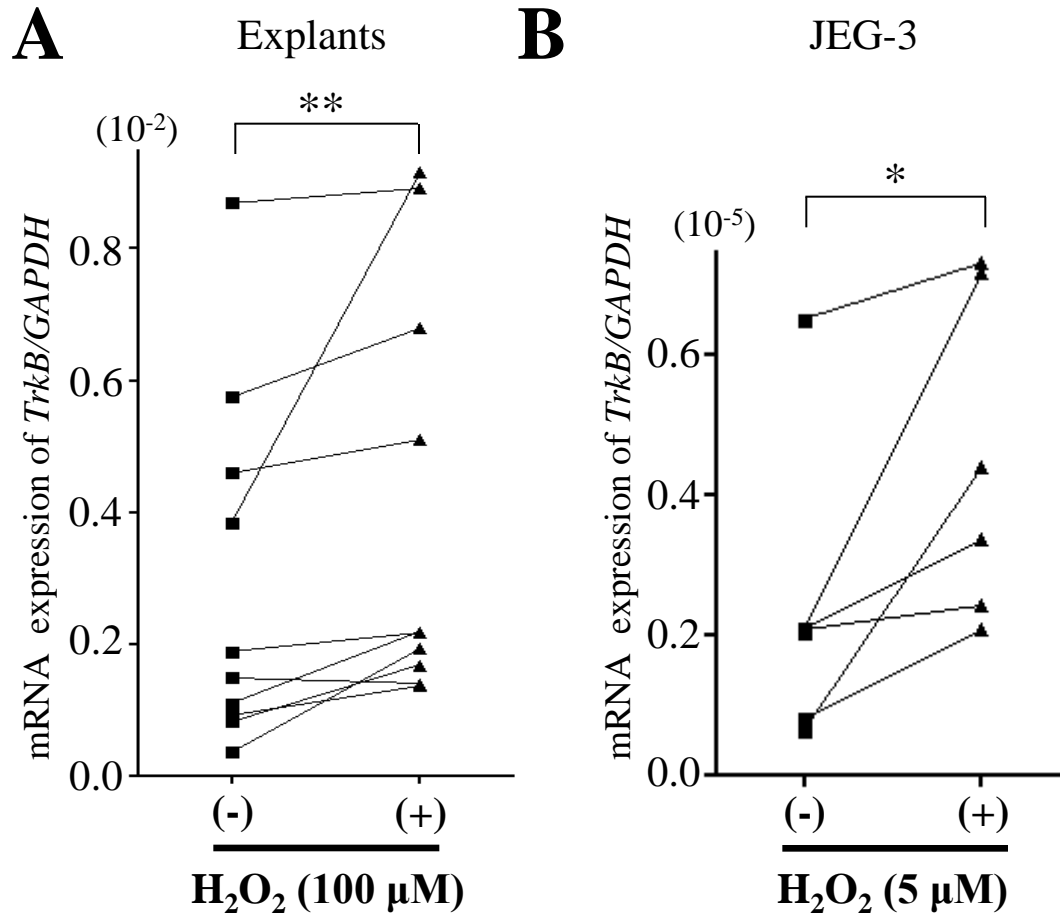


Fig. 5.

