Brain-Derived Neurotrophic Factor Promotes Implantation and Subsequent Placental Development by Stimulating Trophoblast Cell Growth and Survival

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Successful implantation of the blastocyst and subsequent placental development is essential for reproduction. Expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5, together with their receptor, tyrosine kinase B (TrkB), in trophectoderm cells of blastocyst suggests their potential roles in implantation and placental development. Here we demonstrated that treatment with BDNF promoted blastocyst outgrowth, but not adhesion, *in vitro* and increased levels of the cell invasion marker matrix metalloproteinase-9 in cultured blastocysts through the phosphatidylinositol 3-kinase pathway. After implantation, BDNF and neurotrophin-4/5 proteins as well as TrkB were expressed in trophoblast cells and placentas during different stages of pregnancy. Both TrkB and its ligands were also expressed in decidual cells. Treatment of cultured trophoblast cells with the TrkB ectodomain, or a Trk receptor inhibitor K252a, suppressed cell growth as reflected by decreased proliferation and increased apoptosis, whereas an inactive plasma membrane nonpermeable K252b was ineffective. Studies using the specific inhibitors also indicated the importance of the phosphatidylinositol 3-kinase/Akt pathway in mediating the action of TrkB ligands. *In vivo* studies in pregnant mice further demonstrated that treatment with K252a, but not K252b, suppressed placental development accompanied by increases in trophoblast cell apoptosis and decreases in placental labyrinth zone at midgestation. *In vivo* K252a treatment also decreased fetal weight at late gestational stages. Our findings suggested important autocrine/paracrine roles of the BDNF/TrkB signaling system during implantation, subsequent placental development, and fetal growth by increasing trophoblast cell growth and survival. **(*Endocrinology* 150: 3774–3782, 2009)**

In mammals, fertilized oocyte undergoes mitotic cell divisions, eventually developed to the blastocyst with two distinct cell lineages, the inner cell mass (ICM) and a layer of trophectoderm cells surrounding the ICM. The ICM cells form all three germ layers and all tissues of the embryo. They also contribute to the formation of extraembryonic membranes. The trophectoderm cells differentiate during embryonic development to form the invasive trophoblast that mediates implantation of embryos into the uterine wall. Implantation of the blastocyst and subsequent trophoblast growth are crucial steps in the establishment of pregnancy and the development of placenta. Implantation as well as successful placental development and pregnancy is temporally and spatially regulated by multiple growth factors and cytokines, synthesized by different cell types at the maternal-fetal interface, in autocrine, paracrine, and/or juxtacrine manners (1, 2).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of proteins known to activate the high-affinity tyrosine kinase B (TrkB) receptor together with the pan-neurotrophin low-affinity coreceptor p75 (3). Although neurotrophins are widely expressed in the central nervous system and are important for neuronal survival and differentiation (4), they also play important roles in nonneuronal tissues (5). In the ovary, BDNF was found to be essential for the development of early follicles (6–8) and the nuclear and cytoplasmic maturation of

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Abbreviations: BDNF, Brain-derived neurotrophic factor; hCG, human chorionic gonadotropin; ICM, inner cell mass; MMP, matrix metalloproteinase; NT, neurotrophin; PI3K, phosphatidylinositol 3-kinase; TrkB, tyrosine kinase B; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling.

oocytes (9–12). Using *in vitro* and *in vivo* analyses, we recently found the expression of TrkB and its ligands, BDNF and neurotrophin (NT)-4/5 in preimplantation embryos as well as pregnant oviducts and uteri of mice. We demonstrated that BDNF acts on TrkB in the preimplantation embryos to promote early embryonic development and suppress embryo apoptosis in a paracrine/autocrine manner (13).

In blastocysts, we found the expression of TrkB and its ligands in trophectoderm cells and a promotional effect of BDNF on the proliferation of trophectoderm cells before implantation (13). Here we demonstrated the promotion of blastocyst outgrowth, but not adhesion, after BDNF treatment during the periimplantation period. Our RT-PCR and immunoassays further indicated the expression of TrkB and its ligands in placenta and isolated trophoblast cells after implantation. Using soluble ectodomains of TrkB and Trk receptor inhibitors, we demonstrated autocrine/paracrine regulatory roles of the TrkB signaling system in trophoblast cell growth and survival *in vitro*. In pregnant mice, we further showed the important role of the TrkB signaling system for placental development and fetal growth.

Materials and Methods

Animals

To obtain preimplantation embryos, B6D2F1 mice at 25 d of age (CLEA Japan, Tokyo, Japan) were treated sequentially with pregnant mare serum gonadotropin (Calbiochem, Cambridge, MA) and human chorionic gonadotropin (hCG; ASKA Pharmaceutical. Co., Ltd., Tokyo, Japan). The animals were allowed to mate immediately after hCG treatment. At 46–47 h after hCG injection, two-cell-stage embryos were obtained by flushing the oviducts of mated mice for *in vitro* culture as described (14). Plasma was sampled from nonpregnant female mice at 9 wk of age, whereas placenta, amniotic fluid, and maternal plasma were obtained from pregnant animals of comparable age at d 10, 15, or 18 of pregnancy. The presence of a vaginal plug designated d 0 of pregnancy. The care and use of animals was approved by the Animal Research Committee, Akita University School of Medicine.

Blastocyst adhesion and outgrowth assays

For blastocyst adhesion and outgrowth assays, single two-cell-stage embryos were placed in 5-μl drops of KSOM medium (Chemicon, Temecula, CA) in the presence of 3% fetal bovine serum and covered with mineral oil as described (15). The embryos were then transferred to freshly prepared media every 24 h. After 72 h of culture, the embryos developed to expanded blastocyst stage were cultured with or without 10 ng/ml of recombinant human BDNF (Peprotech, Rocky Hill, NJ). Blastocysts that adhered to the culture plate were designated as adhesion blastocysts. When trophoblast cells had grown outward from the adhered blastocysts and the primary giant trophoblast cells became visible, these embryos were designated as outgrowth blastocysts. The proportions of blastocysts undergoing adhesion and outgrowth were estimated at 24 and 48 h of BDNF treatment, respectively (15, 16). The proportions of hatched blastocysts showing adhesion or outgrowth were used to estimate the implantation capacity of the blastocysts *in vitro*. Specificity of the effects of BDNF was confirmed by cotreatment with the soluble ectodomain of TrkB (10 μg/ml; R&D Systems, Minneapolis, MN), a pan-specific Trk receptor inhibitor (K252a, 100 nM; Calbiochem) (17), or the inactive plasma membrane nonpermeable K252b (100 nM; Calbiochem) (18). The doses of BDNF, the TrkB ectodomain, K252a, or K252b chosen for these experiments were based on previous studies (13). For controls, embryos were cultured in KSOM medium only. To analyze the involvement of phosphatidylinositol 3-kinase (PI3K) pathway in the BDNF induction of blastocyst outgrowth, single expanded blastocyst-stage embryos were cultured with 10 ng/ml BDNF with or without a PI3K inhibitor, LY294002 (1–10 μM; Sigma, St. Louis, MO) or its inactive analog, LY303511 (10 μM; Calbiochem). The embryos were cultured and the proportions of blastocysts undergoing outgrowth were estimated as described above. At the end of a 48-h culture period, transcript levels for the cell adhesion marker integrin β-3 (2, 19) and the cell invasion marker matrix metalloproteinase (MMP)-9 (2, 20–22) in blastocysts showing outgrowth were measured using real-time RT-PCR.

Trophoblast cell cultures and apoptosis detection

Isolated trophoblast cells were allowed to attach for 2 h at 37 C in 5% CO_2-95% air, and the culture medium was replaced with a fresh one. Cells were incubated overnight before treatment with different inhibitors. After preincubation, the cells were cultured for 24 h with or without different doses of the TrkB ectodomain, K252a, or K252b in the absence of fetal bovine serum to determine their effects on cell proliferation. To measure the proportion of apoptosis, some cells were subjected to a quantitative enzyme immunoassay at 8 h of culture. The culture period for apoptosis measurement was chosen based on the assumption that DNA fragmentation is an earlier event than eventual cell death. Apoptosis was quantified by detecting cytoplasmic histone-associated DNA fragments (cell death detection-ELISA kit; Roche, Indianapolis, IN). Samples were placed in a streptavidin-coated microtiter plate and incubated with a mixture of biotinylated antihistone antibodies, peroxidase-labeled anti-DNA antibodies, and the incubation buffer [1% BSA (Sigma), 0.5% Tween 20 (Sigma), and 1 mM EDTA (Sigma) in PBS] for 2 h. The antibodies bound to the histone- and DNA-component of apoptotic nucleosomes, respectively. The immunocomplexes were fixed to the microtiter plate by streptavidin-biotin interaction. After removal of unbound antibodies by washing, the retained peroxidase-linked complexes were incubated with a substrate, 2,2′-azino-di(3-ethylbenzthiazolin-sulfonate), resulting in color development. Quantification of the nucleosomes was performed based on photometrical determination of absorbance at 405 nm by using a plate reader (Bio-Rad, Hercules, CA). Results are expressed as apoptotic enrichment factors calculated using the following formula: apoptotic enrichment factor = absorbance of the sample/absorbance of the corresponding control (cells cultured with the medium alone).

Cell proliferation was evaluated by spectrophotometry using a WST-1 colorimetric test kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. This system is based on the cleavage of a sulfonated tetrazolium salt WST-1 by mitochondrial dehydrogenases to form formazan dye for detecting living, but not dead, cells. The augmentation in enzyme activity leads to an increase in the formazan dye formed. Absorbance of the formazan dye was quantified after 30 min of incubation with the WST-1 reagent by using a plate reader at 450 nm (reference wavelength 750 nm).

In vivo analysis

To explore the roles of endogenous TrkB ligands during placental development *in vivo*, female B6D2F1 mice at 9 wk of age were allowed to mate with fertile males. Subcutaneous administration of K252a (10 or 100 μg) was performed every 3 d from d 5 to 18 of pregnancy thus corresponding to the postimplantation period *in vivo*. For negative controls, treatment with K252b (100 μg/injection) or vehicle alone was used. Placental and fetal wet weights were measured at d 10, 15, and 18 of pregnancy. After fixation of placentas with Bouin's solution as described above, placentas were sectioned at the center from maternal to fetal sides. Sections were stained with hematoxylin and eosin to identify the labyrinth and junctional zones according to morphological differences. Thickness of the labyrinth and junctional zones close to the central portion of both sides of halved placentas were measured under a light microscope using a micrometer (Olympus Corp., Tokyo, Japan) (23, 24), and the mean values from two sections per placenta were used for analyses. At d 18 of pregnancy, apoptosis in placenta was assayed by

detecting DNA fragmentation using *in situ* terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) (25).

Statistical analysis

Statistical analysis was carried out by using Mann-Whitney *U* test for paired comparison and the one-way ANOVA followed by Fisher's protected least significant difference for multiple group comparison. *In vivo* data were analyzed using the linear mixed model repeated measures ANOVA. Results are presented as mean ± SEM of at least three separate experiments.

Methods for trophoblast cell dissociation, RT-PCR, immunocytochemistry, immunohistochemistry, ELISA, MMP-9 activity assay, cell-based assay for Akt phosphorylation, and TUNEL assay are described in supplemental *Materials and Methods*, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Results

Effects of BDNF on blastocyst adhesion and outgrowth

To characterize the roles of BDNF during the periimplantation period, blastocyst adhesion and outgrowth were examined in culture. Expanded blastocyst-stage embryos were cultured for 24 h to estimate blastocyst hatching and subsequent attachment. Blastocysts were then cultured for an additional 24 h to evaluate blastocyst outgrowth. Treatment with BDNF promoted blastocyst outgrowth as shown by increases in the proportion of blastocysts showing outgrowth (Fig. 1A). The ability of BDNF to promote blastocyst outgrowth was blocked by cotreatment with either the TrkB ectodomain or K252a but not K252b (Fig. 1A), suggesting mediation by the TrkB receptor. In contrast, BDNF treatment did not affect the adhesion of embryos to the culture substrate (Fig. 1B). In addition, blockage of the actions of endogenous BDNF after treatment with the TrkB ectodomain or K252a did not suppress blastocyst adhesion and outgrowth (Fig. 1, A and B). During the last 24 h of culture, more blastocysts adhered to the culture dish and initiated outgrowth. Thus, in some groups, the proportion of hatched blastocysts showing outgrowth was higher than those showing adhesion. We also determined the effects of BDNF on transcript levels for the cell adhesion marker integrin β-3 and the cell invasion marker MMP-9 in blastocysts showing outgrowth. Although another cell invasion marker, MMP-2 (26, 27), was not detected in blastocysts showing adhesion and outgrowth (Fig. 1C), treatment with BDNF increased the mRNA levels for MMP-9, but not integrin β-3, in cultured blastocysts (Fig. 1D). In the conditioned medium of blastocyst cultures, the levels of both total and activated MMP-9 proteins were increased by treatment with BDNF (Fig. 1E).

We further analyzed the role of the PI3K signaling pathway as downstream mediators of the BDNF/TrkB-induced blastocyst outgrowth. The ability of BDNF to promote blastocyst outgrowth was suppressed by cotreatment with a PI3K inhibitor, LY294002, but not with its inactive analog, LY303511, in a dose-dependent manner (Fig. 1F). Furthermore, treatment with LY294002 inhibited the BDNF stimulation of MMP-9 transcript levels in cultured blastocysts (Fig. 1G).

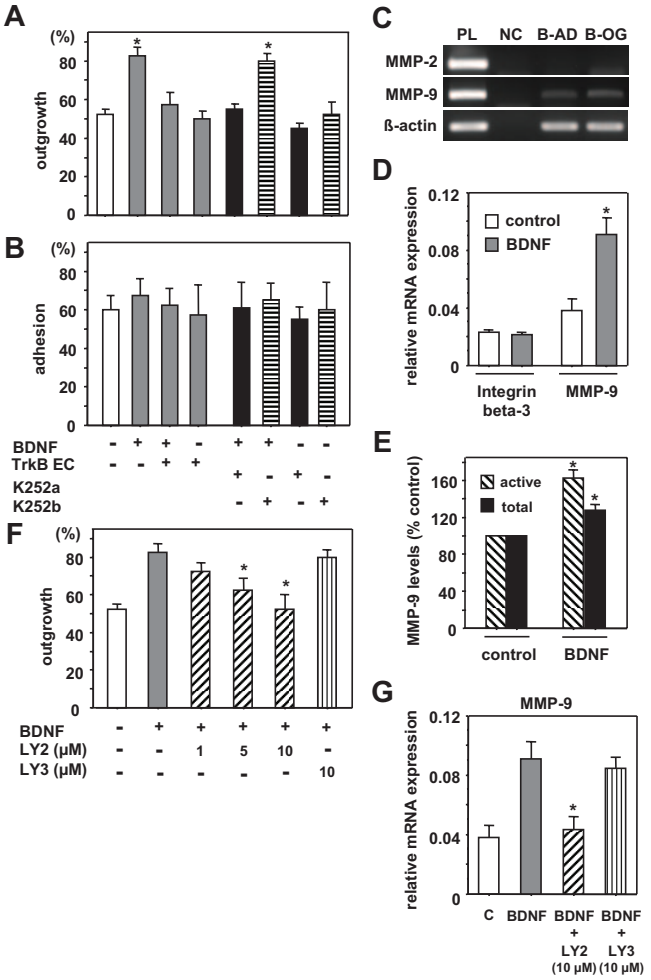

FIG. 1. BDNF stimulation of mouse blastocyst outgrowth through the PI3K pathway. Expanded blastocyst-stage embryos were cultured with or without BDNF (10 ng/ml) to evaluate the effects of BDNF on blastocyst outgrowth (A) and adhesion (B). Specific effects of BDNF on blastocyst outgrowth and adhesion were evaluated by cotreatment with the TrkB ectodomain (TrkB EC; 10 μg/ml), K252a (100 nM), or K252b (100 nM). (Mean ± SEM, n ≥ 4, 40–120 embryos per group). *, $P < 0.05$ *vs.* control group. C, Expression of MMP-2 and MMP-9 in blastocysts. Placentas (PL) at d 15 of pregnancy were used as positive controls. Levels of β-actin served as loading controls. NC, Negative control; B-AD, blastocyst showing adhesion; B-OG, blastocyst showing outgrowth. D, Stimulation of MMP-9 mRNA (D) and protein (E) expression in blastocysts after BDNF treatment. Expanded blastocyst-stage embryos were cultured without (control) or with BDNF (10 ng/ml). After 48 h of culture, integrin β-3 and MMP-9 transcript levels in blastocysts showing outgrowth were quantified using real-time RT-PCR and normalized using those for β-actin in the same sample (mean ± SEM, n = 5). Conditioned medium at 48 h after culture was also collected and subjected to measurement of total and activated MMP-9 proteins by ELISA based the MMP-9 activity assay (mean ± SEM, n = 5). Results are expressed as percent of controls. *, $P < 0.05$. Effects of a PI3K inhibitor on the BDNF stimulation of blastocyst outgrowth (F) and MMP-9 expression in the blastocysts (G) are also investigated. Expanded blastocyst-stage embryos were cultured without [control (C)] or with BDNF (10 ng/ml) in the presence or absence of an inhibitor for PI3K, LY294002 (LY2), or its inactive analog, LY303511 (LY3). At 48 h after culture, the proportion of hatched blastocysts showing outgrowth was evaluated (mean ± SEM, n = 4, 40 embryos per group). MMP-9 transcript levels in blastocysts showing outgrowth were quantified using real-time RT-PCR and normalized using those for β-actin in the same sample (mean ± SEM, n = 5). *, $P < 0.05$ *vs.* BDNF group.

Expression of transcripts for neurotrophins and Trk receptors in placentas and isolated trophoblast cells

BDNF stimulation of the outgrowth of trophoblast cells from cultured blastocysts prompts us to investigate the role of BDNF

in trophoblast cell development during postimplantation stages. Trophoblast cells were isolated from placentas for RT-PCR analyses to determine the expression of neurotrophins and Trk receptors. All Trk ligand-receptor pairs, including BDNF and NT-4/5-TrkB, nerve growth factor-tyrosine kinase A, and neurotrophin-3-tyrosine kinase C, were expressed in both placentas and isolated trophoblast cells (Fig. 2A). In contrast, the pan-neurotrophin low-affinity coreceptor p75 mRNA was not detectable in isolated trophoblast cells (Fig. 2A). The identity of isolated cells was confirmed by immunofluorescent staining for cytokeratin and vimentin as markers for epithelial cells and mesenchymal cells, respectively (28). Nearly all cells were positive for cytokeratin and negative for vimentin (Fig. 2B), indicating that the cultured cells contained minimal fetal fibroblasts or endothelial cells.

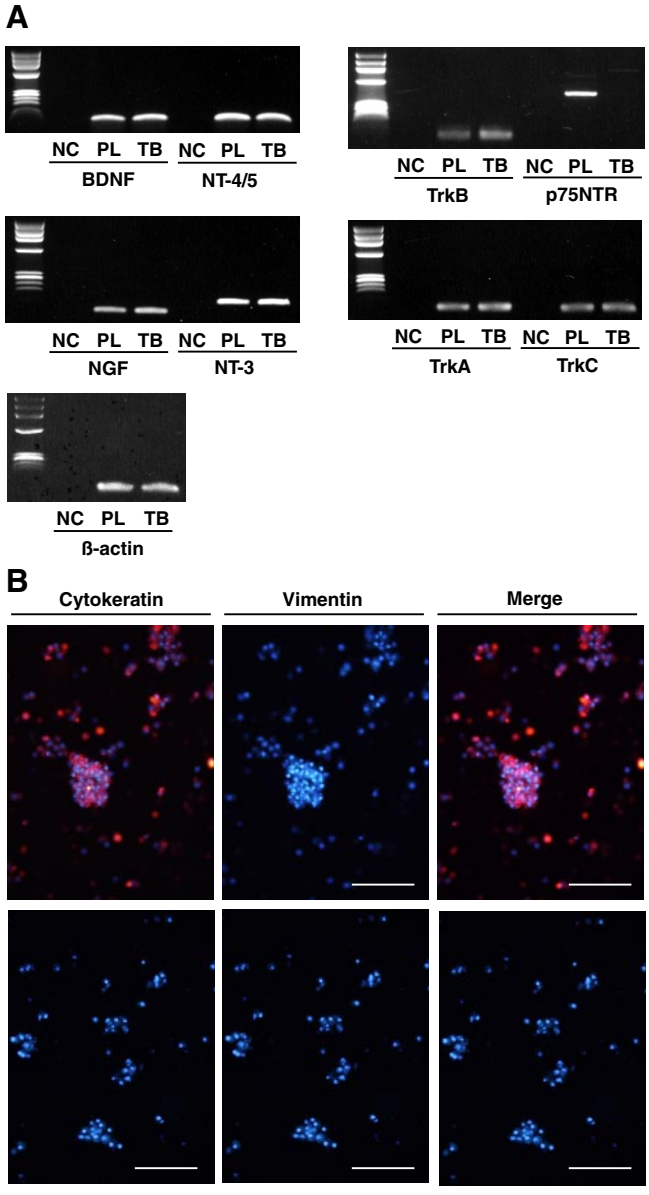

FIG. 2. Expression of BDNF, NT-4/5, and TrkB transcripts in mouse placentas and isolated trophoblast cells. A, Expression of neurotrophins and Trk receptors mRNAs in placentas and isolated trophoblast cells was detected by RT-PCR. Levels of β-actin serve as loading controls. No template DNA was included for negative controls. NGF, Nerve growth factor; NT-3, neurotrophin-3; TrkA, tyrosine kinase A; TrkC, tyrosine kinase C; PL, placenta; TB, isolated trophoblast cell; NC, negative control. Placentas and isolated trophoblast cells were obtained from animals at d 15 of pregnancy. B, Identification of isolated trophoblast cells by coexpression of cytokeratin and vimentin. Most of the cells were positive for an epithelial cell marker, cytokeratin (*red*), but not for a mesenchymal cell marker, vimentin (*green*). Cellular nucleic acids were stained (*blue*) by using Hoechst 33342. Three lower panels depict sections stained with nonimmune IgG and serve as controls. *Scale bars*, 100 μm.

Placental cell types expressing BDNF, NT-4/5, and TrkB proteins

The localization of BDNF, NT-4/5, and TrkB proteins in placenta was determined by using immunohistochemistry. As shown in Fig. 3B, staining for both BDNF and NT-4/5 ligands as well as their receptor TrkB was observed in trophoblast cells of placentas at d 15 of pregnancy in a cell type-specific manner. Strong BDNF signal was detected in spongiotrophoblasts (Fig. 3B, *left panels*), whereas NT-4/5 staining was found in trophoblast giant cells and spongiotrophoblasts (Fig. 3B, *middle panels*). However, NT-4/5 signals in both cell types were weaker than those for BDNF. In contrast, TrkB staining was localized to labyrinth trophoblasts and spongiotrophoblasts (Fig. 3B, *right panels*). In addition, uterine decidual cells were stained with antibodies to both ligands and TrkB (Fig. 3B). We also determined placental cell types expressing BDNF, NT-4/5, and TrkB proteins at different developing stages. At d 10 of pregnancy, staining for both BDNF and NT-4/5 was found in labyrinth trophoblasts in addition to the same placental cell types expressing these ligands at d 15 of pregnancy (Fig. 3A), whereas localization of BDNF and NT-4/5 antigens at d 18 of pregnancy was similar to those at d 15 of pregnancy (data not shown). Placental cell types expressing TrkB protein were not changed at all the pregnant days examined (Fig. 3, A and B).

Temporal expression of BDNF, NT-4/5, and TrkB in placentas during the postimplantation period

The expression of BDNF, NT-4/5, and TrkB in placentas was examined by ELISA and real-time RT-PCR during the postimplantation period. In the placentas, ELISA analyses indicated that BDNF protein levels were 8-fold higher than those of NT-4/5 at all the pregnant days examined, and both BDNF and NT-4/5 protein levels were high in mice at d 10 of pregnancy and gradually decreased during pregnancy progression (Fig. 4A). Similar changes of BDNF and NT-4/5 proteins were detected in amniotic fluid during pregnancy based on the ELISA (Fig. 4B). In contrast, the levels of BDNF protein were very low and the levels of NT-4/5 were under the detection limit (7.8 pg/ml) before and after pregnancy in maternal plasma (Fig. 4C). In contrast, quantitative real-time RT-PCR analyses indicated that TrkB transcript levels in placentas were low in mice at d 10 of pregnancy and increased at d 15 of pregnancy, and maintained at similar levels until d 18 of pregnancy (Fig. 4D).

In vitro suppression of endogenous TrkB signaling on trophoblast cell growth

The expression of both TrkB ligands and receptors in different trophoblast cell types suggests that the TrkB signaling system

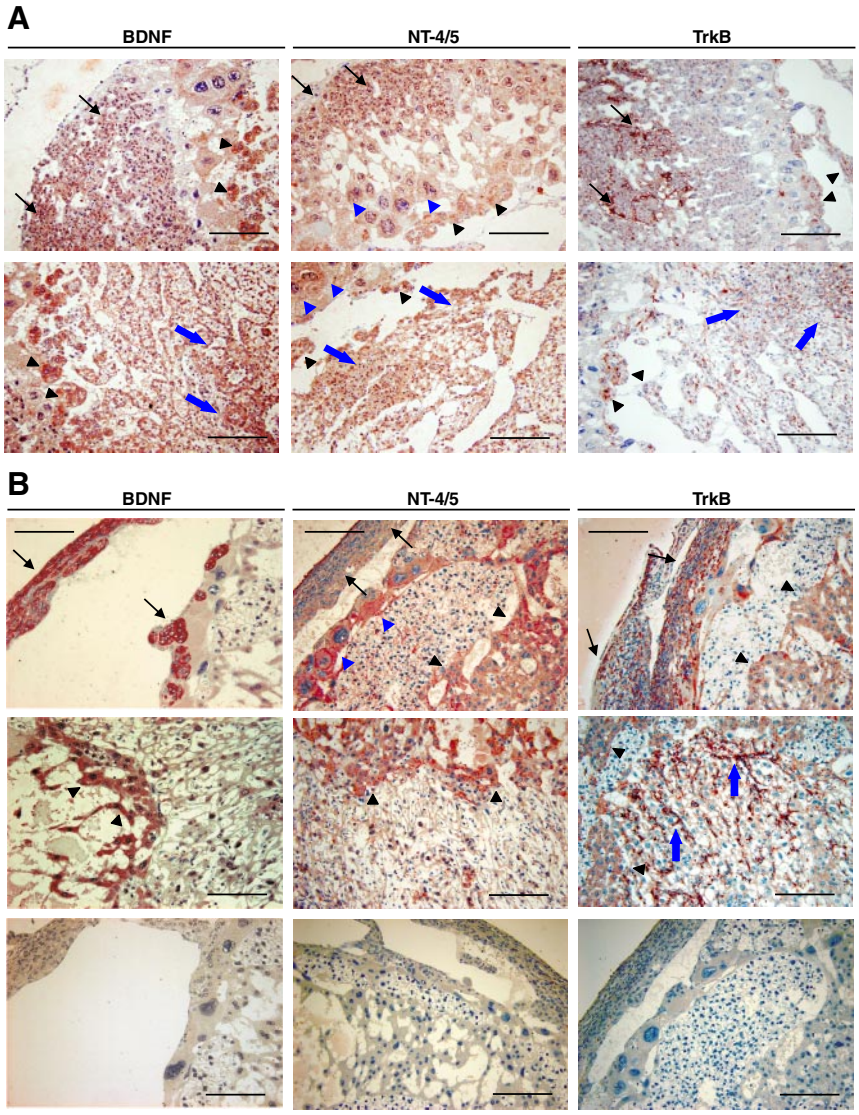

FIG. 3. Localization of BDNF, NT-4/5, and TrkB antigens in mouse placentas. Immunohistochemical detection of BDNF, NT-4/5, and TrkB in placentas were obtained from animals at d 10 (A) and 15 (B) of pregnancy. BDNF was found in spongiotrophoblasts (*black arrowheads*) and decidual cells (*arrows*) at d 10 and 15 of pregnancy and in labyrinth trophoblasts (*blue arrows*) at d 10 of pregnancy. NT-4/5 was found in trophoblast giant cells (*blue arrowheads*), spongiotrophoblasts (*black arrowheads*), and decidual cells (*arrows*) at d 10 and 15 of pregnancy and in labyrinth trophoblasts (*blue arrows*) at d 10 of pregnancy, and its signal was weaker than that of BDNF. In contrast, TrkB was found in labyrinth trophoblasts (*blue arrows*) and decidual cells (*arrows*), whereas weaker staining was found in spongiotrophoblasts (*black arrowheads*) at d 10 and 15 of pregnancy. *Upper* and *middle panels* are specific staining, whereas *lower panels* depict sections stained with nonimmune IgG and serve as controls in B. *Scale bars,* 200 μm.

could play a role through autocrine/paracrine manner during placental development. To determine whether TrkB ligands act as survival factors for trophoblast cells, we evaluated apoptosis and proliferation of cultured trophoblast cells treated with TrkB ectodomain and K252a. As shown in Fig. 5A, treatment with either the TrkB ectodomain or K252a but not the inactive K252b, increased apoptosis in trophoblast cells isolated from the placentas at d 10 of pregnancy in a dose-dependent manner. Using WST-1 assays, we also detected decreases in the absorbance of the formazan dye formed (Fig. 5B), indicating that the suppression of endogenous TrkB signaling led to an inhibition of cell proliferation. The abilities of the TrkB ectodomain and K252a to increase apoptosis and decrease trophoblast cell proliferation were also detected at d 15 of pregnancy (Fig. 5, C and D). Due to extreme viscosity, trophoblast cells were difficult to dissociate from placentas at d 18 of pregnancy.

Akt is a direct downstream target of the PI3K pathway and has prosurvival and cell proliferative activities (29). Thus, we determined Akt activity by quantification of the levels of total Akt and phosphorylated Akt in cultured trophoblast cells. Although changes in total Akt levels were insignificant after treatment with either TrkB ectodomain or K252a, these treatments decreased the levels of phosphorylated Akt (data not shown). Indeed, treatment with either the TrkB ectodomain or K252a, but not the inactive K252b, reduced the phosphorylated Akt to total Akt ratio (Fig. 5E), indicating that the endogenous TrkB signaling is mediated by the Akt pathway.

In vivo effects of a Trk receptor inhibitor on placental growth

By using the Trk receptor inhibitor, K252a, we examined the role of endogenous TrkB ligands during placental development *in vivo*. Consistent with the expression of TrkB in specific cell types of trophoblast cells and decidual cells, the proportion of TUNEL-positive nuclei increased after K252a treatment in labyrinth trophoblasts (Fig. 6A, *upper panel*), some spongiotrophoblasts (Fig. 6A, *middle panel*), and decidual cells (Fig. 6A, *lower panel*). In positive controls treated with deoxyribonuclease I, all nuclei showed TUNEL signals, whereas no TUNEL-positive nuclei was observed in negative controls (data not shown).

We further determined suppressive effect of K252a on placental development throughout gestation. As shown in Fig. 6, B and C, K252a treatment decreased the thickness of the labyrinth zone (Fig. 6B) but not junctional zone (Fig. 6C). Although treatment of pregnant mice with K252a did not alter placental and fetal weights at d 10 of pregnancy (Fig. 6, D and E), K252a treatment decreased placental weight but not fetal weight at d 15 of pregnancy (Fig. 6, D and E). At d 18 of pregnancy, treatment with K252a decreased both placental and fetal weights in a dose-dependent manner (Fig. 6, D and E). The fetal to placental weight ratio was higher in K252a treated mice compared with controls after 15 and 18 d of pregnancy (Fig. 6F). In contrast, treatment with K252b was ineffective for all parameters tested. Because the numbers of implanted embryos were not significantly different among all groups at d 18 of pregnancy (vehicle, 10.7 ± 0.9; 10 μg K252a/injection, 10.3 ± 0.9; 100 μg

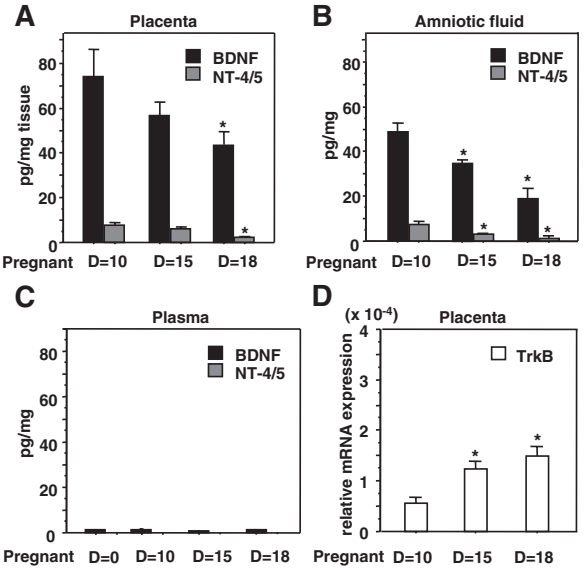

FIG. 4. Temporal expression of BDNF, NT-4/5, and TrkB in mouse placentas. BDNF and NT-4/5 protein or TrkB transcript levels were quantified using ELISA (A: placenta; B: amniotic fluid; C: plasma) or real-time RT-PCR (D: placenta), respectively. Levels of BDNF and NT-4/5 proteins and TrkB mRNA were obtained at different pregnant days (mean ± SEM, n = 5–8 animals). For each pregnant day, samples of placentas and amniotic fluids from individual animals were used (n = 3). Levels of TrkB mRNA were normalized using transcript levels of β-actin in the same sample. *, $P < 0.05$ *vs.* d 10 of pregnancy.

K252a/injection, 10.7 ± 0.3; 100 μg K252b/injection, 11.0 ± 0.6), these data suggest a specific role for the endogenous TrkB signaling system in placental and fetal developments but not fetal survival after implantation.

Discussion

The present study demonstrates the ability of BDNF to promote blastocyst outgrowth, but not adhesion, during the periimplantation period. After implantation, treatment of cultured trophoblast cells with the TrkB ectodomain and the Trk receptor inhibitor, K252a, suppressed cell proliferation and survival. Furthermore, *in vivo* suppression of TrkB signaling by K252a inhibited placental development accompanied by decreases in fetal weight and increases in trophoblast cell apoptosis during pregnancy.

Implantation of blastocysts is a well-organized process regulated by multiple growth factors and cytokines (1, 2). Increasing levels of BDNF in the uterus before implantation and the exclusive expression of TrkB in trophectoderm cells of blastocysts (13) suggest potential paracrine roles of BDNF in blastocyst during implantation. The trophectoderm cells of blastocysts differentiate during embryonic development to form the invasive trophoblasts that mediate implantation of embryos into the uterine wall. The outgrowth of trophoblast cells from cultured blastocysts is believed to reflect the proper differentiation of the embryo, important for trophoblast invasion of the endometrial stroma during implantation *in utero* (30, 31).

During blastocyst attachment, cell adhesion molecules, including integrin β-3, contribute to both cell-cell and cell-extracellular matrix interactions, whereas MMP-9 and other proteases produced by peri-implantation mouse blastocysts are implicated in the degradation of the extracellular matrix to allow invasion of trophoblast cells (32). Although treatment with BDNF stimulated MMP-9 mRNA expression after increases in total MMP-9 protein and its active form in blastocysts showing outgrowth, BDNF had no effect on the expression of integrin β-3, consistent with a lack of effect of BDNF on blastocyst adhesion. Similar to BDNF, it has been demonstrated that TGF-α promotes outgrowth but not the attachment of mouse blastocysts with an increase in the production of MMP-9 or -2 (33, 34). In addition, the stem cell factor stimulated trophoblast outgrowth (35), whereas epidermal growth factor and leukemia inhibitory factor increased production of MMP-9 (20). In contrast to the abundant expression of MMP-9, MMP-2 transcripts

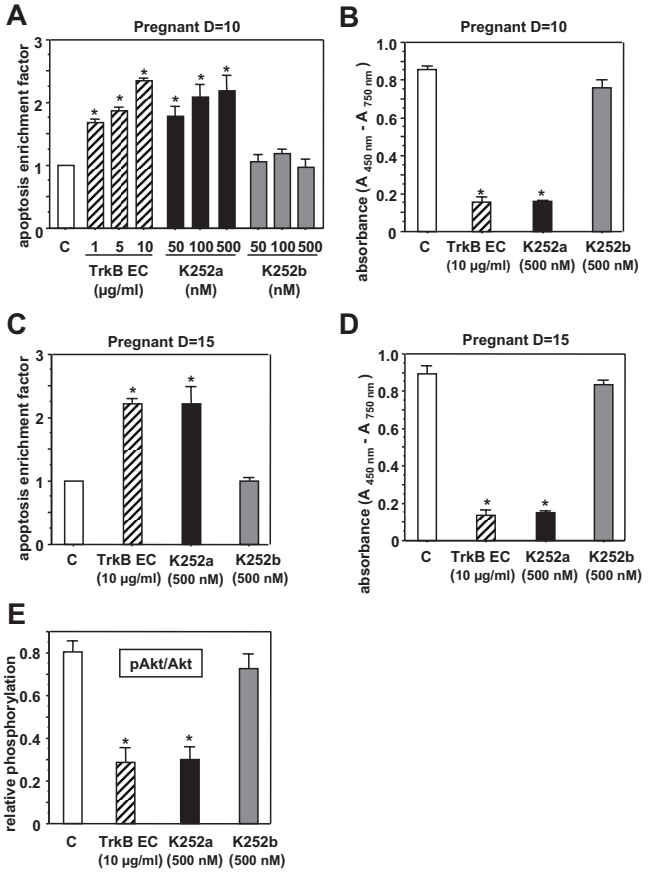

FIG. 5. Roles of endogenous TrkB ligands on *in vitro* growth of mouse trophoblast cells. Effects of suppressing endogenous TrkB ligands on trophoblast cell survival (A and C), proliferation (B and D), and the phosphorylation of Akt (E). Trophoblast cells were isolated from placenta at d 10 or 15 of pregnancy and cultured in NCTC-135 medium alone [control (C)], with different doses of TrkB ectodomain (TrkB EC), with the Trk receptor inhibitor K252a or the plasma membrane nonpermeable K252b. Cells were treated for 8 h to quantify apoptosis using the photometric enzyme immunoassay by detecting cytoplasmic histone-associated DNA fragments. Results were expressed as apoptotic enrichment factor calculated using the following formula: absorbance of the sample/absorbance of the corresponding control (mean ± SEM, n > 4). Some cells were treated for 24 h to evaluate cell proliferation using the WST-1 based colorimetric assay or to assess Akt phosphorylation by quantification of levels of phosphorylated (pAkt) and total Akt (mean ± SEM, n > 4). Results for the WST-1 assay were expressed as absorbance of the formazan dye at 450 nm followed by reference wavelength at 750 nm ($A_{450\ nm}$–$A_{750\ nm}$). *, $P < 0.05$ *vs.* control group.

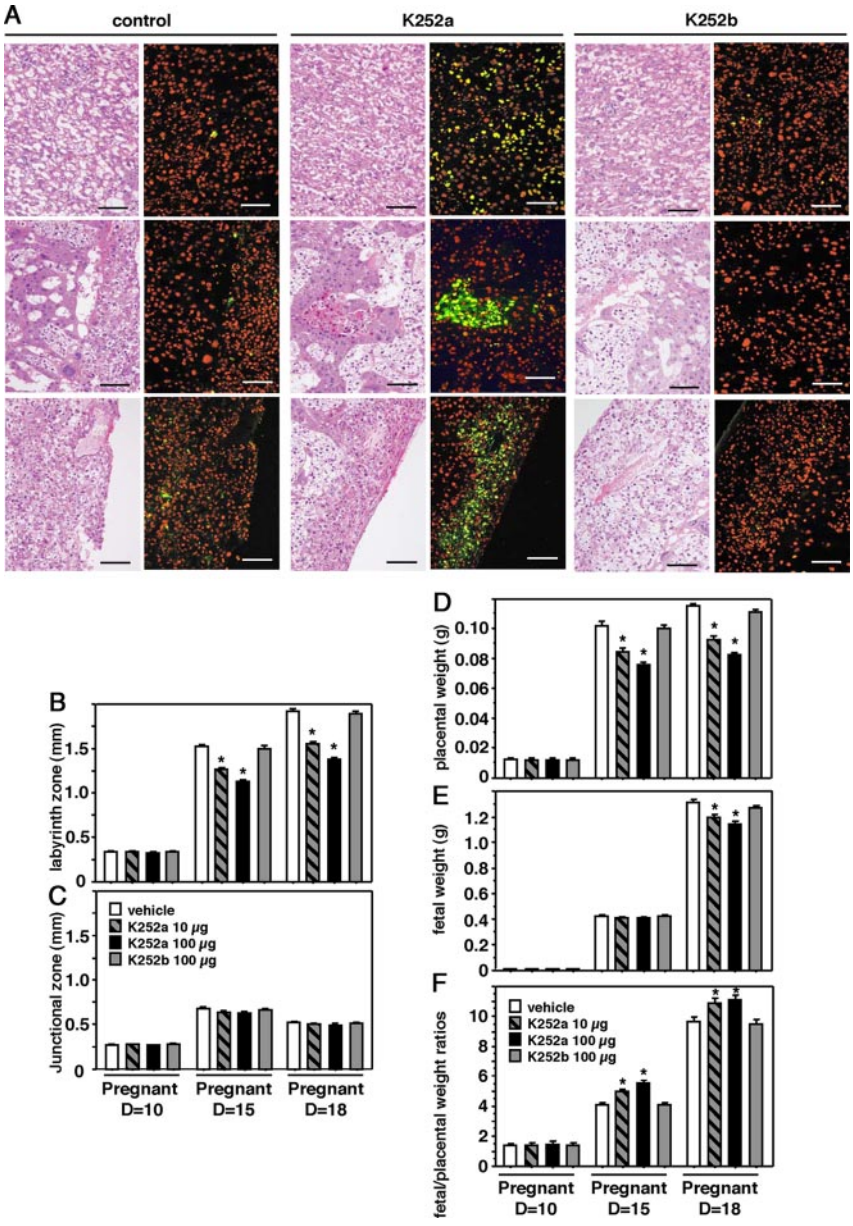

FIG. 6. Roles of endogenous TrkB ligands in *in vivo* growth of mouse placenta. A, Effects of treatment with a Trk receptor inhibitor *in vivo* on placental cell survival. K252a or K252b was administrated every 3 d to 9-wk-old mice from d 5 to 18 of pregnancy (K252a, 10 or 100 μg/injection; K252b, 100 μg/injection). At d 18 of pregnancy, apoptosis in placenta was assayed by detecting DNA fragmentation using *in situ* TUNEL staining. Cellular nucleic acids were stained (*red*) by using propidium iodide. Represented images in three different parts of placentas were obtained from animals treated with or without K252a (100 μg/injection) or K252b. Positive apoptosis signals (*green fluorescence*) are evident in the K252a-treated group showing apoptosis in labyrinth trophoblasts (*upper panels*), some spongiotrophoblasts (*middle panels*), and decidual cells (*lower panels*). *Scale bars*, 100 μm. B–F, Suppression of placental and fetal development *in vivo* after treatment with a Trk receptor inhibitor. K252a or K252b was administrated as described above. At d 10, 15, and 18 of pregnancy, thickness of the labyrinth (B) and junctional (C) zones close to the central portion of placentas as well as placental (D) and fetal (E) wet weights were measured (mean ± SEM, n = 30–32 placentas and fetuses from three animals). Fetal to placental weight ratios (F) were calculated for individual fetus-placenta units of the animals. *, $P < 0.05$ *vs.* vehicle group.

were not detected in blastocysts showing outgrowth based on our RT-PCR analyses, consistent with previous studies showing the lack of MMP-2 expression in blastocysts or the detection of MMP-2 expression only after prolonged culture (72 h) of blastocysts (26, 27). The actions of BDNF on preimplantation embryo development and survival are mediated by the PI3K pathway (13). In this study, blastocyst outgrowth after increases in MMP-9 transcript levels is also mediated by the PI3K pathway, suggesting important roles of the PI3K pathway in BDNF actions during both pre- and periimplantation stages.

Our data further showed the expression of TrkB ligands and receptors in not only trophectoderm cells of blastocysts (13) but also trophoblast cells of placenta after implantation. In placentas, TrkB was expressed in labyrinth trophoblasts and spongiotrophoblasts, whereas the expression of BDNF and NT-4/5 antigens was detected in spongiotrophoblasts and trophoblast giant cells and spongiotrophoblasts, respectively. Thus, TrkB ligands could act on the TrkB receptors expressed in trophoblast cells of placenta via autocrine and/or paracrine mechanisms. Because both TrkB ligands and receptors are expressed in decidual cells, their autocrine/paracrine roles in decidual functions cannot be ruled out. Although both BDNF and NT-4/5 were expressed in trophoblast cells and placentas, BDNF proteins were more abundant in placentas, suggesting endogenous BDNF plays dominant roles in placenta. Although expression of TrkB ligands in labyrinth trophoblasts was lost during placental development, its biological importance remains to be determined. Because there has been no available method to isolate individual cell types of trophoblast cells from mouse placentas, we collected trophoblast cells composed of several cell types for *in vitro* analyses of TrkB ligand actions. Using the TrkB ectodomain and the Trk receptor inhibitor, K252a, the present study demonstrated the ability of endogenous TrkB ligands to promote cell proliferation and survival of the cultured trophoblast cells. Although K252a is a pan-specific Trk receptor inhibitor and the isolated trophoblast cells expressed all paralogous Trk ligand-receptor pairs, the observed inhibitory effect is likely specific for the TrkB receptor because the soluble ectodomains of TrkB exhibited similar effects as K252a on trophoblast cell growth and survival. Akt acts downstream of PI3K to regulate many biological processes (29). Because activation of Akt by phosphorylation induces cell proliferation, survival, and growth (29), suppression of Akt phosphorylation by K252a treatment in cultured trophoblasts indicates an involvement of the PI3K/Akt pathway in mediating the promoting effects of TrkB ligands on cell proliferation and survival.

The important roles of the TrkB signaling system in placental development *in vivo* are underscored by the suppressive effect of

the Trk receptor inhibitor, K252a, on placental growth and trophoblast cell survival in pregnant mice. Of importance, the related plasma membrane nonpermeable K252b was ineffective. The effect of K252a on apoptosis was specific for trophoblast cells expressing TrkB, including labyrinth trophoblasts and spongiotrophoblasts. Labyrinth zone of mouse placenta is composed of labyrinth trophoblasts with underlying blood vessels that provide a large surface area for nutrient, waste, and gas exchange (36). Although the function of the junctional zone is poorly understood, it could act as a structural support for the villous structures of the labyrinth (36). During gestation, the restriction of placental growth accompanied by decreases in thickness of labyrinth zone, but not junctional zone, were apparent after midgestation. In contrast to trophoblast cultures, the effect of K252a on placental growth and thickness of labyrinth zone was insignificant at d 10 of pregnancy, suggesting that one-time injection of K252a and/or the short-term (5 d) treatment protocol is insufficient to suppress placental development *in vivo*. Junctional zone is composed of at least two trophoblast subtypes: spongiotrophoblasts and glycogen trophoblast cells (37). Because glycogen trophoblast cells lacked K252a-induced apoptosis in contrast to spongiotrophoblasts, the minimal effect of K252 on the thickness of junctional zone may be explained by increases in the population of glycogen trophoblast cells in junctional zone after midgestation (37). Future studies using isolated individual trophoblast cell types could reveal the possibilities of different functions of BDNF on different trophoblast lineages.

Placental development is important for fetal growth and pregnancy. Lower placental weights are often associated with intrauterine fetal growth restriction, maternal hypertension, or preeclampsia (38). The major determinant of fetal growth is the placental supply of nutrients, and this process depends on the size, morphology, blood flow, and transporter abundance of the placenta (39). In this study, growth restriction of fetuses after treatment with K252a was evident only toward the end of gestation. Furthermore, the fetal to placental weight ratio was higher in K252a-treated mice. Experimental suppression of placental growth often leads to increased placental efficiency measured as fetal to placental weight ratio (39). Up-regulation of active amino acid transport by placenta is shown to compensate for the decrease in passive permeability for nutrients of the smaller placenta until failure of the compensation after midgestation (40). Thus, suppression of endogenous TrkB ligands may lead to fetal growth restriction only after failure of the placental compensation machinery at late gestational ages.

Accumulating evidences indicate that low birth weight is associated with increased rates of cardiovascular disease, hypertension, insulin resistance, and type 2 diabetes (41). This association appears to be independent of classical adult lifestyle risk factors. It has been hypothesized that a stimulus or insult acting during critical periods of fetal growth and development could permanently alters tissue structure and function, a phenomenon termed fetal programming (41). In the present study, fetal weight was reduced by 15% after treatment with K252a, indicating the essential role of BDNF signaling system in fetal programming. In addition to the determination of neonatal abnormalities, future studies on lifelong observation of the offspring from the K252a-treated pregnant animals would clarify the importance of BDNF for fetal programing.

The effect of endogenous TrkB ligands to promote trophoblast cell growth is associated with its ability to inhibit apoptosis. The apoptosis-suppressing effect of BDNF and NT-4/5 is consistent with earlier studies showing the survival actions on cells of the central nervous system (42) and some peripheral tissues, including human embryonic stem cells (43), preimplantation embryos (13), hair follicles (44), and eosinophils (45). BDNF and NT-4/5 may compensate for each other *in vivo* due to their common actions through the same receptor, TrkB. Thus, the importance of the TrkB ligand signaling system on trophoblast cell growth could be investigated only in TrkB null mice or BDNF and NT-4/5 double-null mice. However, no pregnant mice for either TrkB null or BDNF and NT-4/5 double-null genotypes are available because these animals die shortly after birth (46, 47). Furthermore, studies on the role of TrkB ligands during placental development in pups lacking both BDNF and NT-4/5 are complicated due to compensation by maternal BDNF and/or NT-4/5 produced from decidual cells in heterozygous mutant mothers.

Detection of BDNF and NT-4/5 proteins in the amniotic fluid during the progress of pregnancy is consistent with earlier studies showing BDNF levels in human amniotic fluid during gestation (48). Placenta is likely a source of TrkB ligands in amniotic fluid because maternal plasma levels of BDNF and NT-4/5 were negligible.

Herein we have demonstrated important roles of the BDNF/TrkB signaling system during implantation and placental development by increasing trophoblast cell growth and survival during peri- and postimplantation periods based on *in vitro* and *in vivo* studies. It is becoming apparent that this ligand signaling system, originally found to be essential for the development and differentiation of the neuronal system, is also important for diverse female reproductive processes including the development of early ovarian follicles (6–8), the nuclear and cytoplasmic maturation of oocytes to develop into preimplantation embryos (9–12), early embryonic development (13), and trophoblast cell growth and survival. The present demonstration of an augmenting role of the BDNF/TrkB signaling pathway in trophoblast cell development underscores the importance of this autocrine/paracrine system during peri- and postimplantation. Further understanding of the physiological actions of these neurotrophic factors on trophoblast cell growth could lead to new understanding on the management of abnormal pregnancy and mechanisms of fetal programming.

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