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Regulation of preimplantation embryo development by brain-derived neurotrophic factor

Kazuhiro Kawamura [a,c,*], Nanami Kawamura [a,b], Jun Fukuda [a], Jin Kumagai [a], Aaron J.W. Hsueh [c], Toshinobu Tanaka [a]

[a] Department of Obstetrics and Gynecology, Akita University School of Medicine, Akita, 010-8543, Japan
[b] Department of Dermatology and Plastic Surgery, Akita University School of Medicine, Akita, 010-8543, Japan
[c] Division of Reproductive Biology, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, CA 94305-5317, USA

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Abstract

Hormonal factors secreted by embryos and reproductive tracts are important for successful development of preimplantation embryos. We found expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) transcripts at its highest levels in the blastocyst stages. The transcripts for their receptor, TrkB, were detectable throughout the early embryonic stages with an increase after the early blastocyst stage. Both BDNF and TrkB are expressed in trophectoderm cells, whereas ligand-binding studies indicated specific binding of BDNF to trophectoderm cells. Furthermore, BDNF and NT-4/5 were produced in pregnant oviducts and uteri. Treatment with BDNF promoted the development of two-cell-stage embryos into blastocysts showing increased proliferation and decreased apoptosis. The effects of BDNF were blocked by the TrkB ectodomain or a Trk receptor inhibitor, K252a. Studies using specific inhibitors demonstrated the roles of the PI3K, but not the ERK, pathway in mediating BDNF actions. Under high-density embryo cultures, treatment with the TrkB ectodomain or K252a alone also inhibited embryonic development and survival, suggesting potential autocrine actions of BDNF produced by the embryo. *In vivo* experiments further demonstrated that K252a treatment suppressed early embryo development by inhibiting blastocyst cell numbers, and increasing blastocyst apoptosis. Our findings suggested that BDNF signaling plays important paracrine roles during blastocyst development by promoting the development of preimplantation embryos.

Keywords: Apoptosis; BDNF; Blastocyst; ERK; NT-4/5; PI3K; TrkB

Introduction

Accumulating evidence indicates that a number of growth factors and cytokines act as paracrine and/or autocrine factors during early embryo development and implantation (Brison and Schultz, 1998; Dey et al., 2004; Hardy and Spanos, 2002). Insulin-like growth factor-I, epidermal growth factor, and other hormonal factors secreted by the maternal reproductive tract regulate the development of pre- and peri-implantation embryos through specific receptors (Dey et al., 2004; Hardy and Spanos, 2002; Kane et al., 1997). In addition, developing early embryos produce growth factors that act in an autocrine manner to regulate their own growth and differentiation, or to serve as paracrine factors by regulating endometrial receptivity for blastocyst implantation.

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 co-receptor p75 (Barbacid, 1994). Although neurotrophins are widely expressed in the central nervous system and are important for neuronal survival and differentiation (Jones et al., 1994), they also play important roles in nonneuronal tissues (Ip et al., 1993). In the ovary, BDNF was found to be essential for the development of early follicles (Dissen et al., 1995; Paredes et al., 2004; Spears et al., 2003). Based on DNA microarray

* Corresponding author. 1-1-1 Hondo, Akita-shi, Akita, 010-8543, Japan. Fax: +81 18 884 6447.
E-mail address: kawamura@yf7.so-net.ne.jp (K. Kawamura).

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analyses of ovarian genes during the preovulatory period, we recently found major increases in the expression of BDNF by granulosa and cumulus cells following the preovulatory luteinizing hormone (LH) surge. Consistent with identification of TrkB receptors in the oocyte (Seifer et al., 2006), we demonstrated that BDNF acts on its receptor, TrkB, in the oocyte to enhance first polar body exclusion and to increase the competence of oocytes for development into early embryos (Kawamura et al., 2005c).

Here, we show the expression of BDNF and TrkB in preimplantation embryos and pregnant oviducts and uteri of mice. We demonstrate paracrine and potential autocrine roles of the BDNF/TrkB signaling system in the development of preimplantation embryos using BDNF and Trk receptor blockers, and involvement of PI3K in BDNF signaling.

Materials and methods

Animals

To obtain preimplantation embryos, female B6D2F1 mice at 25 days of age (CLEA Japan, Tokyo, Japan) were treated with a single i.p. injection of 7 IU of human menopausal gonadotropin (HMG; ASKA Pharmaceutical, Tokyo, Japan) followed 48 h later with 10 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical. Co., Ltd.) administrated i.p. Immediately after hCG treatment, animals were allowed to mate and two-cell-stage embryos were obtained by flushing the oviducts of mated mice at 46-47 h after hCG injection for *in vitro* culture as described (Kawamura et al., 2005a). Oviducts and uteri were obtained from immature untreated mice, mice treated with HMG (7 IU), mice treated HMG followed 12 h later by hCG treatment, and from animals at 4 days after mating. Pregnant oviducts were also obtained daily from 1 to 3 days after mating. The care and use of animals was approved by the Animal Research Committee, Akita University School of Medicine.

Isolation of inner cell mass and trophectoderm cells

Inner cell mass and trophectoderm cells were isolated by immunosurgery as described (Kawamura et al., 2005a). After zona removal and lysis using acid Tyrode's solution (Sigma, St. Louis, MO), blastocysts were incubated sequentially at 37 °C in rabbit anti-mouse serum (Sigma) diluted 1:10 in M2 medium (Sigma) for 10 min and then in guinea pig complement serum (Sigma) diluted 1:3 in M2 medium with 10% FBS for 30 min. The lysed trophectoderm cell fraction was subjected to RNA extraction. To remove remaining trophectoderm cells, the inner cell mass was washed through small-bore glass pipettes before RNA extraction. Total RNA was extracted using an RNeasy Micro kit (QIAGEN, Tokyo, Japan) before RT-PCR to determine the spatial expression of TrkB, BDNF, and NT-4/5 in the blastocysts. To confirm purity of cell preparations, RT-PCR was performed to detect the inner cell mass marker Oct-4 (Palmieri et al., 1994) and the trophectoderm marker urokinase plasminogen activator (Robertson et al., 2001).

RT-PCR

For quantitative real-time RT-PCR, two-cell-stage embryos were obtained by flushing the oviducts of mated mice at 46-47 h after hCG injection for *in vitro* culture as described (Kawamura et al., 2005a). Embryos were allowed to continue development to different stages and were then collected after culturing in individual microdrops for 50-52 (four-cell), 59-60 (eight-cell), 70-72 (morula), 94-96 (early blastocyst), 119-120 (expanded blastocyst), and 142-144 (hatched blastocyst) h after hCG injection. Quantitative real-time RT-PCR of transcript levels in preimplantation embryos, oviducts and uteri was performed using a SmartCycler (Takara, Tokyo, Japan) with primers and hybridization probes for TrkB, BDNF, NT-4/5, and histone H2a as described (Kawamura et al., 2005c). Primers for TrkB corresponded to the catalytic kinase domain of the receptor to avoid the amplification of truncated isoforms (Klein et al., 1990). Data were normalized based on histone H2a transcript levels (Robert et al., 2002).

For conventional RT-PCR to study cell types expressing specific genes, primers for β-actin were used as described (Kawamura et al., 2003). PCR reactions comprised 40 cycles of amplification with denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and elongation at 72 °C for 30 sec. For positive controls, mouse ovary cDNA was amplified. For negative controls, no mRNA was included.

ELISA and immunohistochemistry

For ELISA, mouse uteri were homogenized in a buffer containing 137 mM NaCl, 20 mM Tris-HCl, 1% Nonidet P40, 10% glycerol, and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) before centrifugation at 8000×g for 5 min at 4 °C. Quantification of BDNF in the supernatant was performed as described (Kawamura et al., 2005c). Levels of NT-4/5 were quantified using the DuoSet human NT-4 ELISA development system (R and D Systems, Minneapolis, MN) The supernatant was diluted 1:4 in PBS for protein determination using the DC Protein Assay kit (Bio-Rad, Hercules, CA).

Immunofluorescent detection of BDNF and NT-4/5 was performed using mouse blastocysts as described (Kawamura et al., 2005a). Embryos were fixed with 4% paraformaldehyde (Sigma) and then permeabilized in PBS with 0.2% Triton X-100 (Sigma) for 5 min at room temperature. After blocking, embryos were incubated with either rabbit anti-BDNF or NT-4/5 polyclonal antibodies (Chemicon, Temecula, CA) in 1% PBS-BSA with 0.05% Tween 20 (Sigma) at 1:500 dilution for 7 days at 4 °C. After three washes in Tris-buffered saline with 0.1% Tween 20, embryos were incubated with Cy3 conjugated goat anti-rabbit IgG secondary antibodies (Zymed Laboratories, San Francisco, CA) in 1% PBS-BSA with 0.05% Tween 20 at 1:1,000 dilution for 1 h at room temperature followed by three additional washes. Counterstaining was performed for 30 min in 1 μg/ml of Hoechst 33342 dye (Invitrogen, Carlsbad, CA). After five washes in PBS, embryos were analyzed under a confocal laser scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany). For negative controls, the primary antibody was replaced by nonimmune rabbit IgG (Dako, Carpinteria, CA).

To localize BDNF and NT-4/5, oviducts and uteri were obtained from pregnant mice at four days after mating and fixed with Bouin's solution for 1 h at room temperature before transfer to 70% ethanol. Tissues were embedded in paraffin and sectioned at 3-μm intervals. After deparaffinization and dehydration, endogenous peroxidase activities were quenched with 3% hydrogen peroxidase for 10 min. After incubation in the TNB blocking buffer (TSA Biotin System; Perkin-Elmer, Boston, MA) for 30 min, slides were incubated with either rabbit anti-BDNF or NT-4/5 polyclonal antibodies at 1:100 or 1:300 dilution for 48 or 166 h at 4 °C, respectively. After three washes in the TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20), slides were incubated with biotinylated anti-rabbit secondary antibodies (Zymed Laboratories) for 1 h at room temperature. After three washes, BDNF or NT-4/5 signals were amplified using the TSA Biotin System. Bound antibodies were visualized using a Histostain SP kit (Zymed Laboratories). For negative controls, the primary antibody was replaced by nonimmune rabbit IgG.

Binding of fluorescent BDNF to preimplantation embryos

For binding studies, recombinant human BDNF (Peprotech, Rocky Hill, NJ) was labeled with fluorescein isothiocyanate (FITC) using EZ-label FITC protein labeling kit (Pierce Biotechnology, Rockford, IL). Two-cell-stage embryos were cultured in modified M16 medium (MR-010-D; Chemicon) without serum. When embryos reached the expanded blastocyst stage, medium was replaced with the medium containing BDNF conjugated to FITC (FITC-BDNF) and incubated for 30 min at 37 °C in air with 5% CO_2. After three washes, embryos were fixed with 4% paraformaldehyde in PBS for 15 min. To determine background fluorescence, embryos were incubated with the medium alone. Nonspecific binding of FITC-BDNF (30 ng/ml) was estimated by coincubation with a 1,000-fold excess of unlabeled recombinant human

BDNF. After counterstaining with Hoechst 33342, fluorescent signals in embryos were visualized using a confocal laser scanning microscope (LSM 510, Carl Zeiss). Optical sections with intervals of 1 μm were taken with 63X/1.4 Plan Apochromat objective and the fluorescent images were obtained as 8-bit images of TIF format files. Using images without counterstaining, total numbers of fluorescent pixels for individual embryos were calculated using SigmaScan Pro 5.0 (SPSS Japan, Tokyo, Japan) to reflect the number of binding sites for FITC-BDNF as described previously (Kawamura et al., 2003).

Embryo cultures and apoptosis detection

Two-cell-stage embryos were obtained and groups of 10-12 embryos were placed in 50 μl drops of modified M16 medium with or without BDNF and covered by mineral oil. Embryos were cultured for 96 h up to the hatched blastocyst stage with fresh medium replacement every 24 h. Some embryos were cultured with BDNF (10 ng/ml) with or without the TrkB ectodomain (1 or 10 μg/ml; R and D Systems, Minneapolis, MN), a pan-specific Trk receptor inhibitor, K252a (100 nM; Calbiochem, La Jolla, CA) (Tapley et al., 1992), or plasma membrane nonpermeable K252b (100 nM) (Ross et al., 1995). To examine endogenous effects of TrkB ligands on early embryonic development and survival, groups of 40-43 embryos were cultured in 10 μl drops of modified M16 medium with or without TrkB ectodomain (10 μg/ml), K252a (100 nM) or K252b (100 nM). To analyze the involvement of PI3K and ERK pathways in the BDNF induction of early embryonic development and survival, groups of 10-12 two-cell-stage embryos were cultured with BDNF (10 ng/ml) with or without a PI3K inhibitor, LY294002 (1-10 μM; Sigma) or its inactive analog, LY303511 (10 μM; Calbiochem). Some embryos were cultured with BDNF with or without a MEK1/2 inhibitor, U0126 (5-50 μM; Calbiochem) or its inactive analog, U0124 (50 μM; Calbiochem). Embryonic development was monitored after 24, 72, and 96 h of culture to determine the proportion of embryos at morula, expanded blastocyst, and hatched blastocyst stages, respectively. At the end of culture, some embryos were subjected to the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay to detect apoptosis as described (Kawamura et al., 2005b).

Differential labeling of inner cell mass and trophectoderm cells

To examine the preferential effect of BDNF on specific cell lineages in blastocysts, the numbers of inner cell mass and trophectoderm cells in blastocysts were counted by the differential labeling technique using two polynucleotide-specific fluorochromes (Kawamura et al., 2003). After staining, the blastocysts were mounted on a glass slide, and the number of total, inner cell mass and trophectoderm cells in each blastocyst was counted under an epifluorescence microscope (Olympus Corp., Tokyo, Japan).

In vivo analysis

To examine the roles of endogenous TrkB ligands during early embryonic development in vivo, female B6D2F1 mice at 25 days of age were treated with HMG (7 IU) followed 48 h later with 10 IU hCG. Immediately after hCG injection, animals were allowed to mate with fertile males. Because in vitro studies indicated minimal effects of BDNF on early embryos up to the morula stage, K252a was administrated three times (i.p.; 10 μg × 3) at 79, 83, and 87 h after hCG injection thus corresponding to the development of morula to the blastocyst stage in vivo (Kawamura et al., 2005c). For negative controls, K252b or vehicle alone was used. At 90-92 h after hCG injection, expanded blastocysts were obtained from uterine horns. After evaluation of the proportion of embryos that developed to the expanded blastocyst stage, embryos were subjected to the TUNEL assay.

Statistical analysis

Mann-Whitney U test was performed to compare the number of total, inner cell mass and trophectoderm cells in blastocysts. One-way ANOVA, followed by Fisher's protected least significant difference test, was used to evaluate other differences. Results are presented as mean ± s.e.m. of at least three separate experiments.

Results

Temporal and spatial expression of neurotrophins and Trk receptors in preimplantation embryos

Quantitative real-time RT-PCR was performed to examine temporal expression of neurotrophins and the TrkB receptor in preimplantation embryos. Levels of both BDNF and NT-4/5 mRNAs were low from the two-cell stage up to the four-cell stage but increased at the eight-cell stage and reaching its highest levels in the expanded and hatched blastocyst stages (Fig. 1A). Likewise, TrkB mRNA levels were low from the two-cell stage up to the morula stage and then increased at the early blastocyst stage before reaching the highest levels in the hatched blastocyst stage (Fig. 1A).

Inner cell mass and trophectoderm cells were isolated for RT-PCR analyses to determine the cell types expressing BDNF, NT-4/5, and TrkB in blastocysts. All three transcripts were detected in trophectoderm cells and not in inner cell mass cells (Fig. 1B). The expression of BDNF and NT-4/5 in trophectoderm cells was further confirmed using immunofluorescent staining (Fig. 1C).

Binding of fluorescent BDNF to blastocysts

To confirm the expression of functional TrkB receptors in blastocysts, ligand-binding studies were performed using fluorescent-labeled FITC-BDNF. The clustered fluorescent signals were detected in trophectoderm cells but not in cells of the inner cell mass (Fig. 2). The ratios of fluorescent pixels per embryo were saturated at 10 ng/ml of FITC-BDNF by embryos treated with 30 ng/ml of FITC-BDNF showing a similar level of fluorescent signals (Table 1).

Expression of BDNF, NT-4/5, and TrkB in preimplantation oviducts and uteri

We also examined the expression of TrkB and its ligands in oviducts and uteri during the preimplantation period. In oviducts, quantitative real-time RT-PCR analyses indicated that BDNF mRNA levels were high in immature mice at 25 days of age (Fig. 3A, 0 h), and decreased after HMG treatment, whereas BDNF mRNA levels increased at 12 h after treatment with hCG to induce ovulation (Fig. 3A). In contrast, NT-4/5 mRNA levels remained low following gonadotropin treatments, and increased from days 2 of pregnancy (Fig. 3A). However, TrkB mRNA was low in both non-pregnant and pregnant oviducts (Fig. 3A).

In the uterus, levels of BDNF, NT-4/5, and TrkB mRNAs were high in immature mice at 25 days of age (Fig. 4A, 0 h), decreased after HMG treatment, and maintained at low levels until 12 h after hCG treatment (Fig. 4A). However, BDNF and NT-4/5 mRNA levels were increased at days 4 of pregnancy (Fig. 4A). Similar changes of uterine BDNF and NT-4/5 proteins were detected based on ELISA (Fig. 4B). Although the BDNF mRNA levels

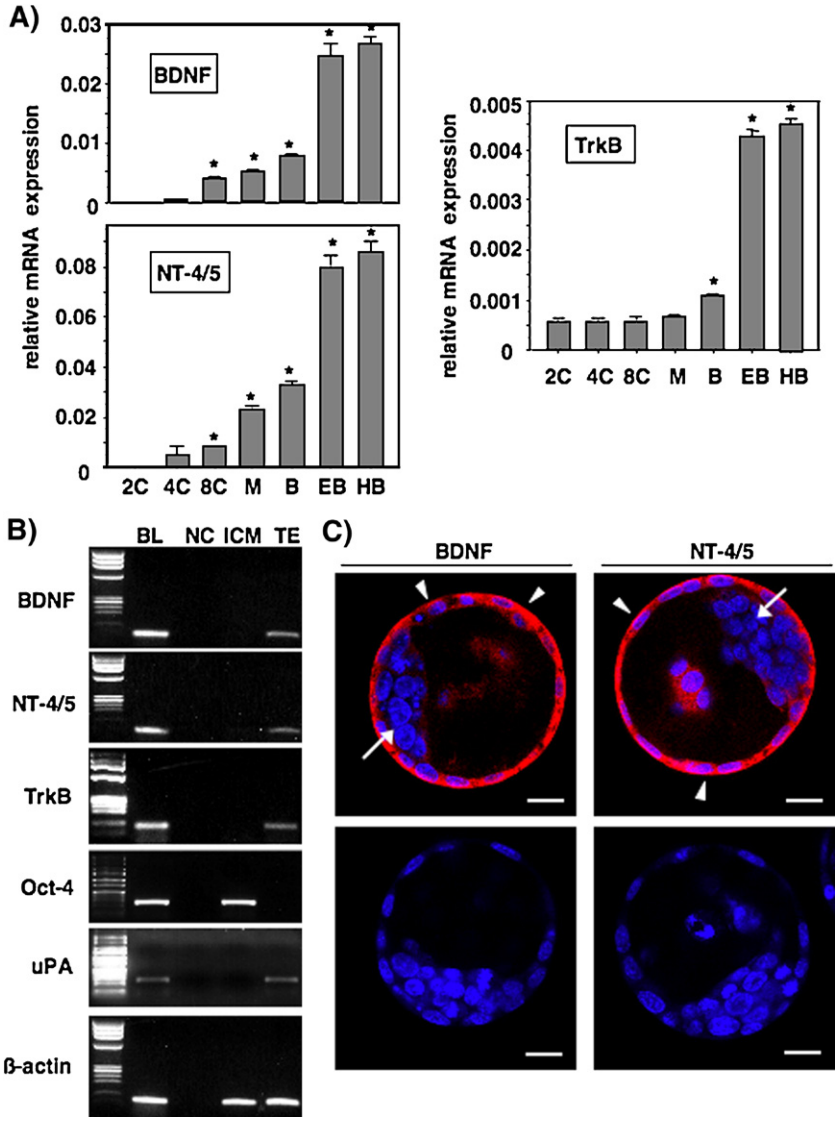

Fig. 1. Temporal and spatial expression of transcripts for BDNF, NT-4/5, and TrkB in mouse preimplantation embryos. (A) Transcript levels for different genes in developing preimplantation embryos were determined using real-time RT-PCR. Levels of all mRNAs were normalized based on those for histone H2a in the same sample (mean ± SEM, n = 3). *, $P < 0.01$ vs. 2C. 2C: 2-cell; 4C: 4-cell; 8C: 8-cell; M: morula; B: early blastocyst; EB: expanded blastocyst; HB: hatched blastocyst. (B) Spatial expression of BDNF, NT-4/5, and TrkB in blastocysts. Oct_4 and urokinase plasminogen activator (uPA) transcripts were used as markers for inner cell mass (ICM) and trophectoderm (TE), respectively. Levels of β–action served as loading controls. BL: whole blastocyst; NC: negative control. (C) Immunohistochemical detection of BDNF and NT-4/5 proteins in blastocysts. Specific BDNF and NT-4/5 signals are localized in TE cells (arrowhead), but not ICM cells (arrow). (Lower) Negative controls. Embryonic nuclei were stained in blue with Hoechst 33342. (Scale bars, 20 μm).

were comparable to those of NT-4/5, uteri expressed higher levels of BDNF than NT-4/5 proteins (Figs. 4A and B).

Immunohistochemical analyses further indicated that both BDNF and NT-4/5 were expressed in the oviductal epithelial cells and the endometrial epithelial cells but not in stromal cells (Figs. 3B and 4C, a and b, BDNF and NT-4/5 staining; c and d, negative controls). BDNF staining was stronger than that of NT-4/5 in both oviducts and uteri (Figs. 3B and 4C).

Effects of BDNF treatment on the development of preimplantation embryos in vitro

The expression of BDNF and TrkB in preimplantation embryos, oviducts and uteri suggests the BDNF/TrkB signaling system could play a role during early embryonic development. We then tested the effect of BDNF treatment on cultured embryos. Although BDNF treatment did not affect the development of two-cell-stage embryos to the morula stage (data not shown), BDNF treatment promoted the development of embryos to the expanded and hatched blastocyst stages evaluated after 72 and 96 h of culture, respectively, when embryos were cultured at a low density (Fig. 5A). At 96 h, BDNF treatment doubled the proportion of blastocysts reaching the hatched stage. The specificity of the effects of BDNF on preimplantation embryos was examined by using the soluble ectodomain of TrkB or a Trk receptor inhibitor, K252a. The ability of BDNF to promote the development of two-cell-stage embryos to the hatched blastocyst stage was blocked by

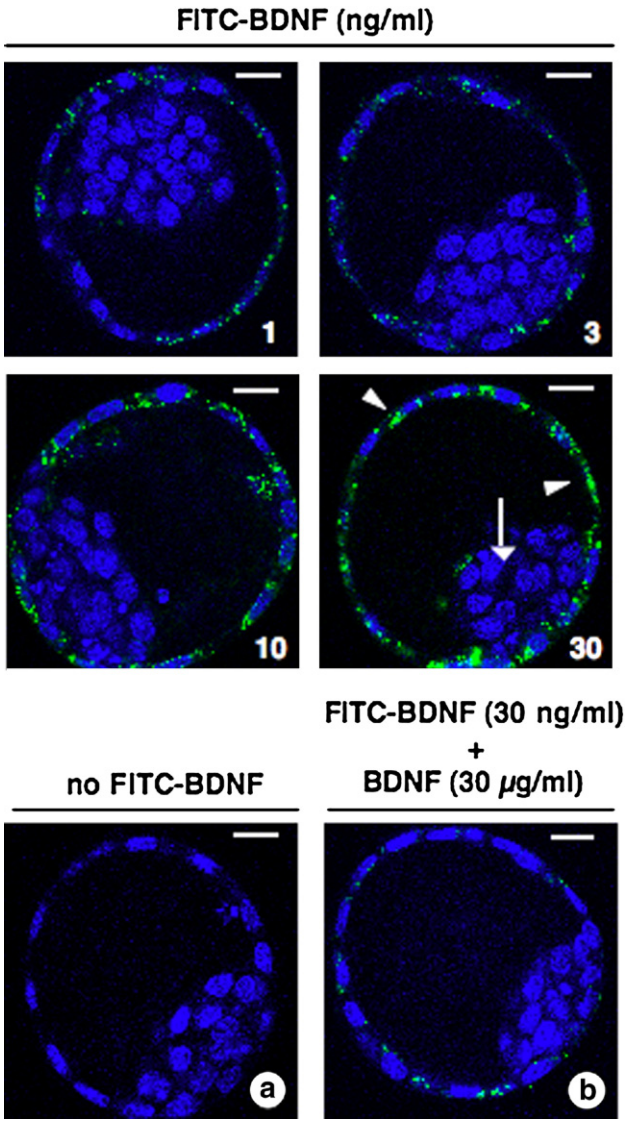

Fig. 2. Binding of BDNF to blastocysts. Blastocysts were treated with 1–30 ng/ml of FITC-BDNF. For nonspecific binding, blastocysts were treated with medium alone (a) or 30 ng/ml of FITC-BDNF and a 1,000-fold excess of unlabeled BDNF (b). The clustered fluorescent signals of FITC-BDNF were detected in TE cells (arrowhead), but not ICM cells (arrow). Embryonic nuclei were stained in blue with Hoechst 33342. (Scale bars, 20 μm).

cotreatment with either the TrkB ectodomain or K252a, but not with the membrane nonpermeable K252b (Fig. 5B).

Because treatment of embryos cultured at a low density with the TrkB ectodomain or K252a alone without BDNF has no effect on the development of two-cell-stage embryos to the hatched blastocyst stage (Fig. 5B), the concentration of endogenously-produced BDNF in the culture medium might not be high enough to exert autocrine effects on embryos. To examine the effect of endogenous TrkB ligands on embryo development, embryos were cultured at higher density to increase the concentration of endogenous TrkB ligands in the culture medium before treatment with the TrkB ectodomain or K252a alone (Fig. 5C). The proportions of embryos at the hatched blastocyst stage in the control group were increased following culture at a high density, whereas the increased development of embryos was inhibited by treatment with the TrkB ectodomain or K252a, but not the inactive K252b (Fig. 5C).

Effects of BDNF treatment on apoptosis of cultured blastocysts

To determine if BDNF acts as a survival factor, we evaluated apoptosis in embryos treated with BDNF. Because hatched blastocysts are difficult to retrieve due to extreme viscosity, expanded blastocysts were used for TUNEL staining (Fig. 6A). Cell counting indicated that BDNF treatment increased the total number of cells in blastocysts cultured at a low density, and the increase of cell number was preferentially observed in trophectoderm cells (Figs. 6B and C). We also detected decreases in the proportion of TUNEL-positive nuclei (Figs. 6A and D), thus suggesting the BDNF suppression of apoptosis. Furthermore, the effects of BDNF on cell number and apoptosis of blastocysts cultured at a low density were blocked by cotreatment with the TrkB ectodomain or K252a, but not with K252b (Figs. 6B and D). Similar to studies on embryonic development, treatment with the TrkB ectodomain or with K252a, but not K252b, decreased cell numbers and increased numbers of TUNEL-positive nuclei in the blastocysts cultured at a high density without BDNF (Figs. 6E and F).

Involvement of the PI3K, but not ERK, pathway in the BDNF promotion of preimplantation embryo development and survival

We further analyzed the roles of PI3K and ERK signaling pathways as downstream mediators of the BDNF/TrkB-induced embryo development and survival. The ability of BDNF to promote the development of two-cell-stage embryos to the hatched blastocyst stage at a low density was suppressed

Table 1
Ratio of flurescent pixels obtained from confocal microscopic eight-bit images of mouse blastocyst

BDNF	Number of fluorescent pixels (A)	Number of pixels in whole embryo's area (B)	Ratio of pixels per embryo (A/B)
1 ng/ml	7471±990	55293±1824	0.1369±0.0207
3 ng/ml	11151±1302	54514±1942	0.2051±0.0252
10 ng/ml	30927±2639	5658±2519	0.5593±0.0514
30 ng/ml	36731±2382	56661±2130	0.6467±0.0262

A total of five embryos were used in each concentration.

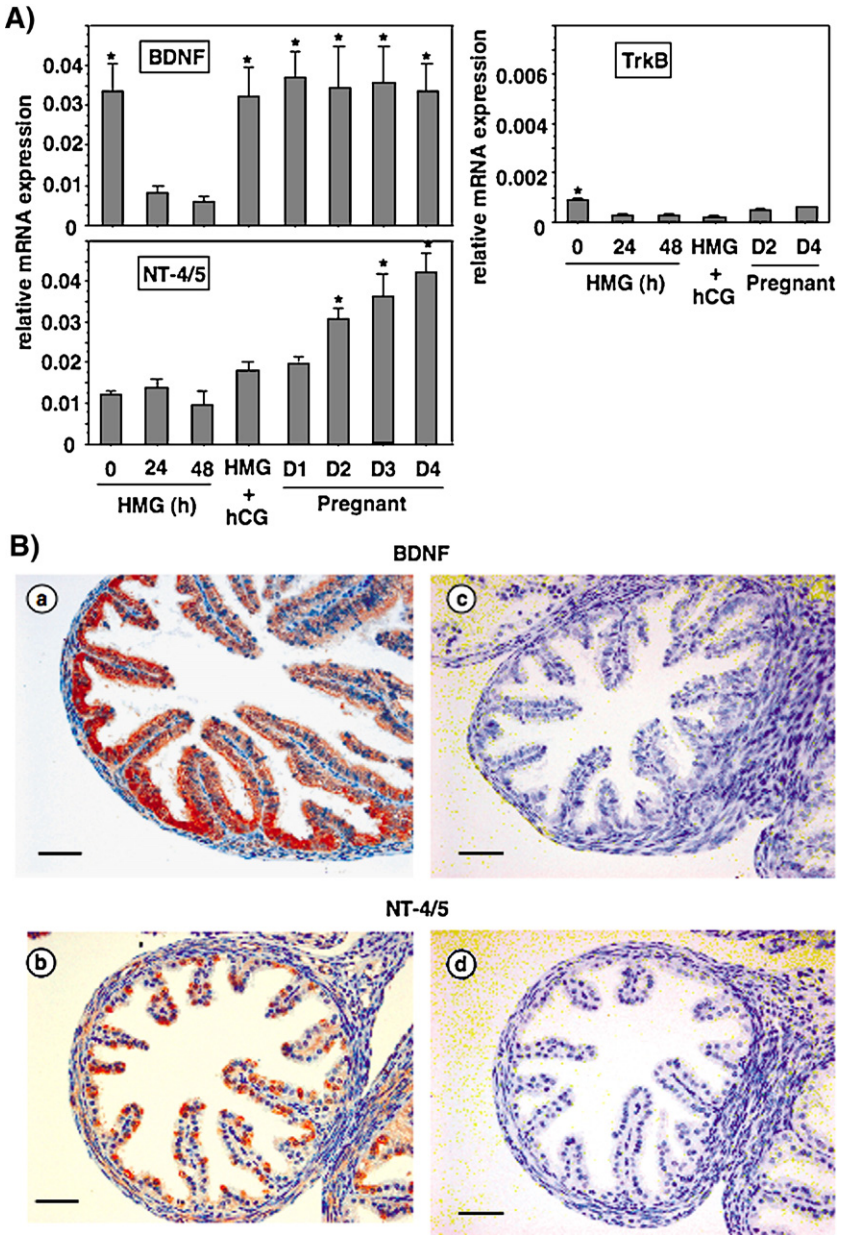

Fig. 3. Expression of BDNF and NT-4/5 in mouse oviducts. (A) Quantification of BDNF, NT-4/5, and TrkB transcripts using real-time RT-PCR. Levels of BDNF, NT-4/5, and TrkB mRNAs were obtained at different times after HMG and hCG treatment and normalized using transcript levels of histone H2a in the same sample (mean ± SEM, n = 3). Some samples were obtained from animals at days 1 through 4 of pregnancy. For each stage, three samples prepared from individual animals were used. *, $P < 0.01$ vs. oviducts from animals treated with HMG for 48 h. (B) Localization of BDNF and NT-4/5 proteins in oviducts. Immunohistochemical analyses showed the expression of BDNF and NT-4/5 proteins in pregnant oviducts at 4 days after mating. a and b: BDNF or NT-4/5 staining. c and d: negative controls. Specific BDNF and NT-4/5 staining was localized to epithelial cells, but not stromal, cells. (Scale bars: 50 μm).

by cotreatment with a PI3K inhibitor, LY294002, but not with its inactive analog, LY303511 in a dose-dependent manner (Fig. 7A). In contrast, cotreatment with different doses of the MEK1/2 inhibitor, U0126, had no effect on the BDNF-induced embryo development (Fig. 7A). Furthermore, treatment with LY294002 inhibited the BDNF stimulation of cell numbers (Fig. 7B) and suppression of the percentage of TUNEL-positive nuclei (Fig. 7C) in blastocysts cultured at a low density. In contrast, U0126 did not alter BDNF actions on cell number and apoptosis (Figs. 7B and C).

In vivo effects of a Trk receptor inhibitor on the development of preimplantation embryos

By using the Trk receptor inhibitor, K252a, we examined the role of endogenous TrkB ligands during early embryonic development *in vivo*. Treatment with K252a inhibited the number of embryos reaching the expanded blastocyst stage (Fig. 8A) without affecting the total number of embryos found (vehicle, 55.2 ± 9.3; K252a, 56.3 ± 3.7; K252b, 58.5 ± 7.9). Assessment of cell numbers indicated that treatment with K252a also decreased

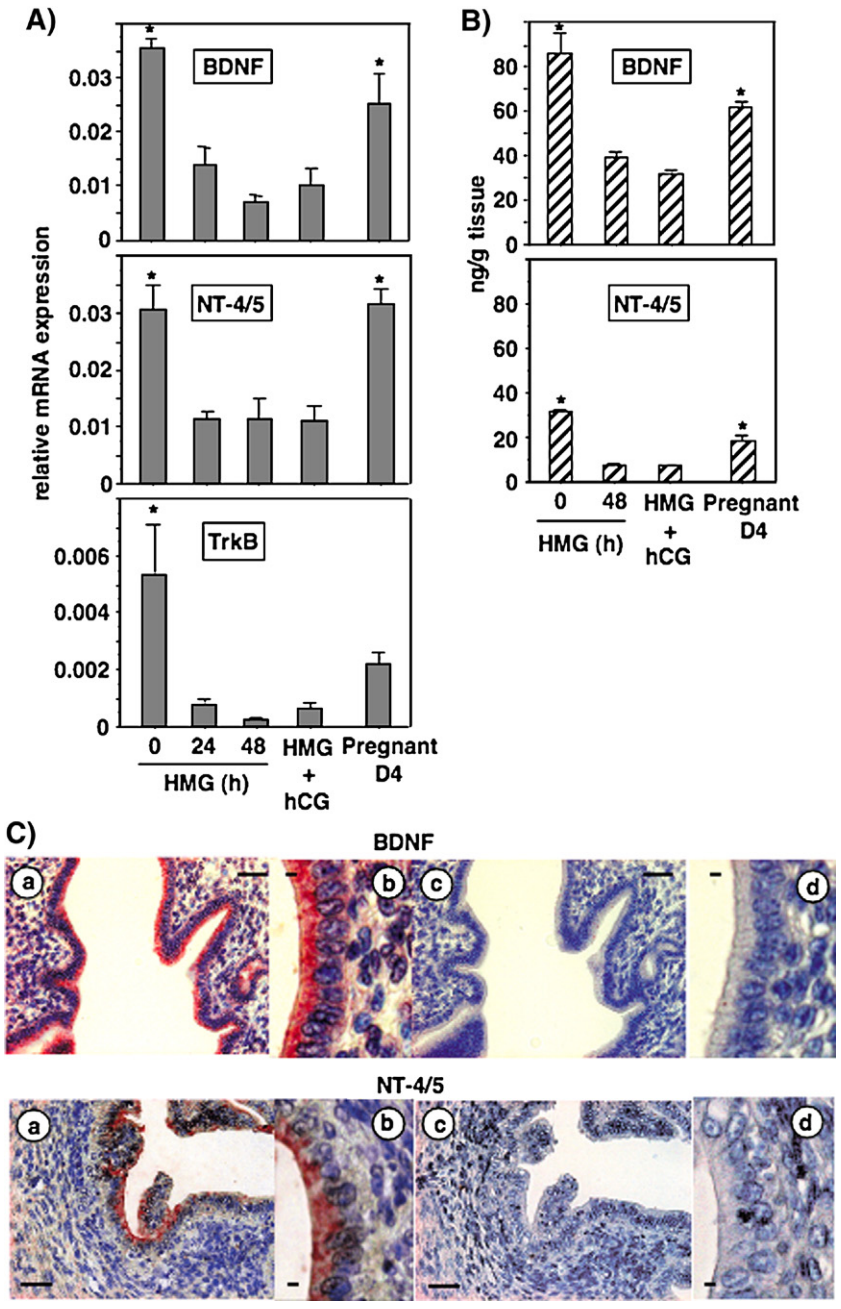

Fig. 4. Expression of BDNF and NT-4/5 in mouse uteri. Quantification of BDNF, NT-4/5, and TrkB levels using real-time RT-PCR (A) and ELISA (B). Levels of BDNF, NT-4/5, and TrkB mRNAs were obtained at different times after HMG and hCG treatment and normalized using transcript levels of histone H2a in the same sample (mean ± SEM, n = 3). Some samples were obtained from animals at days 4 of pregnancy. For each stage, three samples prepared from individual animals were used. *, $P < 0.01$ vs. uteri from animals treated with HMG for 48 h. (C) Localization of BDNF and NT-4/5 proteins in uteri. Immunohistochemical detection of BDNF and NT-4/5 proteins in pregnant uteri at 4 days after mating. a and b: BDNF or NT-4/5 staining. c and d: negative controls. Specific BDNF and NT-4/5 staining was localized to endometrial epithelial, but not stromal, cells. (Scale bars: a and c, 100 μm; b and d, 10 μm).

cell numbers in the embryos (Fig. 8B). Furthermore, the percentage of TUNEL-positive nuclei increased after K252a treatment (Fig. 8C). In contrast, treatment with K252b was ineffective for all three parameters tested.

Discussion

The present study demonstrates the ability of BDNF to promote early embryonic development and to suppress embryo apoptosis, suggesting the inclusion of this factor could be important for *in vitro* embryo cultures and embryonic stem cell derivation. Because our previous (Kawamura et al., 2005c) and unpublished data indicate the absence or very low expression of both BDNF and NT-4/5 mRNA in oocytes, transcription of BDNF and NT-4/5 may be triggered by zygotic genome activation. The high expression of TrkB in trophectoderm cells of expanded and hatched blastocysts is likely responsible for the observed ability of BDNF to promote early embryonic devel-

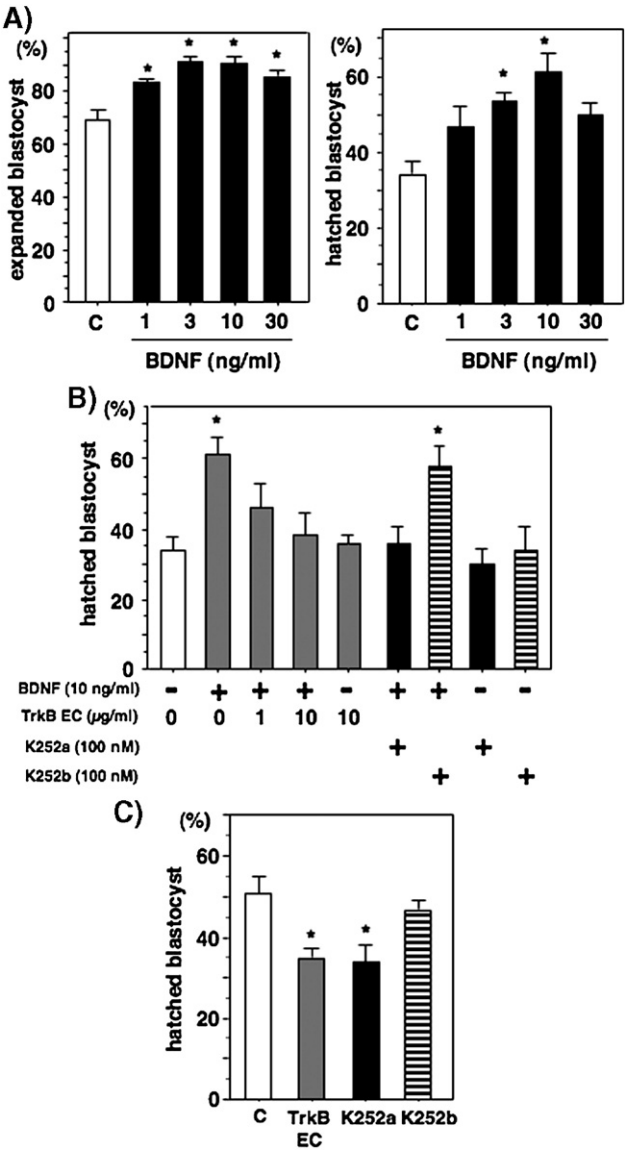

Fig. 5. BDNF promotion of the development of cultured preimplantation embryos. (A) Effects of BDNF treatment on early embryonic development. Two-cell-stage embryos (10–12 embryos/50 μl culture drop) were cultured without (control, C) or with BDNF for 72 and 96 h to evaluate the proportion of embryos at expanded and hatched blastocyst stages, respectively. (Mean±SEM, n≥6, 62–75 embryos per group.) (B) Antagonistic effects of the TrkB ectodomain (TrkB EC) and the Trk receptor inhibitor, K252a on the BDNF stimulation of embryo development. Two-cell-stage embryos were cultured for 96 h with BDNF (10 ng/ml) with or without TrkB ectodomain (10 μg/ml), K252a (100 nM), or K252b (100 nM). (Mean±SEM, n ≥5, 50–72 embryos per group.) (C) Effects of blockade of endogenous TrkB ligands on embryonic development. Two-cell stage embryos in groups at a high density (40–43 embryos/10 μl culture drop) were cultured for 96 h in modified M16 medium alone (C), TrkB ectodomain (10 μg/ml), K252a (100 nM), or K252b (100 nM). (Mean±SEM, n=5, 203–210 embryos per group.) *, $P<0.01$ vs. control group.

opment, and to prevent blastocyst apoptosis. Because treatment with the TrkB ectodomain and the Trk inhibitor, K252a suppressed development and survival of early embryos cultured at a high density, endogenous TrkB ligands (BDNF and NT-4/5) produced by the embryos could play important roles during early embryonic development. Because BDNF and NT-4/5 are also secreted by oviductal and uterine epithelial cells, the observed *in vivo* actions of BDNF on blastocyst development and survival could involve paracrine as well as autocrine actions of BDNF and/or NT-4/5. Using specific inhibitors, we also showed the contribution of the PI3K signaling pathway in the BDNF promotion of early embryonic development.

Treatment with BDNF promoted the development of cultured preimplantation embryos from the two-cell stage to expanded and hatched blastocyst stages, and the observed effects were blocked by the TrkB ectodomain or the Trk receptor inhibitor, K252a. Although the ability of BDNF to promote early embryonic development was not observed prior to the blastocyst stage, BDNF may regulate cellular functions or "condition" the early embryos in preparation for blastocyst development. However, it cannot be ruled out the possibility that the downstream pathways of the BDNF/TrkB signaling are not operational before the blastocyst stage. The effect of BDNF to promote blastocyst development is correlated with its ability to inhibit apoptosis and increase total cell numbers in blastocysts. The apoptosis-suppressing effect of BDNF is consistent with earlier studies showing the survival actions of BDNF on cells of the central nervous system (Han and Holtzman, 2000; Hetman et al., 1999) and some peripheral tissues (Botchkarev et al., 2004; Pyle et al., 2006; Raap et al., 2005).

Both the BDNF promotion of blastocyst development and the suppression of embryonic cell apoptosis were blocked by cotreatment with K252a. Although K252a is a pan-specific Trk receptor inhibitor, the observed inhibitory effect is likely specific for the TrkB receptor because the other two paralogous receptors, TrkA and TrkC, were not detectable in mouse preimplantation embryos (data not shown). Because the concentration of endogenous hormonal ligands in the culture medium are higher when embryos are cultured at a high density (Paria and Dey, 1990), potential autocrine roles of TrkB ligands of embryonic origin during early embryonic development was examined in embryos cultured at a high density following treatment with the TrkB ectodomain or K252a without exogenous BDNF. Because treatment with the TrkB ectodomain or K252a inhibited embryo development and survival under high-, but not low, -density cultures, our findings suggest that the concentration of endogenous BDNF could be increased under high-density cultures to allow demonstration of the inhibitory effects of the TrkB ectodomain or K252a. However, this *in vitro* culture system is artificial and may not entirely reflect exact *in vivo* conditions. The important roles of the BDNF/TrkB signaling system in pregnant mice *in vivo* are underscored by the suppressive effect of the TrK receptor inhibitor, K252a, on blastocyst development and survival when injected into mice during the preimplantation period. Of importance, the related plasma membrane nonpermeable K252b was ineffective. BDNF and NT-4/5 may compensate for each other in vivo due to their actions through the same receptor, TrkB. Thus, the importance of the TrkB ligands signaling system during development of preimplantation embryos could only be investigated

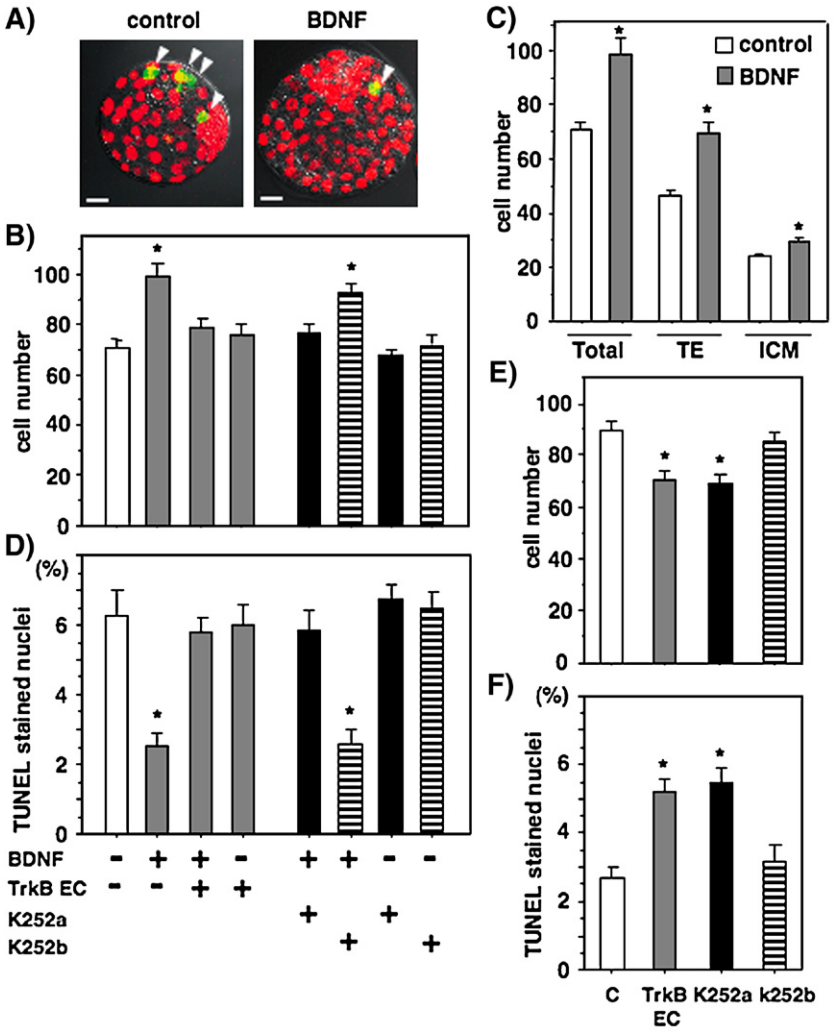

Fig. 6. Treatment with BDNF suppresses apoptosis in cultured blastocysts. (A) Detection of apoptotic nuclei by TUNEL staining in blastocysts with or without treatment with BDNF (10 ng/ml). Two-cell-stage embryos were cultured for 72 h before TUNEL analysis. red: counterstained nuclei, yellow: TUNEL_stained nuclei. (Scale bars, 20 μm.) (B and D) Effects of BDNF treatment on cell number and apoptosis in blastocysts. Two-cell-stage embryos were cultured for 72 h with or without BDNF (10 ng/ml) and the specificity of BDNF actions was evaluated by cotreatment with the TrkB ectodomain (TrkB EC) (10 μg/ml), K252a (100 nM), or K252b (100 nM). (Mean±SEM, total n=20 embryos per group.) (C) Effects of BDNF treatment on the number of total, TE, and ICM cells in blastocysts. Two-cell-stage embryos were cultured for 72 h with or without BDNF (10 ng/ml). (Mean±SEM, total n=20 embryos per group.) (E and F) Effects of blockade of endogenous TrkB ligands on cell number and apoptosis in blastocysts. Two-cell stage embryos in groups at a high density were cultured for 72 h in modified M16 medium alone (CH), TrkB ectodomain (10 μg/ml), K252a (100 nM), or K252b (100 nM). (Mean±SEM, total n=20 embryos per group.) *, $P<0.01$ vs. control group.

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 animals are available because these animals die shortly after birth (Klein et al., 1993; Liu et al., 1995). Furthermore, studies on the role of TrkB ligands during embryonic development in pups lacking both BDNF and NT-4/5 are complicated due to compensation by oviductal and uterine BDNF and/or NT-4/5 in heterozygous mutant mothers. Congenic strain of TrkB null mice of the C57BL/6 line showed defects in embryonic development because only less than 3% of TrkB null pups were delivered at the time of birth (Spears et al., 2003) as compared with the expected ratio of 25%. These findings are consistent with the present findings of paracrine roles of the TrkB ligands during early embryonic development. Future studies on the stage of embryonic arrest in congenic TrkB mutant mice could reveal the exact roles of TrkB and its ligands during embryonic development before and after implantation.

The observed exclusive expression of TrkB and its ligands in trophectoderm cells of blastocysts are consistent with our findings of a preferential effect of BDNF on the cell proliferation of trophectoderm cells. The potential roles of TrkB expressed in trophectoderm cells to mediate BDNF actions are further underscored by the demonstration of the exclusive binding of fluorescence-labeled BDNF to trophectoderm cells, similar to the formation of receptor-ligand complexes found for neuronal cells (Beaudet et al., 1998; Fabry et al., 2000). Because trophectoderm cells of blastocysts differentiate

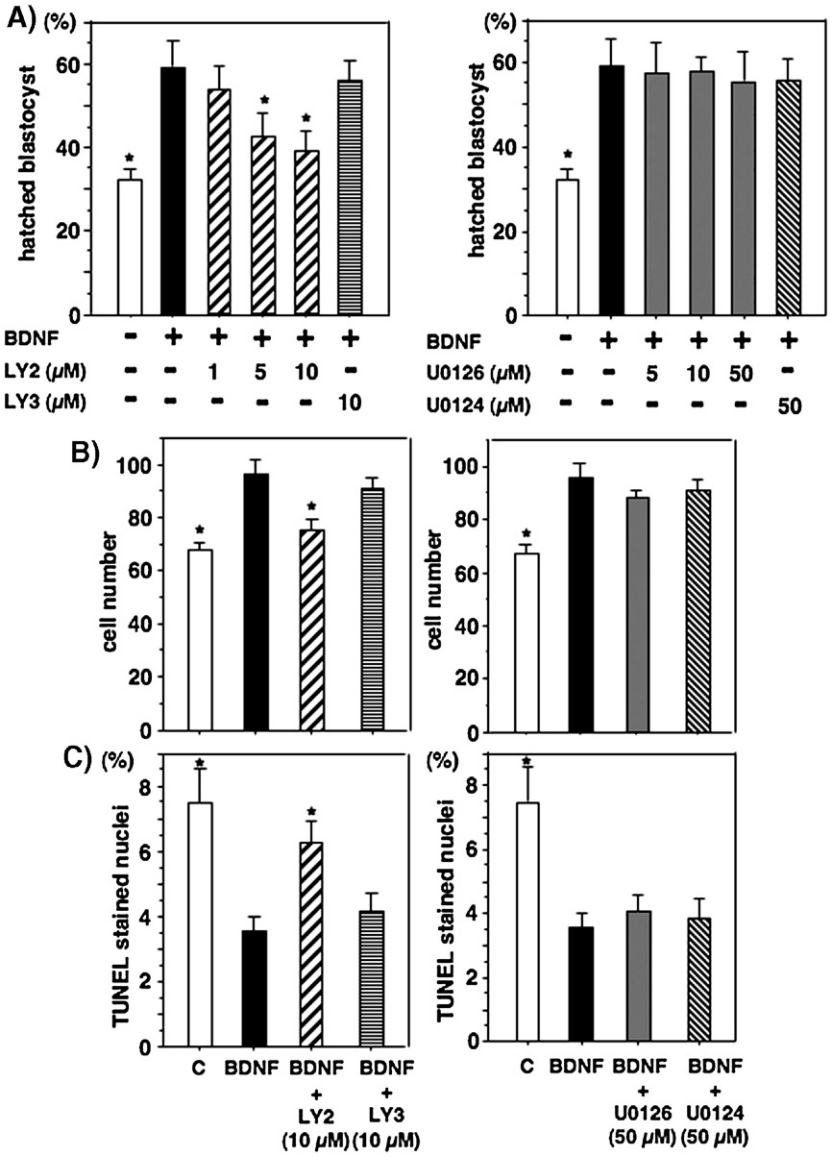

Fig. 7. Involvement of the PI3K pathway in the BDNF promotion of early embryonic development and survival. Effects of PI3K or MEK1/2 inhibitors on the BDNF promotion of preimplantation embryo development (A), stimulation of cell numbers in blastocysts (B), and suppression apoptosis (C). Two-cell-stage embryos were cultured without (control, C) or with BDNF (10 ng/ml) with or without the inhibitor of PI3K, LY294002 (LY2, an inhibitor of PI3K) or its inactive analog, LY303511 (LY3). Some embryos were treated with BDNF with or without U0126 (an inhibitor of MEK1/2), or its inactive analog, U0124. At 96 h after culture, the proportion of embryos at hatched blastocyst stage was evaluated. (Mean±SEM, n=5, 52–53 embryos per group.) Total cell number and TUNEL-positive nuclei in the expanded blastocysts were determined (Mean±SEM, total n=20 embryos per group) at 72 h after culture. *, $P<0.01$ vs. BDNF group.

during embryonic development to form the invasive trophoblasts that mediate embryo implantation into the uterine wall, our findings suggest a potential involvement of BDNF during implantation.

Following binding of BDNF, TrkB induces a complex signaling cascade, including PI3K and ERK pathways in neurons to promote neuronal survival in neurons (Kaplan and Miller, 2000; Yuan and Yankner, 2000). In preimplantation embryos, we demonstrated that the BDNF-promoted embryo development and survival is mediated thorough the PI3K, but not the ERK pathway. Although the ERK pathway is present in preimplantation embryos (Wang et al., 2004), recent study showed that blockade of the JNK or p38 MAPK pathway, but not of the ERK pathway, resulted in the inhibition of the development of mouse preimplantation embryos (Maekawa et al., 2005). Thus, the ERK pathway may not be less important for the early embryonic development.

BDNF and NT-4/5, the two specific ligands for TrkB, are expressed in the trophectoderm cells of blastocysts. They are also detectable in the oviductal and uterine epitheliums based on RT-PCR and immunoassays. Because preimplantation embryos develop in the oviduct until early on day 4 of pregnancy when they reach the late morula/blastocyst stage and then migrate to the uterus, observed increases in levels of TrkB ligands in the oviduct and uterus during the preimplantation period suggest their paracrine roles during early

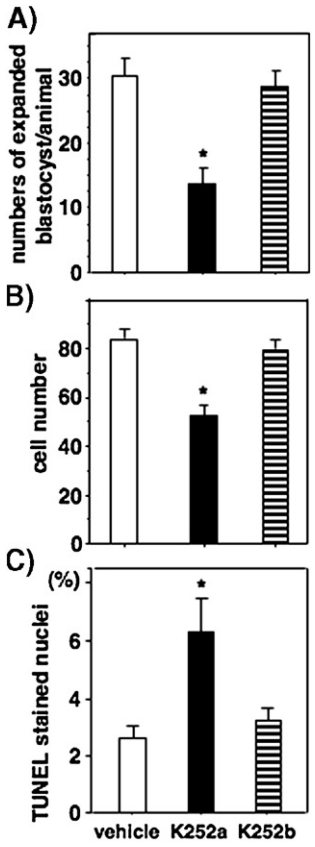

Fig. 8. Roles of endogenous TrkB ligands during embryonic development *in vivo*. Effects of K252a treatment *in vivo* on the development of preimplantation embryos (A), cell numbers in blastocysts (B), and apoptosis (C). HMG-primed immature mice were treated with hCG to induce ovulation before mating. Mice were treated with or without K252a or K252b (10 μg/injection at 79, 83, and 87 h after hCG). At 90–92 h after hCG injection, the number of expanded blastocysts was evaluated ($n \geq 4$ animals). Subsequently, total cell number and TUNEL-positive nuclei in the blastocysts were determined ($n = 20$ embryos). *, $P < 0.01$ vs. vehicle group.

embryonic development. Although mRNA levels for BDNF and NT-4/5 in pregnant oviduct and uterus were comparable, BDNF proteins were more abundant, suggesting oviductal and uterine BDNF could act on TrkB expressed in blastocysts via paracrine mechanisms. Because both TrkB and its ligands are expressed in the oviduct and uterus, their autocrine/paracrine roles in the oviduct and uterus cannot be ruled out.

Herein, we have demonstrated important roles of the BDNF/TrkB signaling system in the development and survival of preimplantation embryos based on *in vitro* and *in vivo* studies. It is becoming clear 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 (Dissen et al., 1995; Paredes et al., 2004; Spears et al., 2003), the nuclear and cytoplasmic maturation of oocytes into preimplantation embryos (Kawamura et al., 2005c), and early embryonic development. The present demonstration of an augmenting role for the BDNF/TrkB signaling pathway in early embryos underscores the importance of diverse autocrine/paracrine systems for early embryonic development. Understanding of these intercellular communication networks could lead to new approaches in the treatment of infertility, and facilitate future formulation for the optimal culture conditions for *in vitro* fertilization and embryonic stem cell derivation.

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