**Abstract.** In contrast with mature brain-derived neurotrophic factor (mBDNF), proBDNF induces cell apoptosis. However, the function of proBDNF in oligodendrocytes remains unclear. In the present study, the OLN-93 oligodendroglia cell line was utilized as an *in vitro* model to analyse the functions of proBDNF in oligodendroglia. p75NTR, sortilin and proBDNF were expressed in cultured OLN-93 cells. It was indicated that proBDNF inhibited OLN-93 cell proliferation in a dose-dependent manner as determined using the MTT assay and BrdU staining. Furthermore, proBDNF suppressed the migration of OLN-93 cells as demonstrated using the scratch assay. proBDNF also decreased cell viability and promoted apoptosis as indicated by activated cysteine-aspartic acid protease-3 (caspase-3) immunocytochemistry. Notably, anti-proBDNF treatment neutralized the effect of proBDNF and resulted in increased cell proliferation and migration and decreased apoptosis. However, these effects were not observed in the presence of recombinant p75NTR extracellular domain-human FC fusion protein and p75NTR antibody, indicating that proBDNF imparts its inhibitory effects on oligodendrocytes through the p75NTR signal pathway.

**Introduction**

Mature brain-derived neurotrophic factor (mBDNF), as well as its precursor proBDNF are widely distributed in the central nervous system (CNS) as a member of the neurotrophin family (NTs). The current view is generally believed that NTs has a protective effect on neurons (1). Interestingly, mature NTs (for example, mBDNF) are not the primary gene products. They are removed from a relatively large neurotrophic factor precursor (for example, proBDNF). The full length protein of proBDNF is about 35 kDa. After shearing, a mature molecule (mBDNF) with a length of about 13.5 kDa and a precursor fragment (pre-domain) of about 20 kDa are obtained. Usually, proBDNF has two processes to be cut by enzyme: One is cleaved endogenously and then secreted to the extracellular matrix (2), the other is directly secreted to the extracellular domain, and then modified in the extracellular environment (3).

Deffer from the mBDNF, which promotes neuron survival and regeneration, which are important to functional recovery after injury to the nerve system (4), no one mentioned whether the exogenous proBDNF may have certain functions. People thought proBDNF was just a middle product. In 2001, Lee *et al* reported the inhibitory effect of proBDNF on neurons (5). Beattie found that full-length proNGF secreted into the extracellular binding to p75 neurotrophin receptor (p75NTR) mediated apoptosis in neurons and glial cells (6). In particular, our previous study found that the inhibitory effect of proBDNF on cells was induced only after nerve injury. After spinal cord injury, proBDNF could inhibit the regeneration of axons (7).

In our previous study, we have shown that endogenous proBDNF can inhibit macrophage infiltration and disturb demyelination and remyelination after SCI (7). This indicates that proBDNF may affect cells other than neurons during post-injury repair.

The present study focused on the functions of proBDNF in proliferation and migration of oligodendroglia. We observed that proBDNF can inhibit proliferation and migration of OLN-93 cells, a permanent oligodendroglia cell line. Moreover, anti-proBDNF treatment could be effective in protecting cells from apoptosis and in promoting cell proliferation and migration.
migration. Therefore, blocking proBDNF may be a therapeutic target for traumatic injuries in CNS.

Materials and methods

Cell culture and maintenance. The OLN-93 oligodendroglia cell line was utilized in this study. OLN-93 cells are known to express several oligodendroglial markers; however, they do not exhibit characteristics of astrocytes (8). Cells were incubated at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM)/F12 with 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Growth medium was changed twice a week. When the cells reached 70% confluency, they were digested with 1% trypsin/EDTA (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 2 min. FBS was added to stop the digestion once the cells were round and floating. Cells were then seeded in plates or flasks and maintained in an incubator.

One subset of cells was directly fixed for fluorescent immunohistochemical staining using primary antibodies against proBDNF, p75NTR, and sortilin, whereas another subset of cells was treated with serial concentrations of recombinant proBDNF (1, 3, 10, 30, 100 ng/ml); bovine serum albumin (BSA; 100 ng/ml) treatment was used as a control. Meanwhile, sheep anti-proBDNF antibody (5, 10 µg/ml), monoclonal proBDNF antibody (PB192E; 100 ng/ml), mouse anti-p75NTR antibody (10 µg/ml; 9,650 from Moses Chao), recombinant p75NTR extracellular domain-human FC fusion protein (p75NTERCD-fc; 3 µg/ml), and normal IgG (10 µg/ml) were also administrated in in vitro observations.

p75NTERCD-fc and the recombinant proBDNF with harbouring an RR-AA mutation on the cleavage site were incubated with corresponding secondary antibody and DAPI at room temperature for 60 min. Finally, after washing the cells three times, fluorescent images of the cells were captured using an Olympus BX-50 (Olympus Corp., Tokyo, Japan) fluorescent microscope equipped with a CCD camera.

Western blot analysis. Cells were seeded at 10^5 cells/well in a 6-well plate, incubated at 37°C and 5% CO2 and subsequently lysed using RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM NaEDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO_4, 1 mM phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin] when they reached a confluency of 70%. After sonication and centrifugation to remove cell debris, the lysate was loaded onto a sodium dodecyl sulphate polyacrylamide gel, containing stacking (0.5%) and a separating gel (10%). On completion of electrophoresis, proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) using the semi-dry method (Bio-Rad, Berkeley, CA, USA) at 20 V, 20 min. The membrane was then washed with PBS three times and blocked with a blocking buffer (5% skim milk in PBS) for 1 h at room temperature. Rabbit anti-p75 (1:1,000; Abcam), sheep anti-proBDNF (5 µg/ml; University of South Australia) and rabbit anti-sortilin (1:1,000; Sigma-Aldrich; Merck KGaA) antibodies were subsequently added as primary antibodies, followed by incubation at 4°C for overnight. Before and after triple washes with PBST, goat anti-rabbit IgG-HRP (1:3,000) and goat-anti-sheep IgG-HRP (1:3,000; both Sigma-Aldrich; Merck KGaA) were co-incubated with the membrane for 1 h at room temperature. ECL substrate solutions A & B (GE Healthcare) were mixed and added onto the membrane in order to observe any potential fluorescent protein bands. Finally, the film was developed in a dark room to obtain results.

Cell viability assay. Cells were seeded at 10^4 cells/well in a 96-well plate, followed by incubation at 37°C and 5% CO2 for overnight to allow the cells to attach to the bottom of the wells. After washing the cells with PBS three times, the cells were cultured in a serial concentration of proBDNF (1, 3, 10, 30, 100 ng/ml) or in a serial concentration of proBDNF and monoclonal proBDNF antibody PB192E (100 ng/ml) in an FBS-free medium. BSA (100 ng/ml) treatment was used as a control. After 20 h, 10 µl of MTT solution (5 mg/ml in PBS; Sigma-Aldrich-Aldrich; Merck KGaA) was added to each well, followed by incubation for an additional 4 h. Subsequently, 100 µl of filter-sterilized solubilisation solution (10% SDS in 0.01 M HCl) was added to each well and incubated overnight at 37°C to dissolve the insoluble purple formazan product in order to produce a coloured solution. Finally, the optical density (OD) of the solution in each well was measured at 595 nm using a multi-well scanning spectrophotometer (Bio-Rad Model 2550 EIA Reader); a wavelength of 620 nm was used as a reference.

BrdU proliferation assay. Cells were at first seeded at 5x10^3 cells per well on a pre-treated glass coverslip in a 24-well plate. Approximately 200 µl of BrdU (80 µM BrdU-medium solution; Sigma-Aldrich; Merck KGaA) was then added to each well, followed by incubation at 37°C and
5% CO₂ for 24 h. The cells were then fixed in 4% PFA and washed with PBS three times. After a 1 h incubation in HCl and neutralization in boric buffer (pH 8.0-8.5), the cells were blocked using a blocking buffer (2% BSA, 0.5% Triton X-100, 0.1% Tween-20, 5% donkey serum). An anti-BrdU antibody (1:1,000; DSHB; University of Iowa, Iowa City, USA) was incubated with the cells as a primary antibody. Then, after washing the cells with PBST three times, cells were subjected to secondary antibody of goat anti-mouse IgG CY3 (1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) and DAPI (1:1,000; Sigma-Aldrich; Merck KGaA). After washing the cells three times, the number of BrdU/DAPI-positive cells was counted at five random fields per slice at magnification, x10 under a fluorescence microscope.

Scratch assay. The scratch assay was performed as previously described (10). Briefly, OLN-93 cells cultured in a 6-well plate were wounded using a sterile P20 pipette tip, followed by washing with culture medium to remove cell debris. The cells were then treated with 1, 3, 10, 30, and 100 ng/ml proBDNF and 100 ng/ml BSA for 24 or 48 h, respectively. Subsequently, cells were fixed by 4% PFA for 10 min then stained by 0.05% crystal violet for 30 min. After washed by PBS, phase contrast images were captured in 6 different fields of every wound using an inverted microscope (Olympus IX-71; Olympus Corp.). Cells that migrated into the wound space were counted in the captured image. The experiments were performed in triplicates.

Cell apoptosis assay. OLN-93 cells at 5x10⁴ cells per well were seeded in 24-well plates. When the cells reached 90% confluency, they were washed three times with PBS and treated with proBDNF, proBDNF antibody and BSA consecutively at 37°C and 5% CO₂ for 24 h. After treatment, cells were fixed by 4% PFA at room temperature for 10 min and then washed with PBS before being blocked using a blocking buffer (2% BSA, 0.5% Triton X-100, 0.1% Tween-20, 5% donkey serum). Next, rabbit anti-activated caspase-3 (1:1,000; EMD Millipore) was co-incubated with the cells at 4°C for overnight. Then, sheep anti-rabbit IgG FITC (1:1,000; Abcam) and DAPI (1:1,000; Sigma-Aldrich; Merck KGaA) were applied and co-incubated with the cells at room temperature for 1 h. After washing the cells 3 times, fluorescent images of the cells were captured using an Olympus BX-50 fluorescent microscope.

Data acquisition and statistical analysis. Five visual fields were randomly selected from superior, inferior and central parts as well as from left-hand and right-hand sides of each slide for fluorescence microscopy analysis. Images at magnifications of x10, x20 and x60 were obtained using an Olympus BX-50 fluorescent microscope.

The total number of cells and positively-stained cells were counted using Image J. Quantitative analysis was performed for all experiments using SPSS for Windows v. 13.0. All data are presented as mean ± standard error of the mean (SEM). Data were analysed using one-way analysis of variance (ANOVA),
followed by Tukey's post-hoc tests, where appropriate, or t-test for paired groups. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least 3 times.

**Results**

**OLN-93 cells express proBDNF and its receptors.** OLN-93 cells were maintained in DMED/F12 1:1 medium. In the presence of 10% FBS, cell proliferation occurred at a high rate (8). All the cells expressed proBDNF, p75NTR and sortilin as shown by fluorescent immunocytochemistry (Fig. 1A-C). The typical oligodendroglia cell marker NG2 (Fig. 1D), which was also used to label oligodendrocytes in spinal cord was also expressed (11). Cell growth was observed to be density-dependent. Cells were mainly bipolar with long cellular extensions at lower confluency; they formed large clumps interconnected with long, thin cellular processes at higher confluency (Fig. 1E). Moreover, Western blot analysis using the cell lysate showed bands of proBDNF, p75NTR and sortilin at the corresponding molecular weights (Fig. 1F). These results indicated that OLN-93 cells expressed endogenous proBDNF and its receptors p75NTR and sortilin.

**proBDNF inhibits OLN-93 cell viability.** To examine the effect of proBDNF on OLN-93 cells, we used a monoclonal antibody (PB192E) that is known to activate proBDNF. We observed that proBDNF inhibits cell viability in a dose-dependent manner, however proBDNF antibody PB192E neutralized the inhibitory effect. **P<0.001 vs. the BSA. **ANOVA, one-way analysis of variance.

![Figure 2: Cell viability assay by MTT method.](image)
proBDNF antibody (PB192E), raised against the pro-domain of BDNF, to neutralise the effects of proBDNF. Cells treated with PB192E at 10 µg/ml were more active than those treated with normal IgG (Fig. 2A), indicating that endogenous proBDNF may have inhibited the cell growth.

MTT assay revealed that proBDNF imparts a toxic effect on cell viability. Treated with serial concentrations of proBDNF (1, 3, 10, 30, 100 ng/ml), the cell growth curve demonstrated a significant growth inhibition at 10 ng/ml, with the highest growth inhibition at 100 ng/ml (Fig. 2B) (P=0.000904), indicating a dose-dependent response.

Then, we treated the cells with serial concentrations of proBDNF (1, 3, 10, 30, 100 ng/ml) and PB192E antibody (100 ng/ml). Cells in this group had significantly higher bioactivity than those treated with normal IgG (Fig. 2B) (P=0.000904), indicating a dose-dependent response.

proBDNF inhibits, but anti-proBDNF promotes, OLN-93 cell proliferation. Quantitative assessment of the proliferative activities in OLN-93 cells were performed (Fig. 3). BrdU/DAPI double-labelling was applied to the OLN-93 cell proliferation assay (Fig. 3C). The proliferating cells were BrdU+/DAPI+, whereas the non-proliferating ones were only DAPI+. The percentage of BrdU+/DAPI+ cells in the proBDNF-treated groups was significantly lower than that in the BSA group, illustrating a dose-dependent inhibition (Fig. 3A) (P=0.0206 in 10; P=0.0420 in 30 ng/ml; P=0.000126 in 100 ng/ml).

To confirm our results, we used polyclonal sheep anti-proBDNF antibodies to neutralise endogenous proBDNF. We found that cells treated with polyclonal antibodies showed higher proliferative activities than those treated with normal sheep IgG (Fig. 3B) (P=0.000105). These experiments indicate that OLN-93 cells secrete endogenous proBDNF, which inhibits their growth.

Inhibitory effect of proBDNF on OLN-93 cell proliferation is blocked by soluble p75 receptor body or p75 antibodies. To investigate the possible pathway by which proBDNF exerts an inhibitory on OLN-93 cells, recombinant fusion molecule of p75NTRECD-fc and p75NTR functional antibody were utilized. These have been used in our previous study as competitive inhibitors to block p75NTR signal transduction extracellularly (12). In this study, we treated OLN-93 cells with 3 µg/ml of p75NTRECD-fc protein and 10 µg/ml of anti-p75NTR for 24 h. Then, BrdU/DAPI double-labelling...
was conducted to check the proliferative activities after 24 h of incubation. Under both the treatments, the percentage of proliferating cells significantly increased in comparison with those in the normal sheep IgG control group (Fig. 3C) (P=0.00206 in p75NTRECD-fc; P=0.0151 in anti-p75NTR).

Moreover, we treated OLN-93 cells with proBDNF and p75NTR antibody. The cells were co-incubated with 100 ng/ml proBDNF and 10 µg/ml of p75NTR antibody for 24 h. Treated cells had better proliferative activities than those in the proBDNF group (Fig. 3C) (P=0.000103) but had no significant differences in comparison with those in the BSA group (Fig. 3C) (P=0.264). This indicated that proBDNF can inhibit OLN-93 cell proliferation via the p75NTR pathway and that proBDNF can be blocked by disruption of p75NTR signal transduction with the soluble receptor body or antibody.
after CNS injury. To investigate the function of proBDNF in OLN-93 cell migration, the scratch assay was used. After scratching, a cell-free wound region was generated in 6-well culture plates (Fig. 4Aa, d, g). Serial concentrations of proBDNF (10, 30, and 100 ng/ml) and proBDNF antibody (5 and 10 µg/ml) were used to treat the cells. After cell growth for 24 and 48 h, a number of cells were observed migrating from the edges into the cell-free scratch region (Fig. 4A). Cells treated with proBDNF showed lower migration activity. A higher concentration of proBDNF resulted in the migration of fewer cells, indicating a dose-dependent inhibition of cell migration by proBDNF (Fig. 4B and C) (P=0.0112 in 10 ng/ml; P=0.000104 in 30 ng/ml; P=0.000229 in 100 ng/ml).

In comparison with the proBDNF group, more cells migrated into the scratch wound region in the proBDNF antibody (5 or 10 µg/ml) treatment group; however, the population of migrated cells between different anti-proBDNF concentrations showed no statistical differences (Fig. 4B and C) (P=0.24 in 24 h; P=0.50 in 48 h).

**proBDNF induces OLN-93 cell apoptosis.** As a member of the cysteine-aspartic acid protease family, caspase-3 is activated when cell apoptosis occurs (13). In this study, we applied the
activated caspase-3 antibody to observe apoptosis of OLN-93 cells that were treated with proBDNF (10, 30, 100 ng/ml) and BSA (100 ng/ml) (Fig. 5A).

As expected, the percentage of apoptotic cells in the proBDNF group was significantly higher than that in the BSA group (Fig. 5B) \((P=0.000384)\). Moreover, with an increase in the dose of proBDNF, more cells were found to be caspase-3-positive (Fig. 5B) \((P=0.024)\).

**Discussion**

Mature BDNF promotes neural differentiation and survival (14) and also plays an important role in proliferation, migration and myelination of oligodendrocytes (15). However, the BDNF precursor, proBDNF, is involved in inhibitory activities in the CNS such as promoting neuron apoptosis, inhibiting neurite outgrowth and cell proliferation and survival via p75NTR and co-receptor sortilin (16-19). The effects of proBDNF on oligodendrocyte have remained unclear. According to our studies, the inhibitory effect of proBDNF on OLN-93 cells is dose-dependent and can be blocked by the proBDNF antibody (Fig. 2). These results indicated the involvement of exogenous and endogenous proBDNF in the viability of oligodendrocyte-like cells.

Endogenous and exogenous proBDNF suppressed OLN-93 proliferation and migration. In our previous studies, we observed that proBDNF inhibited the migration of EDI+ macrophages after SCI (7) and of granule cells in the developing cerebella (20). This result suggests that similar to neurons, proBDNF may exert negative effects on the migration of non-neuronal cells. In order to investigate this, the scratch assay was performed. Under a standardized wound condition and same incubation period, the migration was inhibited by proBDNF in a dose-dependent manner. Higher concentrations of proBDNF had higher inhibitory activities on cell migration, while treatment with proBDNF antibody facilitated the migration (Fig. 4). Further, OLN-93 proliferation, as investigated by BrdU staining, showed similar results (Fig. 3). These results indicate that proBDNF may be a mitosis suppressor, which prevents the proliferation and migration of oligodendrocyte precursor cell (OPC)s after SCI, and that its detrimental effects can be inhibited by proBDNF antibodies.

Both mBDNF and proBDNF mediate their biological activities by binding to the cell surface receptors. It is a common phenomenon that the receptors which combine with mNTs always may combine with proNTs; even though to mediate a different signal. The four main related receptors are tyrosine kinase receptor (Trk) (21), p75NTR (22) tumor necrosis factor (TNF) (23) and sortilin (24). Because of the difference in 3D structure, proNTs cannot combine with the Trk receptor (25), however, it has a higher affinity to p75NTR and sortilin (26).

P75NTR, as a receptor, can be combined with all members of the NTs family. When combined with mNTs, P75NTR shows protective effects on cells, which can promote neuronal survival, myelination and migration. When combined with pro-NTs, P75NTR mainly mediates the apoptosis of cell. It is significant that although the Trk receptor binds to mNTs can mediate cell survival (27), when p75NTR and Trk are both on the cell surface, p75NTR plays a dominant role in apoptosis. This may be due to the stronger affinity of p75NTR to the pro-NTs. It can form a high affinity receptor complex and conduct the apoptosis signal. In this process, sortilin has played a key synergy.

Sortilin is a transmembrane protein that belongs to the Vps10p fragment receptor family (28). It has a high affinity for proBDNF and p75NTR. In fact, sortilin exists in the way of the co-receptor in the reaction between pro-NTs and p75NTR (29). Sortilin can form a stable sortilin/p75NTR complex with p75NTR. In the effect of pro-NTs, the affinity of the compound is 10 times higher than that of mNTs, indicating that the sortilin/p75NTR complex is more inclined to combine with proBDNF rather than mBDNF. Further studies also found that pro-NTs binding to sortilin/p75NTR showed stronger stability to proteolysis and denaturation, and more difficult to decompose and destroy (24). Therefore, sortilin can protect pro-NTs from decomposition and destroy, thus prolonging the time of action of pro-NTs and intensifying the apoptosis process of cells.

In the present study, we found that exogenous proBDNF promotes cell apoptosis in a dose-dependent manner (Fig. 5), indicating that proBDNF induces OLN-93 cell apoptosis. In order to investigate the presence of the p75NTR signal transduction pathway in OLN-93 cells, p75NTR functional antibody and recombinant fusion molecule of p75NTR-RECD-fc were used to inhibit the functions of p75NTR (Fig. 3D). As expected, the obtained results showed specific suppression of proBDNF when p75NTR was blocked, suggesting that the functions of proBDNF are mediated via the p75NTR signal transduction pathway. Considering the important role of oligodendrocytes in vivo, these data suggest that the proBDNF/p75NTR pathway is essential for the functions of oligodendrocytes after CNS injury.

Mature oligodendrocytes are located in the outer rim of the grey matter in the spinal cord (30). After CNS injury and cell loss, cell migration of proliferative OPCs can be observed (31). Our previous studies have shown that proBDNF inhibits the migration of cerebellar granule cells during development (18) in addition to inhibiting macrophage infiltration in the injured spinal cord (7). In this study, the scratch assay performed using OLN-93 cells showed that proBDNF inhibits cell migration in vitro (Fig. 5).

In summary, we demonstrated that exogenous and endogenous proBDNF negatively regulates the proliferation and migration of OPC-like cells in vitro. Antibodies raised against the BDNF pro-domain or p75NTR pathway blocker p75NTR-RECD-fc effectively suppressed the functions of proBDNF and facilitated cell proliferation and migration as well as reduced the apoptosis of OPC-like cells, indicating their therapeutic value for functional recoveries after SCI. However, additional in vivo studies are needed; our research group has been actively conducting further investigations in this field.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
XFZ and SF conceived the study. SL conducted the analyses. WG, HZ, JHZ and LT also analysed the data. All authors contributed to the writing and revisions of the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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