Functional expression of the human BDNF (brain-derived neurotrophic factor) gene

인간 BDNF (brain-derived neurotrophic factor) 유전자의 기능적 발현

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Functional expression of the human BDNF
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A Dissertation

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CONTENTS

CONTENTS OF FIGURES AND TABLES

Functional expression of the human BDNF (brain-derived neurotrophic factor) gene

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ABSTRACT

The brain-derived neurotrophic factor (BDNF) is a small secretory protein and a member of the nerve growth factor (NGF) gene family. It has been shown to influence the survival and differentiation of specific classes of neurons. BDNF binds to the tyrosine kinase TrkB receptor and the low-affinity neurotrophin receptor p75. Binding of BDNF to receptors promotes the activation of signaling pathways at local synaptic targets and also has long-term effects on gene transcription. We have cloned the human BDNF gene using the PCR technique from human brain tissues. The nucleotide sequence of the cloned BDNF gene has determined with an automatic DNA sequencing analyzer and shown an ORF consisting of 744 bp, which corresponding to 248 amino acid residues. In order to investigate the functional aspects of human BDNF, we have studied several different expression systems using both prokaryotic and eukaryotic expression systems. The human

BDNF overexpressed in the prokaryotic system using *E. coli* but did not show the functional activity. Therefore, we have developed the eukaryotic system which produces biologically active protein using the marine microalgae, *Chlorella ellipsoidea*. We also investigated the functional expression *in vivo* of human BDNF using the transgenic techniques into the Medaka, *Oryzias latipes*. The results showed that the transgenic human BDNF gene expresses at several important locations which are critical for the developmental stages. We have applied the valuable *Chlorella* expression system for the mass production of target proteins.

Key word : brain-derived neurotrophic factor, *Chlorella* transformation, expression, transgenesis.

INTRODUCTION

Neurotrophins are growth factors that promote cell survival, differentiation, and cell death (Figure 1). The neurotrophin family of growth factors, including NGF, BDNF, and neurotrophins-3 (NT-3) and -4 (NT-4) regulates neuronal survival, differentiation and synaptic plasticity (Aloe *et al.*, 1999; Aronica *et al.*, 2001; Bonni *et al.*, 1999; Carter *et al.*, 1995; Chan *et al.*, 2001; Gottschalk *et al.*, 1998; Kossel *et al.*, 2001; Murer *et al.*, 2001; Zuccato *et al.*, 2001). They are closely structurally related: NGF, BDNF, NT-3 and NT-4 share approximately 50% sequence identity and the regions of sequence similarity and variation are clustered. Mature BDNF can activate presynaptic and postsynaptic TrkB receptors at the synapse while on endothelial cell surfaces, BDNF promotes TrkB-mediated endothelial cell survival (Kokala *et al.*, 1995; Korte, *et al.*, 1995; Lee *et al.*, 2001). BDNF and other neurotrophins are critically involved in long-term potentiation(LTP). Endogeneous BDNF, via activation of the receptor tyrosine kinase TrkB, can regulate induction of hippocampal LTP. Although the induction of LTP is generally believed to be postsynaptic, the facilitating action of BDNF on LTP induction is assumed to involve presynaptic mechanism (Kohara *et al.*, 2001; Kovalchuk *et al.*, 2002; Weese-Mayer *et al.*, 2002).

BDNF has a functional role in the expression of LTP in the

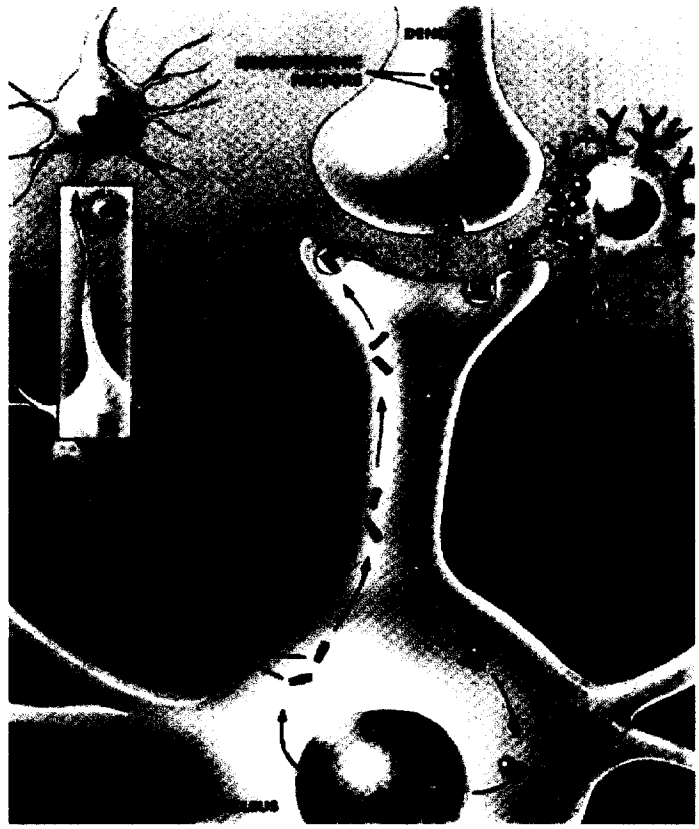


Figure 1. Pathway of the neurotrophic factors in neuron. In the brain, a neurotrophic factor is released by a neuron or a support cell, such as an astrocyte, and binds to a receptor on a nearly neuron. This binding results in the production of a signal which is transported to the nucleus of the receiving neuron where it results in the increased production of proteins associated with neuronal survival and function.

hippocampus and promotes survival through inactivation of components of the cell death machinery. In addition to its role in neuronal survival, BDNF also modulates synaptic activity. At the postsynaptic membrane, BDNF may act as a neurotransmitter itself, as rapid pulsatile application of BDNF to neurons can cause TrkB-dependent membrane deplorization within milliseconds (West *et al.*, 2001). Thus, BDNF treatment may contribute to recovery by limiting tissue damage, augmenting the function of spared neuronal systems, and promoting neural repair (Ankeny *et al.*, 2001). Expression of BDNF represents exciting possibilities for reversing devastating neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and Lou Gehrig's disease, and so on.

In this theme, we describe the molecular cloning of human BDNF and the functional expressions of cloned BDNF using the prokaryotic and eukaryotic systems. *E. coli* is the most widely used heterologous prokaryotic expression system, but it has several limitations such as poor or no expression of some proteins, proteins that lack biological activity, proteins that are toxic to *E. coli*, and formation of insoluble inclusion bodies (Friehs and Reardon, 1993).

Especially, we used the Medaka embryos for the functional expressions *in vivo*. The medaka (*Oryzias latipes, Aplocheilus latipes*), which inhabits fresh and brackish waters from India throughout South-east Asia across Wallace's line to Timor, Sulawesi, Luzon, and Japan, is unique among common laboratory teleost (Iwamatsu, 1994). The Medaka is an oviparous freshwater killifish belonging to the family of toothcarps (*Cyprinodonidae*). It

has also been named as the Geisha-girl fish, topminnow, and ricefish. Indigenous to areas of Japan, Taiwan, and southeastern Asia where air temperatures are 5℃ to 35℃, the medaka is common in rice paddies, feeding extensively on mosquito larvae. The wide strain is brownish-black in color, while cultivated varieties are blue-black, red (golden), white, and red or white variegated with black. Adult medakas are 2 to 4 cm long, and are easily maintained in unheated tanks at ordinary ambient temperature. Sexual dimorphism is externally evident. The male's anal fin is larger than the female's, usually has a convex ventral margin, and the fin rays are rarely branched; the anal fin rays have terminal bifurcations in the female. The dorsal fin of the male is deeply notched between the last two rats, while the female's dorsal fin is smaller and more rounded and has no notch. Breeding females are also distinguishable by the distended abdomen. A healthy female will produce upwards of 3,000 eggs in a single breeding season. *Oryzias latipes* is particulary valuable for embryological purposees because its eggs can be obtained regularly any time of the year and the egg possesses exceptional optical clarity. The progress of development is summarized in Figure 2.

We have developed the eukaryotic system which could produce biologically active protein with mass quantity using the marine microalgae. *Chlorella ellipsoidea* is unicellular green alga, which has been widely used in aquaculture and food industry. It can be inexpensively cultured in large scale because it requires only a limited amount of minerals and sunlight. Some species grow

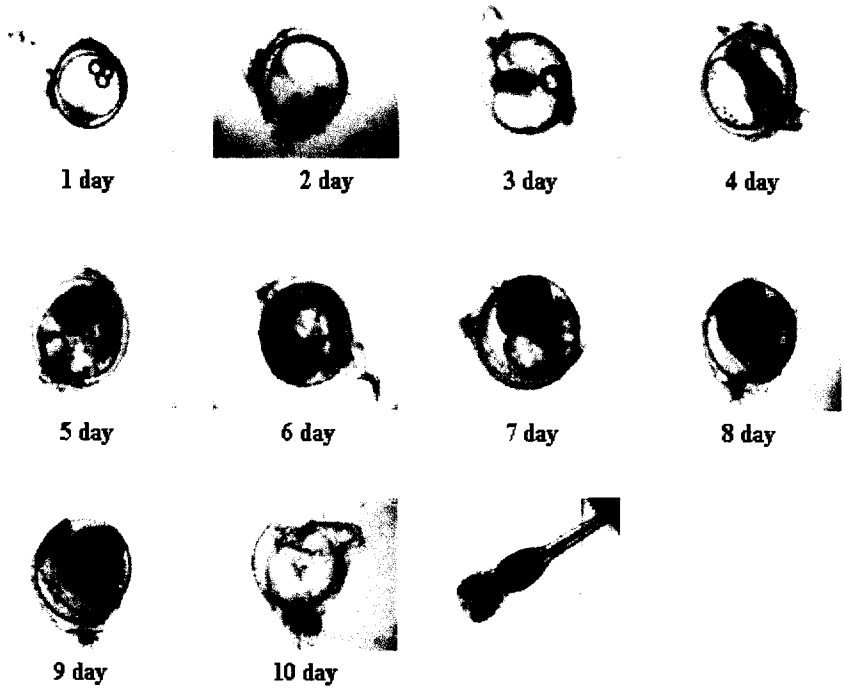


Figure 2. Embryonic development of the medaka egg. 1 day; oviposition, fertilizing, cleavage plane, 2 day; early neurula, 3 day; "blastopore" closed and optic vesicle, 4 day; optic lens and otocyst, 5 day; pectoral fin bed, 6 day; pink blood, 7 day; urinary bladder, 8 day; swim blader, 9 day; jaw movement and yellow coloration, 10 day; hatching

relatively fast, dividing 2-9 times per day depending on the light intensity and temperature. *Chlorella* is an eukaryote and therefore can synthesize complex proteins that require post-translational modification in order to become biologically active. These characteristics provide a rationale for the using *Chlorella* as a new system for foreign protein overexpression using *C. ellipsoidea* table transformant (Kim *et al.*, 2002). In this study we used a plasmid vector system previously developed by Kim *et al.*, (2002) and also applied the valuable *Chlorella* expression system for the mass production of target proteins.

MATERIALS AND METHODS

I . Cloning of human BDNF gene

In order to clone human BDNF gene, specific primers (BDNF-F, BDNF-R) were designed (Table 1) and used for reverse transcription polymerase chain reaction (RT-PCR) using the template RNA isolated from human brain tissue.

I-1. RT-PCR: The RT-PCR was performed using a commercial premix kit (Bioneer) and the reaction mixture contained total RNA 1.0 ng (Bioneer), specific forward primer 20 pmol, and reverse primer 20 pmol in a thermocycler (Perkin Elmer 2400). RT-PCR reaction was RNA denaturation for 10 minutes at 57℃, cDNA synthesis for 60 minutes at 42℃, RTase inactivation for 5 minutes at 94℃ and the profile of thermocycling was one prereaction at 94℃ for 5 minutes and 35 cycling reaction with 94℃ 30 seconds denaturation, 48℃ 30 seconds annealing, 72℃ 1 minutes extension. After reaction, 15 $\mu\ell$ of RT-PCR product was analyzed on 1.0% agarose gel electrophoresis.

I -2. Subcloning in pGEM®-T vector

The amplified DNA fragment was subcloned into pGEM®-T vector system (Promega, USA), described in Figure 3. and transformed to XL1-Blue. The cloned DNA was confirmed by sequencing using T7 and SP6 primers (Table 1).

Table 1. Oligonucleotides used for this study

Primers	Sequence	Description
BDNF-F	5′-TGATGACCATCCTTTTCCTTACTATG-3′	Forward primer for RT-PCR
BDNF-R	5′-TCCACTATCTTCCCCTTTTA-3′	Reverse primer for RT-PCR
T7	5′-TAATACGACTCACTATAGGG-3′	Sequencing
SP6	5′-ATTTAGGTGACACTATAGAAT-3′	Sequencing

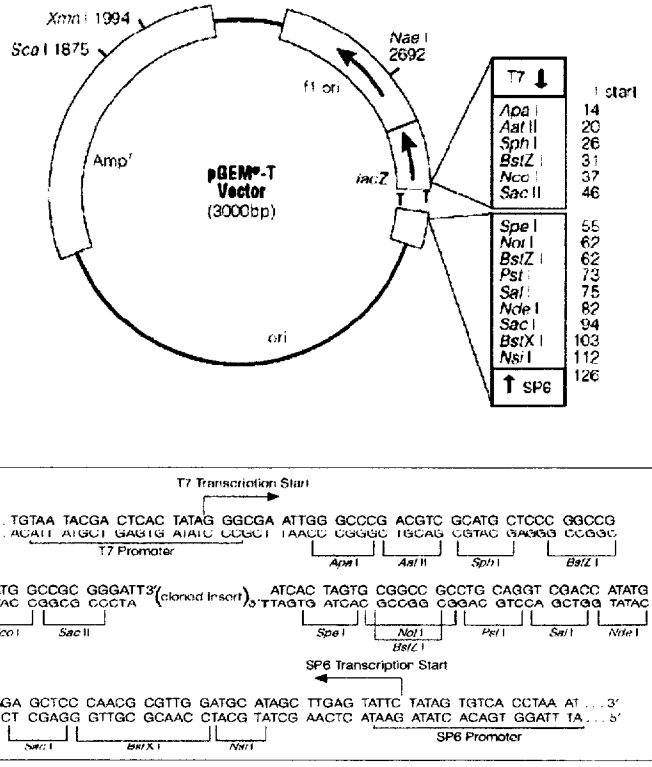


Figure 3. pGEM®-T vector circle map. It contains a T7, SP6 RNA polymerase transcription initiation site and multiple cloning site (MCS). The high copy number pGEM®-T vector contain T7 and SP6 RNA polymerase promoters flaking a multiple cloning region within the α -peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates.

I -3. Plasmid DNA purification and sequencing

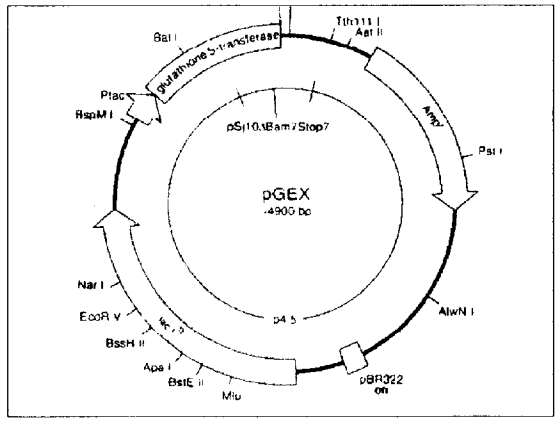
Plasmid DNA was purified using Wizard® plus SV minipreps DNA purification system (Promega). Sequencing reaction was performed using ABI PRISM™ DNA sequencing kit (Perkin Elmer). The reaction mixture was contained 500 ng of template DNA, 3.2 pmole of T7 or SP6 primer. After sequencing PCR reaction was completed and analyzed on using ABI 310 genetic analyzer.

II. Overexpression of human BDNF gene in *E. coli*

For expression in the prokaryotic system, the BDNF gene was subcloned in pGEX-4T-2 expression vector (Amersham bioscience) using restriction sites (BamH I, Xho I). pGEX-4T-2 vector is derived from pGEX plasmid which is applied from GST fusion protein and summarized in Figure 4. In order to produce recombinant pGEX-4T-2-BDNF, specific primer containing enzyme site was designed (Table 2). pGEX-4T-2 and BDNF gene construct was subcloned and transformed BL21(DE3). The cell harboring a plasmid which contains BDNF gene was cultured overnight in 10 ㎖ of LB/amp (30 ㎍/㎖) broth at 37℃ in shaking incubator. The cell was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM at mid-log growth (A_{550} of 0.5-1.0).

II-1. Analysis of expressed protein on SDS-PAGE

At various induction periods, one millilitre of the culture was



pGEX-4T-2 (27-4581-01)

Thrombin

Leu Val Pro Arg Gly Ser Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser

CTG GTT CCG CGT GGA TCC CCA GGA ATT CCC GGG TCG ACT CGA GCG GCC GCA TCG TGA

BamH I EcoR I Sma I Sal I Xho I Not I Stop codon

Figure 4. Map of pGEX-4T-2 (GST fusion) vector. The Glutathion S-transferase (GST) gene fusion system is a versatile system for the expression, purification, and detection of fusion proteins produced in *Eschericia coli*. Glutathione S-transferase fusion vectors have stop codon in three frames downstream from the multiple cloning site. The system is based on inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST.

Table 2. Oligonucleotides used for the construction of pGEX-4T-2

Primers	Sequence	Remarks
B/GST-F	5′-**GGATCC**ATGACCATCCTTTTC-3′	BamH I
B/GST-R	5′-**CTCGAG**CTATCTTCCCCTTTTA-3′	Xho I

centrifuged 14,000 rpm for 1 minutes at room temperature and the supernatant was removed by aspiration. Each pellet was resuspended in 100 ㎕ of 1×SDS-loading buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 2.0% SDS, 100 mM dithiothreitol, 0.1% bromophenol blue), and heated the samples to 100℃ for 3 minutes. The samples were centrifuged 14,000 rpm for 1 minutes at room temperature, and stored them on ice until all of the samples are collected and ready to load on a gel. 20 ㎕ of each samples were loaded on a 12% SDS-polyacrylamide gel (12% seperating gel, pH 8.8 overlayed 4% stacking gel, pH 6.8). Until bromophenol blue reaches the bottom of the gel, run the gel at 200 mA for 2 hours. The gel was stained with coomassie brilliant blue and destained with destaining buffer (7% acetic acid, 15% methanol).

Ⅱ-2. Western blot analysis

After total proteins containing GST-BDNF protein were seperated on a 12% SDS-acrylamide gel electrophoresis, the gel was transferred to nitrocellulose membrane at 100 mA for 16 hours in transfer buffer (25 mM Tris, 120mM glycine, 20% methanol). The nitrocellulose membrane was washed TTBS buffer (20 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 2.5 mM KCl) containing 0.1% Tween 20 and blocked with TBS buffer containing 5% skim milk for 1 hours. The membrane was washed for 5 minutes in TTBS buffer and treated for 1.5 hours with GST primary antibody which diluted 1:10,000 in TTBS containing 5% skim milk. After washing three times for 5 minutes, the membrane was treated for 1 hours with

alkaline phosphatase conjugated anti-mouse IgG in TTBS containing 5% skim milk. The membrane was washed three times for 5 minutes in TTBS and develped by NBT and BCIP solution in alkaline phosphate buffer (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$).

II-3. Isolation of overexpressed GST-BDNF fusion protein

GST-BDNF fusion protein was seperated on a 10% SDS-polyacrylamide gel electrophoresis and stained with 0.3 M $CuCl_2$. The seperated GST-BDNF fusion protein band was sliced out, and the protein was eluted using an electro-seperation system (S&S Elutrap, Schleicher & Schuell) and quantified by Bradford assay.

II-4. Production of polyclonal GST-BDNF antibody

Polyclonal antibodies against GST-BDNF protein were produced from mouse (6 week old female BALB/C) as follows. 30 ㎕ of antigen were mixed 30 ㎕ of Freund's complete adjuvant (Sigma) and emulsion was injected into mouse. 30 ㎕ of antigen mixed with Freund's complete adjuvant were given at 1 week intervals. One week later, 1 ㎖ of 1×10^7 sarcoma cells were injected. Ascitic fluid was collected after 10 days, clarified by overnight incubation at 4℃ and followed by centrifugation at 3000×g for 15 minutes. Collected supernatant were stored before use.

Ⅲ. Transgenesis on Medaka embryos by electroporation

Ⅲ-1. Medaka strain and maintenance

Japanese Medaka (*Oryzias latipes* or *Aplocheilus latipes*) is an oviparous freshwater killifish belonging to the family of toothcarps (*Cyprinodonidae*) and particulary valuable for embryological purposes because its eggs can be obtained regularly any time of the year and the egg possesses exceptional optical clarity. Once detached from the female medaka, eggs may be eaten by adult fish and snails in the aquarium. The eggs, therefore, should be collected as soon as possible after spawning. A cluster can be removed with either a widemouthed pipette or sharp stainless steel forceps while holding the female medaka in a dip net. When using forceps, handle the eggs very gently and avoid injuring the female. Dead or unfertile eggs appear milky, while viable embryos are yellowish in color and nearly transparent.

Ⅲ-2. Subcloning BDNF gene into pIRES2-EGFP expression vector

pIRES2-EGFP vector described in Figure 5 contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green florescent protein (EGFP) cording region. EGFP gene to be translated from a single bicistronic mRNA. pIRES2-EGFP is designed for the efficient selection of transiently transfected mammalian cells expressing EGFP and the protein of the interest. In order to subclone the BDNF gene into pIRES2-EGFP vector, the BDNF gene had to be

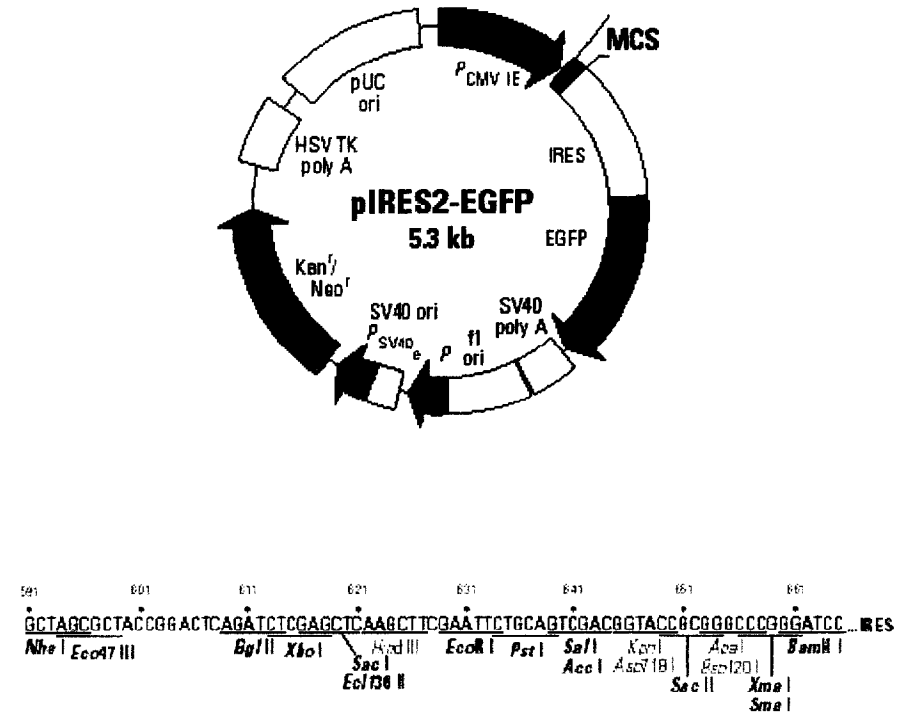


Figure 5. Map of PIRES2-EGFP vector. pIRES2-EGFP contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region. The MCS in pIRES2-EGFP is between the immediate early promoter of cytomegalovirus ($P_{CMV\ IE}$) and the IRES sequence. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the bicistronic mRNA.

contained upstream Xho I , downstream BamH I restriction enzyme sites. Specific primer containing enzyme sites was designed (Table 3) and used for PCR. The BDNF DNA containing enzyme sites was eluted and subcloned to pIRES2-EGFP vector. The resulting pIRES2-EGFP-BDNF construct was transformed and the clone containing BDNF gene in pIRES2-EGFP was confirmed by the sequencing analysis.

Ⅲ-3. Plasmid DNA preparation

The plasmid for electroporation was purified by cesium chloride density gradient ultracentrifugation. DNA was suspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 100 ㎍ was digested overnight with BamH I at 37℃. The digest was extracted once with phenol/chloroform and once with chloroform alone. The DNA was precipitated from the aqueous phase using absolute ethanol. This DNA was then resuspended in calcium-free phosphate buffered saline (PBS/-Ca++) at a concentration of approximately 100 ㎍/㎖ and was used several times for electroporation.

Ⅲ-4. Electroporation

Up to one hundred medaka embryos collected within 30 min after spawning were rinsed in PBS/-Ca^{++} and placed in a 0.4 cm wide cuvette with 800 ㎕ of the DNA solution. The embryos were pulsed three times using the 0.25 ㎌ capacitator of the Gene pulser (Bio-Rad). The pulse field strength was 125 V/cm, and each time constant was approximately 7-10 milisececonds with a pulse

Table 3. Oligonucleotides used for the construction of pIRES2-EGFP

Primers	Sequence	Remarks
B/EGFP-F	5′-CTCGAGATGACCATCCTTTTC-3′	Xho I
B/EGFP-R	5′-GGATCCCTATCTTCCCCTTTTA-3′	BamH I

interval of 1 second. The DNA solution was removed, and the embryos were rinsed in dH_2O and then placed into a 100 ㎜ steriled petri dish at 26℃.

Ⅲ-5. Observation and imaging of GFP fluorescence

An eggs was 1 to 1.5 ㎜ in dimer and was demersal. It has a beautifully clear and transparent chorion (noncellular) with numerous projecting filaments. GFP fluorescence was observed by using a fluorescence microscope equipped with an FITC filter.

Ⅳ. Processing of *Chlorella* transformation

Ⅳ-1. *Chlorella* strain and culture

Chloella ellisodea was cultured in f/2 medium (Table 4) containing 50 mg/㎖ chloramphenicol and streptomycin. Cells were inoculated at the initial concentration of 1×10^6 cells/㎖ and cultured under 3000 lux flurescent lamp at 25℃ with 18:6 hours light:dark cycle.

Ⅳ-2. Subcloning in mpCTV expression vector

The mpCTV vector (Figure 6) described in Kim *et al.* (2002). and BDNF were digested with BamH I and Xho I enzyme, ligated and transformed into *E. Coli*, XL1-Blue strain.

Ⅳ-3. The preparation of *Chlorella* protoplast

The *Chlorella ellipsoidea* cells were harvested when the cell

Table 4. Compositions of f/2 medium

Chemical ingredient	Content (L^{-1})
$NaNO_3$	75 mg
$NaH_2PO_4 \cdot H_2O$	5 mg
$Na_2SiO_3 \cdot 9H_2O$	15-30 mg
$Na_2 \cdot EDTA$	4.36 mg
$FeCL_3 \cdot 6H_2O$	3.15 mg
$CuSO_4 \cdot 5H_2O$	0.01 mg
$ZnSO_4 \cdot 7H_2O$	0.022 mg
$CoCl_2 \cdot 6H_2O$	0.01 mg
$MnCl_2 \cdot 4H_2O$	0.18 mg
$Na_2MoO_4 \cdot 2H_2O$	0.006 mg
Thiamin · HCl	0.1 mg
Biotin	0.5 μg
B_{12}	0.5 μg
Filtered seawater	1 ℓ

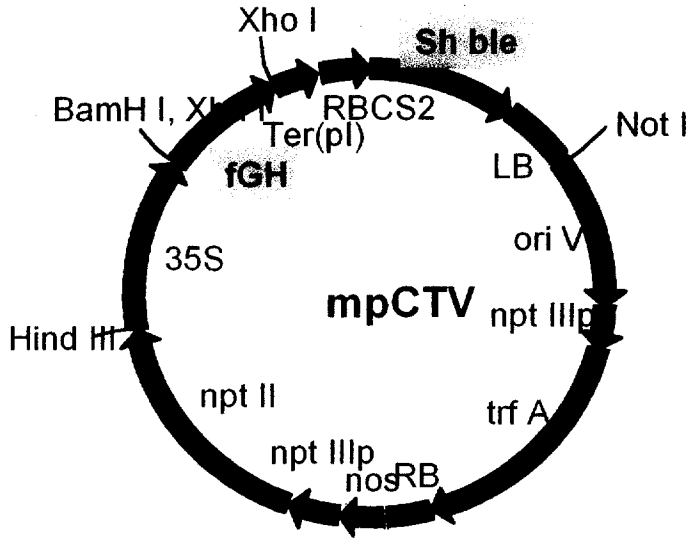


Figure 6. Map of mpCTV vector. The fGH gene was the under the control of 35S promoter and sh ble gene was under the control of RBCS2 gene promoter. Ori V, board host range replication origin, RB, npt II gene under control of nos-npt double promoter.

count reached to 10^7 cells/㎖, usually 1 week after inoculation. 50 ㎖ of incubated cells were harvested centrifuging for 5 min at 1,500×g. The supernatant was removed. and the pellet was washed with 25 mM phosphate buffer (pH 6.0) and then suspended in 5 ㎖ of CMP buffer [0.6 M sorbitol, 0.6 M mannitol, 4%(w/v) cellulase, macerase (CALBIOCHEM, USA), 2%(w/v) macerase, and 1%(w/v) pectinase (Sigma)]. Treated above cells were incubated at 25℃ for 18 hours in a dark shaking incubation.

Ⅳ-4. Transformation of protoplasts

Protoplasts were centrifuged at 400×g for 5 minutes and the supernatant was removed. The cell pelet was suspended in 5 ㎖ of f/2 midium containing 0.6 M sorbitol, 0.6 M mannitol and centrifuged at 400×g for 5 min and resuspended in 1 ㎖ 0.6 M sorbitol/mannitol with 0.05 M $CaCl_2$. 0.4 ㎖ of protoplast was placed in a new microcentrifuge tube and 4 ㎍ DNA, 25 ㎍ carrier DNA (calf thymus DNA, Sigma). When 15 minuts passed, 200 ㎕ PNC buffer (0.8 M NaCl, 0.05 M $CaCl_2$, 40% PEG4000) was added and incubated for 30 min incubation at room temperature. Then 0.6 ㎖ f/2 regeneration medium containing 0.6 M sorbitol/mannitol, 1% yeast extract, 1% glucose was added, and the cells were incubated at 25℃ for 12 hours in dark condition.

Ⅳ-5. Selection of transformants

Chlorella was transformed with mpCTV vector and transformants were transferred into f/2 medium containing

phleomycin (1 ㎍/㎖) and cultured fluorescent lamp at 25℃ with 18:6 hours light:dark cycles.

Ⅳ-6. *Chlorella* genomic DNA isolation

Genomic DNA from *Chlorella* was isolated as described by Dawson *et al.* (1997). Approximately 3×10^8 cells were harvested from 3 ㎖ of culture and resuspended in 500 ㎕ of 54 mM CTAB [(hexadecyltrimethylammonium bromide) buffer, 0.25 mM Tris (pH 8.0), 1.4 M NaCl, 10 mM EDTA, and 2% β-mercaptoethanol]. The mixture was incubated at 65℃ for 1 hour and then extracted with an equal volume of phenol-chloroform. The aqueous was recovered after 5 min of centrifugation at 3,000×g, and extracted several times until the aqueous layer was no longer cloudy. The DNA was precipitated with 0.7 volume of 100% ethanol, centrifuged for 15 min at 17,000×g, washed with 70% ethanol, dried and resuspended in 30 ㎕ TE buffer.

Results and Discussion

Amplification of human BDNF gene using RT-PCR

The condition for annealing temperature was very sensitive and set up a 48℃. The product of RT-PCR was 744 bp and analyzed on 1.0% agarose gel electrophoresis (Figure 7). The RT-PCR product was increased by gradual increases of total RNA template (Figure 8).

Neucleotide sequence of human BDNF gene

To certify nucleotide sequence of human BDNF, cloned DNA was sequenced and analyzed (Figure 9). With comparison to nucleotide sequence of the gene bank reported in NCBI(National Center for Biotechmology Information), a silence mutation (AAG to AAA) was found in the midst of ORF of cloned BDNF, resulting that the altered base was translated into the same amino acid.

Overexpression of human BDNF gene in *E. coli*

In order to overexpress human BDNF gene into the prokaryotic system, we used the GST (Glutathion S-transferase) fusion expression system. The molecular weight of GST-BDNF fusion protein is 53 kDa and GST itself corresponds to 26 kDa, showing that BDNF is 27 kDa. The GST-BDNF fusion protein was overexpressed under the control of the promoter and the expressed proteins were seperated on 12% SDS-PAGE (Figure 10).

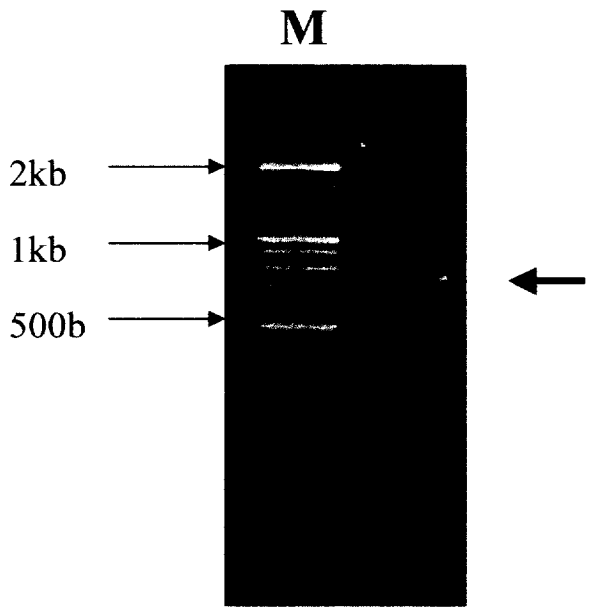


Figure 7. The RT-PCR product. The expected DNA band (744 bp) of BDNF gene was detected.

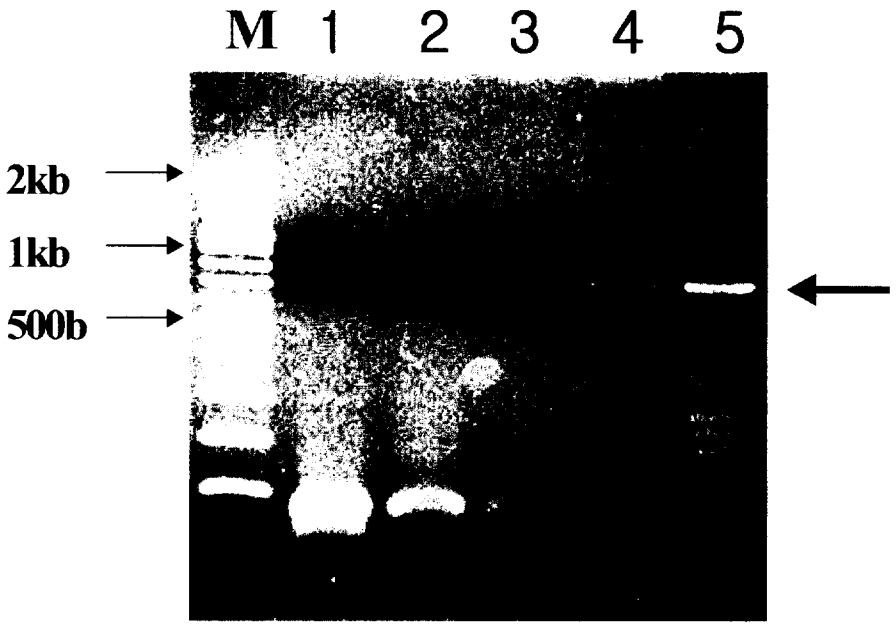


Figure 8. The RT-PCR patterns of increasing total RNA concentrations used as template. lane 1; 0.1 ng, lane 2; 0.3 ng, lane 3; 0.5 ng, lane 4; 0.7 ng, lane 5; 1 ng

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      Met Thr Ile Leu Phe Leu Thr Met Val Ile Ser Tyr Phe Gly Cys Met Lys
  1   ATG ACC ATC CTT TTC CTT ACT ATG GTT ATT TCA TAC TTT GGT TGC ATG AAA
      Ala Ala Pro Met Lys Glu Ala Asn Ile Arg Gly Gln Gly Gly Leu Ala Tyr
 52   GCT GCC CCC ATG AAA GAA GCA AAC ATC CGA GGA CAA GGT GGC TTG GCC TAC
      Pro Gly Val Arg Thr His Gly Thr Leu Glu Ser Val Asn Gly Pro Lys Ala
103   CCA GGT GTG CGG ACC CAT GGG ACT CTG GAG AGC GTG AAT GGG CCC AAG GCA
      Gly Ser Arg Gly Leu Thr Ser Leu Ala Asp Thr Phe Glu His Val Ile Glu
154   GGT TCA AGA GGC TTG ACA TCA TTG GCT GAC ACT TTC GAA CAC GTG ATA GAA
      Glu Leu Leu Asp Glu Asp Gln Lys Val Arg Pro Asn Glu Glu Asn Asn Lys
205   GAG CTG TTG GAT GAG GAC CAG AAA GTT CGG CCC AAT GAA GAA AAC AAT AAG
      Asp Ala Asp Leu Tyr Thr Ser Arg Val Met Leu Ser Ser Gln Val Pro Leu
256   GAC GCA GAC TTG TAC ACG TCC AGG GTG ATG CTC AGT AGT CAA GTG CCT TTG
      Glu Pro Pro Leu Leu Phe Leu Leu Glu Glu Tyr Lys Asn Tyr Leu Asp Ala
307   GAG CCT CCT CTT CTC TTT CTG CTG GAG GAA TAC AAA AAT TAC CTA GAT GCT
      Ala Asn Met Ser Met Arg Val Arg Arg His Ser Asp Pro Ala Arg Arg Gly
358   GCA AAC ATG TCC ATG AGG GTC CGG CGC CAC TCT GAC CCT GCC CGC CGA GGG
      Glu Leu Ser Val Cys Asp Ser Ile Ser Glu Trp Val Thr Ala Ala Asp Lys
409   GAG CTG AGC GTG TGT GAC AGT ATT AGT GAG TGG GTA ACG GCG GCA GAC AAA
      Lys Thr Ala Val Asp Met Ser Gly Gly Thr Val Thr Val Leu Glu Lys Val
460   AAG ACT GCA GTG GAC ATG TCG GGC GGG ACG GTC ACA GTC CTT GAA AAG GTC
      Pro Val Ser Lys Gly Gln Leu Lys Gln Tyr Phe Tyr Glu Thr Lys Cys Asn
511   CCT GTA TCA AAA GGC CAA CTG AAG CAA TAC TTC TAC GAG ACC AAG TGC AAT
      Pro Met Gly Tyr Thr Lys Glu Gly Cys Arg Gly Ile Asp Lys Arg His Trp
562   CCC ATG GGT TAC ACA AAA GAA GGC TGC AGG GGC ATA GAC AAA AGG CAT TGG
      Asn Ser Gln Cys Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp
613   AAC TCC CAG TGC CGA ACT ACC CAG TCG TAC GTG CGG GCC CTT ACC ATG GAT
      Ser Lys Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp Thr Ser Cys Val
664   AGC AAA AAG AGA ATT GGC TGG CGA TTC ATA AGG ATA GAC ACT TCT TGT GTA
      Cys Thr Leu Thr Ile Lys Arg Gly Arg ***
715   TGT ACA TTG ACC ATT AAA AGG GGA AGA TAG
```

Figure 9. Neucleotide sequence of human BDNF gene. A silent mutation (AAG to AAA) of huamn BDNF clone was found (a colored A).

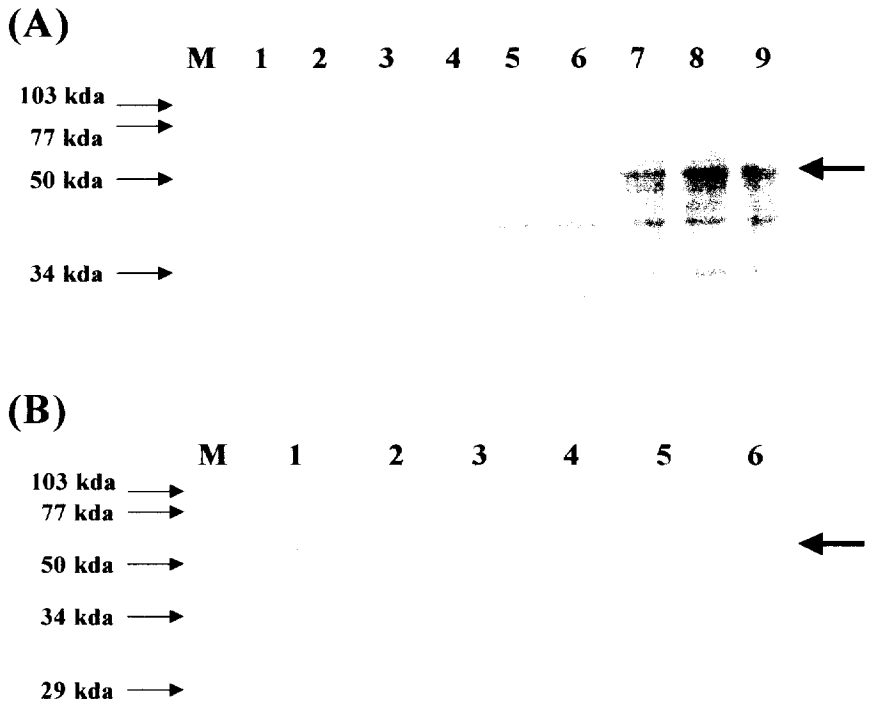

Figure 10. SDS-acrylamide gel electrophoresis of GST-BDNF fusion protein. (A) M; low range molecular weight (Bio-rad), lane 1; BL21(DE3) before induction, lane 2-9: IPTG induction; lane 2; 0 hr; lane 3, 1/2 hr; lane 4, 1 hr lane 5, 1 and 1/2 hr; lane 6, 2 hr; lane 7, 2 and 1/2 hr; lane 8, 3 hr; lane 9, 3 and 1/2 hr (B) M; low range marker, lane 1-6: IPTG induction; 4 hr.

Western blot analysis

After expressed proteins were separated by SDS-PAGE, proteins were transferred to nitrocellulose membrane and the GST-BDNF protein were analyzed by the primary antibody against GST protein. The expression of GST-BDNF fusion protein was identified by immunoblot analyses corresponding to 57 kDa (Figure 11).

Transgenic medaka embryos

In order to construct transgenic medaka, human BDNF was inserted into pIRES2-EGFP vector, resulting pIRES2-EGFP-BDNF (Figure 12), and then injected into fertilized 1-or-2 cell stage of medaka embryos by the electroporation method described in the Material and Method section. The GFP-positive embryos resulted by the BDNF transgenesis were observed under the fluorescence microscope. The GFP fluorescence indicated that functional proteins of foreign genes were expressed into the transgenic body. In early blastomere stages, whole embryos containing normal medaka embryos were shown week green fluorescence images (Figure 13 A, B, C). Bur, from the 5-day-old embryos, embryonic body was observed in green fluorescence images (Figure 13 D, E, F) indicating that BDNF was expressing at this stages. Also, by monitoring the 6- and 7-day-old embryos, GFP fluorescence was clearly detected in transgenemic embryo. The early and strong expression of the BDNF gene in the early stages of embryogenesis suggested that the BDNF contributes an important function in the

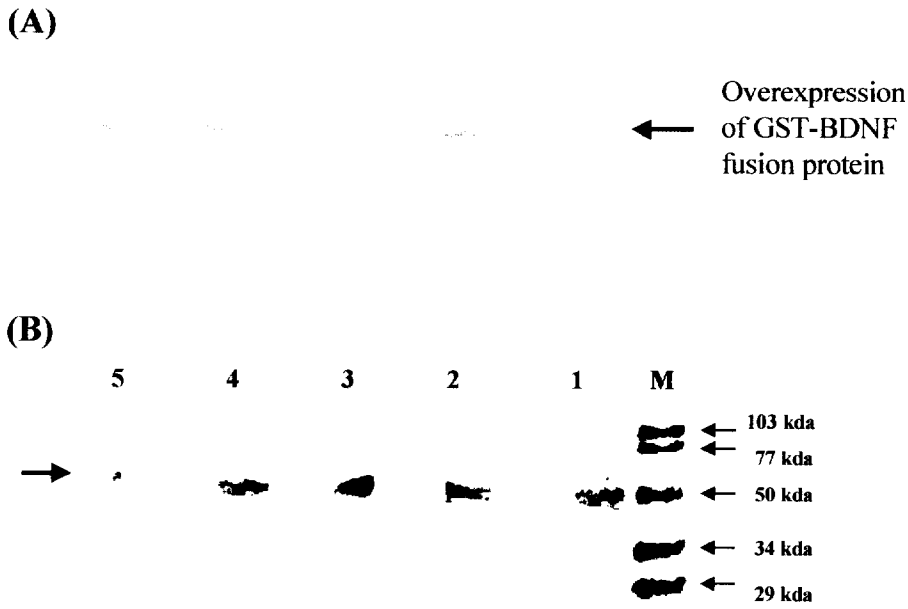

Figure 11. Western blot analysis of GST-BDNF gene. (A) The patterns of SDS-PAGE of the expressed proteins (B) Western blot analysis on transferred membrane.

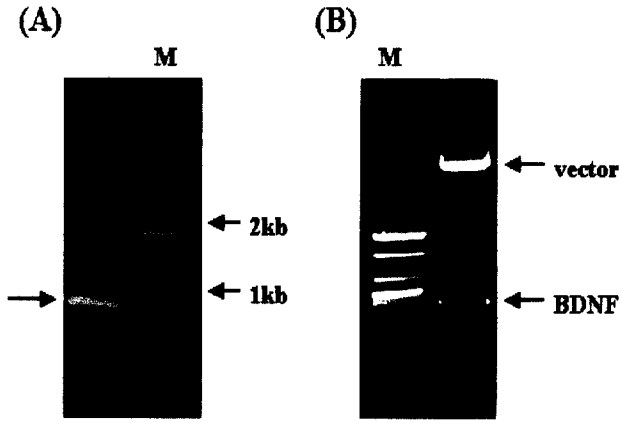

Figure 12. Construction of recombinant BDNF and enzyme digestion. (A) BDNF insert digested with XhoI and BamHI sites (B) After Xho I and BamH I digestion of recombinant BDNF.

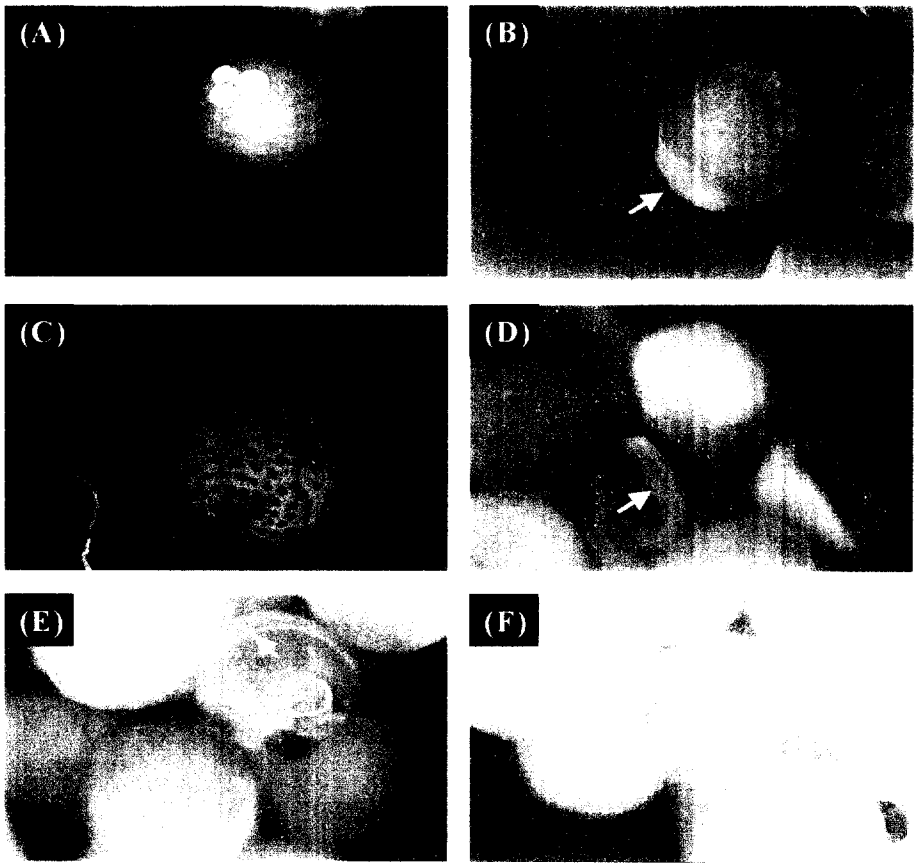

Figure 13. Fluorescent germ cells (1/4). (A), (B) and (C) 4-blastomere stages; (D), (E) and (F) 5-day-old embryos. Embryos were seen week fluorescence during embryogenesis (yellow arrows).

early development of embryos. Yellow spots in the head and on the dorsal midline were autofluorescence (Figure 14). For the embryogenesis, the BDNF gene was expressed from the 4-blastomere stages until continuous developmental stages. After hatching, dorsal region of the intestine showed green fluorescent images. Also, fluorescent images was seen in brain, the periphery of eyes, and other tissues (Figure 15, 16).

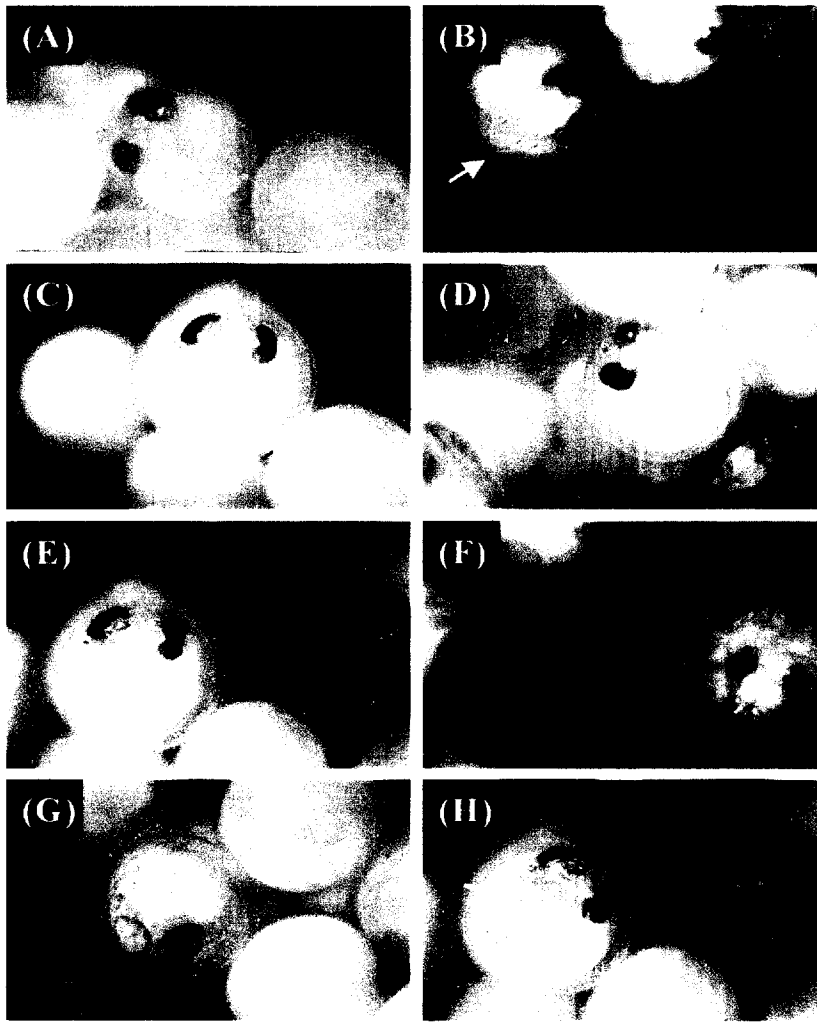

Figure 14. Fluorescent germ cells (2/4). (A) 6-day-old embryo; (B), (C) and (D) 7-day-old embryo. Yellow arrows showed the intrinsic fluorescence. (E), (F), (G) and (H) Contrast with yellow arrows, embryos were seen difference fluorescence image (blue arrows) showing that BDNF-GFP proteins expressed. Yellow spots indicated autofluorescence.

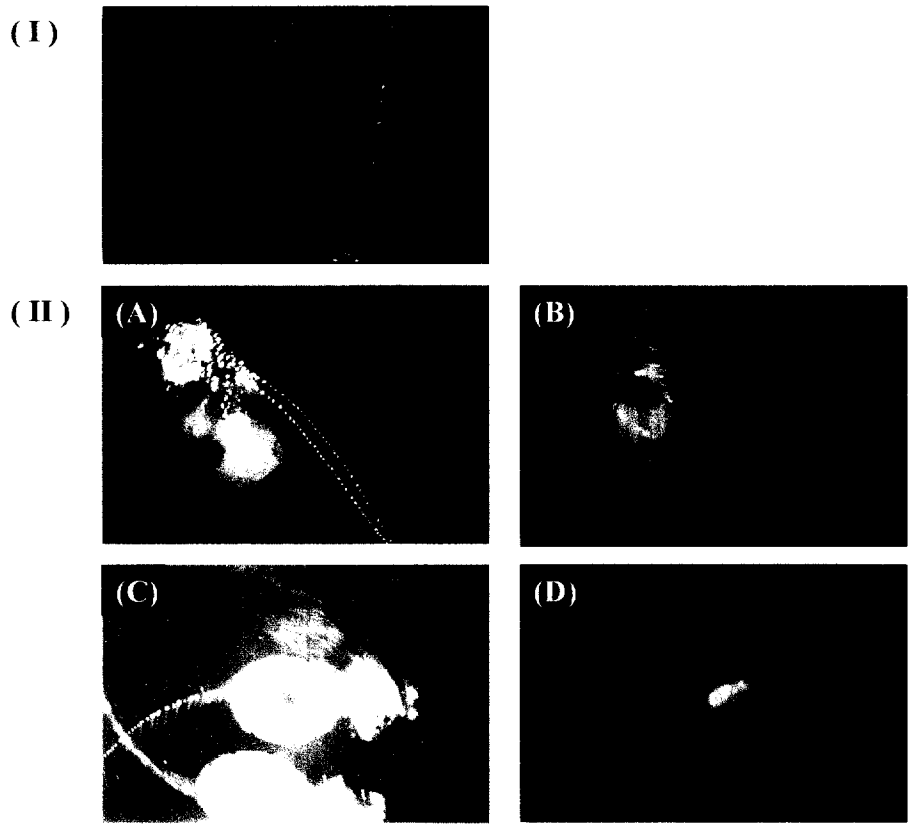

Figure 15. Fluorescent germ cells after hatching (3/4). (I) Nontransgenic, (II) The BDNF transgenic fly. Dorsal region of the intestine were shown strong expression of transgenes. (A), (B), (C) and (D) shown green fluorescence images.

(A)

(B)

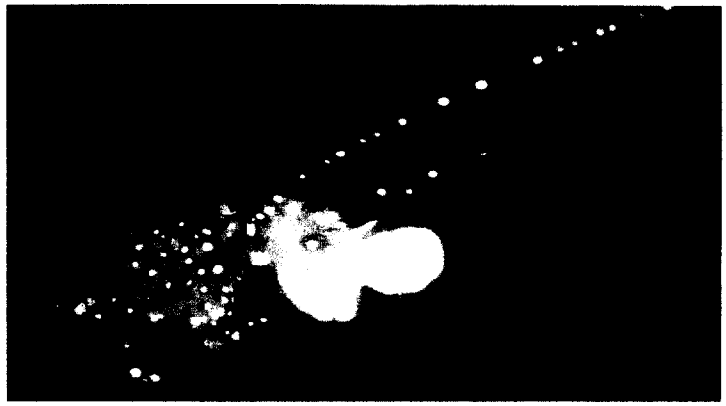

Figure 16. Fluorescent germ cells after hatching (4/4). (A) Green fluorescence images were shown in brain, retina, the periphery of eyes and the rear. (B) The strong fluorescence images were shown in dorsal region.

Transformation of human BDNF into *Chlorella*

Human BDNF gene suncloned into the mpCTV was transformed into *C. ellipsoidea* platoplast. The transformed protoplasts were transferred from regeneration medium to f/2 medium containing 1 ㎕/㎖ of phleomycin. After 5 days of BDNF gene incorporated into the chromosomal DNA into *C. ellipsoidea* was confirmed by PCR amplification of BDNF gene from chromosomal DNA isolated from the transformants (Figure 17).

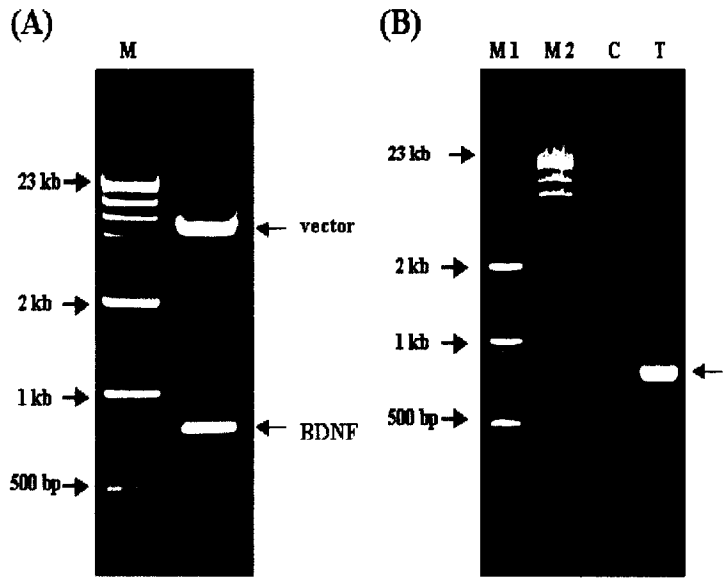

Figure 17. Confirmation by enzyme digestion and PCR. (A) recombinant BDNF enzyme digestion reaction (B) left lane M1; 100bp marker, M2; λ Hind III marker, third lane C; wide type *C. ellipsoidea* (control), lane T; transformed *C. ellipsoidea*.

국문초록

뇌의 신경촉진인자(BDNF:brain-derived neurotrophic factor)는 뇌세포 성장과 생존에 도움을 주는 것으로 알려져 있다. 운동신경 세포의 발달, 재생, 치유에 중요한 대표적인 신경영양인자 물질로 IGF-1, CNTF, BDNF, GDNF 등이 있다. Human brain hippocampus total RNA를 template로 하여 RT-PCR을 수행하여 744 bp의 human BDNF gene을 cloning하였다. BDNF gene의 overexpression을 prokaryotic system인 *Escherichia coli*에 GST (Glutathion S-transferase) fusion system을 이용하여 수행하였다. 대장균을 이용한 발현에서는 functional expression이 불가능하여 functional study로서 BDNF 발현 양상을 eukaryotic system인 medaka에 transgenesis 시켰다. BDNF를 pIRES2-EGFP vector에 construction하여 수정된 medaka egg에 electroporation을 통하여 transgenic fish를 만들었다. embryogenesis 과정을 control과 비교해보면, BDNF gene의 expression 양상은 4-blastomere stage에서부터 fluorescence를 띠는 embryo를 관찰할 수 있었고, 계속적인 발생과정 중에도 whole body에서 fluorescence를 monitoring할 수 있었다. 부화 후의 과정에서도, 뇌, 망막, 배, 눈 등의 특이적인 부위에서 fluorescence를 관찰할 수 있었다. 마지막으로 활성형 BDNF 단백질을 대량 생산하기 위하여 eukaryotic expression system인 marine microalgae, *Chlorella*를 이용하기 위하여 mpCTV expression vector에 recombinant BDNF를 construction하여 transformation을 수행하였다. BDNF gene이 형질전환된 *Chlorella*를 screening하여 genomic DNA를 분리하고, BDNF gene이 incorporation 된 것을 PCR로 확인하였다.

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