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### Effects of Neurotrophic Factors on the Generation of Functional Dopamine Secretory Neurons Derived from *in vitro* Differentiated Human Embryonic Stem Cells

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**Objective:** This study was to examine the *in vitro* neural cell differentiation patterns of human embryonic stem (hES) cells following treatment of various neurotrophic factors [basic fibroblast growth factor (bFGF), retinoic acid (RA), brain derived neurotrophic factor (BDNF) and transforming growth factor (TGF)- ], particularly in dopaminergic neuron formation.

**Methods:** The hES cells were induced to differentiate by bFGF and RA. Group I) In bFGF induction method, embryoid bodies (EBs, for 4 days) derived from hES were plated onto gelatin dish, selected for 8 days in ITSFn medium and expanded at the presence of bFGF (10 ng/ml) for another 6 days followed by a final differentiation in N2 medium for 7, 14 and 21 days. Group II) For RA induction, EBs were exposed of RA ( $10^{-6}$  M) for 4 days and allowed to differentiate in N2 medium for 7, 14 and 21 days. Group III) To examine the effects of additional neurotrophic factors, bFGF or RA induced cells were exposed to either BDNF (10 ng/ml) or TGF- (10 ng/ml) during the 21 days of final differentiation. Neuron differentiation and dopamine secretion were examined by indirect immunocytochemistry and HPLC, respectively.

**Results:** The bFGF or RA treated hES cells were resulted in similar neural cell differentiation patterns at the terminal differentiation stage, specifically, 75% neurons and 11% glial cells. Additionally, treatment of hES cells with BDNF or TGF- during the terminal differentiation stage led to significantly increased tyrosine hydroxylase (TH) expression of a dopaminergic

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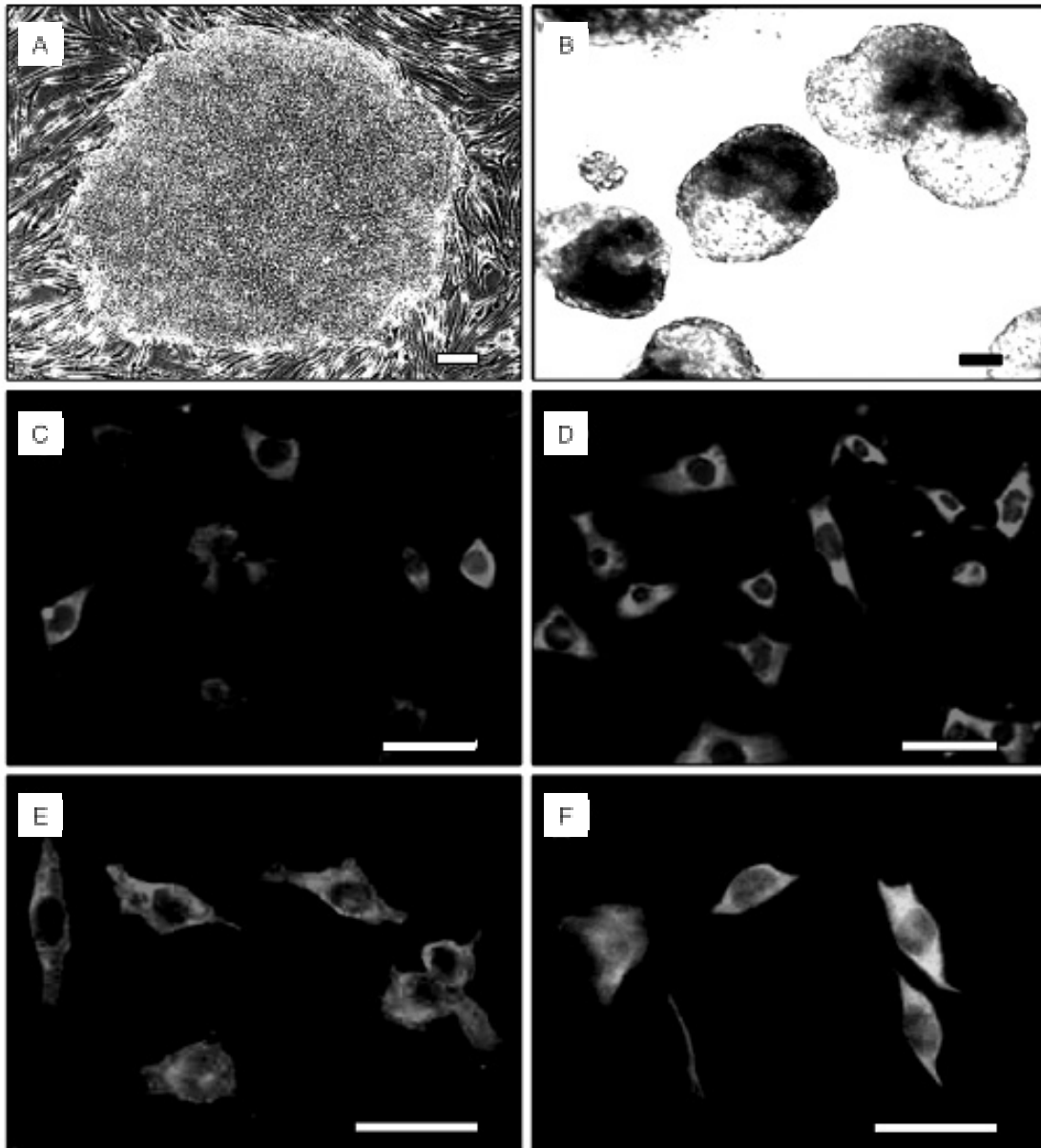
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neuron marker, compared to control ( $p < 0.05$ ). In contrast, no effect was observed on the rate of mature neuron (NF-200) or glutamic acid decarboxylase-positive neurons. Immunocytochemistry and HPLC analyses revealed the higher levels of TH expression (20.3%) and dopamine secretion ( $265.5 \pm 62.8$  pmol/mg) in bFGF and TGF- $\beta$  sequentially treated hES cells than those in RA or BDNF treated hES cells.

**Conclusion:** These results indicate that the generation of dopamine secretory neurons from *in vitro* differentiated hES cells can be improved by TGF- $\beta$  addition in the bFGF induction protocol.

**Key Words:** Human embryonic stem cell, Neural cell differentiation, Basic fibroblast growth factor, Transforming growth factor- $\beta$ , Dopaminergic neuron

가 , 가 . 가  
 , white-matter loss, NIH MB03  
 2,3  
 (cell replacement therapy)  
 4-6  
 (blastocyst) (inner  
 cell mass) (pluripotent 1.  
 cell) MB03 (Figure 1A)  
 가 , 5  
 7,8  
 11  
 ATCC STO (immortalized  
 mouse embryonic fibroblast, CRL-1503)  
 250,000 cells/1.77cm<sup>2</sup> , 10  
 3  
 retinoic acid (RA)<sup>9,10</sup>  
 2,3 ,  
 (IL-1 , 0.1 mM -mercaptoethanol, 1% ribonucleosides, 1%  
 GDNF, neurturin, TGF- $\beta$  3 or db-cAMP) 가 non-essential amino acids 4 ng/ml bFGF 가  
 가 STO 5% Matrigel  
 6 , (Becton Dickinson, Bedford, MA)  
 (conditioned medium) 10  
 가 . STO



**Figure 1.** MB03 human embryonic stem (hES) cells and their *in vitro* neural cell differentiation. (A & B) Phase morphology of established MB03 hES cells and embryoid bodies.; (C) Indirect immunocytochemistry demonstrated positive reactions for neurofilaments (NF) 160 (a marker of immature neurons), (D) NF-200 (a marker of mature neurons), (E) glial fibrillary acid protein (a marker of astrocytes), (F) glutamic acid decarboxylase (a marker of GABAergic neurons). Scale bars = 100  $\mu$ m.

가 (4 × 10<sup>6</sup> cells/75 cm<sup>2</sup>)  
 20% FBS 20% serum replacement (SR; Gibco)가 가 2. bFGF RA , 0.22  $\mu$ l

brain derived neurotrophic factor  
(BDNF) transforming growth factor (TGF)-  
가

1  
0.025% trypsin/EDTA 2  
, bacteriological dish (Falcon, #1007)  
 $3 \times 10^4$  cells/cm<sup>2</sup> 20% SR  
4  
3 embryoid body (EB)  
(Figure 1B).

1) bFGF  
4 EB  
0.1% gelatin (Falcon, #3002)  
insulin/transferrin/selenium/  
fibronectin (ITSFn medium; Sigma, Saint Louis, MO)  
8  
0.025% trypsin/  
EDTA 2 1  
 $\mu$ g/ml laminin (R&D systems Inc, Minneapolis, MN)  
glass coverslip  $2 \times 10^5$  cells/cm<sup>2</sup>  
10 ng/ml bFGF (KOMA biotech Inc.  
Korea)가 N2- 6

bFGF가 N2- 7 , 14  
21 . N2- DMEM/F12  
insulin (5 mg/L, Sigma), putrescine  
(100  $\mu$ M, Sigma), sodium selenite (30 nM, Sigma),  
apo-transferrin (100  $\mu$ g/ml, Sigma), progesterone (20  
nM, Sigma) 가

2) RA  
4 EB RA ( $10^{-6}$ M, Sigma)가 20%  
SR 4 가  
RA laminin glass  
coverslip 7 , 14 21 N2

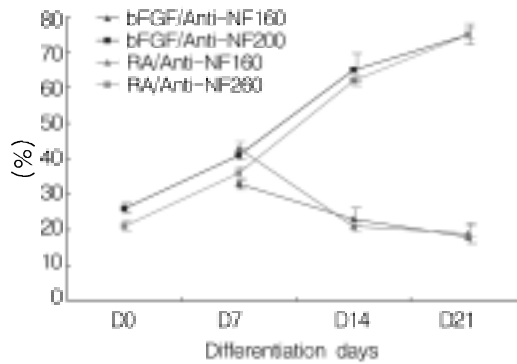
3) 가  
가  
N2- 21 10  
ng/ml BDNF (Chemicon) 10 ng/ml TGF- (R&D  
systems Inc) 가가

3. (Immunocytochemistry)

4% paraformaldehyde (Sigma) 10  
0.02% Triton X-100 (Sigma) 10  
5%  
normal goat serum (Vector) 1  
, 1 4  
1  
anti-neurofilament 160 (NF 160;  
monoclonal antibody, 1:4,000, Sigma, Figure 1C),  
anti-neurofilament 200 (NF200; monoclonal  
antibody, 1:4,000, Sigma, Figure 1D),  
(astrocyte)  
anti-glial fibrillary acidic protein (GFAP;  
polyclonal antibody, 1:500, DAKO, Figure 1E)

anti-tyrosine hydroxylase  
(TH; monoclonal antibody, 1:1,000, Chemicon) GA-  
BAergic anti-glutamic acid decar-  
boxylase (GAD; 1:4,000, Chemicon, Figure 1F)가  
2  
TRITC (tetramethyl rhodamine isothiocyanate)  
conjugated goat anti-mouse anti-rabbit IgG  
(1:800, Jackson Immunoresearch) FITC (fluorescein  
isothiocyanate) conjugated goat anti rabbit IgG (1:200,  
Jackson Immunoresearch)

DAPI (4', 6-diamidino-2-phenylindole  
dihydrochloride, 1:2,000, Roche) 1  
가  
가  
Nikon  
, FITC 520 nm, TRITC  
630 nm . 200  
20 ,



**Figure 2.** Addition of bFGF or retinoic acid during neural progenitor formation of hES cells induced similar neurofilament (NF) expression patterns. As progress of neuronal differentiation of hES cells, immunostaining for NF200 increased, in contrast to that for NF160.

1000 ,

6 .

4. (high performance liquid chromatographic, HPLC)

dopamine HPLC .

bFGF , BDNF 가

TGF- 가

21 5 × 10<sup>6</sup>

PBS buffer ,

0.1 mM EDTA 가 가

0.1M perchloric acid (Sigma-Aldrich, Switzerland) 가

(sonicator) , 12,000 g 10

(nitrocellulose membrane filter, 0.4 μm) HPLC (Gilson) 10 μl

. HPLC (electrochemical detector)

. HPLC Shiseido C18 column 가

(mobile phase) 0.07 mM sodium phosphate monobasic, 1 mM sodium octanesulfonic acid, 0.1 mM EDTA, 8% acetonitrile (pH 4.0) 0.7 ml/min .

2 μM dihydroxy-benzylamine (DHBA; Sigma) 가

(internal standard) .

5.

8.02 (TS level 02M0) SAS release , HPLC

one-way ANOVA test Mann-Whitney U test

p 0.05 .

1.

bFGF RA 가

21 ,

NF200 bFGF

26.2 ± 2.7% (D0) 75.1 ± 4.0% (D21) , RA 21.4 ± 2.0% (D0)

75.3 ± 2.7% (D21) 가 ,

bFGF NF160

33.1 ± 1.3% (D7) 18.2 ± 4.2% (D21) RA 43.3 ± 2.2% (D7)

19.4 ± 1.3% (D21)

(Figure 2). ,

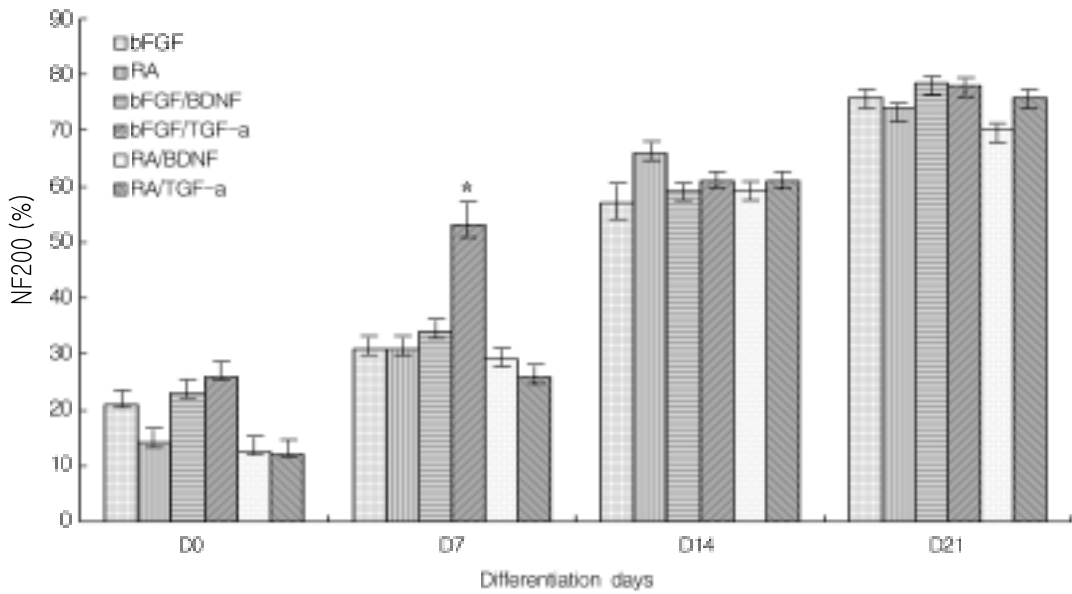
GFAP , 7 30~34%, 14 16~22%, 21 9~12%

가

2.

가가

BDNF TGF-



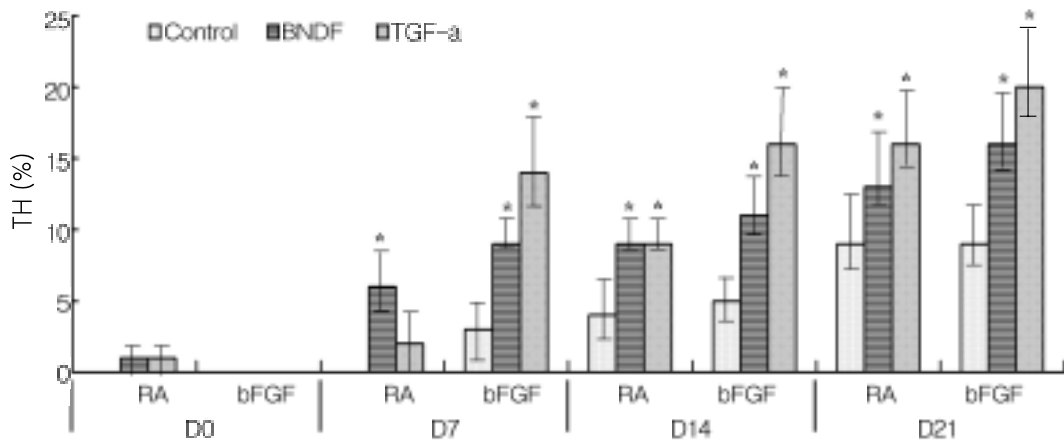
**Figure 3.** Effects of neurotrophic factors (BDNF and TGF- ) on the *in vitro* differentiation of hES cells. Data are presented as the mean±SD of six independent experiments. \*indicates significantly different from the other treatment groups at day 7 (SAS release 8.02 TS level 02M0, p <0.05)

21 가 NF-200 (p<0.05)

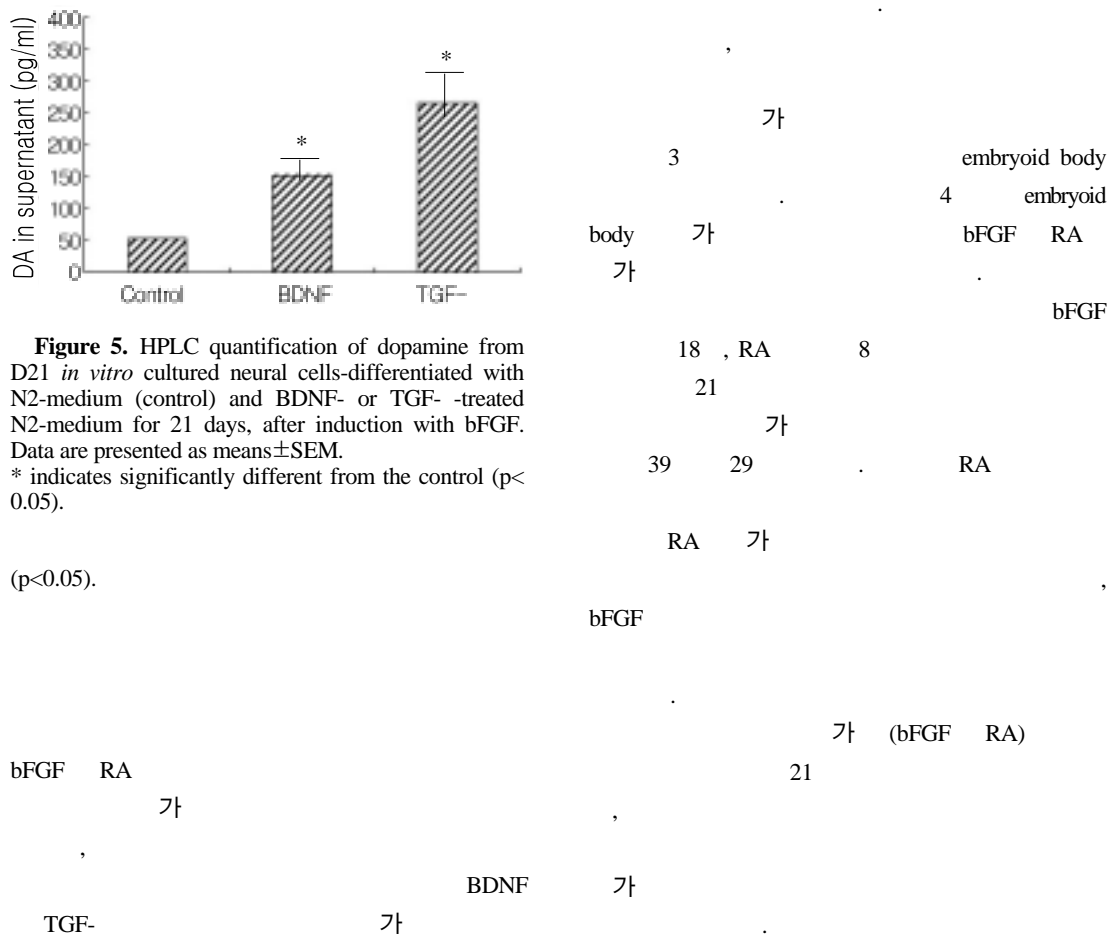
bFGF 78.1 ± 2.7% 78.4 ± 3.3% bFGF RA TH (Figure 4). bFGF TGF- (D7; 14.0 ± 2.7%, D14; 16.2 ± 2.7%, D21; 20.4 ± 2.3%) BDNF (D7; 9.2 ± 2.2%, D14; 11.3 ± 3.2%, D21; 16.2 ± 2.3%) (D7; 3.4 ± 1.7%, D14; 5.2 ± 1.8%, D21; 9.3 ± 2.3%) TH 가 , GABAergic GAD 21 26~30% BDNF TGF- 가

3. 가 가 TH BDNF TGF- 가 TH 가

4. TH bFGF dopamine HPLC , BDNF (151.4 ± 36.5 pmol/mg) TGF- (265.5 ± 62.8 pmol/mg) 가 (52.3 ± 8.4 pmol/mg)

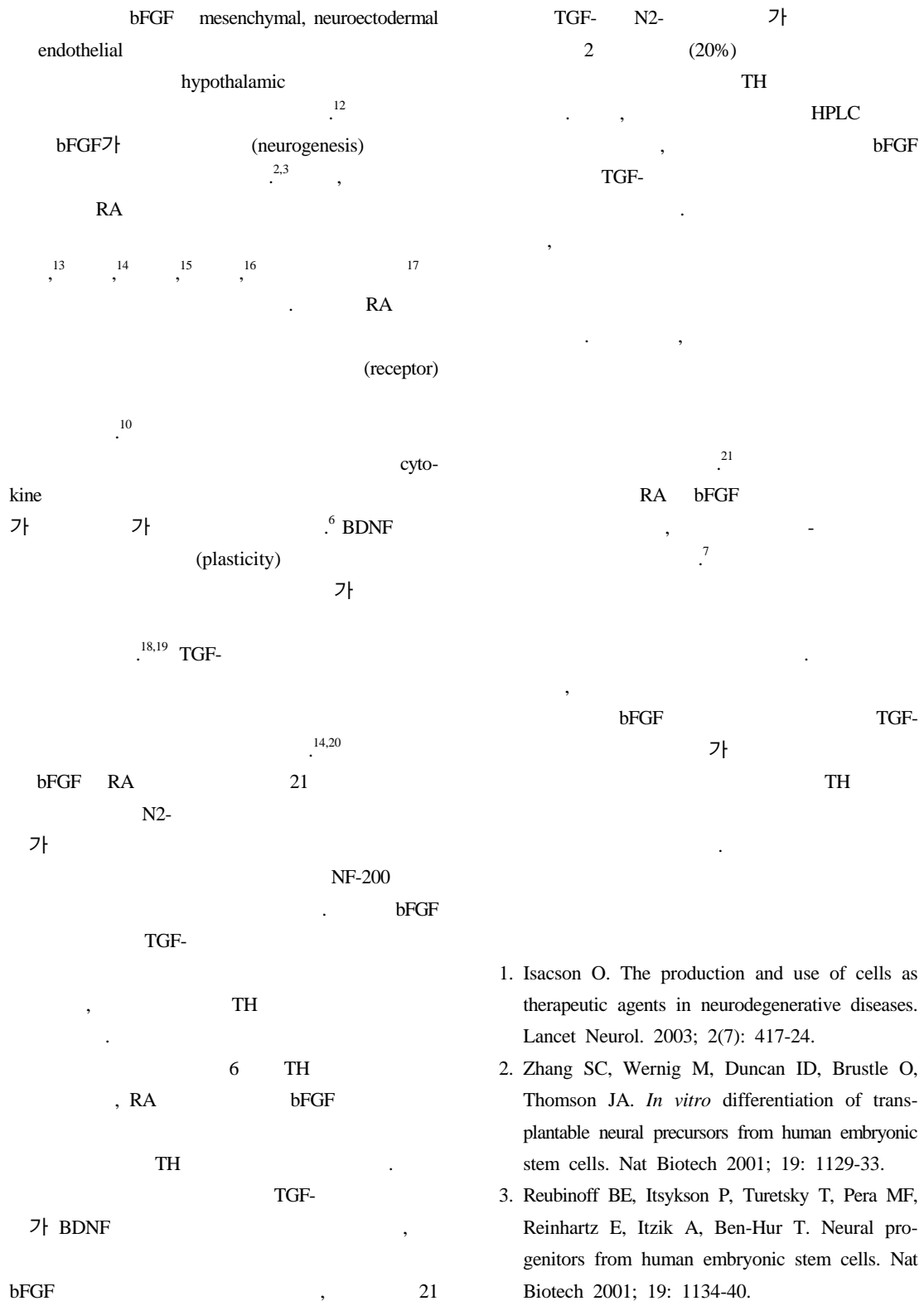


**Figure 4.** TH expression from *in vitro* differentiated hES cells, following RA or bFGF induction, and BDNF or TGF- $\alpha$  treatment, respectively. Data are presented as mean $\pm$ SD of six independent experiments. \* indicates significantly differences different in the values obtained from the control group in the same induction method and culture days ( $p < 0.05$ ).



**Figure 5.** HPLC quantification of dopamine from D21 *in vitro* cultured neural cells-differentiated with N2-medium (control) and BDNF- or TGF- $\alpha$ -treated N2-medium for 21 days, after induction with bFGF. Data are presented as means $\pm$ SEM. \* indicates significantly different from the control ( $p < 0.05$ ).

( $p < 0.05$ ).



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