

## Tyrosine Hydroxylase 가

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### ***In vitro* Neural Cell Differentiation of Genetically Modified Human Embryonic Stem Cells Expressing Tyrosine Hydroxylase**

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**Objective:** This study was to examine *in vitro* neural cell differentiation pattern of the genetically modified human embryonic stem cells expressing tyrosine hydroxylase (TH).

**Materials and Methods:** Human embryonic stem (hES, MB03) cell was transfected with cDNAs coding for TH. Successful transfection was confirmed by western immunoblotting. Newly transfected cell line (TH#2/MB03) was induced to differentiate by two neurogenic factors retinoic acid (RA) and b-FGF. Exp. I) Upon differentiation using RA, embryoid bodies (EB, for 4 days) derived from TH#2/MB03 cells were exposed to RA ( $10^{-6}$  M)/AA ( $5 \times 10^{-2}$  mM) for 4 days, and were allowed to differentiate in N2 medium for 7, 14 or 21 days. Exp. II) When b-FGF was used, neuronal precursor cells were expanded at the presence of b-FGF (10 ng/ml) for 6 days followed by a final differentiation in N2 medium for 7, 14 or 21 days. Neuron differentiation was examined by indirect immunocytochemistry using neuron markers (NF160 & NF200).

**Results:** After 7 days in N2 medium, approximately 80% and 20% of the RA or b-FGF induced Th#2/MB03 cells were immunoreactive to anti-NF160 and anti-NF200 antibodies, respectively. As differentiation continued, NF200 in RA treated cells significantly increased to 73.0% on 14 days compared to that in b-FGF treated cells (53.0%,  $p < 0.05$ ), while the proportion of cells expressing NF160 was similarly decreased between two groups. However, throughout the differentiation, expression of TH was maintained (~90%). HPLC analyses indicated the increased levels of L-DOPA in RA treated genetically modified hES cells with longer differentiation time.

**Conclusion:** These results suggested that a genetically modified hES cells (TH#2/MB03)

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(01-PJ10-PG8-01EC01-0010)

could be efficiently differentiated in vitro into mature neurons by RA induction method.

**Key Words:** Human embryonic stem cell, TH, Differentiation, RA, b-FGF

Parkinson's disease (PD) retinoic acid (RA) basic fibroblast growth factor (b-FGF) TH가 가 가 L-DOPA

blood-brain-barrier L-dopa

motor symptoms

rate-limiting enzyme tyrosine MB03 5

hydroxylase (TH) (chemotherapeutics) 가 가 MB03 10 misogynic C STO (ATCC CRL-1503, 250,000 cells/1.77cm<sup>2</sup>, #3653, Becton Dickinson, NJ, USA) feeder Knockout-Dulbecco's modified Eagle's medium (KO-DMEM; Gibco, Grand Island, New York) 20% fetal bovine serum (FBS; Hyclone, Logan, UT), 1 mM glutamine, 0.1 mM -mercaptoethanol, 1% ribonucleosides, 1% non-essential amino acids (NEAA) 4 ng/ml b-FGF 가 가

PD TH viral vector vectors가

lipid-based vectors TH가

(pluripotency)

STO 5% Matrigel (Becton Dickinson, Bedford, MA) (conditioned medium) 10 가

10

가 ,<sup>7-11</sup> Zhang<sup>12</sup> TH Reubinoff<sup>13</sup>

GABA Glutamate MB03 5×10<sup>4</sup>

35% 15% 10 cm (Falcon # 3003)

TH <1% pcDNA3.1 vector 6 μg TH

cDNA restriction enzyme linearize

FuGene 6 (Pharmacia) MB03 가

. 24

가 (250 µg/ml) (Fisher) , 4-well dish (Nunc) 3 cm dish (Falcon, #3001), 6 cm dish (Falcon, #3002) 가 TH MB03 western blotting (immunocytochemistry) 3. Western blotting 가 MB03 TH Nonidet P-40 buffer [10 mM Tris (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 100 µM PMSF] 50 µl lysis Bradford (Bio Rad) 2X SDS western buffer [130 mM Tris (pH 6.8), 20% glycerol, 4.6% SDS, 10% mercaptoethanol] 가 5 10% SDS-PAGE well , PVDF membrane transfer anti-TH antibody (Chemicon) 5% skim milk 가 10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% Triton X-100 (TBST) blotting . TBST , transfer membrane HRP-conjugated goat anti-rabbit antibody 1 TBST band chemiluminescence (ECL, Amersham) X-ray film 4. TH 가 0.025% trypsin/EDTA 2 , bacteriological dish (Falcon, #1007) 3×10<sup>4</sup> cells/cm<sup>2</sup> 20% serum replacement (SR) 3 4 5 6 7 , 14 21 5. 10 0.02% Triton X-100 (Sigma) 10 0.025% trypsin/EDTA 2 1 µg/ml laminin (R&D systems Inc, Minneapolis, MN) glass coverslip 2×10<sup>5</sup> cells/cm<sup>2</sup> 7 , 14 21 N2 DMEM/F12 N2 가 insulin (Sigma, 5 mg/L), putrescine (Sigma, 100 µM), sodium selenite (Sigma, 30 nM), apo-transferrin (Sigma, 100 µg/ml), progesterone (Sigma, 20 nM) 가 b-FGF , 4 EB 0.1% gelatin insulin/transferrin/selenium/fibronectin (ITSFn medium; Sigma, Saint Louis, Missouri) 8 0.025% trypsin/EDTA 2 1 µg/ml laminin glass coverslip 10 ng/ml b-FGF (KOMA biotech Inc.)가 N2 6 . b-FGF가 N2 7 , 14 21 5. TH 10 4% paraformaldehyde (Sigma) , 0.02% Triton X-100 (Sigma) 10 5% normal goat serum (Vector) 1 , 1 4 overnight . 1 EB

anti-neurofilament 160 (NF 160; monoclonal antibody, 1:4,000, Sigma, Figure 2 A-C),  
anti-neurofilament 200 (NF200; monoclonal antibody, 1:4,000, Sigma, Figure 2 D-F), TH (1:1000, Chemicon)

2 Rhodamine (TRITC)-Conjugated Affini Pure F(ab')<sub>2</sub> Fragment Goat Anti-mouse IgG (H + L) (Jackson Immunoresearch, 1:800)

1 DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, 1:2,000, Roche)

1 sample mounting 590nm TRITC 가 Nikon

6. (high performance liquid chromatographic, HPLC)

TH 가 L-DOPA HPLC

7, 14, 21 5×10<sup>6</sup> PBS buffer

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

가 (sonicator) lysis 12,000 g

10 (nitrocellulose membrane filter; 0.4 μm) HPLC (Gilson)

HPLC (electrochemical detector) HPLC

Shiseido C18 column (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

7. SAS release

8.02 (TS level 02M0) HPLC

one-way ANOVA test p 0.05 Mann-Whitney U test

1. TH

pcDNA3.1 vector TH

, 9 가 , 2 (TH#2/MB03, TH#8/MB08) TH

western blotting TH#2/MB03 TH

MB03 TH TH#2/MB03 TH

가 TH TH

2. TH#2/MB03

TH#2/MB03 RA b-FGF 가

21 TH

TH , Figure 1

가 21 80~90%

TH가

, b-FGF

NF160 7 70.0% 가

14 21 51.0% 29.8%

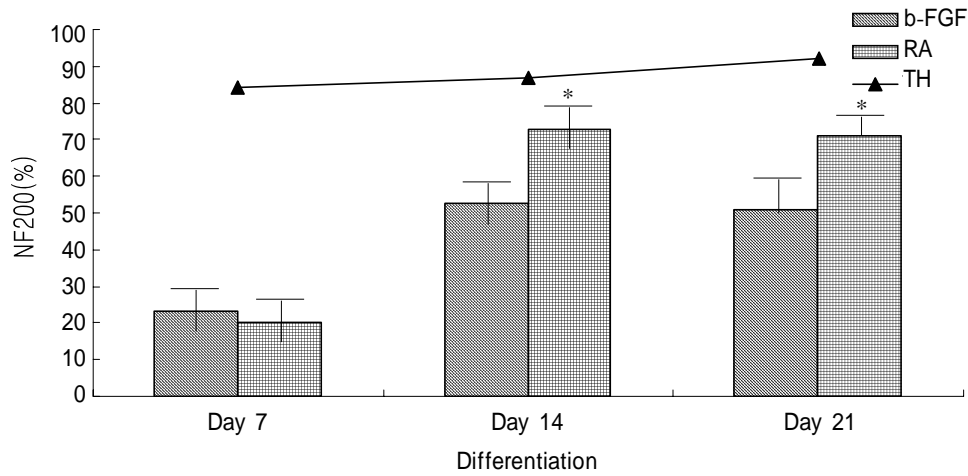
(Figure 1),

NF200 7 23.3% 가 14 52.8% 가 21 51.0%

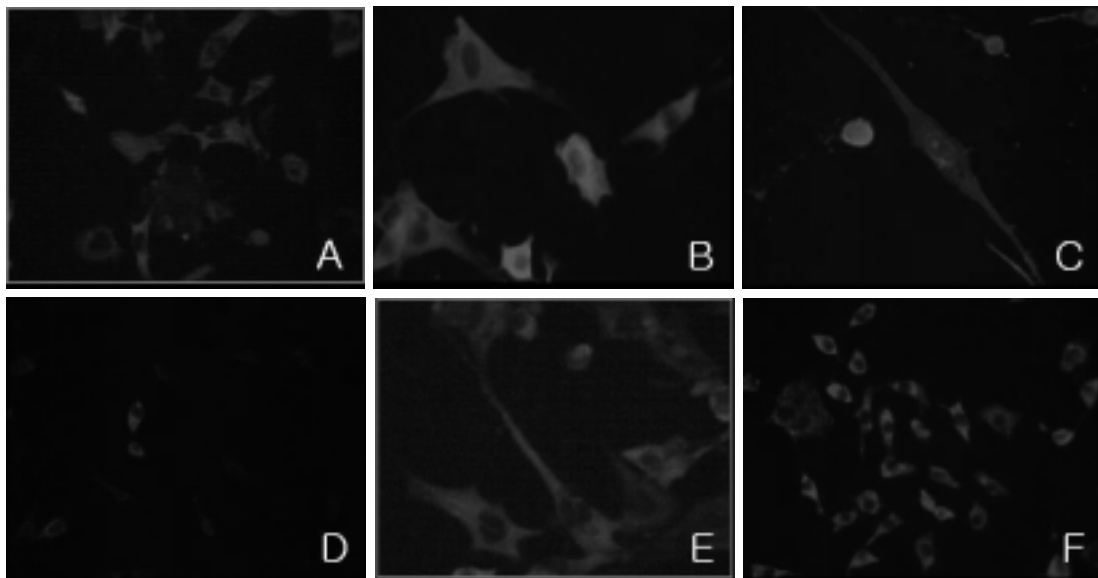
(Figure 1). RA , NF160 7 , 14

21 80.0%, 20.0% 10.0% b-FGF NF200

20.0%, 73.0% 71.0%



**Figure 1.** Stage-specific influence of b-FGF or RA on TH#2MB03 cells differentiation. The percentage of cells expressing TH and NF200 were assayed by immunocytochemistry. \* indicates significantly different from the b-FGF treatment group ( $p < 0.05$ ).



**Figure 2.** In vitro neural differentiation of TH#2/MB03. Stage-specific influence of b-FGF on TH#2/MB03 cells differentiation. (A)~(C) Immunostaining for NF160 of 7, 14 and 21 days cells plated on coverslip shows that proportion of cells expressing NF160 decreased rapidly at 21 days, (D)~(F) Immunostaining for NF200 of 7, 14 and 21 days cells plated on coverslip shows that proportion of cells expressing NF200 decreased rapidly at 21 days.

(Figure 1), TH#2/MB03

3. HPLC L-DOPA

RA

14

TH#2/MB03

가

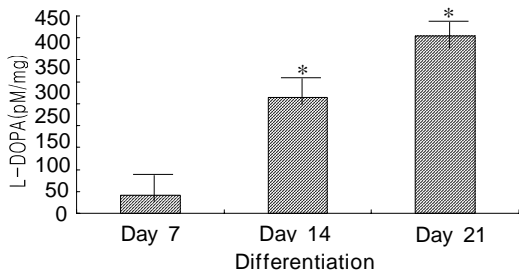
RA L-dopa

, 7 41

( $p < 0.05$ ).

pmole/mg protein, 14

264 pmole/mg protein, 21



**Figure 3.** HPLC quantification of L-DOPA from *in vitro* cultured neural cells-differentiated with N2 medium for 21 days, after induction with RA. \* indicates significantly different from the control ( $p < 0.05$ ).

405 pmole/mg protein (19 pmole/mg protein) (p < 0.05).

TH (TH#2/MB03)가 RA가 TH가 L-DOPA가

가<sup>9-11</sup>,  
 가<sup>12-13,15-16</sup>,  
 가<sup>21</sup> 10~20% TH가  
 (data not shown).  
 limiting enzyme  
 TH  
 TH  
 TH PD

TH cDNA가  
 vectors가  
 Adenoviral vector episome, retroviral vector infection  
 Adenoviral vector가, liposome vectors가, vector

3,4 pcDNA3.1 vector  
 TH가, western blotting TH가 90%  
 TH가 100%  
 cytokine가  
 가  
 TH  
 factor (RA b-FGF) RA  
 FGF mesenchymal, neuroectodermal b-endothelial<sup>15</sup>  
 hypothalamic<sup>12,13</sup>

b-FGF  
 ,<sup>12,13,16</sup> EGF, PDGF IGF  
 가 ,<sup>13,16</sup>  
 10<sup>-6</sup> M RA  
 Schuldiner (2001)<sup>15</sup>  
 TH#2/MB03  
 b-FGF RA 가  
 21  
 ,  
 NF200 50~70% 가  
 ,  
 NF160 10~30%  
 ,  
 RA  
 14  
 21  
 TH 80~90% TH#2/  
 MB03 RA  
 , TH dopamine L-  
 DOPA tyrosine  
 .  
 가  
 , L-DOPA  
 가 L-DOPA  
 가  
 가  
 , TH 가  
 (TH#2/MB03) RA  
 L-DOPA 가  
 가

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