Identification of differentially expressed genes (DEGs) under the proliferation and differentiation conditions of human neural stem cells (NSCs) using annealing control primers (ACPs)

MiYeong Kim

Department of Medical Science

The Graduate School, Yonsei University

Identification of differentially expressed genes (DEGs) under the proliferation and differentiation conditions of human neural stem cells (NSCs) using annealing control primers (ACPs)

Directed by Professor Kook In Park

The Master's Thesis submitted to the Department of Medical Science, The Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

MiYeong Kim

June 2007

This certifies that the Master's Thesis
of MiYeong Kim is approved

Thesis Supervisor : Kook In Park

Thesis Committee Member#1 : Jin sung Lee

Thesis Committee Member#2 : Dong-Wook Kim

The Graduate School

Yonsei University

June 2007

감사의 글

2년이란 짧은 시간 안에 이 논문이 완성될 수 있도록 격려해주시고 열정적으로 지도해 주신 은사 박국인 교수님께 진심으로 감사 드립니다.

그리고 부족한 논문을 지켜봐 주시고 심사해주신 이진성 교수님과, 김동욱 교수님께도 감사 드립니다.

논문이 완성되기까지 옆에서 힘이 되어주고 많은 도움을 주었던 김형태 선생님과 실험실 식구들 김일선, 이일신, 정광수, 박성주, 윤석환, 이혜진, 박태훈, 황규진, 이지윤에게도 고마운 마음을 전합니다.

마지막으로 이 논문이 완성되기까지 사랑과 믿음으로 아낌없는 격려와 지원을 해주시고 버팀목이 되어주신 나의 사랑하는 가족 아버지, 어머니, 동생 미지 와 광인이에게 마음 속 깊이 감사하고 이 기쁨을 함께 나누고 싶습니다.

2007년 6월

<TABLE OF CONTENTS>

<LIST OF FIGURES>

<LIST OF TABLES>

ABSTRACT

Identification of differentially expressed genes (DEGs) under the proliferation and differentiation conditions of human neural stem cells (NSCs) using annealing control primers (ACPs)

Mi-Yeong Kim

Department of Medical science
The Graduate School, Yonsei University

(Directed by professor Kook In Park)

Proliferating single cells were isolated from the telencephalic brain region of human fetal cadavers at 13 weeks of gestation and grown as neurospheres in long-term cultures. Human neurospheres derived from the telencephalon were found to contain cells of different subtypes, suggesting that multipotent neural stem cells (NSCs), progenitors, or radial glial cells co-exist with restricted neuronal or glial progenitors within the spheres. These cells also had specific regional and temporal characteristics with regard to their growth, differentiation, and region-specific gene expression.

In order to identify genes that are involved under the proliferation and

differentiation conditions of human neural stem cells (NSCs), we employed a new and accurate reverse transcription-polymerase chain reaction (RT-PCR) technique using annealing control primers (ACPs). This method could detect differentially expressed genes (DEGs) that were expressed specifically or prominently under the proliferation and differentiation conditions of human NSCs in culture. RT-PCR and/or real time PCR were used to validate these DEGs.

Using 110 ACPs, the RT-PCR analysis of human NSCs resulted in approximately 500 amplicons. Among them, we identified and sequenced 34 amplicons of DEGs; 4 amplicons were highly expressed under the proliferation condition and 30 amplicons were highly expressed under the differentiation condition of human NSCs. To find regions of local similarity between these DEG sequences, we used the Basic Local Alignment Search Tool (BLAST). The results showed that the expression of 2 known and 2 unknown genes were highly increased under the proliferation condition and the expression of 25 known and 5 unknown genes were highly increased under the differentiation condition of human NSCs. These data would provide some insights into the genetic mechanisms involving molecular events and biological functions in the proliferation and differentiation of human NSCs.

Key words: differentially expressed genes (DEGs), Human neural stem cells (NSCs), annealing control primers (ACPs), proliferation, differentiation

Identification of differentially expressed genes (DEGs) under the proliferation and differentiation conditions of human neural stem cells (NSCs) using annealing control primers (ACPs)

Mi-Yeong Kim

Department of Medical science
The Graduate School, Yonsei University

(Directed by professor Kook In Park)

I. INTRODUCTION

Neural stem cells(NSCs) are defined as self-renewing and primordial immature cells with the capacity to give rise to differentiated progeny within all neural lineages in all the regions of the neuraxis. These cells are also posited to exist in embryonic and fetal germinal zones, where they participate in CNS organogenesis.[1] It is unclear whether NSCs are more defined by their tissues of origin or by their multipotentiality. The standard method of isolating NSCs *in vitro* is to remove a region of fetal

or adult brain that has been demonstrated to contain dividing cells *in vivo*. For example, the subventricular zone (SVZ) or the hippocampus in the adult brain may contain these dividing cells, or they can be found in a larger variety of structures in the developing brain [2].

Though the study of human NSCs is actively progressing, it is still poorly understood what conditions are needed for the differentiation of NSCs. In order to study what genes are differentially expressed under the proliferation and differentiation conditions of hNSCs, we isolated proliferating single cells from the telencephalon of gestational age 13 weeks fetal central nervous system (CNS) and induced these cells to proliferate as free-floating neurospheres (HFT13 cells). Many changes occur following the induction of differentiation of human neurospheres in culture; these are changes in the morphology of HFT13 cells, the percentage of neuronal or glial markers + cells, and the patterns of neurotransmitter expression.

To analyze the cellular composition of HFT13 cells, we assayed for the expression of proteins that are characteristic of immature cells, such as the NSC and radial glial marker nestin, and the radial glial protein recognized by vimentin, GFAP, Pax6, and the glutamate astrocyte-specific transporter (GLAST). In addition, we analyzed the expression levels of GFAP, a protein that is expressed by astrocytes, neuron-specific TUJ1, and neuronal markers NF and NeuN. All of these results indicate that, although

NSCs are operationally characterized on the basis of functional criteria due to the lack of definitive markers, multipotent NSCs, progenitors, and radial glial cells may co-exist with some restricted neuronal or glial progenitors in HFT13 cells derived from human fetal CNS tissue.[3]

In this study, we investigated which genes were differentially expressed under the proliferation and differentiation conditions of human NSCs. Various methods have been used to identify differentially expressed genes. However, the current methods employed to identify these genes that are expressed at a specific developmental stage are labor intensive and have high rates of false positives. We employed a new and accurate reverse transcription-polymerase chain reaction (RT-PCR) method that uses annealing control primers (ACPs) to identify these genes [4]. The ACP system is based on the unique tripartite structure of the primers, which have a distinct 3'- and 5'-region that are separated by a polydeoxyinosine linker [poly(dI)]. The interaction of each of these regions with a specific primer occurs during the high annealing temperatures in a two-stage PCR. This ACP anneals specifically to the template and allows only genuine product to be amplified, thus eliminating false-positive results. We used this ACP system to find specifically or prominently expressed genes during the proliferation and differentiation states of human NSCs.

We confirmed the expression patterns of DEGs by RT-PCR

assay, and quantified the expression of these genes by real time RT-PCR. The results suggest that this new PCR-based technique is a very useful tool for the identification of stage-specific DEGs. Analysis of identified DEGs in human NSCs under the proliferation and differentiation conditions will provide a stepping stone for developing an account of the basic gene expression patterns or in determining the genetic background of human NSCs. It will also be the framework for the putative network of linking cell cycle control to the cell fate pathway using gene expression data.

II. MATERIALS AND METHODS

1. Culture of human NSCs (HFT13 cells)

Human fetal tissue from therapeutically aborted embryos at 13 weeks of gestation was obtained with full patient consent and the approval of the research ethics committee of Yonsei University College of Medicine, Seoul, Korea. The methods of acquisition conformed to NIH and Korean Government guidelines. Human fetal telencephalon (HFT) was isolated from the telencephalon of gestational age 13 weeks fetal CNS and was induced to proliferate as free-floating neurospheres. Neurospheres were cultured in serum-free medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12; Gibco Invitrogen, Carsbad, CA, USA), and was supplemented with penicillin/ streptomycin (1% vol/vol; Gibco) and N2 formulation (1% vol/vol; Gibco). Mitogens were added to maintain the proliferation state, 20 ng/ml basic fibroblast growth factor (bFGF; R&D,Minneapolis, MN, USA) and 8 ㎍/ml heparin (Sigma, St. Louis, MO, USA) were added to stabilize the FGF-2 and also to increase initial growth rates [5], and 10 ng/ml leukemia inhibitory factor (LIF; Sigma) was added to allow the growth of a long-term self

renewing neural stem cell [6]. To maintain the differentiation state the cells were cultured in a PLL (poly-L-Lysine) coated plate without mitogens. All cultures were maintained in a humidified incubator (37ºC, 5% CO2 in air) and half the growth medium was replenished every 3~4 days. Neurospheres were passed every 7~9 days by treating the cells with 0.05% Trypsin/0.53mM EDTA (T/E).

2. RNA Extraction

Total RNA was extracted from the proliferation and differentiation conditions of human NSCs, respectively using the RNeasy mini kit (QIAGEN, Valencia,CA). The buffer RLT was used to harvest and lyse the cells. Seventy percent ethanol was added to the homogenized lysate and the sample was then applied to an RNeasy mini column. Buffer RW1 was added to the column, which was then washed with the buffer RPE. The RNA was eluted from the column with RNase-free water. The mRNA of HFT13 cells was treated with DNAse, the OD (optical density) was determined (Table 1), and 3 μg of the RNA preparation was electrophoresed (Figure 1). 20 μg of total RNA from the proliferation and differentiation stages of HFT13 cells, respectively was used for RT-PCR assay with the ACPs.

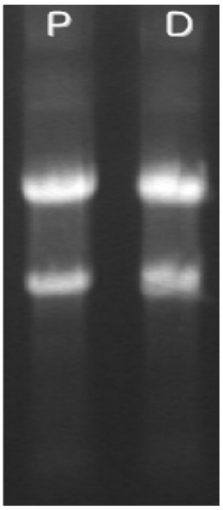

Figure 1. Electrophoresis of extracted RNA. Representative RNA band patterns under the proliferation (P) and differentiation (D) conditions of HFT13 cells.

Table 1. RNA OD determination

Sample	A260	A260/A280	Conc.(ug/ul)	Total(ug)
Proliferation	0.036	1.333	0.72	20
Differentiation	0.029	1.381	0.58	20

3.ACP-based GeneFishing™ PCR

RNAs extracted from the samples were used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42℃ in a final reaction volume of 20 ㎕, which contained the following: 3 ㎍ of the purified RNA, 4 ㎕ of 5 × reaction buffer (Promega, Madison, WI, USA), 5 ㎕ of dNTPs (each 2 mM), 2 ㎕ of 10 μM dT-ACP1 (5'-CGTGAATGCTGCGACTACGA TIIIIIT(18)-3'), 0.5 ㎕ of RNasin® RNase Inhibitor (40 U/㎕; Promega), and 1 ㎕ of Moloney murine leukemia virus reverse transcriptase (200 U/㎕; Promega). First-strand cDNAs were diluted by the addition of 180 ㎕ of ultra-purified water for the GeneFishingTM PCR and were stored at -20℃ until used.

Differentially expressed genes were screened by the ACP-based PCR method [4,7] using the GeneFishingTM DEG kits

(Seegene, Seoul, South Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of the first-stage PCR in a final reaction volume of 20 ㎕, which contained 3-5 ㎕ (about 50 ng) of diluted first-strand cDNA, 1 ㎕ of 10 μM dT-ACP2 (5'-CGTGAATGCTGCGAC TACGATIIIIIT(18)-3'), 1 ㎕ of 10 μM arbitrary ACP, and 10 ㎕ of 2 × Master Mix (Seegene, Seoul, South Korea). The PCR protocol for second-strand synthesis was one cycle at 94℃ for 1 min, followed by 50℃ for 3 min, and 72℃ for 1 min. After the second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65℃ for 40 s, 72℃ for 40 s, followed by a 5 min final extension at 72℃. The amplified PCR products were then separated on a 2% agarose gel stained with ethidium bromide.

4. Cloning and sequencing

The differentially expressed bands were extracted from the gel using the GENCLEAN® II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly cloned into a TOPO TA cloning vector (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The cloned plasmids were sequenced with ABI PRISM 7700 Analyzer (Applied Biosystems,

Foster City, CA, USA). Complete sequences were analyzed by searching for similarities using the BLASTX and BLASTN search program available at the National Center for Biotechnology Information (NCBI) GenBank website (http://www.ncbi. nlm.nih.gov /BLAST/)

5. RT-PCR and Real Time RT-PCR Quantification

To confirm the results of the ACP differential display and to determine the relative abundance of target sequences, both the RT-PCR and the real time quantitative PCR were performed on the samples. First, we synthesized cDNA for the RT-PCR. The extracted RNAs were then used for the synthesis of the first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1 h at 37℃ in a final reaction volume of 20 ㎕, which contained 4 ㎍ of purified RNA, 4 ㎕ of 5 X First strand buffer (Invitrogen), 1 ㎕ of dNTPs (each 10 mM), 1 ㎕ of oligo dT (500 ng /㎕), 0.5 ㎕ of Ribonuclease Inhibitor (40 U/㎕; Takara), 2 ㎕ of 0.1 M DTT (Invitrogen), and 1 ㎕ of Moloney murine leukemia virus reverse transcriptase (200 U/㎕; Invitrogen). First-strand cDNAs were diluted by the addition of 80 ㎕ of ultra-purified water and stored at -20℃

until use. The same expressed genes were also confirmed by RT-PCR. The cDNA was synthesized from mRNA using the reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Specific primers were designed using the ProbeWiz Server (H. B. Nielsen et al., 2002). Primer sequences are shown in Table 2. For optimal quantification, the parameters were set to design primer sequences with melting temperatures (Tm) between 55–65℃, to avoid 3'-end complementarity (to avoid primer-dimer formation), and a product size of 150–300 bps. Human GAPDH mRNA was used as an internal standard. The GAPDH primer sequences were for the forward primer 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for the reverse primer. The RT-PCR products were separated on a 1.5% agarose gel stained with ethidium bromide.

The real time PCRs were conducted in ABI Prism 7700 (Applied Biosystems) and were detected with SYBR Green, a double-stranded DNA-specific fluorescent dye, which is included in the SYBR premix Ex Taq (TAKARA). Prior to the quantification of the results, optimization procedures were performed by running PCRs, with and without the purified template, to identify the melting temperatures of the primer dimers and of the specific product. Serial dilutions of the

GAPDH plasmid DNA (102–109 molecules) were used for the standard curves. PCR conditions were 10 s at 94℃ followed by 50 cycles of 5 s at 94℃, 30 s at 58℃ and 30 s at 72℃. Data was collected during the extension step of the SYBR Green reaction.

Table 2. DEG primer sequences and cycling conditions used in semi-quantitative RT-PCR and/or Real-Time RT-PCR.

Primer number	Accession number	Primer	Product length	Annealing temperature(℃)
17	BC018036	F – CTCCCAGTTAATTGGCATGC R -	254	58
20	BC021174	F-GAGATCATGCAAGAAAAGCAGA R-	275	60
21	BX649038	F-CCCTGACTCAGTTTGGAATAGG R-	198	56
21	AL121980	F-ACAGGAATGGGGAGTAATCCTT R-	283	56
34	BC046114	F-AAAAGAAGAAACCCCTGAGGTC R-	345	56
41	BC032565	F-GTGCATATGCGTGAGTTTCATT R-	290	60
44	XM_379510	F-TGCACACTTGTAATCACAGCAC R-	248	58
45	NM_015329	F-CGTTTATCAGAAACACAGGCAA R-	283	60
46	AY337516	F- TGCATTTGTGAGCCAAAGAG R-	316	58
50	AL136721	F-CACCAGTTGACACTACATCGGT R-	264	56
52	AL353648	F-TCCTATATTACGGTTTGCCCC R-	259	58
55	NM_001004333	F-CCTTTACGAGCAAGTCAGCT R-	259	58
58	NM_002489	F-AGCTGAAGAAGGAACGTCCA R-	445	58
58	BC042486	F-AAGAAAAAGAGGCTACGCAGTG R-	288	56
60	BC036957	F-TGTGAGTAAAATTCTGGGCAGG R-	563	58
60	NM_080820	F- CGCATGTGGTAGAAGGTGTG R-	230	58
62	NM_198427	F-GTTGCTTAAGGCTCTGTGGG R-	301	58
65	BC025296	F-ATGGCGACCCTACTCTTACAAA R-	219	56
65	BC040436	F- AAGATCTCCTAAAAAGCCTCGG R-	243	56
66	BC003385	F-TGCTTCTGGAAATTAAACGGAG R-	214	56
77	BC028311	F- AACCAAAGCCACCAGTGTTC R-	308	58
78	NM_006227	F-TGTCCAGCATGACTATGGAC R-	254	58
83	NM006317	F-GAGTTGGGAGTGATGCGTTT R-	314	58

86	AK023048	F- ACGACCCACATGGTTTCAAT	R-	186	58
87	AY339566	F-TTCACTATCATATTCATCGGCG	R-	285	58
87	BC017265	F-CTTTGAGGAAGCGGTTCGAA	R-	256	58
87	BC065711	F- CCTTCGAGTCGTTCTTGCTC	R-	284	58
94	BC024240	F- TGAGGTCCTGCTTTGTCCTT	R-	261	58
96	BC053630	F-CTCAGGCCGTGGACTTTTTA	R-	322	56

(Table2 continue)

Primer number	Accession number	primer		Product length	annealing temperature(℃)
98	AK223612	F-GGCAAAAGATCTTCAAGAAGTGA	R-	170	56
101	BX647311	F-CGTGTGCTGCAGAACCTAAA	R-	277	58
104	BC050637	F-ATTCCTCTCCTTCTTCCTCACC	R-	302	60
117	M77774	F -TCTCTCACAGCTTGAAAAGCCT	R-	300	60
120	AL160251	F-AACCTCAAAAACGTGAGCGTAT	R-	268	60

6.Real time PCR Analysis

The Ct value is the fractional cycle number at which the fluorescence generated by the reporter dye exceeds a fixed level above baseline. The selected genes' signals were normalized against the relative quantity of GAPDH and the relative amount of each cDNA was calculated by determining the $\triangle C_T$ value; $\triangle C_T = C_{T,target} - C_{T,GAPDH}$. The fold difference in the target gene relative to the GAPDH endogenous control gene was determined by the $2^{-\triangle(\triangle CT}$) method [8]. The $2^{-\triangle(\triangle CT)}$ method is a convenient way to analyze the relative change in gene expression from a real time PCR;$\triangle(\triangle C_T) = \triangle C_{T,sample} - \triangle C_{T,control}$. Analysis of variance was followed by the nonparametric t- test and the Wilcoxon rank sum test to analyze the results of these studies [9], and a value of $P < 0.001$ was considered significant.

III.RESULTS

1. Morphology of human NSCs (HFT13 cells) under the proliferation and differentiation conditions and RNA extraction

The typical morphology of HFT13 cells under the proliferation and differentiation conditions is represented in Figure 2. Proliferating HFT13 cells gave rise to free-floating spheres or cell clusters that could be identified by their phase-bright appearance, regular cell membranes, and diffraction rings (Fig. 2). To analyze the cellular composition of HFT13 cells at the proliferation stage, the immunocytochemistry with various immature neural stem cells or progenitors markers, and differentiated neuronal and glial markers was performed. These results indicate that, although NSCs are operationally characterized on the basis of functional criteria due to the lack of definitive markers, multipotent NSCs, progenitors, and radial glial cells may co-exist with some restricted neuronal or glial progenitors in HFT13 cells derived from human fetal CNS tissue.[3]

To induce the differentiation of HFT13 cells, whole neurospheres of HFT13 cells were dissociated into a single cell

suspension in T/E and plated directly onto poly-L-lysine (10 μg/ml; Sigma)-coated, 100mm dish(corning) in serum-free medium (DMEM/F-12) that contained N2 supplement but without mitogens. Over the 7-day period following plating, the cells represented differentiation associated morphological changes of human NSCs (Fig. 2).

Total RNA was isolated under the proliferation and differentiation conditions of HFT13 cells, respectively and 20 ㎍ of total RNA from the proliferation and differentiation stages of HFT13 cells, respectively was used for RT-PCR assay with the ACPs.

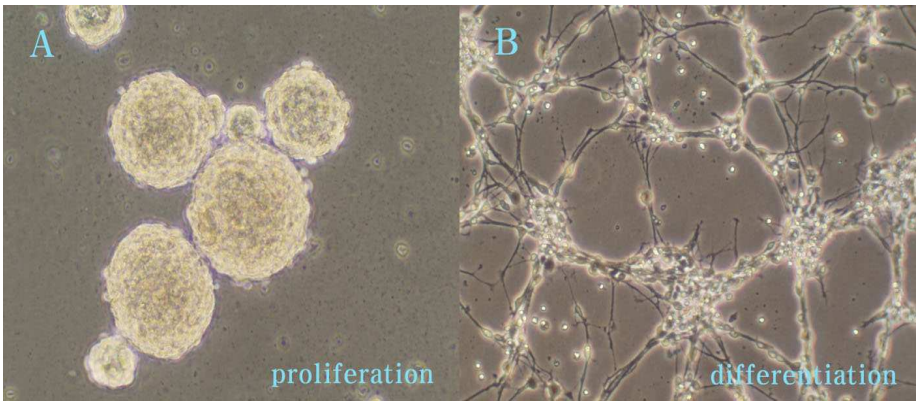

Figure 2. Representative morphological characteristics under the

proliferation and differentiation conditions of human NSCs (HFT13 cells). HFT13 could be identified by their phase-bright appearance (A) HFT13 expand as free-floating neurospheres and (B) show the extension of many cellular processes during the induction of the differentiation in PLL-coated dishes.

2. Screening of DEGs using ACPs

To identify genes that are specifically or differentially expressed under the proliferation and differentiation conditions of human NSCs, we used the ACP (Seegene, Seoul, Korea) [4]. The mRNA isolated from the proliferation and differentiation stages of human NSCs was subjected to ACP RT-PCR analysis using a combination of 110 arbitrary primers and two anchored oligo (dT) primers (dT-ACP 1 and dT-ACP 2). This method is described in the Materials and Methods section. The analysis generated approximately 500 amplicons.

3. Identification of DEGs

We found 34 differentially expressed amplicons under the proliferation and differentiation conditions of human NSCs. 4

amplicons were highly increased under the proliferation condition and 30 amplicons were increased under the differentiation condition (Figure 3). 34 DEGs were totally extracted from the gel and were cloned into a TOPO TA vector and sequenced. Sequences results were searched by using BLASTN and BLASTX for sequence similarity in the NCBI GenBank and revealed that these DEGs showed significant similarity with known genes, unknown genes or ESTs (Expressed Sequence Tags). Therefore, 4 genes were found to be differentially highly expressed under the proliferation condition and 30 genes were identified to be differentially highly expressed under the differentiation condition of human NSCs. All of these genes and ESTs identified and characterized in this study have already been submitted to Genbank and assigned accession numbers. Under the proliferation condition of human NSCs, 2 DEGs are known genes; BCAN and HLA-B, and the other 2 DEGs are unknown genes. Under the differentiation condition of human NSCs, 25 DEGs are known genes; C13orF17, SERF1A, CSMD2, HMGB1, Pax6, SFRS11, SPOP, DCT, PNN, tubulin alpha 3, TSPAN7, MAP1B, LASS5, GAD1, KIF3C, NDUFA4, HARS2, ODC1, PLTP, BASP1, RAB9A, POLR2J, COX1, COX5A, and GNG7, and the other 5 DEGs are unknown genes. The identity and size of these DEGs are summarized in Table 3-A and B.

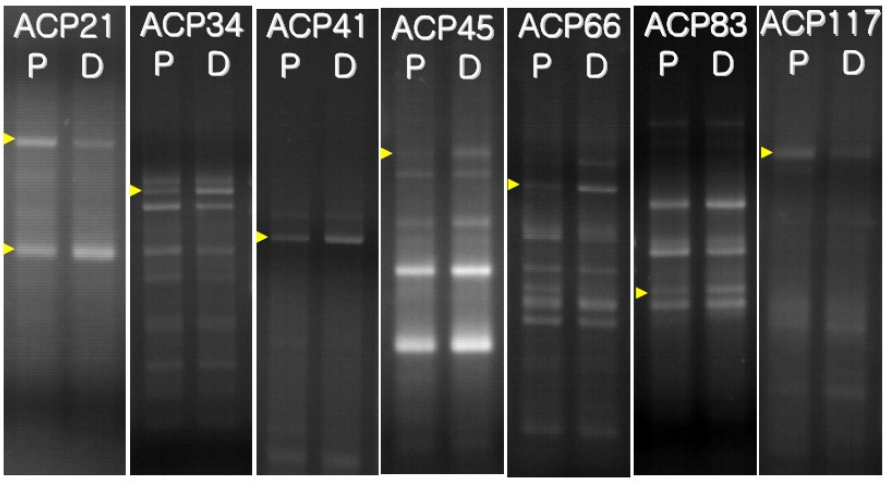

Figure 3. Representative band patterns for DEGs under the proliferation(P) and differentiation(D) conditions of HFT13 cells after annealing control primer-polymerase chain reaction (ACP-PCR). The PCR products were separated by electrophoresis on 2% agarose gels. Yellow arrows indicate DEGs

Table 3-A. Identity and size of DEGs that encode known proteins and their homology to sequences in GenBank

Primer number	Accession number	Base pairs sequenced	Identity	Homology (human)	E-value*
Increased in Proliferation					
62	NM 198427	330	brevican isoform 2(BCAN)	254/254(100%)	8E-140
117	M77774	1251	Human HLA-B*5401	1151/1177(97%)	0.0
Increased in differentiation					
17	BC018036	873	tetraspanin 7(TSPAN7)	428/439(97%)	0.0
20	BC021174	726	Small EDRK-rich factor 1A(SERF1A)	635/650 (97%)	0.0
21	AL121980	214	CUB and Sushi multiple domains 2(CSMD2)	144/145 (99%)	E-72
34	BC046114	708	microtubule-associated protein 1B(MAP1B)	410/488 (84%)	0.0
41	BC032565	1076	LAG1 longevity assurance homolog 5(LASS5)	948/962 (98%)	0.0
46	AY337516	225	glutamate decarboxylase 1(GAD1)	133/138(96%)	8E-59
52	AL353648	190	high-mobility group box 1(HMGB1)	117/119(98%)	e-54
58	BC042486	1288	Kinesin family member 3C(KIF3C)	1055/1073 (98%)	0.0
58	NM_002489	341	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex(NDUFA4)	273/273(100%)	4E-151
60	NM_080820	1059	histidyl-tRNA synthetase 2(HARS2)	959/960(99%)	0.0
60	BC036957	396	Paired box gene 6(Pax6)	291/319(91%)	2E-101
65	BC040436	531	splicing factor, arginine/serine-rich 11(SERS11)	130/153 (84%)	3E-79
65	BC025296	588	ornithine decarboxylase 1(ODC1)	512/513 (99%)	0.0
66	BC003385	1153	speckle-type POZ protein(SPOP)	554/562 (98%)	0.0
77	BC028311	787	dopachrome tautomerase (DCT)	667/667(100%)	0.0
78	NM_006227	916	phospholipid transfer protein isoform a precursor(PLTP)	843/845(99%)	0.0
83	NM006317	449	brain abundant, membrane attached signal 1(BASP1)	212/212(100%)	1E-114
87	AY339566	749	cytochrome c oxidase subunit I(COX1)	662/664 (99%)	0.0
87	BC017265	723	RAB9A, member RAS oncogene family	472/474(99%)	0.0
87	BC065711	662	Polymerase(RNA)II(DNA directed) polypeptides(POLR2J)	588/591(99%)	0.0
94	BC024240	580	cytochrome c oxidase subunit Va, precursor(COX5A)	498/498(100%)	0.0
96	BC053630	529	guanine nucleotide binding protein (G protein), gamma 7(GNG7)	461/462(99%)	0.0
98	AK223612	393	Pinin(PNN)	280/304 (92%)	e-169
104	BC050637	613	tubulin, alpha 3	527/530 (99%)	0.0
120	AL160251	619	chromosome 13 open reading frame 17(C13orF17)	529/550 (96%)	0.0

Table 3-B. Identity and size of DEGs that encode unknown proteins and their homology to sequences in GenBank

Primer number	Accession number	Base pairs	Identity	Homology (human)	E-value*
Increased in Proliferation					
21	BX649038	351	uncharacterized	280/280	e-155
50	AL136721	760	hypothetical protein	670/687(97%)	0.0
Increased in differentiation					
44	XM_379510	334	hypothetical protein	257/265 (96%)	e-131
45	NM_015329	933	hypothetical protein LOC23383	641/659 (97%)	0.0
55	NM_00100433	241	hypothetical protein LOC440400	197/197(100%	6E-106
86	AK023048	563	unnamed protein product	489/491(99%)	0.0
101	AF190901	366	hypothetical protein	294/294(100%	1E-163

*Expectation value (E-value) represents the number of different alignments with scores equivalent to or better than the alignment in question and that would be expected to occur in the database by chance alone. E-value is generated by BLAST and is a convenient way to create a significance threshold for reporting results. The lower the E-value, the more significant the match

4. Confirmation of DEGs by RT-PCR and Real Time RT-PCR

To confirm the efficacy of the ACP system, the semi-quantitative RT-PCR using specific primers for selected DEGs was assayed. The primer sequences and the annealing

temperatures used for the different DEGs are summarized in Table 2.

This RT-PCR analysis revealed that all of identified DEGs were differentially expressed in the human NSCs (Figure 4), which confirms the results of the ACP differential display analysis. We further analyzed the expression patterns of the 34 selected DEGs under the proliferation and differentiation conditions by using the real-time RT-PCR method (Table 4-A and B). To normalize the RT-PCR and real time RT-PCR reaction efficiency, GAPDH was used as an internal standard. In real time RT-PCR, we represented the expressed levels using a fold change and a P-value of < 0.001. Genes with the increased expression under the proliferation condition did not show a high gap fold change. The most highly expressed gene under the proliferation condition was the one with the accession number AL136721 and a fold change of 2.46. Under the differentiation condition, the gene with the accession number AK023048 was the most highly expressed and its fold change was 11.31. Other genes with high fold changes under the differentiation condition were BASP1 with a fold change of 9.92, NM_015329 with 9.7, ODC1 with 7.78, GAD1 with 6.8, and KIF3C with 5.49.

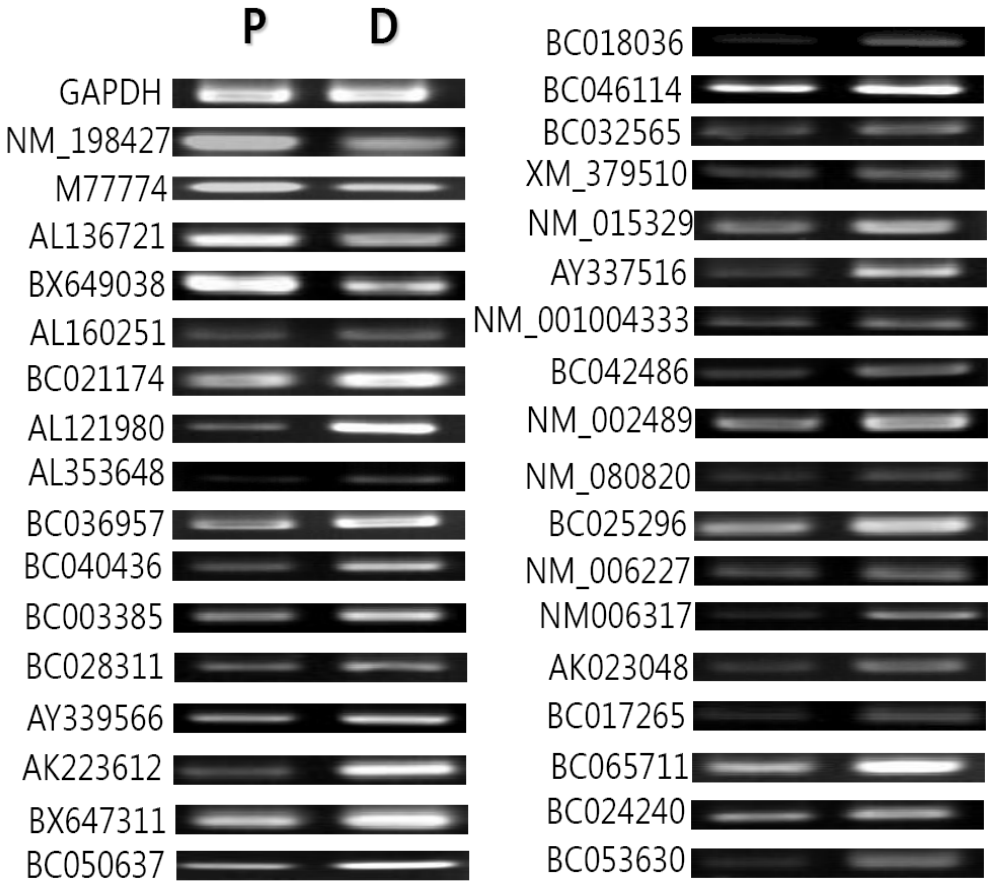

Figure 4 Comparison of the expression patterns of DEGs under the proliferation and differentiation conditions of human NSCs (HFT 13 cells) by semi-quantitative RT-PCR

Table 4-A. Quantitative real-time PCR analysis showing increased expression of genes under the proliferation condition of human NSCs (HFT13 cells)

Accession	Identity	Fold change*
known		
NM 198427	brevican isoform 2(BCAN)	1.43±0.29
M77774	Major histocompatibility complex, class I,	2.35±0.075
hypothetical		
AL136721	hypothetical protein	2.46±0.094
BX649038	uncharacterised	1.4±0.066

* Relative expression levels of DEGs after normalization with human GAPDH mRNA levels are presented as an n-fold expression difference compared to the other party. P-value is <0.001

Table 4-B. Quantitative real-time PCR analysis showing increased expression of genes under the differentiation condition of human NSCs (HFT13 cells)

Accession number	Identity	Fold
known		

AL160251	chromosome 13 open reading frame	3.7±0.112
BC021174	Small EDRK-rich factor 1A(SERF1A)	2.3±0.277
AL121980	CUB and Sushi multiple domains 2(CSMD2)	2.27±0.081
AL353648	high-mobility group box 1(HMGB1)	2.35±0.075
BC036957	Paired box gene 6(Pax6)	1.67±0.136
BC040436	splicing factor, arginine/serine-rich	2.73±0.17
BC003385	speckle-type POZ protein(SPOP)	3.81±0.043
BC028311	dopachrome tautomerase (DCT)	1.65±0.066
AY339566	cytochrome c oxidase subunit I(COX1)	3.05±0.051
AK223612	Pinin(PNN)	4.23±0.051
BC050637	tubulin, alpha 3	2.06±0.021
BC018036	tetraspanin 7(TSPAN7)	1.79±0.107
BC046114	microtubule-associated protein 1B(MAP1B)	3.44±0.04
BC032565	LAG1 longevity assurance homolog 5(LASS5)	3.55±0.090
AY337516	glutamate decarboxylase 1(GAD1)	6.8±0.067
BC042486	Kinesin family member 3C(KIF3C)	5.49±0.30
NM_002489	NADH dehydrogenase (ubiquinone) 1 alpha	1.9±0.094
NM_080820	histidyl-tRNA synthetase 2	2.59±0.222
BC025296	ornithine decarboxylase 1	7.78±0.038
NM_006227	phospholipid transfer protein isoform a	2.2±0.007
NM006317	brain abundant, membrane attached signal	9.92±0.173
BC017265	RAB9A, member RAS oncogene family	2.91±0.136
BC065711	POLR2J protein	3.27±0.165
BC024240	cytochrome c oxidase subunit Va, precursor	2.23±0.079
BC053630	guanine nucleotide binding protein (G	2.10±0.115
hypothetical		
BX647311	hypothetical protein	2.10±0.115
XM_379510	hypothetical protein	3.9±0.063
NM_015329	hypothetical protein LOC23383	9.7±0.24
NM_001004333	hypothetical protein LOC440400	1.69±0.117
AK023048	unnamed protein product	11.31±0.213

DISCUSSION

To study differentially expressed genes under the different conditions, various methods such as Microarrary [10, 11], DD-PCR, SAGE and subtractive hybridization are currently using. But those techniques have some limitations to investigate DEGs because they have high false positive results. In this study, we tried new approach termed Genefishing, which is the RT-PCR technique using ACPs. The Microarrary technique which is currently the most popular used identifies the expression of known and expected genes. But the Genefishing method could find not only known and expected genes, but also unexpected and unknown genes. The success of PCR is based solely on the specificity and the sensitivity of the primer binding to the target sequence. This depends on the annealing temperatures of the primers. ACP has inosine between the 3'- and 5'- ends of the primer so the PCR is specific and sensitive to the target sequence in high annealing temperatures [4, 7]. We found 70 differentially expressed bands using this ACP technique. All of the 70 bands were cloned, sequenced and analyzed. We performed RT-PCR for 53 genes that might be expected to have the importance; but the RT-PCR results showed that 19 genes

had no difference in expression levels (data not shown). Some of the 34 DEGs under the proliferation and differentiation conditions of human NSCs have been studied in relation to neurogenesis, while this relation has not yet been studied in others.

Among highly expressed genes under the proliferation condition of human NSCs, BCAN is known as one of the most abundant extracellular matrix proteoglycans in the mammalian brain. Brevican produced by gray matter astrocytes constitutes a major component of perineuronal extracellular matrix in the adult brain. Expression of brevican in oligodendrocytes and white matter astrocytes is differently regulated during the development of brain. Brevican is not synthesized by mature oligodendrocytes; it is produced by astrocytes in the mature adult [12, 13]. HLA-B is known to be related to immunity but not to neurogenesis. HLAB*5401 is not studied well.

Among highly expressed genes under the differentiation condition of human NSCs, TSPAN7 is implicated in cell proliferation, differentiation and tumor invasion, but the mechanism is still unknown [14-17]. The basic function of SERF1A (telomeric) is unknown, but the down-regulation of telomerase activity was found to be a general response to the induction of differentiation [18]. MAP1B which controls neurites extension and

growth cone motility via modulating microtubule dynamics, is the first MAP gene to be expressed during brain development. Despite discrepancies between some studies, MAP1B plays a principal role in the development of the nervous system [19, 20] and is required for the regeneration of axons that control the directionality of growth cone migration and the axonal branching in regeneration of adult dorsal root ganglia neurons [21]. HMGB1 previously was thought to function only as a nuclear factor that enhances transcription, but was recently discovered to be a crucial cytokine that mediates the response to infection, injury, inflammation[22], necrosis[23, 24] and apoptosis[25]. HMGB1 was also reported to stimulate the proliferation of vessel-associated embryonic stem cells [26]. But, the expression of HMGB1 in HFT13 cells is highly increased under the differentiation condition which may reflect the characterization of human NSCs or cells are not fully differentiated under the current differentiation condition. Pax6 is involved in cell proliferation and/or cell differentiation in hippocampal neurogenesis [27]. In the developing mammalian brain, Pax6 plays a crucial role in the regionalization of the major divisions of the telencephalon and diencephalons [28]. Pinin is associated with the mature desmosomes of the epithelia [29], is related to a tumor suppressor [30], and has been reported as a differentiation-

specific desmosomal protein[31]. BASP1 is involved in the maturation and/or maintenance of the synapse rather than in the process of the axonal outgrowth by controlling the cholesterol-dependent membrane dynamics and signal proteins participating in the neurite outgrowth and synaptic plasticity [32, 33]. GAD1 is the nerve terminal vesicle-associated antigen that catalyzes the production of GABA and relates to the common disease schizophrenia and bipolar affective disorder. KIF3C plays a role in various intracellular transport and spindle formation mechanisms and is reported to show expression during neural development and neural differentiation [34].

There are still a number of DEGs involved in the proliferation and differentiation stages of HFT13 cells that have not been characterized in details. Based on our observations, further functional studies are warranted to determine the role of DEGs in human NSCs.

CONCLUSION

NSCs are defined as self-renewing and primordial immature cells with the capacity to give rise to differentiated progeny within all neural lineages in all the regions of the neuraxis. Though the study of human NSCs is actively progressing, it is still poorly understood what conditions are needed for the differentiation of NSCs. So we approached with a genetic tool to investigate which genes were differentially expressed under the proliferation and differentiation conditions of human NSCs.

We tried a new and accurate reverse transcription-polymerase chain reaction (RT-PCR) method which uses annealing control primers (ACPs). We identified 34 DEGs through RT-PCR technique using ACPs. The expression of 2 known and 2 unknown genes were highly increased under the proliferation condition and the expression of 25 known and 5 unknown genes were highly increased under the differentiation condition of human NSCs. We confirmed the expression patterns of DEGs by RT-PCR assay and quantified the expression of these genes by real time RT-PCR. Some of the 34 DEGs under the proliferation and differentiation conditions of human NSCs have been studied in relation to neurogenesis, while this relation has not yet been studied in others. Analysis of identified DEGs in human NSCs under the proliferation and differentiation conditions will

provide a stepping stone for developing an account of the basic gene expression patterns or in determining the genetic background of human NSCs. It will also be the framework for the putative network of linking cell cycle control to the cell fate pathway using gene expression data.

REFERENCES

1. Ourednik V, Ourednik J, Flax JD, Zawada WM, Hutt C, Yang C, et al. Segregation of human neural stem cells in the developing primate forebrain. Science 2001 Sep 7;293(5536):1820-4.

2. Gage FH. Mammalian neural stem cells. Science 2000 Feb 25;287(5457):1433-8.

3. Kim HT, et al. human neurospheres derived from the fetal central nervous system are regionally and temporally specified, but are not committed. Exp Neurol 2006.

4. Hwang IT, Kim YJ, Kim SH, Kwak CI, Gu YY, Chun JY. Annealing control primer system for improving specificity of PCR amplification. Biotechniques 2003 Dec;35(6):1180-4.

5. Caldwell MA, Svendsen CN. Heparin, but not other proteoglycans potentiates the mitogenic effects of FGF-2 on mesencephalic precursor cells. Exp Neurol 1998 Jul;152(1):1-10.

6. Wright LS, Li J, Caldwell MA, Wallace K, Johnson JA, Svendsen CN. Gene expression in human neural stem cells: effects of leukemia inhibitory factor. J Neurochem 2003 Jul;86(1):179-95.

7. Kim YJ, Kwak CI, Gu YY, Hwang IT, Chun JY.

Annealing control primer system for identification of differentially expressed genes on agarose gels. Biotechniques 2004 Mar;36(3):424-6, 8, 30 passim.

8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001 Dec;25(4):402-8.

9. Troyanskaya OG, Garber ME, Brown PO, Botstein D, Altman RB. Nonparametric methods for identifying differentially expressed genes in microarray data. Bioinformatics 2002 Nov;18(11):1454-61.

10. Geschwind DH, Ou J, Easterday MC, Dougherty JD, Jackson RL, Chen Z, et al. A genetic analysis of neural progenitor differentiation. Neuron 2001 Feb;29(2):325-39.

11. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 2002 Oct 18;298(5593):597-600.

12. Ogawa T, Hagihara K, Suzuki M, Yamaguchi Y. Brevican in the developing hippocampal fimbria: differential expression in myelinating oligodendrocytes and adult astrocytes suggests a dual role for brevican in central nervous system fiber tract development. J Comp Neurol 2001 Apr 9;432(3):285-95.

13. Aya-ay J, Mayer J, Eakin AK, Muffly BG, Anello M, Sandy JD, et al. The effect of hypoxic-ischemic brain injury in perinatal rats on the abundance and proteolysis of brevican and NG2. Exp Neurol 2005 May;193(1):149-62.

14. Hemler ME. Specific tetraspanin functions. J Cell Biol 2001 Dec 24;155(7):1103-7.

15. Berditchevski F. Complexes of tetraspanins with integrins: more than meets the eye. J Cell Sci 2001 Dec;114(Pt 23):4143-51.

16. Carloni V, Mazzocca A, Ravichandran KS. Tetraspanin CD81 is linked to ERK/MAPKinase signaling by Shc in liver tumor cells. Oncogene 2004 Feb 26;23(8):1566-74.

17. Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annu Rev Cell Dev Biol 2003;19:397-422.

18. Yamada O, Takanashi M, Ujihara M, Mizoguchi H. Down-regulation of telomerase activity is an early event of cellular differentiation without apparent telomeric DNA change. Leuk Res 1998 Aug;22(8):711-7.

19. Lu R, Wang H, Liang Z, Ku L, O'Donnell W T, Li W, et

al. The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. Proc Natl Acad Sci U S A 2004 Oct 19;101(42):15201-6.

20. Gonzalez-Billault C, Jimenez-Mateos EM, Caceres A, Diaz-Nido J, Wandosell F, Avila J. Microtubule-associated protein 1B function during normal development, regeneration, and pathological conditions in the nervous system. J Neurobiol 2004 Jan;58(1):48-59.

21. Bouquet C, Soares S, von Boxberg Y, Ravaille-Veron M, Propst F, Nothias F. Microtubule-associated protein 1B controls directionality of growth cone migration and axonal branching in regeneration of adult dorsal root ganglia neurons. J Neurosci 2004 Aug 11;24(32):7204-13.

22. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005 Apr;5(4):331-42.

23. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002 Jul 11;418(6894):191-5.

24. Rovere-Querini P, Capobianco A, Scaffidi P, Valentinis B, Catalanotti F, Giazzon M, et al. HMGB1 is an endogenous

immune adjuvant released by necrotic cells. EMBO Rep 2004 Aug;5(8):825-30.

25. Bell CW, Jiang W, Reich CF, 3rd, Pisetsky DS. The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol 2006 Dec;291(6):C1318-25.

26. Palumbo R, Sampaolesi M, De Marchis F, Tonlorenzi R, Colombetti S, Mondino A, et al. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol 2004 Feb 2;164(3):441-9.

27. Maekawa M, Takashima N, Arai Y, Nomura T, Inokuchi K, Yuasa S, et al. Pax6 is required for production and maintenance of progenitor cells in postnatal hippocampal neurogenesis. Genes Cells 2005 Oct;10(10):1001-14.

28. Manuel M, Price DJ. Role of Pax6 in forebrain regionalization. Brain Res Bull 2005 Sep 15;66(4-6):387-93.

29. Ouyang P, Sugrue SP. Identification of an epithelial protein related to the desmosome and intermediate filament network. J Cell Biol 1992 Sep;118(6):1477-88.

30. Shi Y, Ouyang P, Sugrue SP. Characterization of the gene encoding pinin/DRS/memA and evidence for its potential tumor suppressor function. Oncogene 2000 Jan 13;19(2):289-97.

31. Brandner JM, Reidenbach S, Franke WW. Evidence that "pinin", reportedly a differentiation-specific desmosomal protein, is actually a widespread nuclear protein. Differentiation 1997 Dec;62(3):119-27.

32. Bomze HM, Bulsara KR, Iskandar BJ, Caroni P, Skene JH. Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nat Neurosci 2001 Jan;4(1):38-43.

33. Iino S, Taguchi K, Maekawa S, Nojyo Y. Motor, sensory and autonomic nerve terminals containing NAP-22 immunoreactivity in the rat muscle. Brain Res 2004 Mar 26;1002(1-2):142-50.

34. Navone F, Consalez GG, Sardella M, Caspani E, Pozzoli O, Frassoni C, et al. Expression of KIF3C kinesin during neural development and in vitro neuronal differentiation. J Neurochem 2001 May;77(3):741-53.

ABSTRACT (IN KOREAN)

결합조절 프라이머 (ACPs)를 사용하여 인간 신경줄기 세포의 증식과 분화조건에서 차별되게 발현되는 유전자 (DEGs) 확인

<지도교수 박 국 인>

연세대학교 대학원 의과학과

김미영

인간 신경줄기세포를 배양하기 위하여 재태연령 13주에 자연 유산되어 사망한 태아 사체의 종뇌 (telencephalon) 조직에서 증식능력을 보이는 단일 신경세포들을 분리하여 생체 외에서 장기간 신경구 (neurospheres)상태로 배양하였는데, 본 세포들은 신경줄기세포, 신경전구세포 (neural progenitors), 방사교세포 (radial glial cells), 신경원 전구세포 (neuronal progenitor) 및 신경교 전구세포 (glial progenitor) 등 다양한 종류의 세포형태로 구성되어 있으며, 특이적인 성장, 증식, 분화 및 지역 특이 유전자 발현 형태를 보였다.

신경구 형태로 배양되는 미성숙 인간 신경줄기세포 혹은 전구세포의 증식 및 분화관련 유전자를 확인하기 위하여 본 연구에서는 결합조절 프라이머(ACPs)를 이용한 정확하고 새로운 역전사 중합효소연쇄반응(RT-PCR)기술을 사용하였는데, 인간 신경줄기세포의 증식 및 분화 조건에

서 특이적으로 차별 발현되는 유전자들(DEGs)을 확인하였고, 본 DEGs들의 정확성 및 정량성은 정량적 중합효소연쇄반응과 실시간 효소연쇄반응 방법을 통해서 검증하였다. 110개의 ACPs를 사용하여 증식 및 분화조건의 인간 신경줄기세포에서 추출한 RNAs를 RT-PCR로 분석한 결과 약 500개의 반응물 (amplicons)을 얻었고, 이 반응물들 중에서 34개의 DEGs를 찾았으며, 염기서열을 분석하고 확인한 결과 4개의 amplicons은 증식상태에서 높게 발현되었고 30개의 amplicons은 분화상태에서 높게 발현됨을 확인하였다. 확인된 34개의 DEGs 연기서열과 유사성을 가진 유전자 부분을 찾기 위하여 Basic Local Alignment Search Tool(BLAST)를 사용하였는데, 증식상태에서 높게 발현되는 4개의 DEGs 중 2개는 알려진 유전자이고 2개는 알려지지 않은 유전자였으며, 분화상태에서 높게 발현되는 30개의 DEGs 중 25개는 알려진 유전자였고 5개는 알려지지 않은 유전자로 확인되었다. 따라서 본 연구결과는 유전자 차원에서 인간 신경줄기세포의 증식과 분화에 관련된 분자학적 기전을 이해하는데 사용될 수 있으며, 관련 유전자의 생물학적 기능 연구에도 도움을 줄 수 있다.

인간 신경줄기세포, 차별 발현되는 유전자(DEGs),결합조절 프라이머(ACPs), 증식, 분화