Expression Profiles of Subtracted Genes During Neural Differentiation from Human Embryonic Stem Cells using Suppression Subtractive Hybridization

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Abstract

Human embryonic stem cells (human ES cells) are pluripotent and self-renewing cells that can be isolated from the inner cell mass at the blastocyst stage. Human ES cells differentiate into specific cell lineages according to the expression of related genes. During neural development, specific gene expressions induced human ES cells some morphological changes. We used suppression subtractive hybridization to identify genes which shows altered expression during neural differentiation from human ES cells. We identified 90 genes as downregulated and 64 genes as upregulated in neural precursor (NP) cells derived from human ES cells compared with human ES cells. To obtain further information about differentiation, we performed expression profiling of subtracted genes between human ES cells and NP cells. Of the subtracted genes in human ES cells, the 19 genes showed decreased expression in NP cells. Meanwhile, of the subtracted genes in NP cells, the 20 genes were upregulated. Among them, COPZ1, CPSF6, MATR3 and TOMD3 were specific gene expressions that they significantly expressed up and down-regulation during neural development derived from ES cells. These genes have not previously been associated with neural differentiation, but they may be potentially participated in neurogenesis.

Introduction

Stem cells can self-renew and can differentiate into specific cell types to produce various organs. Stem cells can be subdivided into few groups, such as embryonic stem cells (ES cells), adult stem cells, induced pluripotent stem cells (iPS cells) and somatic cell nuclear transfer ES cells (SCNT-ES cells) (Tachibana et al., 2013). ES cells originate in the inner cell mass of a blastocyst, the stage 5 ~ 7 days after a human ovum is fertilized. ES cells maintain stemness and specifically express stem cell markers, including Oct4, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, and are pluripotent (Rippon and Bishop, 2004). Research using ES cells comes with ethical issues associated with using donor eggs or pre-implantation embryos (de Wert and Mummery, 2003). On the other hand, adult stem cells can be induced from adult cells, including bone marrow, cord blood, brain, and testis. Adult stem cells also show self-renewal and multi-differentiation but are more limited in both of these functions compared with ES cells (Ramalho-Santos et al., 2002; Yoo et al., 2010). Patient-specific pluripotent stem cells are produced by modifying gene regulation via pluripotency-related genes such as Oct4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007). However, Klf4 and c-Myc have shown tumorigenic potential because these genes activate oncogenic transcription (Evans and Liu, 2008). Therefore, iPS cells are considered unsuitable for use in regenerative medicine. SCNT-ES cells are generated by replacing the nucleus of the host cell with that of a somatic cell, as in the case of Dolly the sheep (Tachibana et al., 2013). This cell type is a good source for patient-specific regenerative medicine applications but has some problems related to donors and ethical issues, similar to those for ES cells (Zacharias et al., 2011). Despite these problems, because stem cells can differentiate into specific tissues or organs, many researchers dedicate time to use in regenerative medicine applications (Zacharias et al., 2011).

During neural differentiation from ES cells, ES cell markers decrease and neural lineage markers such as Nestin and PAX-6 increase in expression (Yap et al., 2015). For the differentiation of NP (neural precursor) cells, most approaches are based on embryoid body (EB) formation. Next, NP cells are generated via differentiation from EBs using various methods (Yap et al., 2015). Recently, NP cells have become an important source for clinical applications of regenerative medicine. The central nervous system (CNS) is not regenerate tissues after birth. Damage to these areas produces CNS disorder (Carpenter et al., 1999). NP cells represent a good source for cell transplantation in therapy for CNS disorders (Wilcox et al., 2014) because NP cells are components of the CNS and show the ability to differentiate into neurons, astrocytes and oligodendrocytes (Goldman, 1995). Although there are known markers of neural differentiation (Yap et al., 2015), they are not strongly expressed specifically in neural cells. In this research, we aimed to identify novel markers of neural differentiation.

PCR based numerous techniques have been used for identification of differentially expressed genes (DEGs), including expressed sequence tag (EST) sequencing, serial analysis of gene expression (SAGE), arbitrary primed-PCR, representational difference analysis (RDA), differential display polymerase chain reaction (DD-PCR) and suppression subtractive hybridization (SSH) (Jung et al., 2009). Benefit of SSH is that it can find novel genes and has a low false-positive rate (Yoo et al., 2010). In our previous study, we reported that the expression of TPX2 gradually decrease during neurogenesis (Noh et al., 2007). Here, we used SSH to identify DEGs during neurogenesis from human ES cells. We then characterized those DEGs and categorized their functions using a bioinformatics approach. Among these genes, specifically expressed genes may be useful as new biomarkers of neural development.

Materials And Methods

Cell culture of human ES cells

The human ES cell line CHA4 was cultivated by using a method described in a previous study (Kim et al., 2007). Human ES cells were cultured on mitotically inactivated STO cells (CRL-1503; ATCC, Manassas, VA, USA) in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1; Gibco BRL, Gaithersburg, MD, USA) supplemented with 20% serum replacement (Gibco) and standard human ES cell medium components, including 1 mM L-glutamine (Gibco), 1% nonessential amino acids (Gibco), 100 mM β -mercaptoethanol (Gibco), and basic fibroblast growth factor (b-FGF, Sigma, USA). The medium was changed every 24 h, and the human ES cells were transferred to new feeder cells every 5-7 days using a dissecting pipette. The CHA4 human ES cell were provided by Stem Cell Research Laboratory of CHA University, respectively

Differentiation of NP cells from human ES cells

The technique for differentiating NP cells is described in detail in our previous study (Kim et al., 2004; Noh et al., 2007). Before differentiation of NP cells, human ES cells were induced to form EBs by culturing the ES cells in suspension media without bFGF for 20 days. Neuroepithelial cells with a rosette structure appeared after 7 days in culture and were manually isolated from the culture and transferred into new plates containing F-127 in neurosphere (NS) medium. Neurospheres were formed in suspension culture within 24 h and were subcultured every 14 days. Neurosphere medium consisted of DMEM/F12 (1:1) supplemented with 2 mM L-glutamine, 25 μ g/ml human insulin (Sigma, St. Louis, MO, USA), 100 μ g/ml human transferrin (Sigma), 20 nM progesterone (Sigma), 60 μ M putrescine (Sigma), 30 nM sodium selenite (Sigma), 2 μ g/ml heparin (Sigma), recombinant human epidermal growth factor (EGF; R&D Systems) and 20 ng/ml FGF-2. The medium was changed every day and characterized NP cells using RT-PCR (S1 fig).

RNA isolation

Total RNA was isolated from human ES cells and NP cells using Trizol reagent (Life Technology) and quantified using a DN-1000 spectrophotometer (Nanodrop Technologies Inc., Waltham, MA, USA). The A_{260}/A_{280} ratio was in the range of 1.8-2.0. To confirm integrity, approximately 1 μ g of total RNA was electrophoresed on a 1.2% denaturing agarose gel.

cDNA synthesis from total RNA

cDNA was synthesized using the BD SMARTTM cDNA Synthesis Kit (BD Bioscience Clontech, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μ g of total RNA and reverse-transcribed in a 10 μ l reaction mixture containing PowerScript reverse transcriptase (BD Bioscience) using 3' BD SMART CDS primer II A (5'- AAG CAG TGG TAT CAA CGC AGG GTA CTT -3') and BD SMARTTM A oligonucleotide primer (5'- AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG -3'). The first-strand cDNA was diluted to a final volume of 40 μ l with 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and used to generate second-strand cDNA by long-distance PCR with BD Advantage 2 polymerase mix (BD Bioscience) using PCR primer II A (5'- AAG CAG TGG TAT CAA CGC AGA GT -3') and following the manufacturer's instructions. The PCR-amplified second-strand cDNA was purified using column chromatography and then digested with 10 U/ μ l RsaI in a final volume of 50 μ l at 37°C for 3 h to obtain blunt-ended fragments. The final concentration of digested second-strand cDNA was 300 μ g/ μ l.

Suppression subtractive hybridization

Purified two-target cDNA was used for SSH using a Clontech PCR selected cDNA subtraction kit (BD Bioscience) according to the manufacturer's instructions. Tester cDNA (human ES cell or human NPC) was ligated to adaptor 1 and adaptor 2R in separate ligation reactions at 16°C overnight using 400 U/ μ l T4 ligase. Driver cDNA (human NPC or human ES cells) was not ligated to adaptors. After ligation, two samples were subjected to hybridization. In the first sample, an excess of driver mRNA was added to each adaptor-ligated tester cDNA in the hybridization buffer, heat-denatured at 98°C for 1.5 min and then allowed to anneal at 68°C for 8 h. Next, two primary hybridization samples were mixed together without denaturing, and freshly denatured driver cDNA was added to the sample and allowed to anneal at 68°C overnight. After a second hybridization, samples were diluted in 200 μ l dilution buffer (20 mM HEPES, 50 mM NaCl, 0.2 mM EDTA) at 68°C for 7 min. This hybrid cDNA was used in PCR to amplify the desired differentially expressed sequences using 50X Advantage cDNA Polymerase PCR Kit (BD Bioscience). First-round PCR amplifications were performed using PCR primer 1, and second-round PCR amplifications were performed using nested primer 1 and nested primer 2R. First-round PCR was performed using the following cycling parameters. Then, amplified second-round PCR product was cloned directly into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5 α cells. Plasmid DNA from each clone was purified, and the inserts were confirmed by digestion with EcoRI (NEB, England). Successfully cloned cDNA fragments were sequenced using the T7 promoter primer and the MacroGen sequencing service (<http://dna.macrogen.com/kor>).

Bioinformatics analysis

The nucleotide sequences were compared against all sequences in the non-redundant nucleotide database at NCBI (National Center for Biotechnology Information) using the Basic Local Alignment Search Tool program (<http://www.ncbi.nlm.nih.gov/BLAST>). Subtracted genes distributed ontology using NCBI database (nucleotide database).

RT-PCR (Reverse transcription PCR)

To validate differential expression between the cell types, we also performed RT-PCR. Full-length cDNA was synthesized using the SuperScriptII First-strand Synthesis system (Invitrogen, Carlsbad, CA, USA). The list of primers used for RT-PCR is provided (S1 table).

Quantitative RT-PCR

Real time PCR was performed using a QuantiTect SYBR green PCR kit (QIAGEN, Hilden, Germany) and the Real time PCR Detected System (Bio-rad). The list of primers used for quantitative RT-PCR is provided (S2table).

Statistical analysis

The data are presented as the mean $\pm$ S.E.M. from at least three independent experiments. The significant differences were analyzed using Student's t-test. P-values < 0.05 were considered statistically significant.

Results

SSH analysis of subtracted clones from human ES cells compared with NP cells derived from human ES cells

We performed SSH and identified and characterized subtracted clones of one hundred twenty genes, including known genes (77%), redundant genes (10%) and ribosomal RNA (12%) (Fig. 1A). To analyze these data, we categorized these genes according to cellular process in human ES cells *in silico* analysis. Human ES cell subtracted genes were associated with molecular functions including apoptosis, cell adhesion, cell division, development, immune response, metabolism, proliferation, RNA processing, transcription and non-coding RNA (Fig. 2A). Most of the subtracted genes in human ES cells involved metabolism (37%) or development (18%). The subtracted genes of human ES cells are summarized in Table 1.

Table 1
Summary of subtracted clones in human ES cells

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
1	<i>FASTKD1</i>	Homo sapiens FAST kinase domains 1 (FASTKD1), transcript variant 1, mRNA	NM_024622.4	Apoptosis	2q31		
2	<i>FXR1</i>	Homo sapiens fragile X mental retardation, autosomal homolog 1 (FXR1), transcript variant 1, mRNA	NM_005087.3	Apoptosis	3q28		20197067
3	<i>JADE1</i>	Homo sapiens jade family PHD finger 1 (JADE1), transcript variant 1, mRNA	NM_199320.3	Apoptosis	4q26-q27		
4	<i>NACA</i>	Homo sapiens nascent polypeptide-associated complex alpha subunit (NACA), transcript variant 1, mRNA	NM_001113203.2	Apoptosis	12q23-q24.1		22154550
5	<i>TERF1</i>	Homo sapiens telomeric repeat binding factor (NIMA-interacting) 1 (TERF1), transcript variant 1, mRNA	NM_017489.2	Apoptosis	8q21.11		15614775
6	<i>TMA7</i>	Homo sapiens translation machinery associated 7 homolog (<i>S. cerevisiae</i>) (TMA7), mRNA	NM_015933.4	Apoptosis	3p21.31		
7	<i>TMBIM6</i>	Homo sapiens transmembrane BAX inhibitor motif containing 6 (TMBIM6), transcript variant 1, mRNA	NM_003217.2	Apoptosis	12q13.12		22906541
8	<i>WNK3</i>	Homo sapiens WNK lysine deficient protein kinase 3 (WNK3), transcript variant 1, mRNA	NM_020922.4	Apoptosis	Xp11.22		26069258
9	<i>YWHAZ</i>	Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ), transcript variant 1, mRNA	NM_003406.3	Apoptosis	8q23.1		21802640
10	<i>PCDH15</i>	Homo sapiens protocadherin-related 15 (PCDH15), RefSeqGene on chromosome 10	NM_001142763.1	Cell adhesion	10q21.1		16904662
11	<i>PCDH18</i>	Homo sapiens protocadherin 18 (PCDH18), transcript variant 1, mRNA	NM_019035.4	Cell adhesion	4q31	2	18468594, 225671294
12	<i>PTPRF</i>	Homo sapiens protein tyrosine phosphatase, receptor type, F (PTPRF), transcript variant 1, mRNA	NM_002840.3	Cell adhesion	1p34		16784531
13	<i>STOM</i>	Homo sapiens stomatin (STOM), transcript variant 1, mRNA	NM_004099.5	Cell adhesion	9q34.1		19379682
14	<i>ASUN</i>	Homo sapiens asunder spermatogenesis regulator (ASUN), mRNA	NM_018164.2	Cell division	12p11.23		
15	<i>AURKA</i>	Homo sapiens aurora kinase A (AURKA), transcript variant 1, mRNA	NM_198433.1	Cell division	20q13		23011139, 24019927

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
16	<i>HAUS1</i>	Homo sapiens HAUS augmin-like complex, subunit 1 (HAUS1), transcript variant 1, mRNA	NM_138443.3	Cell division	18q21.1		
17	<i>HSP90AA1</i>	Homo sapiens heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1), transcript variant 1, mRNA	NM_001017963.2	Cell division	14q32.33	2	26071189
18	<i>ORC3</i>	Homo sapiens origin recognition complex, subunit 3 (ORC3), transcript variant 1, mRNA	NM_181837.2	Cell division	6q14.3-q16.1		10402192, 23110156, 23349620
19	<i>SKIL</i>	Homo sapiens SKI-like proto-oncogene (SKIL), transcript variant 1, mRNA	NM_005414.4	Cell division	3q26	2	17202138
20	<i>SMC3</i>	Homo sapiens structural maintenance of chromosomes 3 (SMC3), mRNA	NM_005445.3	Cell division	10q25		26438359
21	<i>YTHDF3</i>	Homo sapiens YTH domain family, member 3 (YTHDF3), transcript variant 1, mRNA	NM_152758.5	Cell division	8q12.3		
28	<i>ACVR2A</i>	Homo sapiens activin A receptor, type IIA (ACVR2A), transcript variant 1, mRNA	NM_001278579.1	Development	2q22.3		18436533, 19342382
24	<i>ARL6IP1</i>	Homo sapiens ADP-ribosylation factor-like 6 interacting protein 1 (ARL6IP1), mRNA	NM_015161.1	Development	16p12-p11.2		
25	<i>CD9</i>	Homo sapiens CD9 molecule (CD9), mRNA	NM_001769.3	Development	12p13.3	2	15742316, 11950938
26	<i>CDH1</i>	Homo sapiens cadherin 1, type 1, E-cadherin (epithelial) (CDH1), mRNA	NM_004360.3	Development	16q22.1		20473026, 24266635
27	<i>DPYSL2</i>	Homo sapiens dihydropyrimidinase-like 2 (DPYSL2), transcript variant 1, mRNA	NM_001197293.2	Development	8p22-p21		19926752
28	<i>FANCD2</i>	Homo sapiens Fanconi anemia, complementation group D2 (FANCD2), transcript variant 1, mRNA	NM_033084.3	Development	3p26		22683204, 21458466
29	<i>IARS</i>	Homo sapiens isoleucyl-tRNA synthetase (IARS), transcript variant 1, mRNA	NM_002161.5	Development	9q21		
30	<i>NDC1</i>	Homo sapiens NDC1 transmembrane nucleoporin (NDC1), transcript variant 1, mRNA	NM_018087.4	Development	1p32.3	2	
31	<i>PGK1</i>	Homo sapiens phosphoglycerate kinase 1 (PGK1), mRNA	NM_000291.3	Development	Xq13.3		18447643
32	<i>RB1CC1</i>	Homo sapiens RB1-inducible coiled-coil 1 (RB1CC1), transcript variant 1, mRNA	NM_014781.4	Development	8q11		22184008, 15968549

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
33	<i>SORL1</i>	Homo sapiens sortilin-related receptor, L (DLR class) A repeats containing (SORL1), mRNA	NM_003105.5	Development	11q23.2-q24.2		25772071
34	<i>SPEN</i>	Homo sapiens spen family transcriptional repressor (SPEN), mRNA	NM_015001.2	Development	1p36		26190100
35	<i>TFRC</i>	Homo sapiens transferrin receptor (TFRC), transcript variant 1, mRNA	NM_003234.2	Development	3q29	2	22595340
36	<i>UGCG</i>	Homo sapiens UDP-glucose ceramide glucosyltransferase (UGCG), mRNA	NM_003358.2	Development	9q31		19745600, 10430909
37	<i>USP9X</i>	Homo sapiens ubiquitin specific peptidase 9, X-linked (USP9X), transcript variant 3, mRNA	NM_001039590.2	Development	Xp11.4	4	19176755
38	<i>ZNF281</i>	Homo sapiens zinc finger protein 281 (ZNF281), transcript variant 1, mRNA	NM_012482.4	Development	1q32.1		22963690
39	<i>IFITM1</i>	Homo sapiens interferon induced transmembrane protein 1 (IFITM1), mRNA	NM_003641.3	Immune response	11p15.5		22634173, 25896322
40	<i>HSPD1</i>	Homo sapiens heat shock 60kDa protein 1 (chaperonin) (HSPD1), transcript variant 2, mRNA	NM_199440.1	Immune response	2q33.1		18047272, 20954177
41	<i>SLC39A10</i>	Homo sapiens solute carrier family 39 (zinc transporter), member 10 (SLC39A10), transcript variant 1, mRNA	NM_001127257.1	Immune response	2q32.3		
42	<i>AASS</i>	Homo sapiens aminoadipate-semialdehyde synthase (AASS), mRNA	NM_005763.3	Metabolism	7q31.3		
43	<i>ACP1</i>	Homo sapiens acid phosphatase 1, soluble (ACP1), transcript variant 2, mRNA	NM_007099.3	Metabolism	2p25		25159695
44	<i>ATP5C1</i>	Homo sapiens ATP synthase, H ⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1 (ATP5C1), transcript variant 1, mRNA	NM_001001973.1	Metabolism	10p15.1		
45	<i>AZIN1</i>	Homo sapiens antizyme inhibitor 1 (AZIN1), transcript variant 1, mRNA	NM_015878.5	Metabolism	8q22.3		
46	<i>CCT2</i>	Homo sapiens chaperonin containing TCP1, subunit 2 (beta) (CCT2), transcript variant 1, mRNA	NM_006431.2	Metabolism	12q15		21640791, 21404461
46	<i>CECR2</i>	Homo sapiens cat eye syndrome chromosome region, candidate 2 (CECR2), transcript variant 1, mRNA	NM_001290047.1	Metabolism	22q11.2		
47	<i>COPZ1</i>	Homo sapiens coatomer protein complex, subunit zeta 1 (COPZ1), transcript variant 1, mRNA	NM_016057.2	Metabolism	12q13.2-q13.3		

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
48	<i>DEK</i>	Homo sapiens DEK proto-oncogene (DEK), transcript variant 1, mRNA	NM_003472.3	Metabolism	6p22.3		21943234, 10391928
49	<i>EEF1A1</i>	Homo sapiens eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), mRNA	NM_001402.5	Metabolism	6q14.1		15540898, 19157425
50	<i>EIF4A2</i>	Homo sapiens eukaryotic translation initiation factor 4A2 (EIF4A2), mRNA	NM_001967.3	Metabolism	3q28		
51	<i>GARS</i>	Homo sapiens glycyl-tRNA synthetase (GARS), mRNA	NM_002047.2	Metabolism	7p15		22791832
52	<i>G3BP1</i>	Homo sapiens GTPase activating protein (SH3 domain) binding protein 1 (G3BP1), transcript variant X1, mRNA	NM_005754.2	Metabolism	5q33.1		
53	<i>GPN1</i>	Homo sapiens GPN-loop GTPase 1 (GPN1), transcript variant 1, mRNA	NM_007266.3	Metabolism	2p23.3		
54	<i>JMJD1C</i>	Homo sapiens jumonji domain containing 1C (JMJD1C), transcript variant 1, mRNA	NM_032776.2	Metabolism	10q21.3		24318875
55	<i>LRBA</i>	Homo sapiens LPS-responsive vesicle trafficking, beach and anchor containing (LRBA), transcript variant 1, mRNA	NM_001199282	Metabolism	4q31.3		
56	<i>LYPLA1</i>	Homo sapiens lysophospholipase I (LYPLA1), transcript variant 1, mRNA	NM_006330.3	Metabolism	8q11.23		
57	<i>MYL6</i>	Homo sapiens myosin, light chain 6, alkali, smooth muscle and non-muscle (MYL6), transcript variant 2, mRNA	NM_079423.3	Metabolism	12q13.2	2	
58	<i>NDUFS5</i>	Homo sapiens NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase) (NDUFS5), transcript variant 1, mRNA	NM_004552.2	Metabolism	1p34.2-p33		
59	<i>NECAB1</i>	Homo sapiens N-terminal EF-hand calcium binding protein 1 (NECAB1), mRNA	NM_022351.4	Metabolism	8q21.3		24616509
60	<i>OTUD4</i>	Homo sapiens OTU deubiquitinase 4 (OTUD4), transcript variant 3, mRNA	NM_001102653.1	Metabolism	10p12.2		
61	<i>PDK1</i>	Homo sapiens pyruvate dehydrogenase kinase, isozyme 1 (PDK1), transcript variant 1, mRNA	NM_001278549.1	Metabolism	2q31.1		21540297, 18514190
62	<i>PLOD2</i>	Homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), transcript variant 1, mRNA	NM_182943.2	Metabolism	3q24		17553463
63	<i>PSMD2</i>	Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 2 (PSMD2), transcript variant 1, mRNA	NM_002808.4	Metabolism	3q27.1		

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64	<i>RASSF3</i>	Homo sapiens Ras association (RalGDS/AF-6) domain family member 3 (RASSF3), transcript variant 1, mRNA	NM_178169.3	Metabolism	12q14.2		
65	<i>RPL14</i>	Homo sapiens ribosomal protein L14 (RPL14), transcript variant 2, mRNA	NM_003973.4	Metabolism	3p22-p21.2		
66	<i>SEPHS1</i>	Homo sapiens selenophosphate synthetase 1 (SEPHS1), transcript variant 1, mRNA	NM_012247.4	Metabolism	10p14		19128516
67	<i>SERINC5</i>	Homo sapiens serine incorporator 5 (SERINC5), transcript variant 1, mRNA	NM_001174072.2	Metabolism	5q14.1		
68	<i>SLC2A3</i>	Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA	NM_006931.2	Metabolism	12p13.3	2	18948350, 16221986
69	<i>SLC44A1</i>	Homo sapiens solute carrier family 44 (choline transporter), member 1 (SLC44A1), transcript variant 1, mRNA	NM_080546.4	Metabolism	9q31.2		
70	<i>SSR2</i>	Homo sapiens signal sequence receptor, beta (translocon-associated protein beta) (SSR2), mRNA	NM_003145.3	Metabolism	1q21-q23	2	
71	<i>UBE2H</i>	Homo sapiens ubiquitin-conjugating enzyme E2H (UBE2H), transcript variant 1, mRNA	NM_003344.3	Metabolism	7q32		
72	<i>UBE2W</i>	Homo sapiens ubiquitin-conjugating enzyme E2W (putative) (UBE2W), transcript variant 1, mRNA	NM_001001481.2	Metabolism	8q21.11		
73	<i>UGP2</i>	Homo sapiens UDP-glucose pyrophosphorylase 2 (UGP2), transcript variant 1, mRNA	NM_006759.3	Metabolism	2p14-p13		
74	<i>CD24</i>	Homo sapiens CD24 molecule (CD24), transcript variant 1, mRNA	NM_013230.3	Proliferation	6q21		15614775, 17204602, 19579288
75	<i>GPC4</i>	Homo sapiens glypican 4 (GPC4), mRNA	NM_001448.2	Proliferation	Xq26.1		22761013
76	<i>NAP1L1</i>	Homo sapiens nucleosome assembly protein 1-like 1 (NAP1L1), transcript variant 1, mRNA	NM_139207.2	Proliferation	12q21.2		23260146
77	<i>NPM1</i>	Homo sapiens nucleophosmin (nucleolar phosphoprotein B23, numatrin) (NPM1), transcript variant 1, mRNA	NM_002520.6	Proliferation	5q35.1		24798243
78	<i>SLC35F2</i>	Homo sapiens solute carrier family 35, member F2 (SLC35F2), mRNA	NM_017515.4	Proliferation	11q22.3		
79	<i>CPSF6</i>	Homo sapiens cleavage and polyadenylation specific factor 6, 68kDa (CPSF6), transcript variant 1, mRNA	NM_007007.2	RNA processing	12q15		

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80	<i>HNRNPA2B1</i>	Homo sapiens heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), transcript variant A2, mRNA	NM_002137.3	RNA processing	7p15		23495120
81	<i>HNRNPA3</i>	Homo sapiens heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3), mRNA	NM_194247.2	RNA processing	2q31.2		
82	<i>HSPA8</i>	Homo sapiens heat shock 70kDa protein 8 (HSPA8), transcript variant 1, mRNA	NM_006597.5	RNA processing	11q24.1		16100000
83	<i>LARP7</i>	Homo sapiens La ribonucleoprotein domain family, member 7 (LARP7), transcript variant 2, mRNA	NM_006597.5	RNA processing	4q25		24768001
84	<i>MATR3</i>	Homo sapiens matrin 3 (MATR3), transcript variant 1, mRNA	NM_199189.2	RNA processing	5q31.2		
85	<i>DPPA4</i>	Homo sapiens developmental pluripotency associated 4 (DPPA4), mRNA	NM_018189.3	Transcription	3q13.13	2	25421462
86	<i>DPY30</i>	Homo sapiens dpy-30 homolog (C. elegans) (DPY30), mRNA	NM_032574.2	Transcription	2p22.3		
87	<i>EGR1</i>	Homo sapiens early growth response 1 (EGR1), mRNA	NM_001964.2	Transcription	5q31.1		19798744
88	<i>H2AFZ</i>	Homo sapiens H2A histone family, member Z (H2AFZ), mRNA	NM_002106.3	Transcription	4q24		21752451
89	<i>ESRG</i>	Homo sapiens embryonic stem cell related (non-protein coding) (ESRG), long non-coding RNA	NR_027122.1	Non-coding RNA	3p14.3		
90	<i>MALAT1</i>	Homo sapiens metastasis associated lung adenocarcinoma transcript 1 (non-protein coding) (MALAT1), long non-coding RNA	NR_002819.2	Non-coding RNA	11q13.1		23485710, 25868726

SSH analysis of subtracted clones from NP cells derived from human ES cells compared with human ES cells

In addition, we performed the inverse SSH, which yielded eighty-five subtracted clones, including known genes (73%), redundant genes (9%) and ribosomal RNA (18%) (Fig. 1B). Next, we used an *in silico* approach to categorize the genes specific to NP cells derived from human ES cells based on cellular processes. Human ES cell-derived NP cell subtracted genes were associated with molecular functions including cell adhesion, cell division, development, metabolism, proliferation, RNA processing, transcription and non-coding RNA (Fig. 2B). Most of the subtracted genes in NP cells derived from human ES cells were associated with metabolism (31%) and development (26%), similar to the subtracted genes in human ES cells. The NP cell subtracted genes are summarized in Table 2.

Table 2
Summary of subtracted clones from NP cells derived from human ES cells

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
1	<i>CAPZA1</i>	Homo sapiens capping protein (actin filament) muscle Z-line, alpha 1 (CAPZA1), mRNA	NM_006135.2	Cell adhesion	1p13.2		
2	<i>CASK</i>	Homo sapiens calcium/calmodulin-dependent serine protein kinase (MAGUK family) (CASK), transcript variant 1, mRNA	NM_003688.3	Cell adhesion	Xp11.4		16842202
3	<i>CCDC80</i>	Homo sapiens coiled-coil domain containing 80 (CCDC80), transcript variant 1, mRNA	NM_199511.2	Cell adhesion	3q13.2		22384085
4	<i>CCDC90B</i>	Homo sapiens coiled-coil domain containing 90B (CCDC90B), transcript variant 1, mRNA	NM_021825.4	Cell adhesion	11q14.1		
5	<i>SPTBN1</i>	Homo sapiens spectrin, beta, non-erythrocytic 1 (SPTBN1), transcript variant 1, mRNA	NM_003128.2	Cell adhesion	2p21		
6	<i>TMOD3</i>	Homo sapiens tropomodulin 3 (ubiquitous) (TMOD3), mRNA	NM_014547.4	Cell adhesion	15q21.1-q21.2		
7	<i>TRIQQ</i>	Homo sapiens triple QxxK/R motif containing (TRIQQ), transcript variant 1, mRNA	NM_001171796.1	Cell adhesion	8q22.1	2	
8	<i>TUBA1A</i>	Homo sapiens tubulin, alpha 1a (TUBA1A), transcript variant 1, mRNA	NM_006009.3	Cell adhesion	12q13.12	2	23231350
9	<i>CCNB2</i>	Homo sapiens cyclin B2 (CCNB2), mRNA	NM_004701.3	Cell division	15q22.2		15318809
10	<i>CUL1</i>	Homo sapiens cullin 1 (CUL1), mRNA	NM_003592.2	Cell division	7q36.1	2	16396913
11	<i>IST1</i>	Homo sapiens increased sodium tolerance 1 homolog (yeast) (IST1), transcript variant 1, mRNA	NM_001270975.1	Cell division	16q22.2		
12	<i>PSMA5</i>	Homo sapiens proteasome (prosome, macropain) subunit, alpha type, 5 (PSMA5), transcript variant 1, mRNA	NM_002790.3	Cell division	1p13		
13	<i>PSMD1</i>	Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 1 (PSMD1), transcript variant 1, mRNA	NM_002807.3	Cell division	2q37.1	2	19282972
14	<i>PSMD14</i>	Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 14 (PSMD14), mRNA	NM_005805.5	Cell division	2q24.2		
15	<i>ADAM10</i>	Homo sapiens ADAM metalloproteinase domain 10 (ADAM10), mRNA	NM_001110.3	Development	15q22		24404179
16	<i>CD46</i>	Homo sapiens CD46 molecule, complement regulatory protein (CD46), transcript variant a, mRNA	NM_002389.4	Development	1q32		9386776

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
17	<i>DAD1</i>	Homo sapiens defender against cell death 1 (DAD1), mRNA	NM_001344.3	Development	14q11.2		21305469
18	<i>DAZAP2</i>	Homo sapiens DAZ associated protein 2 (DAZAP2), transcript variant 1, mRNA	NM_014764.3	Development	12q12		19555680
19	<i>EIF4E</i>	Homo sapiens eukaryotic translation initiation factor 4E (EIF4E), transcript variant 1, mRNA	NM_001968.3	Development	4q23		16705177
20	<i>GGNBP2</i>	Homo sapiens gametogenetin binding protein 2 (GGNBP2), mRNA	NM_024835.4	Development	17q12		
21	<i>HMGCS1</i>	Homo sapiens 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) (HMGCS1), transcript variant 1, mRNA	NM_001098272.2	Development	5p14-p13		9425122
22	<i>ILDR2</i>	Homo sapiens immunoglobulin-like domain containing receptor 2 (ILDR2), mRNA	NM_199351.2	Development	1q24.1		
23	<i>MEIS1</i>	Homo sapiens Meis homeobox 1 (MEIS1), mRNA	NM_002398.2	Development	2p14		23756022
24	<i>NIPBL</i>	Homo sapiens Nipped-B homolog (Drosophila) (NIPBL), transcript variant A, mRNA	NM_133433.3	Development	5p13.2		
25	<i>PYG01</i>	Homo sapiens pygopus family PHD finger 1 (PYG01), mRNA	NM_015617.2	Development	15q21.3		
26	<i>SOX4</i>	Homo sapiens SRY (sex determining region Y)-box 4 (SOX4), mRNA	NM_003107.2	Development	6p22.3	2	23894955, 20646169
27	<i>SOX11</i>	Homo sapiens SRY (sex determining region Y)-box 11 (SOX11), mRNA	NM_003108.3	Development	2p25		23874955
28	<i>STMN1</i>	Homo sapiens stathmin 1 (STMN1), transcript variant 3, mRNA	NM_005563.3	Development	1p36.11	3	19526456
29	<i>TAF8</i>	Homo sapiens TAF8 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 43kDa (TAF8), mRNA	NM_138572.2	Development	6p21.1		
30	<i>TPM1</i>	Homo sapiens tropomyosin 1 (alpha) (TPM1), transcript variant Tpm1.3, mRNA	NM_001018020.1	Development	15q22.1		23918370
31	<i>WDR19</i>	Homo sapiens WD repeat domain 19 (WDR19), RefSeqGene on chromosome 4	NM_025132.3	Development	4p14		
32	<i>ALDH18A1</i>	Homo sapiens aldehyde dehydrogenase 18 family, member A1 (ALDH18A1), transcript variant 1, mRNA	NM_002860.3	Metabolism	10q24.3		
33	<i>CBR4</i>	Homo sapiens carbonyl reductase 4 (CBR4), mRNA	NM_032783.4	Metabolism	4q32.3		

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
34	<i>CHMP2B</i>	Homo sapiens charged multivesicular body protein 2B (CHMP2B), transcript variant 1, mRNA	NM_014043.3	Metabolism	3p11.2		23284619
35	<i>ERCC1</i>	Homo sapiens excision repair cross-complementation group 8 (ERCC8), transcript variant 1, mRNA	NM_000082.3	Metabolism	19q13.32		20362516, 18221731
36	<i>EXOSC8</i>	Homo sapiens exosome component 8 (EXOSC8), mRNA	NM_181503.2	Metabolism	13q13.1		
37	<i>GBE1</i>	Homo sapiens glucan (1,4-alpha-), branching enzyme 1 (GBE1), mRNA	NM_000158.3	Metabolism	3p12.3		
38	<i>HPRT1</i>	Homo sapiens hypoxanthine phosphoribosyltransferase 1 (HPRT1), mRNA	NM_000194.2	Metabolism	Xq26.1		24130677
39	<i>NDUFS2</i>	Homo sapiens NADH dehydrogenase (ubiquinone) Fe-S protein 2, 49kDa (NADH-coenzyme Q reductase) (NDUFS2), transcript variant 1, mRNA	NM_004550.4	Metabolism	1q23		
40	<i>NIPA2</i>	Homo sapiens non imprinted in Prader-Willi/Angelman syndrome 2 (NIPA2), transcript variant 1, mRNA	NM_030922.6	Metabolism	15q11.2		
41	<i>OSTC</i>	Homo sapiens oligosaccharyltransferase complex subunit (non-catalytic) (OSTC), transcript variant 1, mRNA	NM_021227.3	Metabolism	4q25		
42	<i>PAICS</i>	Homo sapiens phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS), transcript variant 1, mRNA	NM_001079525.1	Metabolism	4q12		22155494
43	<i>PSMB3</i>	Homo sapiens proteasome (prosome, macropain) subunit, beta type, 3 (PSMB3), transcript variant 1, mRNA	NM_002795.3	Metabolism	17q12		
44	<i>RPL24</i>	Homo sapiens ribosomal protein L24 (RPL24), mRNA	NM_000986.3	Metabolism	3q12		
45	<i>RPLP0</i>	Homo sapiens ribosomal protein, large, P0 (RPLP0), transcript variant 1, mRNA	NM_001002.3	Metabolism	12q24.2	2	
46	<i>RPLP1</i>	Homo sapiens ribosomal protein, large, P1 (RPLP1), transcript variant 1, mRNA	NM_001003.2	Metabolism	15q22		
47	<i>SH3BGRL</i>	Homo sapiens SH3 domain binding glutamate-rich protein like (SH3BGRL), mRNA	NM_003022.2	Metabolism	Xq13.3		
48	<i>TBC1D15</i>	Homo sapiens TBC1 domain family, member 15 (TBC1D15), transcript variant 1, mRNA	NM_022771.4	Metabolism	12q21.1		

	Gene	Full name	Accession Num.	Function	Location	Redundant	PMID
49	<i>TRA2B</i>	Homo sapiens transformer 2 beta homolog (Drosophila) (TRA2B), transcript variant 1, mRNA	NM_004593.2	Metabolism	3q26.2-q27		24586484, 23818142
50	<i>UGDH</i>	Homo sapiens UDP-glucose 6-dehydrogenase (UGDH), transcript variant 1, mRNA	NM_003359.3	Metabolism	4p15.1		
51	<i>CREG1</i>	Homo sapiens cellular repressor of E1A-stimulated genes 1 (CREG1), mRNA	NM_003851.2	Proliferation	1q24		20857207
52	<i>PELI1</i>	Homo sapiens pellino E3 ubiquitin protein ligase 1 (PELI1), mRNA	NM_020651.3	Proliferation	2p13.3		26131354
53	<i>SETD5</i>	Homo sapiens SET domain containing 5 (SETD5), mRNA	NM_001080517.1	Proliferation	3p25.3		
54	<i>HNRNPA1</i>	Homo sapiens heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), transcript variant 1, mRNA	NM_002136.2	RNA processing	12q13.1	2	
55	<i>HNRNPA2B1</i>	Homo sapiens heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), transcript variant A2, mRNA	NM_002137.3	RNA processing	7p15		25124043
56	<i>TPRKB</i>	Homo sapiens TP53RK binding protein (TPRKB), mRNA	NM_016058.2	RNA processing	2p24.3-p24.1		
57	<i>TRDMT1</i>	Homo sapiens tRNA aspartic acid methyltransferase 1 (TRDMT1), mRNA	NM_004412.5	RNA processing	10p15.1		9592134
58	<i>PRELID3B</i>	Homo sapiens PRELI domain containing 3B (PRELID3B), transcript variant 1, mRNA	NM_016045.2	Signal transduction	20q13.32		
59	<i>MRPS21</i>	Homo sapiens mitochondrial ribosomal protein S21 (MRPS21), transcript variant 2, mRNA	NM_018997.3	Transcription	1q21		
60	<i>PNRC2</i>	Homo sapiens proline-rich nuclear receptor coactivator 2 (PNRC2), mRNA	NM_017761.3	Transcription	1p36.11		
61	<i>POLR2D</i>	Homo sapiens polymerase (RNA) II (DNA directed) polypeptide D (POLR2D), mRNA	NM_004805.3	Transcription	2q21		
62	<i>SNHG16</i>	Homo sapiens small nucleolar RNA host gene 16 (non-protein coding) (SNHG16), transcript variant 1, long non-coding RNA	NR_038108.1	Non-coding RNA	ch. 17		
63	<i>FAM91A1</i>	Homo sapiens family with sequence similarity 91, member A1 (FAM91A1), mRNA	NM_144963.2	Unknown function	8q24.13		
64	<i>TEX9</i>	Homo sapiens testis expressed 9 (TEX9), transcript variant 1, mRNA	NM_198524.2	Unknown function	15q21.3		

Expression profiles of subtracted genes in human ES cells

We performed expression profile analysis of subtracted genes in human ES cells. Some subtracted genes have been reported previously, including, FXR1, NACA, TERF1, TMBIM6, WNK3, YWHAZ, PCDH15, PCDH18, PTPRF, STOM, AURKA, HSP91AA1, ORC3, SKIL, SMC3,

ACVR2A, CD9, CDH1, DPYSL2, FANCD2, PGK1, RB1CC1, SORL1, SPEN, TFRC, UGUG, USP9X, ZNF281, IFITM1, HSPD1, ACP1, CCT2, DEK, EEF1A1, GARS, JMJD1C, NECAB1, PDK1, PLOD2, SEPHS1, SLC2A3, CD24, GPC4, NAP1L1, NPM1, HNRNPA3, HSPA8, LARP7, DPPA4, EGR1, H2AFZ, and MALAT1. Instead of the previously reported genes, we used RT-PCR to analyze the expression profiles of thirty eight genes: FASTKD1, JADE1, TMA7, ASUN, HAUS1, YTHDF3, ARL61P1, IARS, NDC1, SLC39A10, AASS, ATP5C1, AZIN1, CECR2, COPZ1, G3BP1, GPN1, EIF4A2, LRBA, LYPLA1, MYL6, NDUFS5, OTUD4, PSMD2, RASSF3, RPL14, SERINC5, SLC44A1, SSR2, UBE2H, UBE2W, UGP2, SLC35F2, CPSF6, HNRNPA3, MATR3, DPY30, and ESRG (Fig. 3). According to RT-PCR data, human ES cells showed significantly higher expression level than NP cells derived from human ES cells for the following nineteen genes: FASTK1, JADE1, ASUN, ATP5C1, CECR2, COPZ1, EIF4A2, G3BP1, GPN1, MYL6, NDUFS5, OTUD4, PSMD2, SERINC5, SLC44A1, UGP2, CPSF6, MATR3, and DPY30. We re-analyzed expressed pattern of nineteen genes using quantitative RT-PCR. Specially, COPZ1, CPSF6, MATR3 were expressed human ES cell, not NP cells (Fig. 5A). These result suggest that three gene seem to be related proliferation or stemness of human ES cells.

Expression profiles of subtracted genes in NP cells derived from human ES cells

Furthermore, we performed expression profiling for subtracted genes of NP cells derived from ES cells. Similar to the subtracted genes of human ES cells, some subtracted genes have been reported in previous studies of neural development, including CASK, CCDC80, TUBA1A, CCNB2, CUL1, PSMD1, ADAM10, CD46, DAD1, DAZAP2, EIF4E, HMGC1, MEIS1, SOX4, SOX11, STMN1, TPM1, CHMP2B, ERCC1, HPRT1, PAICS, TRA2B, CREG1, PELI1, SETD5, HNRNPA1, HNRNPA2B1, TRDMT1 and PRELID3. Excluding those twenty-nine genes, we analyzed the expression levels of subtracted genes of NP cells derived from human ES cells during neural differentiation from human ES cells using RT-PCR, specifically CAPZA1, CCDC90B, SPTBN1, TMOD3, TRIQK, IST1, PSMA5, PSMD14, GGNBP2, ILDR2, NIPBL, PYGO1, TAF8, WDR19, ALDH18A1, CBR4, EXOSC8, GBE1, NDUFS2, NIPA2, OSTC, PSMB3, RPL24, RPLP0, RPLP1, SH3BGRL, TBC1D15, UGDH, TPRKB, MRPS21, PNRC2, POLR2D, SNHG16, FAM91A1, and TEX9 (Fig. 4). Notably, NP cells derived from human ES cells showed significantly lower expression than human ES cells for twenty subtracted genes: CAPZA1, CCDC90B, TMOD3, TRIQK, IST1, PSMD14, GGNBP2, NIPBL, WDR19, ALDH18A1, CBR4, GBE1, PSMB3, RPLP0, TBC1D15, UGDH, TPRKB, POLR2D, SNHG16 and FAM91A1. Likewise, we re-analyzed expression level of twelve genes using quantitative RT-PCR. In Fig. 5B, TMOD was not expressed human ES cells, but it was express NP cells. These result suggest that TMOD may play a role on development in NP cells derived from human ES cells.

Discussion

NP cells endogenously develop into neural lineages and are derived from NSCs (neural stem cells) in the spinal cord, retina or cortex (Conti and Cattaneo, 2010). Like other stem cells, NSCs are capable of both self-renewal and differentiation (Goldman, 1995). However, although NSCs are good cell source for regenerative medicine, there are only few NSCs to be used broadly (Conti and Cattaneo, 2010). Many researchers have produced NSCs in vitro using human ES cells, iPS cells or SCNT-ES cells, which can be applied in regenerative medicine (Tounson and McDonald, 2015). During the differentiation and neural development of NP cells, they adopt a specific morphology called the neural rosette. Neural rosette cells can differentiate into various cell types within the neural lineage, including neurons, astrocytes and oligodendrocytes (Gaspard and Vanderhaeghen, 2010).

In this study, we identified ninety downregulated genes using SSH during NP cell differentiation derived from ES cells. Functional analysis of subtracted genes in human ES cells showed that these genes were related to metabolism, development, apoptosis, cell division, RNA processing, proliferation, transcription, cell adhesion and the immune response. Metabolism-related genes play important roles in the self-renewal and differentiation of stem cells (Westermann, 2010). Human ES cells require energy metabolism, which depends on the regulation of glycolysis (Vander Heiden et al., 2009). Stimulation of glycolysis facilitates pluripotency and supports the maintenance of stemness (Kondoh et al., 2007). Aside from the previously reported genes, we used RT-PCR to analyze the expression profiles of thirty eight genes, FASTKD1, JADE1, TMA7, ASUN, HAUS1, YTHDF3, ARL61P1, IARS, NDC1, SLC39A10, AASS, ATP5C1, AZIN1, CECR2, COPZ1, G3BP1, GPN1, EIF4A2, LRBA, LYPLA1, MYL6, NDUFS5, OTUD4, PSMD2, RASSF3, RPL14, SERINC5, SLC44A1, SSR2, UBE2H, UBE2W, UGP2, SLC35F2, CPSF6, HNRNPA3, MATR3, DPY30, and ESRG. These genes have not previously been reported to be involved in ES cell maintenance. Importantly, 19 of these 38 subtracted genes showed specifically decreased expression patterns. Below, we describe these 19 subtracted genes. FASTKD1 is a member of the FASTKD family, which is characterized by a Fas-activated serine/threonine phosphoprotein domain. Members of the FASTKD family suppress FAS- or UV-induced apoptosis (Simarro et al., 2010). JADE1 has a PHD zinc finger, which is typically involved in cell proliferation and embryogenesis (Foy et al., 2008). ASUN, also called GCT1, plays critical roles in spermatogenesis and mitotic division (Jodoin et al., 2012). ATP5C1 synthesizes ATP from ADP in mitochondria and is composed of five subunits (Itoh et al., 2004). CECR2 is a chromatin-remodeling protein involved in neurogenesis and retinal development (Dawe et al., 2011). COPZ1 is one of the secretory vesicle coat proteins and is involved in cell proliferation, apoptosis and metabolism (Shtutman et al., 2011). EIF4A2 is highly expressed in skeletal muscle cells, and its expression level is related to the cell

survival rate in NSCLC (Shaoyan et al., 2013). G3BP1 has an mRNA-stabilizing effect and regulates cancer cells, including cell growth and mortality (Winslow et al., 2013). GPN1 is a GTPase characterized by a glycine/proline/asparagine motif (Mendez-Hernandez et al., 2014). MYL6 is myosin light chain type 6 and is involved in cytoskeletal organization (Goossens et al., 2007). NDUFS5 is a member of NADH dehydrogenase family, and mutation of NDUS5 induces mitochondrial disease (Iuso et al., 2006). OTUD4 regulates the resistance to alkylation damage in humans (Zhao et al., 2015). PSMD2 upregulates cell proliferation, and blocking PSMD2 signaling causes accumulation of cells in G1 (Matsuyama et al., 2011). SERINC5 is expressed in most eukaryote cells and underlies the incorporation of serine into phosphatidylserine and sphingolipids (Usami et al., 2015). SLC44A1 is related to the transfer of choline into the mitochondria (Michel and Bakovic, 2009). UGP2 is UDP-glucose pyrophosphorylase 2; it catalyzes glucose transfer and is important for carbohydrate metabolism (Fuhling et al., 2013). CPSF6 is regulated by the hTERT promoter and is strongly expressed in lung adenocarcinoma (Chen et al., 2014). MART3 is an inner nuclear matrix protein and contributes to various functions including chromatin organization; DNA replication, transcription, and repair; and RNA processing and transport (Salton et al., 2010). DPY30 shows highly increased expression levels in gastric cancer cells, in which it promotes proliferation and migration (Lee et al., 2015).

In this study, we also identified sixty-four subtracted genes from NP cells derived from human ES cells. Similarly, we analyzed the functional distribution of these genes. These genes are related to metabolism, development, cell adhesion, cell division, proliferation, RNA processing, and transcription. In addition, metabolism-related genes are belonged to multiple pathways, as in the results from ES cell subtraction. When ES cells differentiate, glycolysis dramatically decreases, but mitochondrial oxidative phosphorylation increases (Cho et al., 2006). Metabolic processes are important mechanisms for living cells (Cho et al., 2006; Kondoh et al., 2007; Shyh-Chang et al., 2013; Vander Heiden et al., 2009; Westermann, 2010). Neural differentiation from ES cells changed the expression of various genes, including Nestin and Pax-6 (Gaspard and Vanderhaeghen, 2010; Yap et al., 2015). In this study, sixty-four subtracted genes showed increased expression levels in NP cells derived from human ES cells. Setting aside the already known genes, we analyzed the thirty-five upregulated genes, CAPZA1, CCDC90B, SPTBN1, TMOD3, TRIQK, IST1, PSMA5, PSMD14, GGNBP2, ILDR2, NIPBL, PYGO1, TAF8, WDR19, ALDH18A1, CBR4, EXOSC8, GBE1, NDUFS2, NIPA2, OSTC, PSMB3, RPL24, RPLP0, RPLP1, SH3BGRL, TBC1D15, UGDH, TPRKB, MRPS21, PNRC2, POLR2D, SNHG16, FAM91A1, and TEX9, that were not previously associated with neural differentiation. Among them, twenty subtracted genes showed gradually increased expression during neural development. Below, we describe the subtracted genes showing higher expression in NP cells derived from ES cells than in human ES cells. CAPZA1 is upregulated in patients with gastric cancer and is a good marker for early stages of gastric cancer (Lee et al., 2013). CCDC90B is a mitochondrial integral membrane protein and is related to ES cell differentiation (Brandenberger et al., 2004). TMOD3 binds actin filaments, and the TMOD3-null mutant is embryonic lethal in mice (Sui et al., 2014). TRIQK is a membrane protein in the endoplasmic reticulum and is involved in regulating embryogenesis (Onuma et al., 2008). IST1 plays essential roles in cytokinesis during mammalian cell division (Agromayor et al., 2009). PSMD14 are two proteasomal subunits that contribute to the cleavage of ubiquitinated peptides. The lack of PSMD14 promotes cell cycle arrest and cellular senescence (Brandenberger et al., 2004). GGNBP2 is a tumor suppressor gene and is used for novel diagnosis and therapeutic applications in breast cancers (Lan et al., 2016). NIPBL is a cohesion-loading factor that is important in the G1/S transition (Zuin et al., 2014). WDR19 is overexpressed in prostate cancer and may be a new cancer biomarker (Lin et al., 2008). ALDH18A1 is mainly involved in an unusual metabolic pathway and encodes the protein P5CS, which is important for the first step in proline and ornithine biosynthesis (Bicknell et al., 2008). CBR4 belongs to the short-chain dehydrogenase/reductase superfamily, whose members play roles in steroid metabolism (Endo et al., 2008). GBE1 is a major factor in glycogen storage, and deficiency in this gene leads to genetic disorders such as glycogen storage disease type IV (Said et al., 2016). PSMB3 is another type of proteasomal subunit and is involved in genome stabilization (Gemoll et al., 2013). RPLP0 is a ribosomal protein that interacts with rRNA and is involved with translation in eukaryotic cells (Artero-Castro et al., 2011). TBC1D15 shows oncoprotein activity and promotes self-renewal in tumor-initiating stem-like cells (Feldman et al., 2013). UGDH shows strongly increased expression in breast cancer cells, in which it promotes cell proliferation (Hwang et al., 2008). TPRKB, also called CGI-121, potentially inhibits the binding of PRPK to p53 (Miyoshi et al., 2003). POLR2D is type of RNA polymerase II subunit that is related to transcription regulation (Khazak et al., 1998). SNHG16 affects lipid metabolism and has an oncogenic effect in colorectal cancer (Christensen et al., 2016). The functions of FAM91A1 are unknown.

Conclusions

In summary, we identified genes that are differentially expressed during neural differentiation from human ES cells. We used SSH to identify and characterize genes in NP cells showing either upregulated or downregulated expression in comparison to the human ES cells from which the NP cells were derived. Analysis of the subtracted genes showed that 90 genes were downregulated and 64 genes were upregulated in NP cells compared with human ES cells. Several genes (subtraction of hES cells; 52 genes, subtraction of NP cells; 29 genes) have already been reported in neural development from human ES cells. Aside from those known genes, the remaining subtracted genes might be related to neurogenesis. Many of the subtracted genes in this study were involved in metabolism (subtraction of human

ES cells; 37%, subtraction of NP cells; 30%) or development (subtraction of human ES cells; 18%, subtraction of NP cells; 26%). This study suggests that the subtracted genes (COPZ1, CPSF6, MART3 and TMOD3) will provide insight into their specific function in differentiation of NP cells from human ES cells.

Declarations

Author Contributions

JK Yoo and JK Kim designed the study. JK Yoo, HM Noh and SJ Choi differentiated NP and performed in vitro experiments. JK Yoo, HM Noh, JH Kim and JK Kim analyzed the data. SJ Choi created figures for the results. JK Yoo, SH Lim, SJ Choi and JK Kim wrote the manuscript with inputs from all the authors and reviewed the manuscript. All authors read and approved the final manuscript.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors

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Figures

Figure 1

Pie chart showing the categories into which the subtracted clones are distributed. (A) Subtracted clones in human ES cells. (B) Subtracted clones in NP cells derived from human ES cells.

Figure 2

Pie charts showing the functional classification of subtracted genes. The functions of the subtracted genes were determined using the NCBI database. (A) Subtracted genes in human ES cells (92 of the subtracted genes showed lower expression levels in NP cells derived from human ES cells than in human ES cells). (B) Subtracted genes in NP cells derived from human ES cells (64 of the subtracted genes showed higher expression levels in NP cells).

Figure 3

Expression profiles of subtracted genes from human ES cells. Subtracted genes of human ES cells showing downregulated expression during neural development compared with human ES cells. The expression profiles were obtained from three independent RT-PCR amplifications. Lane 1: human ES cells. Lane 2: NP cells derived from human ES cells.

Figure 4

Expression profiles of subtracted genes from NP cells derived from human ES cells. Subtracted genes of NP cells showed upregulated expression levels, and expression profile data were obtained from three independent experiments using RT-PCR. Lane 1: human ES cells. Lane 2: NP cells derived from human ES cells.

A

B

Figure 5

Re-analyzed expressing pattern of subtracted genes using quantitative RT-PCR. (A) Expression profiles of human ES cell specific genes (B) Expression profiles of NP cell specific genes. The data represent the average of three independent experiment and are shown as the mean $\pm$ S.E.M.; *P<0.05

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