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Identification of Genetic Networks during Mesenchymal Stem Cell Transformation into Neurons

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Abstract

The aim of this experiment is to identify related genes for human umbilical mesenchymal stem cells transformation into nervous cells. After the human umbilical mesenchymal stem cells were treated with neuronal conditioned medium (NCM) for 9 days, the gene expression groups are compared to those only treated with DMEM. The related genes for cell cycles, the human umbilical mesenchymal stem cells treated with DMEM increases the amount of cells that remain in the G2/M phase and S phase, including CAV1, EBF, NRG1, CDH13, MLH1. After treatment, the human umbilical cord mesenchymal stem cells with NCM for 9 days, gene expression related to the G_0/G_1 phase are also increased, including MYC, CSF3, PETN. Gene expressions related to neural regeneration and neural stem cells also increase significantly, such as CXCL1, BMP2, NRCAM, FGF2, SPG7. This study thereby provides a foundation for a more detailed understanding of HUMSCs neuronal differentiation.

Key Words: mesenchymal stem cell, neurogenesis, neuronal differentiation

Introduction

Human mesenchymal cells from the Wharton's jelly of the umbilical cord (HUMSCs) possess stem cell properties (5, 10, 21). HUMSCs are capable of differentiating into osteogenic, chondrogenic, adipogenic, and myogenic cells *in vitro* (21). We also found that the transformed HUMSCs in the striatum were still viable 4 months after transplantation without the need for immunological suppression, suggesting that HUMSCs might be a good stem cell source for transplantation (4).

We previously demonstrated that human umbilical mesenchymal stem cells (HUMSCs) could be induced to differentiate into neuron-like cells (about 87%) which could express neurofilaments, kainate receptor subunits and glutamate decarboxylase as well as generate an inward

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current in response to evocation by glutamate (5). In this study, we aimed to unmask the related gene changes during the transformation of HUMSCs into nerve cells by using gene microarray. Moreover, we aimed to analyze the global genetic network alternations during the neurogenesis of HUMSCs. Our data could provide new clues into a refined molecular pathway picture and a better understanding of the neuronal differentiation. Manipulating the expression of related genes will eventually benefit the clinical usages of HUMSCs.

Materials and Methods

Preparation of Human Umbilical Mesenchymal Stem Cells (HUMSCs)

Human umbilical cords were collected in HBSS (Gibco 14185-052, USA) at 4°C. Following disinfection in 75% ethanol for 30 sec, the umbilical cord vessels were cleared off while still in HBSS. The mesenchymal tissue (in Wharton's jelly) was then diced into cubes of about 0.5 cm^3 and centrifuged at 250 ×g for 5 min. Following the removal of the supernatant fraction, the precipitate (mesenchymal tissue) was washed with serum-free DMEM (Gibco 12100-046) and centrifuged at 250 ×g for 5 min. After the aspiration of the supernatant fraction, the precipitate (mesenchymal tissue) was treated with collagenase at 37°C for 18 hours, washed, and further digested with 2.5% trypsin (Gibco 15090-046) at 37°C for 30 min. Fetal bovine serum (FBS; Hyclone SH30071.03, USA) was then added to the mesenchymal tissue to neutralize the excess trypsin. The dissociated mesenchymal cells were further dispersed by treatment with 10% FBS-DMEM and counted under the microscope and with the aid of a hemocytometer. The mesenchymal cells were then used directly for cultures or stored in liquid nitrogen for later use.

Preparation of Neuronal Conditioned Medium (NCM)

Seven-day postnatal (P7) Sprague-Dawley rats were anaesthetized by intraperitoneal injection of over-dosed pentobarbital. The brain was removed, placed in Ca^{2+}/Mg^{2+} free buffer (Gibco 14185-052), and centrifuged at 900 rpm for 5 minutes. Following the removal of the supernatant fraction, 10% FBS-DMEM was added to the precipitate (brain tissue). The brain tissue suspension was triturated 15 times for dispersal into single cells. The cells were suspended in 10% FBS-DMEM and incubated at 37°C in 5% CO_2 and 95% O_2. To inhibit the growth of glial cells, 2 μM AraC (Sigma, C-6645) was added on the next day. On the 5th day of culture, the culture medium was removed (neuronal conditioned medium, NCM) to be used for the culture of umbilical mesenchymal cells. The HUMSCs were cultured in NCM alone, which was replaced every other day.

Microarray Hybridization

Human umbilical cord stromal cells that were cultured with 10% FBS DMEM for 9 days or with NCM for 9 days were collected, and total RNA were extracted. The extraction process mainly followed the manual of the RNAeasy® mini kit (cat. No. 74104, Qiagen, Germany). First, 600 μ1 RLT solution was added to 5 × 10^6 ~ 10^7 cells. A micro pipette or syringe to homogenize the cells, and then equal amount of 70% alcohol was added. The micropipette was homogenized again to ensure the solution to be even. Then it was put into an RNAeasy® mini kit spin column with a collection tube. All liquids within the collection tube was removed by high revolution centrifuge (8000 ×g, 30 sec). Add 700 μl RW1 (rinsing effect) to the RNAeasy® mini kit column and again remove the liquids by high revolution centrifuge (8000 ×g, 30 sec). DNase I working buffer (80 μl) was added and left to act under room temperature for 15 minutes (remove DNA). Add 700 μl RW1 and repeat high revolution centrifuge (8000 ×g, 30 sec). Afterwards, 500 μl RPE buffer (containing alcohol) was added and centrifuged at high revolution (8000 ×g, 30 sec) for a short time, then a 500 μl RPE buffer was added and centrifuged at high revolution (8000 ×g, 30 sec) for 2 minutes. In this step, the RPE BUFFER was completely removed; to prevent its influencing the dissolving ability of the RNA. Change the collection tube to a 1.5 ml microcentrifuge, add RNase free water. Wait for about one minute to allow the RNA to dissolve completely. Centrifuge again at high revolution (8000 ×g, 30 sec). The liquid thus extracted would contain high purity total RNA. After testing the concentration of the RNA, it was stared in a -80°C refrigerator. After QC, the extracted RNA was then sent to the National Yang-Ming University VGH-YM Genome Research Center for cRNA probe preparation and array hybridization. See related texts on core facilities for microarray and gene expression analysis at the Microarray Core, National Yang-Ming University Genome Research Center at http://www.ym.edu.tw/microarray. The chips used were Affymetrix Chips (Human genome U133 plus 2.0).

Array Probe Preparation, Data Analysis and Function Network Analyses

Data analyses were done as previously described (22). RMA log expression units were calculated from Affymetrix GeneChip array data using the 'affy' package of the Bioconductor (http://www.bioconductor.org) suite of software for the R statistical programming language (http://www.r-project.org). The default RMA settings were used as the background correction, normalization and summarization of all expression values. Significant difference between sample groups was identified using the method described by Storey

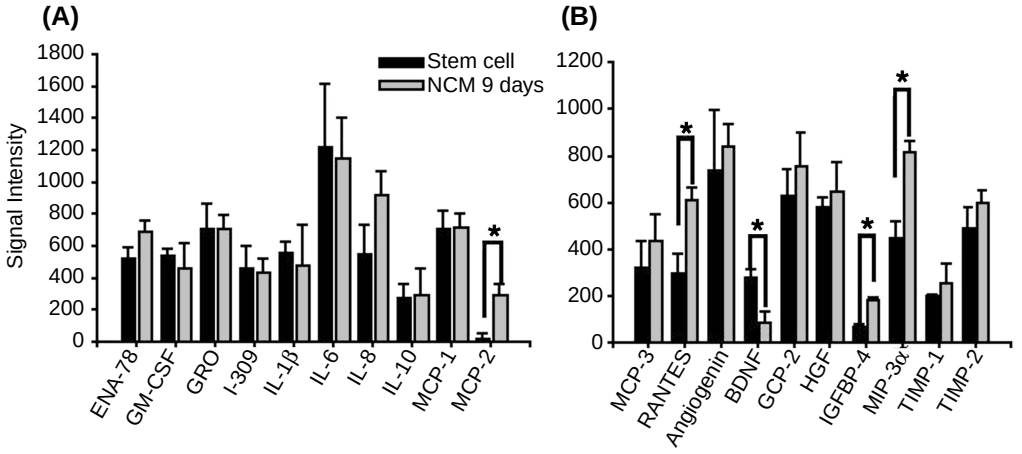

Fig. 1. Human cytokines expression during neurogenesis of HUMSCs. Seventy nine human cytokines were blotted onto a membrane and the intensities of the relative expression levels of cytokines secreted from different cells were quantified by densitometry. The expression of human MIP-3α (CCL20), ENA78 (CXCL5), and MCP2 (CCL8) in cells cultured in NCM were higher than those in DMEM. (*, $P < 0.05$).

& Tibshirani (22). Briefly, a *t*-statistic was calculated as normal for each gene and a *P*-value was then calculated using a modified permutation test (22). To control the multiple testing error, a false discovery rate (FDR) algorithm was then applied to these *P*-values to calculate a set of *q*-values: thresholds of the expected proportion of false positives, or false rejections of the null hypothesis. Heatmaps were created by the dChip software (www.dchip.org).

Gene annotation was performed by the ArrayFusion web tool (http://microarray.ym.edu.tw/tools/arrayfusion/). The Ingenuity Pathway Analysis (IPA) web tool developed by the Ingenuity Co. (http://www.ingenuity.com/) and the Pathway Studio pathways analysis software (http://www.ariadnegenomics.com/products/pathway-studio/) were used to construct functional regulatory networks of gene profiles. IPA uses the Ingenuity Pathways Knowledge Base to identify known interactions between signature genes and other genes which are not present in the gene list. A Fisher's exact test is used to calculate *P* values to determine the probability that the biological function assigned to a gene list is explained by chance alone. The significance/*P* value calculations are based on the hypergeometric distribution calculated *via* the right-tailed Fisher's exact test for 2×2 contingency tables. Right-tailed refers to the fact that IPA shows only over-represented functional/pathway annotations. Principal component analysis (PCA) plots were drawn by the Partek® Genomics Suit 6.2 software (www.partek.com).

Human Protein Cytokine Array

To find out which human cytokines might be involved in the transformation into neurons, a human protein cytokine kit (RayBio® Human Cytokine Antibody Array V Kit protein cytokine assay (H0108005) was used. The culture medium centrifuged in 1x cell lysis buffer at 1,500 ×g to remove cell debris. The harvested supernatant was used for assay of cytokines proteins using a human protein cytokine array kit. The membrane included in the human protein cytokine array kit were blocked with a blocking buffer, and then 1 ml of sample supernatant was individually added and incubated at room temperature for 2 h. The membranes were then analyzed according to the manufacturer's instructions.

Statistical Analyses

All data were presented as means ± standard error (SE). One-way or two-way ANOVA were used to compare all means, and least significance difference (LSD) was used for the posterior test. In all statistical analyses, $P < 0.05$ was considered significant.

Results

Change of Cytokine Patterns after Luman Umbilical Mesenchymal Stem Cell Treatment with Neuronal Conditioned Medium

Cell media from human umbilical mesencymal stem cell cultured in DMEM and neuronal conditioned medium were prepared and incubated with membranes containing an array of 79 human protein cytokine antibodies. Autoradiographs were scanned, and the density of each cytokine at the corresponding position was determined. The relative intensities of each cytokine were normalized to control spots on the same membrane. Human MIP-3α (CCL20), ENA78 (CXCL5), and MCP2 (CCL8) increase in cells incubated with NCM than those with the DMEM (Fig. 1, A and B). However, BDNF

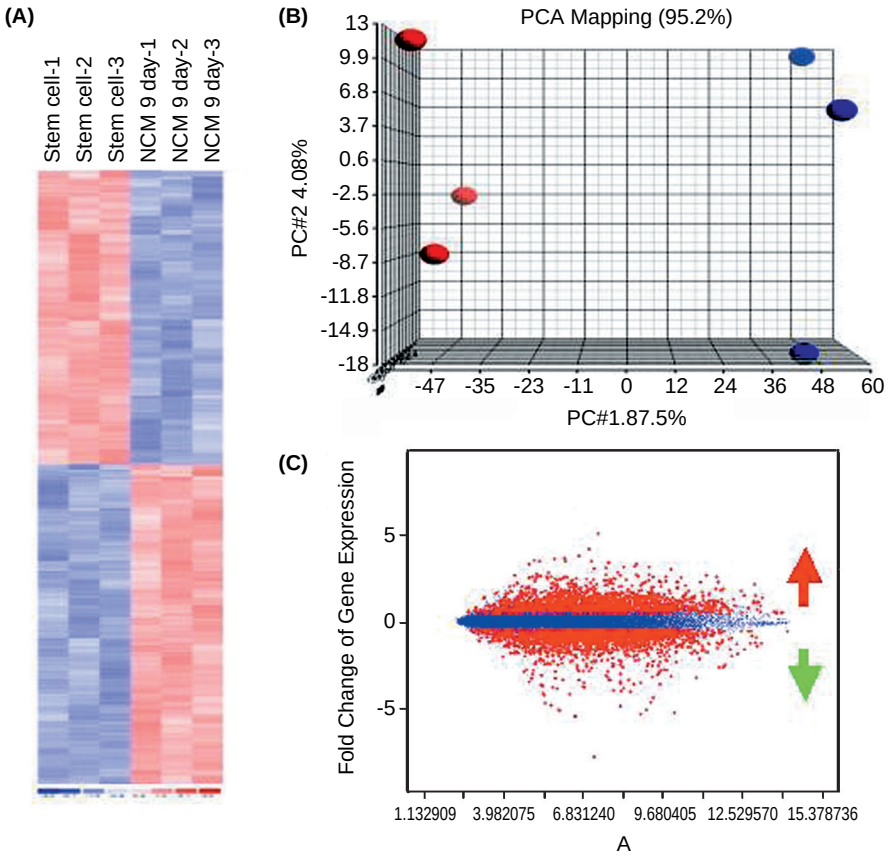

Fig. 2. Gene expression microarray analysis of umbilical MSCs cultured in NCM or DMEM. (A) A heatmap of human umbilical mesenchymal stem cells processed in 10% FBS DMEM, or NCM, for 9 days. The diagram shows the gene changes that are resulted from the two different cultivating conditions. Genes in red increased expression, in blue decreased expression. (B) A Principal Component Analysis (PCA) plot shows the differences for cell groups. All (~54,600) probe sets on the Affymetrix plus 2.0 chips were used for PCA analysis. Each spot represents a single array sample. (C) A MvA plot displays the log intensity ratio M versus the mean log intensity A of the microarray data of the human umbilical mesenchymal stem cells after processing with 10% FBS DMEM or NCM for 9 days.

concentration was lower in cells cultured in the NCM compared with those in the DMEM (Fig. 1B).

Genes Differentially Expressed in Luman Umbilical Mesenchymal Stem Cells Cultured in Neuron Condition Medium (NCM) for 9 Days

The human umbilical mesenchymal stem cells cultivated in 10% FBS DMEM for 9 days and those cultivated in NCM showed a great difference (Fig. 2, A and B). About 3462 genes were differentially expressed (false discovery rate (FDR), $P < 0.05$) before and after treatment with NCM (Fig. 2C).

Analyses of the human umbilical mesenchymal stem cells showed that the one hundred genes that have decreased the most, including CDKN2B, COL1A1, and CDH10 (Table 1). These genes are related to stem cells. Meanwhile, genes connected to cell adherence were also found, such as ITGA4 (CD49D), DCH10, PKP2, PCDH7, JUP, ADAM12, ADAM23, and CLDN11 (Table 1). Among these, ADAM12, ADAM23 and ITGA4 belong to the integrin mediate pathway. Organelle organisation and biogenesis genes include CPA4, NAP1L3, KRT8, EGF. Genes related to differentiation included PDLIM7, DCAMK1, CSPG4, MGP. Transcription genes on the other hand included NFE2L3, SSBP3, GATA6, BHLHB3. Protein metabolism genes included PCSK7, CPA4, ALPK2, SYT1, SKP2, SH3RF2, ST8SI4, AMARCH4, DCAMKL1, LOXL1, ADAM23, NAP1L3, SULF1, ADAM12, TUBA3, MYLK, and LMO7. Other genes related to cellular assembly and organisation include KRT8, KRT18, and TPM1 (Table 1). It was also found that when human umbilical mesenchymal stem cells are cultivated in 10% FBS DMEM, genes of the G2 phase of the cell cycle will be highly activated, such as NRG1, EGF, MLH1, and CDH13 (Table 4).

In the human umbilical mesenchymal stem cells that were processed in NCM for 9 days (*i.e.*, in neuronal differentiating cells), a large surge in signal transduction

Table 1. The first 100 cells that increased significantly after processing human umbilical mesenchymal stem cells with 10% FBS DMEM for 9 days, compared to those processed in NCM for 9 days. N = 3

Representative Public ID	Gene Symbol	Gene Title	Fold	*P*-values
NM_002521	NPPB	natriuretic peptide precursor B	33.21026	0.000991
NM_000916	OXTR	oxytocin receptor	17.82624	4.03E-05
NM_030786	SYNC1	syncoilin, intermediate filament 1 ///syncoilin, intermediate filament 1	13.75018	1.87E-05
AI422986	ST8SIA4	ST8alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	12.62167	6.15E-06
NM_022406	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	12.23241	0.000113
AI583530	RHOJ	ras homolog gene family, member J	11.40624	2.81E-06
NM_001963	EGF	epidermal growth factor (beta-urogastrone)	10.98877	0.000372
AI088063		CDNA FLJ44429 fis, clone UTERU2015653	9.884129	0.000345
AW444761	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	8.966246	0.000146
NM_005824	LRRC17	leucine rich repeat containing 17	8.785859	0.00202
AI743621	COL1A1	collagen, type I, alpha 1	8.715329	0.000379
NM_016352	CPA4	carboxypeptidase A4	8.40144	0.002912
AW242720	LOC143381	hypothetical protein LOC143381	8.158648	0.000241
AW264204	CLDN11	claudin 11 (oligodendrocyte transmembrane protein)	7.874965	2.17E-06
NM_000366	TPM1	tropomyosin 1 (alpha)	7.08331	0.000538
AI376433	KIAA1912	KIAA1912 protein	6.995371	0.000507
BE674466	ADAM23	ADAM metallopeptidase domain 23	6.614905	0.000132
BE551416	ALPK2	alpha-kinase 2	6.505015	0.00041
AI803088	C9orf94	chromosome 9 open reading frame 94	6.449632	0.000333
AA886870	ANKRD37	ankyrin repeat domain 37	6.428761	2.65E-05
NM_004338	C18orf1	chromosome 18 open reading frame 1	6.311975	0.000691
NM_000961	PTGIS	prostaglandin I2 (prostacyclin) synthase /// prostaglandin I2 (prostacyclin) synthase	6.259274	0.001067
AI129626	DCAMKL1	Doublecortin and CaM kinase-like 1	6.122112	0.00088
NM_021827	FLJ23514	hypothetical protein FLJ23514	6.069252	0.002915
NM_005613	RGS4	regulator of G-protein signalling 4	6.068264	0.000144
AW009747	SYNPO2	Synaptopodin 2	5.986467	5.96E-05
BC005935	NIPSNAP3A	nipsnap homolog 3A (C. elegans) /// nipsnap homolog 3A (C. elegans)	5.982805	2.95E-05
NM_002402	MEST	mesoderm specific transcript homolog (mouse)	5.955466	0.001232
AI250910	FLJ44635	TPT1-like protein	5.927919	8.35E-07
AI129628	SAMD3	sterile alpha motif domain containing 3	5.874851	0.000122
NM_005264	GFRA1	GDNF family receptor alpha 1	5.846506	0.000549
NM_001299	CNN1	calponin 1, basic, smooth muscle	5.833425	3.40E-05
AA100793	LMO7	LIM domain 7	5.814941	0.002967
BF344237	---	CDNA clone IMAGE: 4152983	5.808768	3.10E-05
NM_000224	KRT18	keratin 18	5.808549	1.28E-07
BF678830	LOC152485	Hypothetical protein LOC152485	5.807365	0.001822
BF726212	ANK2	ankyrin 2, neuronal	5.61541	7.30E-07
BF111214	ADAMTSL1	ADAMTS-like 1	5.601237	0.002843
NM_025239	PDCD1L	G2 programmed cell death 1 ligand 2	5.526444	0.000188
BE857703	CSPG4	Chondroitin sulfate proteoglycan 4 (melanoma-associated)	5.514886	2.12E-05
N66571	MRVI1	Murine retrovirus integration site 1 homolog	5.494207	2.87E-06
NM_006727	CDH10	cadherin 10, type 2 (T2-cadherin)	5.491877	0.000222
AV724192	KIAA0644	KIAA0644 gene product	5.478429	0.00154

Table 1. The first 100 cells that increased significantly after processing human umbilical mesenchymal stem cells with 10% FBS DMEM for 9 days, compared to those processed in NCM for 9 days. N = 3 (Continue)

Representative Public ID	Gene Symbol	Gene Title	Fold	*P*-values
BF221547	PDE5A	phosphodiesterase 5A, cGMP-specific	5.416482	3.64E-05
W46291	ADAM12	ADAM metallopeptidase domain 12 (meltrin alpha)	5.32363	0.002177
NM_002380	MATN2	matrilin 2	5.226645	0.000994
U89281	HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6	5.212976	0.002753
NM_000900	MGP	matrix Gla protein	5.175001	0.002334
M64571	MAP4	microtubule-associated protein 4	5.149331	0.001368
AW207243	---	CDNA FLJ38181 fis, clone FCBBF1000125	5.132558	0.00429
NM_000885	ITGA4 i	ntegrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	5.108575	0.00159
AI082237	PCSK7	Proprotein convertase subtilisin/kexin type 7	5.083663	0.000361
NM_004538	NAP1L3	nucleosome assembly protein 1-like 3	4.944568	0.000554
AF329092	DOC1	downregulated in ovarian cancer 1	4.741286	0.000267
AI475902	---	---	4.666336	2.46E-05
BG496998	FAM33A	family with sequence similarity 33, member A	4.640111	2.32E-05
AI807681	SH3RF2	SH3 domain containing ring finger 2	4.634953	2.92E-06
AL050069	DOK5	docking protein 5	4.612831	0.001566
AA166965	LOC440416	hypothetical gene supported by BC072410	4.557965	9.36E-07
AW206786	PDLIM7	PDZ and LIM domain 7 (enigma)	4.547026	1.49E-06
BF000162	FRMD4A	FERM domain containing 4A	4.532086	0.000593
AI659477	---	CDNA FLJ34873 fis, clone NT2NE2014950	4.49915	0.000503
BF112171	ODZ4	odz, odd Oz/ten-m homolog 4 (Drosophila)	4.458693	7.05E-07
AI088622	PRKCDBP	protein kinase C, delta binding protein	4.418909	0.001667
AA716165	JPH2	junctophilin 2	4.387901	1.97E-05
AI655611	EGFL3	EGF-like-domain, multiple 3	4.364839	0.000185
NM_001613	ACTA2	actin, alpha 2, smooth muscle, aorta	4.336284	0.000164
BC001875	MFI2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	4.322861	0.003879
NM_024589	FLJ22386	leucine zipper domain protein	4.223763	7.67E-05
BE857425	BHLHB3	basic helix-loop-helix domain containing, class B, 3	4.168584	1.90E-05
NM_000810	GABRA5	gamma-aminobutyric acid (GABA)A receptor, alpha 5	4.150093	9.95E-05
U76549	KRT8	keratin 8 /// keratin 8	4.149173	0.000213
AF329841	C1QTNF5	C1q and tumor necrosis factor related protein 5	4.13593	9.70E-05
NM_005576	LOXL1	lysyl oxidase-like 1	4.099149	0.000184
BF197655	CAV2	caveolin 2	4.011456	2.52E-05
AF169974	SRR	serine racemase	4.001308	8.95E-06
NM_021991	JUP	junction plakoglobin	3.994538	0.003012
AV752215	SRI	sorcin	3.985667	0.000155
AA831192	FAM36A	family with sequence similarity 36, member A	3.961881	1.96E-05
AA227879		Transcribed locus weakly similar to NP_055301.1 neuronal thread protein AD7c-NTP	3.926125	0.002576
NM_004572	PKP2	plakophilin 2	3.916326	8.55E-06
D87811	GATA6	GATA binding protein 6	3.906662	0.000157
NM_002589	PCDH7	BH-protocadherin (brain-heart)	3.89948	1.73E-05
AA284532	C9orf19	chromosome 9 open reading frame 19	3.841264	0.000422
AV731490	SYT1	synaptotagmin I	3.815522	0.001282
W74476	---	CDNA FLJ10196 fis, clone HEMBA1004776	3.794774	7.68E-05
U36501	SP100	nuclear antigen Sp100	3.756049	5.91E-05
AK000776	---	Full-length cDNA clone CS0DD009YB17 of Neuroblastoma Cot 50-normalized of Homo sapiens (human)	3.752761	1.12E-05

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Representative Public ID	Gene Symbol	Gene Title	Fold	*P*-values
AB037820	MARCH4,	membrane-associated ring finger 4 (C3HC4)	3.752328	0.000914
NM_005965	MYLK	myosin, light polypeptide kinase /// myosin, light polypeptide kinase	3.737045	0.00014
AF141347	TUBA3	tubulin, alpha 3	3.703982	3.72E-05
AI813331	DIAPH3	Diaphanous homolog 3 (Drosophila)	3.703811	5.59E-05
BF032500	C20orf133	chromosome 20 open reading frame 133	3.663786	0.003862
	SSBP3	single stranded DNA binding protein 3	3.627456	5.07E-06
AW043713	SULF1	sulfatase 1	3.615333	5.03E-06
BF002195	GALNT5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5) (GALNT5)	3.612044	5.16E-05
BG105365	SKP2	S-phase kinase-associated protein 2 (p45)	3.592571	1.91E-05
AJ227860	COTL1	Coactosin-like 1 (Dictyostelium)	3.562477	0.001803
AK026494	MBNL1	Muscleblind-like (Drosophila)	3.553716	0.000753
NM_003326	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	3.513587	0.002507

Table 2. The first 100 cells that increased significantly after processing human umbilical mesenchymal stem cells with NCM for 9 days, compared to those processed in 10% FBS DMEM for 9 days. N=3 (Continue)

Representative Public ID	Gene Symbol	Gene Title	Fold	*P*-values
NM_002612	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4	226.051	5.11E-11
NM_014059	RGC32	response gene to complement 32	45.3077	2.23E-06
M33376	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III) /// aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha h	41.6747	6.54E-09
NM_001353	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	38.2562	9.15E-09
AI492388	---	Transcribed locus	35.5617	2.10E-07
NM_003856	IL1RL1	interleukin 1 receptor-like 1	34.6923	1.07E-05
AW276078	LOC387763	hypothetical LOC387763	29.2309	6.51E-07
NM_001657	AREG	amphiregulin (schwannoma-derived growth factor)	29.1424	1.23E-07
NM_005525	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	21.5035	6.84E-07
NM_002425	MMP10	matrix metallopeptidase 10 (stromelysin 2)	19.7193	0.00037
NM_004049	BCL2A1	BCL2-related protein A1	18.3392	0.000155
AA594609	---	CDNA FLJ33809 fis, clone CTONG2001858	17.7061	9.14E-06
BF244402	---	---	16.5832	3.29E-05
NM_000759	CSF3	colony stimulating factor 3 (granulocyte)	16.2835	3.70E-06
U16996	DUSP5	dual specificity phosphatase 5	16.0019	0.00024
X62009	FBN2	fibrillin 2 (congenital contractural arachnodactyly)	15.0258	3.89E-05
NM_004591	CCL20	chemokine (C-C motif) ligand 20	14.6547	7.14E-05
BF589322	RSPO3	R-spondin 3 homolog (Xenopus laevis)	14.2313	1.14E-06

Table 2. The first 100 cells that increased significantly after processing human umbilical mesenchymal stem cells with NCM for 9 days, compared to those processed in 10% FBS DMEM for 9 days. N=3 (Continue)

Representative Public ID	Gene Symbol	Gene Title	Fold	*P*-values
AI146848	DPT	dermatopontin	14.0271	0.000175
NM_000591	CD14	CD14 antigen /// CD14 antigen	13.5489	0.000202
AI935096	NR4A2	nuclear receptor subfamily 4, group A, member 2	13.3606	1.05E-05
NM_006006	ZBTB16	zinc finger and BTB domain containing 16	12.9392	2.61E-07
AF069506	RASD1	RAS, dexamethasone-induced 1	12.4152	0.000173
NM_002090	CXCL3	chemokine (C-X-C motif) ligand 3	12.3666	0.000186
AI828007	HTRA3	HtrA serine peptidase 3	11.9611	9.59E-05
BF055343	GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	11.9079	4.48E-06
AL136653	C10orf10	chromosome 10 open reading frame 10	11.6925	5.44E-05
NM_004915	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	11.3818	3.12E-05
NM_015714	G0S2	G0/G1switch 2	11.1397	0.001857
AI633559	MAP3K4	Mitogen-activated protein kinase kinase kinase 4	10.5086	0.00111
BE326919	SAT	Spermidine/spermine N1-acetyltransferase	10.3723	6.79E-06
NM_002422	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	10.3204	0.000608
AA904430	WDR69	WD repeat domain 69	10.2738	1.23E-05
NM_001432	EREG	epiregulin	9.95637	0.000891
AW006123	FBXO32	F-box protein 32	9.14851	8.24E-06
AF169312	ANGPTL4	angiopoietin-like 4	9.12655	0.000764
M57731	CXCL2	chemokine (C-X-C motif) ligand 2	8.91301	5.20E-05
BG166705	CXCL5	chemokine (C-X-C motif) ligand 5	8.82223	0.003674
AL582836	PEG10	paternally expressed 10	8.54046	1.04E-05
BE856336	C8orf13	chromosome 8 open reading frame 13	8.18187	1.83E-05
NM_002424	MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	8.10755	0.004004
AW188198	TNFAIP6	tumor necrosis factor, alpha-induced protein	6 7.72443	3.12E-06
AW244016	---	Transcribed locus	7.53874	1.83E-05
NM_022136	SAMSN1	SAM domain, SH3 domain and nuclear localisation signals, 1	7.51087	0.003858
NM_014033	DKFZP586A0522	DKFZP586A0522 protein	7.29995	0.000994
AK023795	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	7.21924	9.04E-05
NM_003855	IL18R1	interleukin 18 receptor 1	7.0044	0.00011
NM_005010	NRCAM	neuronal cell adhesion molecule	6.93772	0.00164
NM_000641	IL11	interleukin 11	6.89887	3.57E-06
L47125	GPC3	glypican 3	6.54238	0.00191
BC020691	PBEF1	pre-B-cell colony enhancing factor 1	6.29809	3.26E-05
NM_001124	ADM	adrenomedullin	6.20956	7.88E-05
NM_030751	SNF1LK	SNF1-like kinase /// SNF1-like kinase	6.13944	1.51E-05
BF511276	AKAP12	A kinase (PRKA) anchor protein (gravin) 12	6.12595	0.001339
AL110191	TSC22D3	TSC22 domain family, member 3	5.94471	0.000296
NM_000240	MAOA	monoamine oxidase A	5.93972	4.93E-05
AW052044	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	5.91401	0.000122
NM_002450	MT1X	metallothionein 1X	5.89015	3.92E-05
NM_012417	PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1	5.78511	6.42E-06
AF043337	IL8	interleukin 8	5.73546	0.000918
NM_006981	NR4A3	nuclear receptor subfamily 4, group A, member 3	5.56549	6.36E-05
L10343	PI3	peptidase inhibitor 3, skin-derived (SKALP)	5.56101	0.000801
AL049369	DSCR1	Down syndrome critical region gene 1	5.55007	1.65E-06

Table 2. The first 100 cells that increased significantly after processing human umbilical mesenchymal stem cells with NCM for 9 days, compared to those processed in 10% FBS DMEM for 9 days. N=3 (Continue)

Representative Public ID	Gene Symbol	Gene Title	Fold	*P*-values
NM_025001	MTHFD2L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	5.52967	5.19E-07
AF212995	CUL4B	cullin 4B	5.36564	0.003256
AU154504	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	5.34137	0.002385
AL080169	DKFZP434C171	DKFZP434C171 protein	5.32299	0.000966
NM_003364	UPP1	uridine phosphorylase 1	5.11413	0.000155
NM_005923	MAP3K5	mitogen-activated protein kinase kinase kinase 5	5.1004	0.001596
AI990816	LAMA1	laminin, alpha 1	5.0851	0.002939
AI949549	FGD4	FYVE, RhoGEF and PH domain containing 4	5.04626	0.000833
NM_001874	CPM	carboxypeptidase M	5.03355	5.65E-05
Z83838	ARHGAP8 /// LOC553158	Rho GTPase activating protein 8 /// PRR5-ARHGAP8 fusion	4.98428	1.00E-05
NM_001674	ATF3	activating transcription factor 3	4.96849	0.002077
NM_014338	PISD	phosphatidylserine decarboxylase	4.91244	1.79E-07
H79306	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	4.90141	5.58E-05
BE672313	C8orf72	chromosome 8 open reading frame 72	4.86464	1.27E-05
AL832409	TGIF	TGFβ-induced factor (TALE family homeobox)	4.83789	0.000173
NM_000877	IL1R1	interleukin 1 receptor, type I	4.83742	0.000301
AI246590	IRAK2	interleukin-1 receptor-associated kinase 2	4.83435	8.26E-05
BF576710	PTP4A1	protein tyrosine phosphatase type IVA, member 1	4.82026	0.004278
NM_005692	ABCF2	ATP-binding cassette, sub-family F (GCN20), member 2	4.74367	4.38E-05
AI377389	CSNK1A1	Casein kinase 1, alpha 1	4.73916	0.002285
NM_000956	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	4.60412	0.001331
AL109935	KIAA1434	hypothetical protein KIAA1434	4.59872	0.000539
AA115106	NEGR1	neuronal growth regulator 1	4.5834	0.000153
AK022804	---	CDNA FLJ12742 fis, clone NT2RP2000644	4.44949	0.000169
U46768	STC1	stanniocalcin 1	4.41542	0.002651
AI949179	BCL2L11	BCL2-like 11 (apoptosis facilitator)	4.40892	0.000415
NM_002084	GPX3	glutathione peroxidase 3 (plasma)	4.32148	0.000535
AF277181	C1orf79	chromosome 1 open reading frame 79	4.28178	0.003525
NM_005228	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	4.16159	0.000121
NM_000167	GK	glycerol kinase	4.13127	0.002161
AI202201	JPH1	junctophilin 1	4.11734	0.003436
BF515913	FLJ35696	FLJ35696 protein	4.06442	0.000177
NM_015623	DKFZP564D166	putative ankyrin-repeat containing protein	4.05794	1.37E-05
NM_006820	IFI44L	interferon-induced protein 44-like	4.05121	0.000507
BC005127	ADFP	adipose differentiation-related protein	4.04142	0.000203
NM_001523	HAS1	hyaluronan synthase 1	4.025	0.000123
AA046439	SFRS1	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	-3.99321	4.36E-06

genes could be observed among the top 100 genes, which included MIP-3α, CSF3, CPM, ANGPTL4, IL11, IL8, CXCL3, ENA78, MMP3, MMP8, MMP10, NRCAM, FBN2, PBEF1, AKAP12, IL1RL1, and MAP3K4 (Table 2). Genes related to cell differentiation included NRCAM, ZBTB16, EREG, SNF1LK, CSF3, IL11 (Table 2). It was also discovered that after 9 days of cultivating human umbilical mesenchymal stem cells in NCM, genes of the G1 cell cycle phase were greatly increased, including CDKN1C, MXD1, MYC,

Table 3. Gene groups related to neural regeneration or nerve cells that showed changes after processing human umbilical mesenchymal stem cells with NCM for 9 days.

Representative Public ID	Gene Symbol	Gene Title	Fold	*p*-values
NM_005010	NRCAM	neuronal cell adhesion molecule	6.93772	0.00164
NM_001511	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	3.09683	0.000218
AA583044	BMP2	bone morphogenetic protein 2	2.87208	0.000875
AW074143	PBX1	Pre-B-cell leukemia transcription factor 1	2.76753	0.000805
M27968	FGF2	fibroblast growth factor 2 (basic)	2.42624	1.88E-05
AI571166	NR2C2	Nuclear receptor subfamily 2, group C, member 2	2.27279	2.20E-05
BE670386	SPG7	spastic paraplegia 7, paraplegin (pure and complicated autosomal recessive)	2.16897	0.000327
AK027184	FALZ	fetal Alzheimer antigen	1.94852	0.00139
NM_016339	RAPGEFL1	Rap guanine nucleotide exchange factor (GEF)-like 1	1.65914	0.003058
BF337329	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	1.57128	0.002071
BF059512	DNER	delta-notch-like EGF repeat-containing transmembrane	1.52761	0.00192
NM_000454	SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	-1.422014	0.001988
AA769818	CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	-1.956339	0.003885
AB028641	SOX11	SRY (sex determining region Y)-box 11	-2.294519	0.000179
AW051856	FLNA	filamin A, alpha (actin binding protein 280)	-2.296763	0.000459
M69148	MDK	midkine (neurite growth-promoting factor 2)	-2.328064	0.001184
NM_000399	EGR2	early growth response 2 (Krox-20 homolog, Drosophila)	-2.350717	0.001738
AL117593	FEZ1	fasciculation and elongation protein zeta 1 (zygin I)	-2.644674	4.17E-05
NM_018534	NRP2	Neuropilin 2	-2.660377	0.000739
AW956580	THBS1	Thrombospondin 1	-2.745862	0.004248
U62325	APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2 (Fe65-like)	-2.994027	2.32E-06
NM_000216	KAL1	Kallmann syndrome 1 sequence	-3.065674	4.96E-05
NM_000430	PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	-3.158756	7.16E-06
NM_013959	NRG1	neuregulin 1	-3.276965	0.001537
AK026494	MBNL1	Muscleblind-like (Drosophila)	-3.553716	0.000753
NM_001963	EGF	epidermal growth factor (beta-urogastrone)	-10.98877	0.000372

and PTEN. Genes related to the G0/G1 phase included CSF3, FOX03A, STAT1 (Table 4).

Further discovery showed that after processing the human umbilical mesenchymal stem cells with NCM for 9 days, the changed genes that were related to neural regeneration or nerve cell growth included NRCAM, CXCL1, BMP2, PBX1, FGF2, NR2C2, SPG7, FALZ, RAPGEFL1, NAB2, and DNER (Table 3).

After processing the umbilical mesenchmyal stem cells in NCM for 9 days, the changed genes were entered in Ingenuity 3.0, and groups with higher expression amount were selected according to their function. Function groups included gene expression, small molecule biochemistry, organismal injury and abnormalities, lipid metabolism, embryonic development, connective tissue disorders, protein synthesis, skeletal and muscular disorders, organ development, endocrine system disorders, inflammatory disease, and cardiovascular disease (Fig. 3). Details of the genes in each group are listed in Table 5.

Coordinated Changes in Function Networks during the Neuronal Differentiating of HUMSCs HUHU

To understand how the changed genes are related, we performed function network analyses for these

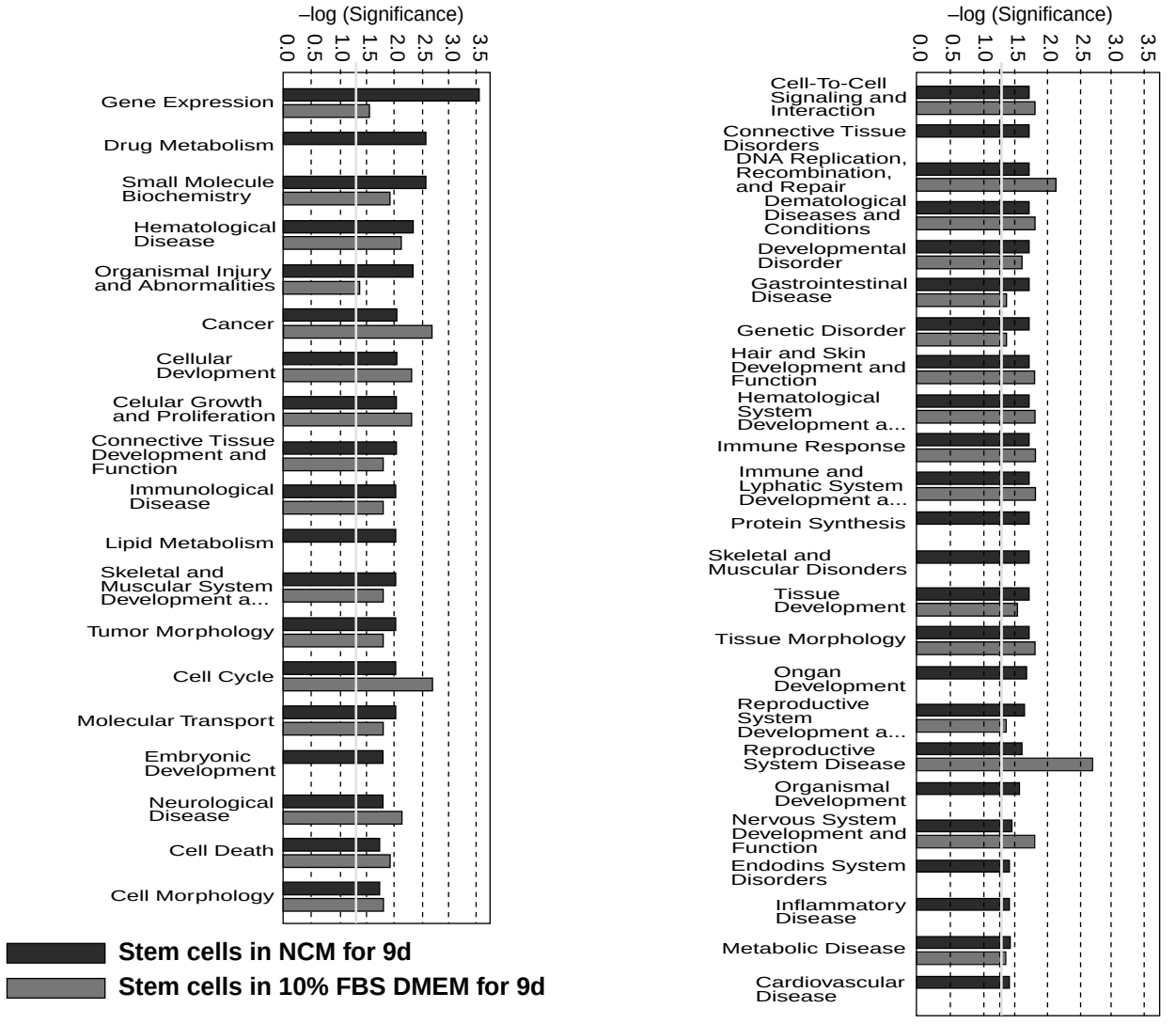

Fig. 3. Biological processes enriched in human umbilical mesenchymal stem cells or differentiated cells. Genes differentiating stem cells and differentiated cells were subjected into the Ingenuity 3.0 web tool for further functional grouping. Biological functions enriched in stem cells or neuronal differentiated cells are shown ($P < 0.05$ by a Fisher's exact test).

genes using the Pathway Studio network tool. When genes involved in neurogenesis (from the above IPA analysis) and cytokines (from cytokine array) were analyzed, a major network was identified (Fig. 4). Several transcription factors, such as SP1, MYC, EGR1 and CEBPB, were at the centre of the network (Fig. 4). These genes have also appeared repeatedly in the pathway networks mentioned above. SP1 connected with CXCL5, CCL20, ESRRA, ERCC3, IFRD1, SOD2, PTEN, FGF2, NRF1, CEBPB and TP53 (Fig. 4). Among these, CXCL5 and CCL20 are cytokines that increased after the NCM processing of the human umbilical mesenchymal stem cells. Genes that connect with MYC, on the other hand, include LIF, BMP2, CSF3, FGF2, MME, RAB9A, PIMI, DLAGL2, IER3, ERCC3, TP53, IFRD1, SOD2, PTEN, STAT5A, STAT5B, PBX1, DYRK1 and EGF2.

Discussion

In this study, we compared the relationships between genes that changed during MSC neurogenesis. We found several genes occupied important positions in the genetic network: SP1, EGR1, MYC, PPARA, FGF2, and IL8 (Fig. 4). It is possible for us to take the cytokines that showed differences from the cytokine array study and the genes that showed change from the gene expression microarray experiments, and then integrate them into genetic networks to find the key genes for neuronal differentiation. For cytokines released after processing the human umbilical mesenchymal stem cells in NCM for 9 days, macrophage inflammatory

Table 4. Genes related to cell cycles that showed changes after processing human umbilical mesenchymal stem cells with NCM for 9 days.

Representative Public ID	Gene Symbol	Gene Title	Cell cycle	Fold	*P*-values
NM_000759	CSF3	colony stimulating factor 3 (granulocyte)	G0/G1	16.28347	3.70E-06
AI242583	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	G1, BS	2.17703	0.002718
AW071793	MXD1	MAX dimerization protein 1	G1	2.172389	0.002246
AF329841	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	S	2.053832	0.000199
NM_007315	STAT1	signal transducer and activator of transcription 1, 91kDa	G0/G1	1.933238	0.000238
U96180	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	G1	1.919779	0.000353
NM_000076	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	G1	1.905643	0.003687
NM_020310	MNT	MAX binding protein	S	1.805798	0.003022
AA749262	CD320	CD320 antigen	Cell cycle progression	1.572136	0.001574
BE888885	FOXO3A	forkhead box O3A	G0/G1	1.511103	0.003005
NM_017902	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	Cell cycle progression	1.496032	0.000545
NM_006538	BCL2L11	BCL2-like 11 (apoptosis facilitator)	Cell cycle progression	1.376922	0.004033
NM_006807	CBX1	chromobox homolog 1 (HP1 beta homolog Drosophila)	Cell cycle progression	-1.37942	0.0038
NM_000389	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	G2/M, G2, G1	-1.50759	0.003781
NM_004517	ILK	integrin-linked kinase	Cell cycle progression	-1.658279	0.001272
NM_000249	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	G2	-1.6643	0.003648
BC004153	PCBP4	poly(rC) binding protein 4	G2/M	-1.93866	0.000215
AI471375	PRKCA	protein kinase C, alpha	Cell cycle progression	-1.95246	0.003485
AI560305	CDH13	Cadherin 13, H-cadherin (heart)	G2	-1.96606	0.00104
K03199	TP53	tumor protein p53 (Li-Fraumeni syndrome)	G2/M, G2, S, G1, Senescence	-2.66588	0.000332
NM_005572	LMNA	lamin A/C	Cell cycle progression	-2.80438	0.000209
NM_001753	CAV1	caveolin 1, caveolae protein, 22kDa	S	-3.18023	0.001642
NM_013959	NRG1	neuregulin 1	G2	-3.27697	0.001537
NM_001963	EGF	epidermal growth factor (beta-urogastrone)	G2	-10.9888	0.000372

protein 3 alpha (CCL20) (MIP-3α) and ENA78 (epithelial neutrophil-activating peptide-78) (CXCL5) are both modulated by SP1 (7, 8). Both of these cytokines showed significant differences in the statistics, and it can be assumed that SP1 plays an important role in differentiation. Discussions on genes related to cell differentiation generally refer to target genes under the mechanism of NMDA (19), NRSF (16), REST (3). In 2002, Okamoto S. and other scholars suggested that SP1 participates in nerve cell differentiation in the NR1 mechanism under NMDA, inducing downstream reaction (11). Other scholars have pointed out that SP1 participates in a special expression on the protein BM88 on neural progenitor cells, and work with the promoter on its gene, accelerating the differentiation of the neural progenitor cells (13). The diagrams show that SP1 modulated many genes. For example, it merged with EGR1, partaking in the differentiation of Dmrt1 in Sertoli cells (9). MYC

Table 5. Changed genes after the human umbilical mesenchymal stem cells were processed for 9 days using NCM, listed according to function.

Relevant Functions & Diseases	***P* value**
Gene Expression	2.89E-4 – 3.64E-2
expression	2.89E-4 – 8.25E-3
expression of endoplasmic reticulum stress element	2.89E-4
→ **ERN1, SP1, SP3, SP4, YY1**	
expression of Egr-1 binding site	2.77E-3
→ **CD44, FGF2, NAB2**	
expression of camp response element	8.25E-3
→ **ATF1, ATF3, CEBPB, DYRK1A, FGF2, SP1, SP3**	
activation	4.61E-3 – 1.98E-2
activation of PPAR response element	4.61E-3
→ **ABCC3, NFKBIA, PPARA, RXRA**	
activation of Egr-1 binding site	9.93E-3
→ **EGR1, SP1, SP3**	
activation of Hypoxia response element	1.70E-2
→ **EPAS1, HIAF1A, PIK3CA, PLAGL2**	
activation of heat shock element	1.98E-2
→ **FGF2, HSF1**	
activation of NRF-1 binding site	1.98E-2
→ **NRF1, PPRC1**	
activation of nuclear receptor response element-1	1.98E-2
→ **ESRRA, PPARA**	
activation of Nutrient-sensing response element	1.98E-2
→ **SP1, SP3**	
inhibition	9.93E-3 – 9.93E-3
inhibition of Sp1 binding site	9.93E-3
→ **BCL6, SP3, ZBTB16**	
transcription	9.93E-3 – 3.64E-2
transcription of STAT5 binding site	9.93E-3
→ **CENPJ, STAT5A, STAT5B**	
transcription of camp response element	1.21E-2
→ **ATF1, CEBPB, DYRK1A, SP1, SP3**	
transcription of Nutrient-sensing response element	1.98E-2
→ **SP1, SP3**	
initiation of transcription of gene	3.64E-2
→ **CDK7, ERCC3, FUBP1, MYC, YY1**	
Drug Metabolism	2.77E-3 – 2.77E-3
adhesion	
adhesion of hyaluronic acid	2.77E-3
→ **CD44, MIME, TNFAIP6**	
Protein Synthesis	1.98E-2 – 1.98E-2
translation	1.98E-2 – 1.98E-2
Translation of reporter protein	1.98E-2
→ **ERN1, NCK1**	
Organ development	2.22E-2
development	
Delay in development of mice	
→ **DYRK1A, NR4A3, POR, WASL**	

Table 5. Changed genes after the human umbilical mesenchymal stem cells were processed for 9 days using NCM, listed according to function. (Continue)

Inflammatory Disease	3.99E-3 – 4.04E-2
Dermatitis	3.99E-2 – 4.04E-2
Dermatis of skin	3.99E-2
→ **NFBIZ, NR4A1, RELB**	
Dermatis of organ	4.04E-3
→ **MMP3, NFKBIZ, NR4A1, RELB**	
Cardiovascular Disease	4.08E-2 – 4.08E-2
hypertrophy	4.08E-2 – 4.08E-2
Hypertrophy of heart cells	4.08E-2
→ **CTSC, CTSD, DUSP1, EPAS1, FBXO32, GPX3, IER3, IL11, LIF, MAP2K1, MAP2K7, MNT, NFKBIAPIM1, PTEN, SP3, TIMP1**	
Neurological Disease	1.70E-2 – 1.98E-2
G1 phase	1.70E-2 – 1.98E-2
G1 phase of brain cancer cell lines	1.70E-2
→ **CDKN1C, MXD1, MYC, PTEN**	
demyelination	1.98E-2
Demyelination of mice	
→ **CSF3, TNFRSF21**	
glioblastoma	1.98E-2 – 1.98E-2
glioblastoma	1.98E-2
→ **EGFR, PTEN**	
Endocrine System Disorder	3.99E-2 – 3.99E-2
hyperproliferation	3.99E-2 – 3.99E-2
Hyperproliferation of beta islet cells	3.99E-2
→ **IRS2, MEN1, MYC**	
Small Molecular Biochemistry	2.77E-3 – 4.85E-2
Transport	3.99E-2 –4.85E-2
Transport of oleic acid	3.99E-2
→ **ACSL1, ACSL4, NPC1**	
Transport of fatty acid	4.85E-2
→ **ACSL1, ACSL4, ADFP ACSL4, CPT1B, NPC1**	
removal	9.55E-3 – 9.55E-3
Removal of chelesterol	9.55E-3
→ **ABCG1, HSD11B11, NPC1, RAB9A**	
production	1.98E-2 –1.98E-2
Production of 5-hydroxyecicosatetraenoic acid	1.98E-2
→ **IL8, LTB4R**	
modification	1.83E-2 – 1.83E-2
Modification of phosphatidylinositol	1.83E-2
→ **PIK3CA, PIP5K3, PITPNA, PTEN, RAB5A**	

Table 5. Changed genes after the human umbilical mesenchymal stem cells were processed for 9 days using NCM, listed according to function. (Continue)

catabolism	1.98E-2 – 1.98E-2
Catabolism of spermine	
→ **SAT, SMDX**	
degradation	
Degradation of Heme	2.77E-3 – 2.77E-3
→ **HMOX1, POR**	
adhesion	
Adhesion of hyaluronic acid	9.93E-3 – 9.93E-3
→ **CD44, MME, TNFAIP6**	
accumulation	
Accumulation of fat	
→ **CEBPB, IFRD1, PPARA**	9.55E-3 – 4.85E-2
esterification	
Accumulation of fat	4.85-2 –4.85E-2
→ **CEBPB, IFRD1, PPARA**	
Lipid Metabolism	
removal	9.55E-3 – 9.55E-3
removal of cholesterol	9.55E-3
→ **ABCG1, HSD11B1, NPC1, RAB9A**	4.85E-2
accumulation	9.93E-3 –9.93E-3
accumulation of fat	9.93E-3
→ **CEBPB, IFRD1, PPARA**	
modification	1.83E-2 –1.83E-2
modification of phosphatidylinositol	1.83E-2
→ **PIK3CA, PIP5K3, PITPNA, PTEN, RAB5A**	
production	1.98E-2 –1.98E-2
production of 5-hydroxyeicosatetraenoic acid	1.98E-2
→ **IL8, LTB4R**	
adiposis	3.99E-2 – 3.99E-2
adiposis	3.99E-2
→ **LEPR, PPARA, SOD2**	
transport	3.99E-2 –4.85E-2
transport of oleic acid	3.99E-2
→ **ACSL1, ACSL4, NPC1**	
transport of fatty acid	4.85E-2
→ **ACSL1, ACSL4, ADFP, CPT1B, NPC1**	
esterification	4.85E-2 – 4.85E-2
esterification of cholesterol	4.85E-2
→ **ABCG1, HSD11B1, OSBPL2, PPARA, SOAT1**	
Embryonic Development	1.70E-2 – 2.22E-2
tumorigenesis	1.98E-2 – 1.98E-2
tumorigenesis of embryonic cell lines	1.98E-2
→ **CD44, EGFR**	
proliferation	1.70E-2 – 1.70E-2
proliferation of embryonic stem cells	1.70E-2
→ **HIAF1A, LIF, PTEN TIAL1**	
morphology	1.98E-2 – 1.98E-2
morphology of conotruncus	1.98E-2
→ **PDGFRA, RXRA**	
colony formation	2.22E-2 – 2.22E-2
colony formation of embryonic cells	2.22E-2
→ **LIF, MYC, PIGA**	

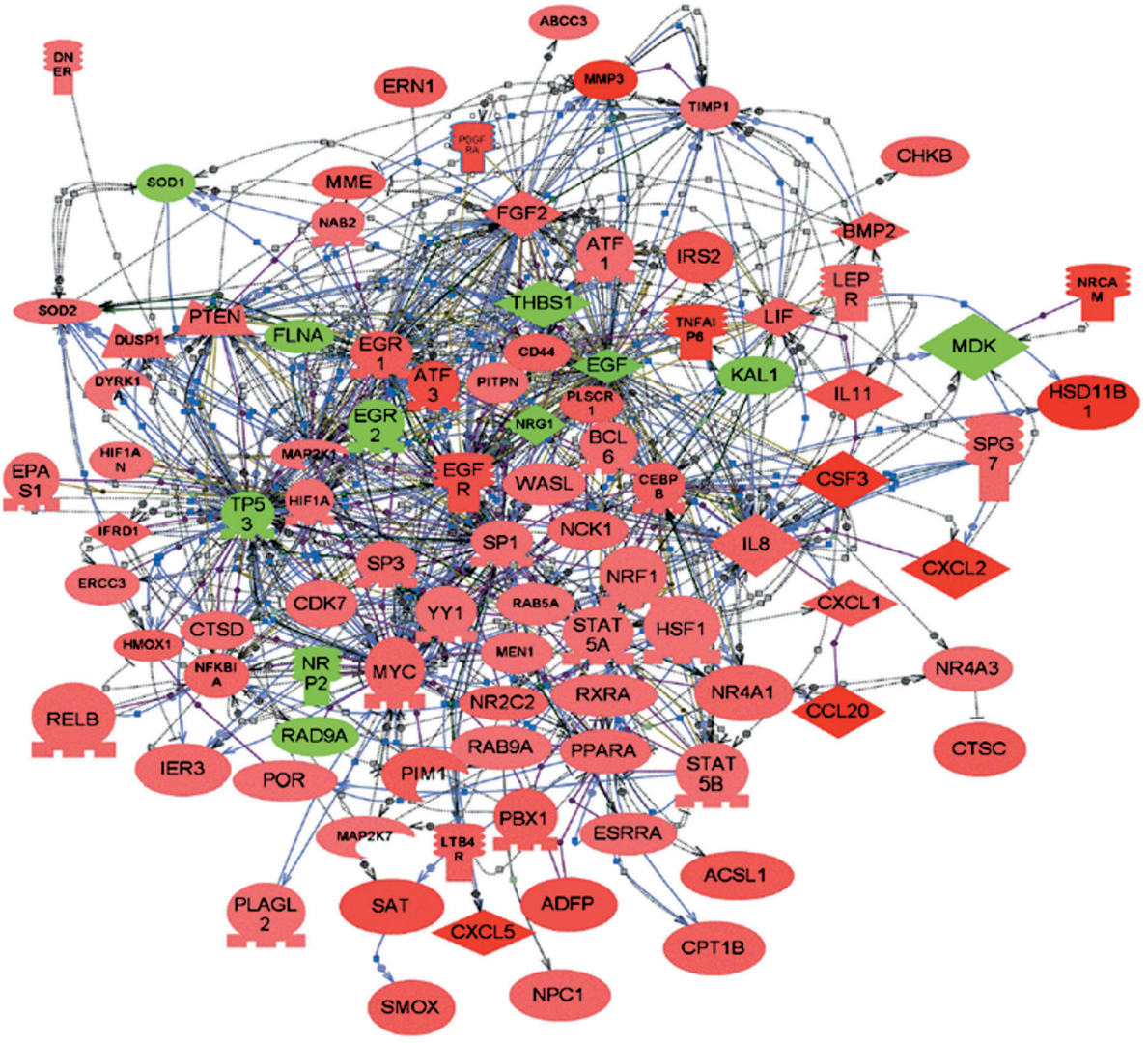

Fig. 4. Protein-protein interaction networks as a framework for the interpretation of neuronal differentiation. Genes (from gene expression microarray) and cytokines (from cytokine array) involved in neurogenesis were organized into a genetic network by the Pathway Studio software. These networks are displayed graphically as nodes (gene products) and edges (biological relationships between nodes). The intensity of the node color indicates the degree of up-regulation.

also interacted with SP1. SP1 will act together with MYC, causing a reaction in telomerase genes, giving stem cells the ability to differentiate endlessly (1).

Another important transcription factor is MYC. MYC has great influence over cell proliferation, cell cycle progression, differentiation, and cell survival (14). Research shows that mutations in MYC hamper the differentiation of neural stem/progenitor cells, in turn affecting cerebellar development (18). Other studies show that MYC is also crucial for the development of the neural crest (2).

When cultivating umbilical mesenchymal stem cells in 10% FBS DMEM, we could see that the key gene modulating the cell cycle is TP53. After differentiation, the expression of TP53 dropped. By merging with SP1, TP53 can suppress downstream reaction (12), and it can also suppress MYC action, thus controlling cell cycles (17). By suppressing the expression of FGF2 mRNA, TP53 can affect the growth of new blood veins on tumours (23), and the change in IL8 triggered when cells are damaged (6).

TP53 is involved in the G1 phase checkpoint in the cell cycle, and that function loss of TP53 might be related to the formation of cancer (15). Besides, TP53 is also related to the modulation of cell regeneration, apoptosis, and mitogenesis (20).

Therefore, we can conclude that the transcription factors TP53, MYC and SP1 seem to be connected in some way. Therefore, it is also possible to imagine the pathways through which the differentiation of nerve

cells will be taking after the human umbilical mesenchymal stem cells have been processed with NCM for 9 days.

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