Table 1 Primers for PCR for RT-PCR and cloning utilized in the present study

Genes	GenBank accession No.	Sense primers	Antisense primers	Application
NES	NM_006617	5′ctgctcaggagcagcactcttaac3′	5′cttagcctatgagatggagcaggc3′	Real-time RT-PCR
MSI1	NM_002442	5′caaagtgtctatctgggtgtgggc3′	5′acagctgaggcctgcaagcttaca3′	Real-time RT-PCR
GFAP	NM_002055	5′atgaggaggaaggagagaaggga3′	5′ccttccctttcctgtctgagtctc3′	Real-time RT-PCR
NFH	NM_021076	5′gagaaaggaacatccggaacagcc3′	5′tgggagtgccctctcttgctaaca3′	RT-PCR
MBP	NM_002385	5′gttccggaatcctgtcctcagctt3′	5′taactgttggccggaaattgccgg3′	RT-PCR
ID1	NM_002165	5′aattacgtgctctgtgggtctccc3′	5′gtctctggtgactagtaggtgtgc3′	Real-time RT-PCR
ID1	NM_002165	5′atcatggaagtcgccagtggcagc3′	5′tcagcgacacaagatgcgatcgtc3′	Cloning for luciferase assay
Myc-tagged ID1	NM_002165	5′cggaattccgaaagtcgccagtggcagcac3′	5′gaagatcttctcagcgacacaagatgcgat3′	Cloning for CoIP assay
ID3	NM_002167	5′aacttcgccctgcccacttgactt3′	5′cacctccacgctctgaaaagacct3′	Real-time RT-PCR
NPTX1	NM_002522	5′ttgtcctcatgcacacgaagcagc3′	5′acacgcacacacagatcctctcac3′	Real-time RT-PCR
FOS	NM_005252	5′gagctggtgcattacagagaggag3′	5′ggacttgagtccacacatggatgc3′	Real-time RT-PCR
DLL1	NM_005618	5′acgaatgctgctgaagaggaggga3′	5′aactgtccatagtgcaacggcgac3′	Real-time RT-PCR
MASH1	NM_004316	5′tgagtaaggtggagacactgcgct3′	5′tcagaaccagttggtgaagtcgagaag3′	Real-time RT-PCR
Flag-tagged MASH1	NM_002165	5′cggaattccgaaagctctgccaagatggag3′	5′cgggatcccgtcagaaccagttggtgaagt3′	Cloning for CoIP assay and luciferase assay
G3PDH	NM_002046	5′ccatgttcgtcatgggtgtgaacca3′	5′gccagtagaggcagggatgatgttc3′	Real-time RT-PCR
DLL promoter #1	AF222310	5′ggggtacccctcctgacactaggtggcaaga3′	5′gaagatcttcctcggcctccctccgcctggt3′	Cloning for luciferase assay
DLL promoter #2	AF222310	5′ccgctcgagcggaccccctcggctgccgccgcgg3′	5′gaagatcttccggacagcgcggcggcggcgac3′	Cloning for luciferase assay

NES nestin, *MSI1* musashi homolog 1, *GFAP* glial fibrillary acidic protein, *NFH* neurofilament heavy polypeptide, *MBP* myelin basic protein, *ID1* inhibitor of DNA binding 1, *ID3* inhibitor of DNA binding 3, *NPTX1* neuronal pentraxin 1, *FOS* cellular oncogene c-fos, *DLL1* delta-like 1, *MASH1* mammalian achaete scute homolog 1, *G3PDH* glyceraldehyde-3-phosphate dehydrogenase, *CoIP* coimmunoprecipitation. The underlined sequences represent restriction enzyme sites

Coimmunoprecipitation Analysis

The open-reading frame (ORF) of the human ID1 and MASH1 (ASCL1) genes were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and primer sets listed in Table 1. They were then cloned into the mammalian expression vector pCMV-Myc (Clontech, Mountain View, CA, USA) or p3XFLAG-CMV7.1 (Sigma) to express a fusion protein with an N-terminal Myc or Flag tag. At 48 h after co-transfection of the vectors in HEK293 cells by Lipofectamine 2000 reagent (Invitrogen), the cells were homogenized in M-PER lysis buffer (Pierce) supplemented with a cocktail of protease inhibitors (Sigma). After preclearance, the supernatant was incubated at 4°C for 3 h with rabbit polyclonal anti-Myc-conjugated agarose (Sigma), mouse monoclonal anti-Flag M2 affinity gel (Sigma), or the same amount of normal mouse or rabbit IgG-conjugated agarose (Santa Cruz Biotechnology). After several washes, the immunoprecipitates were processed for Western blot analysis using rabbit polyclonal anti-Myc antibody (Sigma) and mouse monoclonal anti-FLAG M2 antibody (Sigma).

Dual Luciferase Assay

The ORF of the human ID1 gene, amplified by PCR using PfuTurbo DNA polymerase and primer sets listed in Table 1, was cloned into the mammalian expression vector pEF6/V5-His TOPO (Invitrogen) by designing omission of V5 and His tags. The web search on Database of Transcriptional Start Sites (DBTSS; dbtss.hgc.jp) indicated that several E-box (CANNTG) sequences were clustered in the approximately 3,000 bp promoter region of the human DLL1 gene. Two non-overlapping regions of the DLL1 promoter, consisting of the region #1 spanning −1,253 and −254 containing two E-box sequences or the region #2 spanning −2,946 and −1,786 containing 10 E-box sequences, when the first amino acid of the initiation codon is defined as the position zero, were separately amplified by PCR using GC-RICH PCR system (Roche Diagnostics) and primer sets listed in Table 1. They were then cloned into the Firefly luciferase reporter vector pGL4.14-luc2-Hygro (Promega, Madison, WI, USA). The Renilla luciferase reporter vector pGL4.74-hRluc-TK (Promega) was used for an internal control that normalizes variability caused by differences in transfection efficacy. They were co-transfected in HEK293 cells, which were introduced with MASH1 and/or ID1 expression vectors at 36 h before transfection of the luciferase reporter vectors. At 16 h after transfection of the luciferase reporter vectors, cell lysate was processed for dual luciferase assay on a 20/20 Luminometer (Promega). All the assays were performed in triplicate.

Results

Human Neural Progenitor Cells (NPC) in Culture

Human NPC were capable of proliferating for several months by forming free floating or loosely attached growing spheres, when incubated in the NPC medium under the serum-free culture conditions (Fig. 1a). When human NPC spheres were incubated in the NPC medium supplemented with 10% FBS, they rapidly attached on the plastic surface, followed by vigorous outgrowth of a sheet of adherent cells from the attachment face (Fig. 1b). By RT-PCR analysis, NPC cells expressed the transcripts of nestin (NES), musashi homolog 1 (MSI1), and GFAP at high levels, whereas they displayed fairly low levels of NFH and MBP mRNA under culture conditions with or without inclusion of the serum (Fig. 1c, lanes 1–10).

When incubated in the serum-free NPC medium, the great majority of the cells forming the core of NPC spheres exhibited an intense immunoreactivity for nestin, and expressed less intensely immunoreactivity for GFAP (Fig. 2a). In contrast, when incubated in the 10% FBS-containing NPC medium, virtually all of adherent cells with a polygonal shape, growing out from the NPC spheres, expressed very strongly both GFAP and nestin immunoreactivities (Fig. 2b and d–f). None of the cells expressed the oligodendrocyte marker O4 or O1 in the serum-free and serum-containing culture conditions (data not shown). These results suggest that adherent cells growing from NPC spheres at the attachment face represent the cells that underwent astrocyte differentiation.

Upregulated Genes in Human NPC Following Exposure to the Serum

NPC spheres were harvested, replated on a non-coated plastic surface, and incubated further for 72 h in the NPC medium with (S+) or without (S−) inclusion of 10% FBS. Then, total cellular RNA was processed for microarray analysis. Exposure of NPC spheres to the serum elevated the levels of expression of 45 genes (Table 2). They include tropomodulin 1 (TMOD1), inhibitor of DNA binding 1 (ID1), connective tissue growth factor (CTGF), Kruppel-like factor 9 (KLF9), inhibitor of DNA binding 3 (ID3), fibroblast growth factor binding protein 2 (FGFBP2), zinc finger protein 436 (ZNF436), transforming growth factor alpha (TGFA), tumor protein D52 (TPD52), sulfatase 1 (SULF1), regulator of G-protein signaling 4 (RGS4), collectin sub-family member 12 (COLEC12), angiotensinogen (AGT), solute carrier family 16, member 9 (SLC16A9), meteorin (METRN), cathepsin H (CTSH), growth arrest and DNA-damage-inducible beta (GADD 45B), sterile alpha motif domain containing 11 (SAMD11),

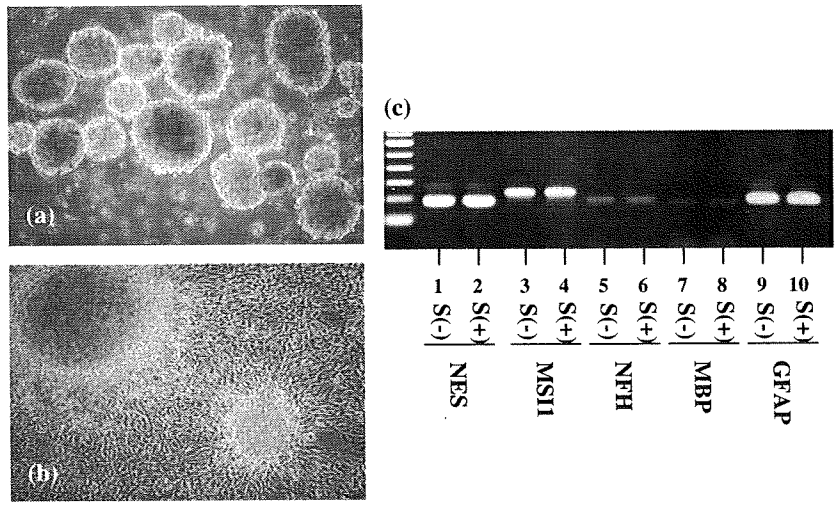

Fig. 1 Human neural progenitor cells (NPC) in culture. **a** Human NPC maintained under the serum-free culture conditions formed free floating growing spheres. **b** Human NPC spheres exposed to 10% FBS rapidly attached on the plastic surface, followed by vigorous outgrowth of a sheet of adherent cells from the attachment face. **a**, **b** Phase-contrast photomicrographs. **c** RT-PCR amplified for 32 cycles of nestin (NES, lanes 1 and 2), musashi homolog 1 (MSI1, lanes 3 and 4), neurofilament heavy polypeptide (NFH, lanes 4 and 6), myelin basic protein (MBP, lanes 7 and 8), and glial fibrillary acidic protein (GFAP, lanes 9 and 10) expressed in human NPC under the serum-free (S−) and the 10% FBS-containing (S+) culture conditions

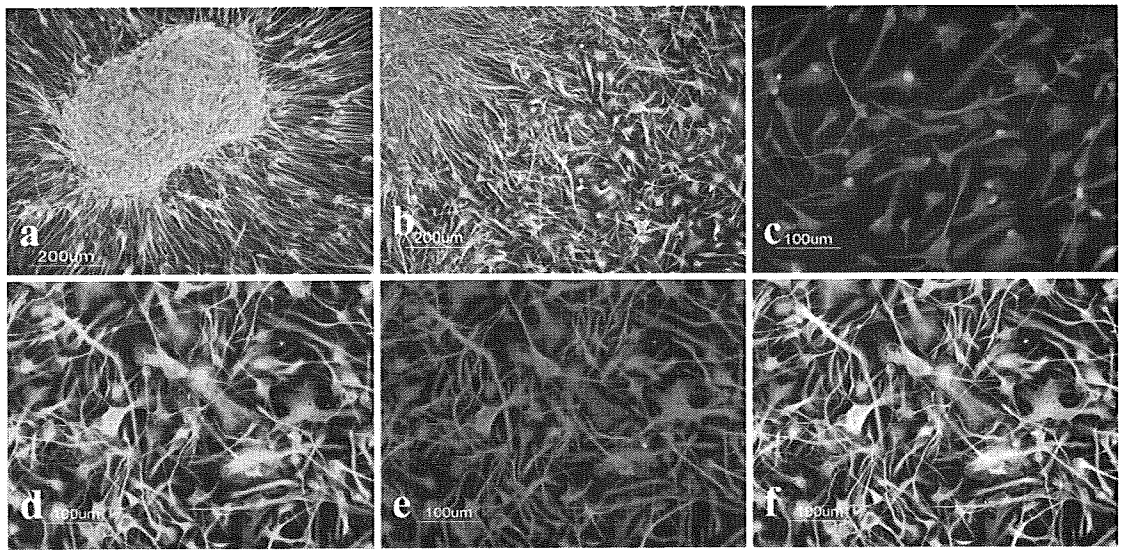

Fig. 2 Nestin, GFAP, and ID1 expression in human NPC in culture. Human NPC spheres attached on poly-L-lysine-coated cover glasses were incubated for 72 h in the NPC medium with (S+) or without (S−) inclusion of 10% FBS, and processed for double-labeling immunocytochemistry for nestin, GFAP, or ID1. **a** S−, NPC sphere, merge of nestin (green) and GFAP (red), **b** S+, vigorous outgrowth of adherent cells from the attachment face of the sphere, merge of nestin (green) and GFAP (red), **c** S+, outgrowth of adherent cells, merge of ID1 (green) and GFAP (red), **d** S+, outgrowth of adherent cells, nestin (green), **e** the same field as **d**, GFAP (red), and **f** merge of **d** and **e**

adenomatosis polyposis coli 2 (APC2), solute carrier family 2 member 5 (SLC2A5), GFAP, coiled-coil domain containing 103 (CCDC103), chromosome 9 open reading frame 58 (C9orf58), chitinase 3-like 2 (CHI3L2), complement factor I (CFI), chemokine C-X-C motif ligand 14 (CXCL14), annexin A1 (ANXA1), regulator of calcineurin 1 (RCAN1), retinal pigment epithelium-specific protein 65 kDa (RPE65), serine/threonine kinase 17a (STK17A), chromosome 4 open reading frame 30 (C4orf30), alpha B crystallin (CRYAB), transmembrane protein 132B (TMEM132B), frizzled homolog 1 (FZD1), inhibitor of DNA binding 2 (ID2), CDC42 effector protein 4

Table 2 Upregulated genes in human neuronal progenitor cells (NPC) following exposure to the serum

Rank	Gene symbol	Gene ID	Ratio	Gene name	Putative function
1	TMOD1	7111	13.05	Tropomodulin 1	A modulator of association between tropomyosin and the spectrin-actin complex
2	ID1	3397	9.00	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	A HLH protein that acts as a dominant negative regulator of bHLH family transcription factors
3	CTGF	1490	5.17	Connective tissue growth factor	A secreted mitogenic protein with insulin-like growth factor-binding capacity
4	KLF9	687	4.43	Kruppel-like factor 9	A transcription factor that binds to GC box elements
5	ID3	3399	4.08	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	A HLH protein that acts as a dominant negative regulator of bHLH family transcription factors
6	FGFBP2	83888	3.76	Fibroblast growth factor binding protein 2	A protein of unknown function secreted by T lymphocytes
7	ZNF436	80818	3.67	Zinc finger protein 436	A trancriptional factor that represses transcriptional activities of SRE and AP-1
8	TGFA	7039	3.60	Transforming growth factor, alpha	A growth factor that competes with EGF for binding to EGF receptor
9	TPD52	7163	3.35	Tumor protein D52	A coiled-coil domain bearing protein involved in calcium-mediated signal transduction and cell proliferation
10	SULF1	23213	3.23	Sulfatase 1	An endosulfatase that modulates signaling by heparin-binding growth factors
11	RGS4	5999	3.13	Regulator of G-protein signaling 4	A member of RGS family that deactivates G protein subunits of heterotrimeric G proteins
12	COLEC12	81035	2.93	Collectin sub-family member 12	A C-lectin family protein that acts as a scavenger receptor binding to carbohydrate antigens
13	AGT	183	2.90	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Angiotensinogen cleaved by renin to produce angiotensin I
14	SLC16A9	220963	2.82	Solute carrier family 16, member 9 (monocarboxylic acid transporter 9)	A monocarboxylic acid transporter
15	METRN	79006	2.79	Meteorin, glial cell differentiation regulator	A glial cell differentiation regulator
16	CTSH	1512	2.75	Cathepsin H	A lysosomal cysteine proteinase
17	GADD45B	4616	2.70	Growth arrest and DNA-damage-inducible, beta	An environmental stress-inducible protein that activates p38/JNK signaling
18	SAMD11	148398	2.69	Sterile alpha motif domain containing 11	A protein with a SAM motif of unknown function
19	APC2	10297	2.67	Adenomatosis polyposis coli 2	A negative regulator of Wnt signaling
20	SLC2A5	6518	2.63	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Glucose/fructose transporter GLUT5
21	GFAP	2670	2.62	Glial fibrillary acidic protein	An intermediate filament protein of astrocytes
22	CCDC103	388389	2.59	Coiled-coil domain containing 103	A coiled-coil domain bearing protein of unknown function
23	C9orf58	83543	2.55	Chromosome 9 open reading frame 58 (ionized calcium binding adapter molecule 2; IBA2)	A calcium binding protein of unknown function
24	CHI3L2	1117	2.52	Chitinase 3-like 2	A secreted chitinase-like protein of unknown function
25	CFI	3426	2.46	Complement factor I	A proteolytic enzyme that inactivates cell-bound, activated C3

Table 2 continued

Rank	Gene symbol	Gene ID	Ratio	Gene name	Putative function
26	CXCL14	9547	2.45	Chemokine (C-X-C motif) ligand 14	A chemoattractant for monocytes and dendritic cells
27	ANXA1	301	2.30	Annexin A1	An annexin family protein with phospholipase A2 inhibitory activity
28	RCAN1	1827	2.29	Regulator of calcineurin 1	A negative regulator of calcineurin signaling
29	RPE65	6121	2.24	Retinal pigment epithelium-specific protein 65 kDa	A protein abundant in retinal pigment epithlium cells involved in the 11-cis retinol synthesis
30	STK17A	9263	2.22	Serine/threonine kinase 17a (apoptosis-inducing)	DAP kinase-related apoptosis-inducing protein kinase DRAK1
31	C4orf30	54876	2.22	Chromosome 4 open reading frame 30 C4orf30	Hypothetical protein LOC27146
32	CRYAB	1410	2.21	Crystallin, alpha B	A small HSP family protein
33	TMEM132B	114795	2.11	Transmembrane protein 132B	A transmembrane protein of unknown function
34	FZD1	8321	2.10	Frizzled homolog 1	A fizzled gene family protein that acts as a receptor for Wnt
35	ID2	3398	2.10	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	A HLH protein that acts as a dominant negative regulator of bHLH family transcription factors
36	CDC42EP4	23580	2.09	CDC42 effector protein (Rho GTPase binding) 4	A CDC42-binding protein that interacts with Rho family GTPases
37	NCAN	1463	2.08	Neurocan	Chondroitin sulfate proteoglycan 3 involved in modulation of cell adhesion and migration
38	NAV2	89797	2.07	Neuron navigator 2	A helicase regulated by all-trans retinoic acid that plays a role in neuronal development
39	ENOX1	55068	2.06	Ecto-NOX disulfide-thiol exchanger 1	An enzymes with a hydroquinone (NADH) oxidase activity and a protein disulfide-thiol interchange activity
40	CLSTN2	64084	2.06	Calsyntenin 2	A postsynaptic membrane protein with Ca^{2+}-binding activity
41	NMB	4828	2.03	Neuromedin B	An amidated bombesin-like decapeptide
42	PCSK5	5125	2.02	Proprotein convertase subtilisin/kexin type 5	A member of the subtilisin-like proprotein convertase family
43	MAN1C1	57134	2.02	Mannosidase, alpha, class 1C, member 1	Alpha-1,2-mannosidase IC involved in N-glycan biosynthesis
44	GRAMD1C	54762	2.02	GRAM domain containing 1C	A protein with a GRAM motif of unknown function
45	VAT1	10493	2.01	Vesicle amine transport protein 1	An integral membrane protein of cholinergic synaptic vesicles involved in vesicular transport

Whole Human Genome Microarray (41,000 genes) was hybridized with Cy5-labeled cRNA of NPC incubated in the 10% FBS-containing culture medium and Cy3-labeled cRNA of NPC incubated in the serum-free culture medium. Upregulated genes in NPC by exposure to the serum are listed in order of greatness of the Cy5/Cy3 signal intensity ratio. The results of ID1, ID3, and GFAP (underlined) were validated by real-time RT-PCR analysis (see Fig. 3)

(CDC42EP4), neurocan (NCAN), neuron navigator 2 (NAV2), ecto-NOX disulfide-thiol exchanger 1 (ENOX1), calsyntenin 2 (CLSTN2), neuromedin B (NMB), proprotein convertase subtilisin/kexin type 5 (PCSK5), mannosidase alpha class 1C member 1 (MAN1C1), GRAM domain containing 1C (GRAMD1C), and vesicle amine transport protein 1 (VAT1).

It is worthy to note that three members of ID family genes, ID1, ID2, and ID3, were upregulated coordinately in the serum-treated NPC spheres. The ID family proteins that have an HLH domain but lack the DNA binding domain act as a dominant negative regulator of bHLH transcription factors (Ruzinova and Benezra 2003). Real-time RT-PCR and Western blot analysis validated marked upregulation of ID1, ID3, and GFAP in NPC following exposure to the serum (Fig. 3a–c, g, h). By immunocytochemistry, ID1 was located in the nucleus of GFAP-positive polygonal cells under the serum-containing culture condition

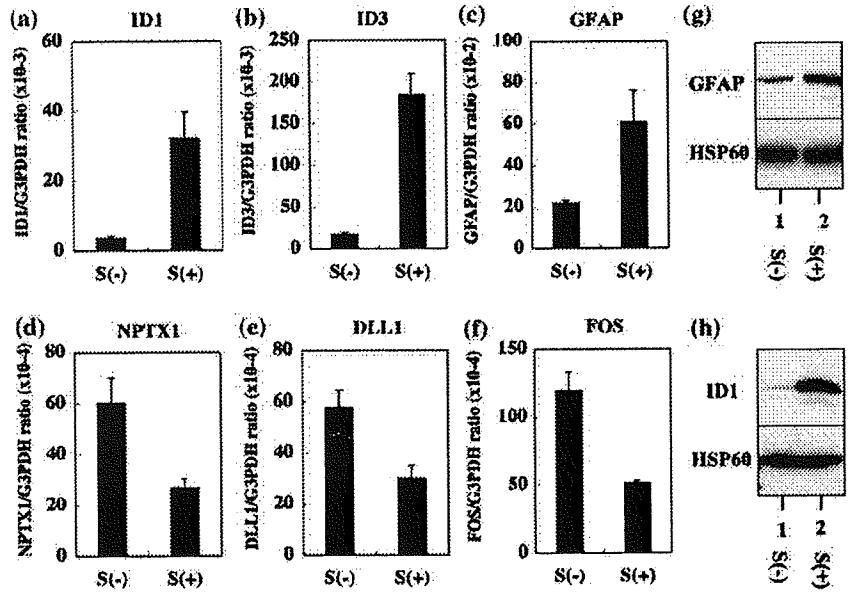

Fig. 3 Validation of microarray data by real-time RT-PCR and western blot analysis. Human NPC spheres were incubated for 72 h in the NPC medium with (S+) or without (S−) inclusion of 10% FBS, and then total cellular RNA or protein extract was processed for real-time RT-PCR and western blot analysis. a–f Real-time RT-PCR. The levels of target genes were standardized against the levels of the G3PDH gene. **a** ID1, **b** ID3, **c** GFAP, **d** NPTX1, **e** DLL1, and **f** FOS. **g**, **h** Western blot. The blots were reprobed with anti-HSP60 antibody to serve HSP60 for an internal control. **g** GFAP and **h** ID1

(Fig. 2c). Because GFAP is a defining marker of astrocytes, the results of microarray, RT-PCR, and Western blot verified that the serum promotes astrocyte differentiation of NPC.

Downregulated Genes in Human NPC Following Exposure to the Serum

Exposure of NPC to the serum reduced the levels of expression of 23 genes (Table 3). They include neuronal pentraxin I (NPTX1), cerebellin 4 (CBLN4), delta-like 1 (DLL1), cellular oncogene c-fos (FOS), SPARC related modular calcium binding 1 (SMOC1), matrilin 2 (MATN2), platelet-derived growth factor receptor alpha (PDGFRA), ryanodine receptor 3 (RYR3), transferrin receptor (TFRC), pleckstrin homology domain containing family H member 2 (PLEKHH2), delta-like 3 (DLL3), SRY-box 4 (SOX4), myosin VC (MYO5C), protocadherin 8 (PCDH8), ankyrin repeat domain 10 (ANKRD10), glutamate receptor ionotropic kainate 1 (GRIK1), chondroitin sulfate proteoglycan 4 (CSPG4), cystatin C (CST3), secreted frizzled-related protein 1 (SERP1), ryanodine receptor 1 (RYR1), growth arrest-specific 1 (GAS1), cystatin D (CST5), and hairy and enhancer of split 5 (HES5).

It is worthy to note that the list of downregulated genes included two Notch ligand Delta family members, DLL1 and DLL3, and a Notch effector HES5. It is well known that Notch signaling regulates cell fate specification and multipotency of NSC and NPC (Yoshimatsu et al. 2006). Real-time RT-PCR analysis validated substantial downregulation of NPTX1, DLL1, and FOS in the serum-treated NPC (Fig. 2d–f).

Functional Annotation of the Serum-Responsive Genes in Human NPC

To investigate the functional annotation of the serum-responsive genes in human NPC identified by microarray analysis, the list of Entrez Gene IDs of 45 serum-upregulated genes and 23 serum-downregulated genes was uploaded onto the DAVID database. Top 5 most significant biological processes relevant to the panel of these genes consisted of developmental process (GO:0032502; 32 genes; *P*-value = 2.0E-9), anatomical structure development (GO:0048856; 26 genes; *P*-value = 4.2E-9), multicellular organismal development (GO:0007275; 26 genes; *P*-value = 2.5E-8), system development (GO:0048 731; 20 genes; *P*-value = 2.2E–6), and anatomical structure morphogenesis (GO:0009653; 16 genes; *P*-value = 3.2E–6). The genes involved in the category GO:0032502 include the serum-upregulated genes such as IDI, ID2, ID3, CTGF, TGFA, METRN, KLF9, SULF1, AGT, GADD45B, ANXA1, RCAN1, RPE65, STK17A, CRYAB, FZD1, CDC42EP4, and VAT1, and the serum-downregulated genes such as DLL1, DLL3, HES5, NPTX1, FOS, PDGFRA, RYR1, RYR3, SOX4, PCDH8, GRIK1, CSPG4, SERP1, and GAS1. Thus, the genes whose expression levels were drastically changed in NPC by exposure to the serum are clustered in GO functional categories termed "development."

ID1 Acts as a Negative Regulator of DLL1 Expression

Since the serum-induced astrocyte differentiation of human NPC was followed by remarkable upregulation of ID1, ID2,

Table 3 Downregulated genes in human neuronal progenitor cells (NPC) following exposure to the serum

Rank	Gene symbol	Gene ID	Ratio	Gene name	Putative function
1	NPTX1	4884	0.26	Neuronal pentraxin I	A member of the neuronal pentraxin gene family involved in synaptic plasticity
2	CBLN4	140689	0.36	Cerebellin 4 precursor	A glycoprotein with sequence similarity to precerebellin
3	DLL1	28514	0.38	Delta-like 1	A Notch ligand involved in intercellular communication
4	FOS	2353	0.39	v-fos FBJ murine osteosarcoma viral oncogene homolog	A component of the AP-1 transcription factor complex
5	SMOC1	64093	0.41	SPARC related modular calcium binding 1	A secreted modular calcium-binding glycoprotein in basement membrane
6	MATN2	4147	0.43	Matrilin 2	A filament-forming protein widely distributed in extracellular matrices
7	PDGFRA	5156	0.44	Platelet-derived growth factor receptor, alpha polypeptide	A PDGF receptor component
8	RYR3	6263	0.44	Ryanodine receptor 3	An intracellular calcium release channel
9	TFRC	7037	0.44	Transferrin receptor (p90, CD71)	A gatekeeper for regulating iron
10	PLEKHH2	130271	0.45	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 2	A cytoskeletal protein involved in cell growth
11	DLL3	10683	0.46	Delta-like 3	A Notch ligand involved in intercellular communication
12	SOX4	6659	0.46	SRY (sex determining region Y)-box 4	A member of the SOX family transcription factor involved in the regulation of embryonic development
13	MYO5C	55930	0.46	Myosin VC	A myosin superfamily protein involved in transferrin trafficking
14	PCDH8	5100	0.47	Protocadherin 8	A member of the protocadherin gene family involved in cell adhesion
15	ANKRD10	55608	0.48	Ankyrin repeat domain 10	A protein with ankyrin repeats of unknown function
16	GRIK1	2897	0.48	Glutamate receptor, ionotropic, kainate 1	Ionotropic glutamate receptor subunit GluR5
17	CSPG4	1464	0.48	Chondroitin sulfate proteoglycan 4 (melanoma-associated; NG2)	Chondroitin sulfate proteoglycan that plays a role in stabilizing cell-substratum interaction
18	CST3	1471	0.48	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	An extracellular inhibitor of cycteine proteases
19	SFRP1	6422	0.49	Secreted frizzled-related protein 1	A soluble inhibitor for Wnt signaling
20	RYR1	6261	0.49	Ryanodine receptor 1 (skeletal)	A calcium release channel of the sarcoplasmic reticulum
21	GAS1	2619	0.49	Growth arrest-specific 1	A GPI-anchored protein expressed at growth arrest
22	CST5	1473	0.50	Cystatin D	An extracellular inhibitor of cysteine proteases
23	HES5	388585	0.50	Hairy and Enhancer of split 5 (Drosophila)	bHLH transcription factor downstream of Notch signaling

Whole Human Genome Microarray (41,000 genes) was hybridized with Cy5-labeled cRNA of NPC incubated in the 10% FBS-containing culture medium and Cy3-labeled cRNA of NPC incubated in the serum-free culture medium. Downregulated genes in NPC by exposure to the serum are listed in order of smallness of the Cy5/Cy3 signal intensity ratio. The results of NPTX1, DLL1, and FOS (underlined) were validated by real-time RT-PCR analysis (see Fig. 3)

and ID3, and concomitant downregulation of DLL1 and DLL3, we studied the possible inverse relationship between ID family and Delta family genes with respect to regulation of gene expression. First, by real-time RT-PCR, we determined the levels of ID1 and DLL1 expression in various human neural and non-neural cell lines. The levels of ID1 expression are high but those of DLL1 are very low in HMO6, and HeLa, HepG2, U-373MG, and SK-N-SH, whereas the levels of DLL1 expression are high but those of ID1 are much lower in NTera2 N and IMR-32 (Fig. 4a, b).

Next, we investigated the molecular network of ID1, ID2, ID3, DLL1, and DLL3 by KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database. The "N-points to N-points" search of KeyMolnet illustrated the shortest route connecting the start point molecules of ID1, ID2, and ID3 and the end point molecules DLL1 and DLL3 (Fig. 5). The pathway based on the molecules showed a significant relationship with canonical pathways of KeyMolnet library, such as transcriptional regulation by SMAD (P-value = 6.6E–12), transcriptional regulation by CREB (P-value = 7.8E–11), and Notch signaling pathway (P-value = 9.7E–9). Although no direct interaction was identified between ID family and Delta family genes,

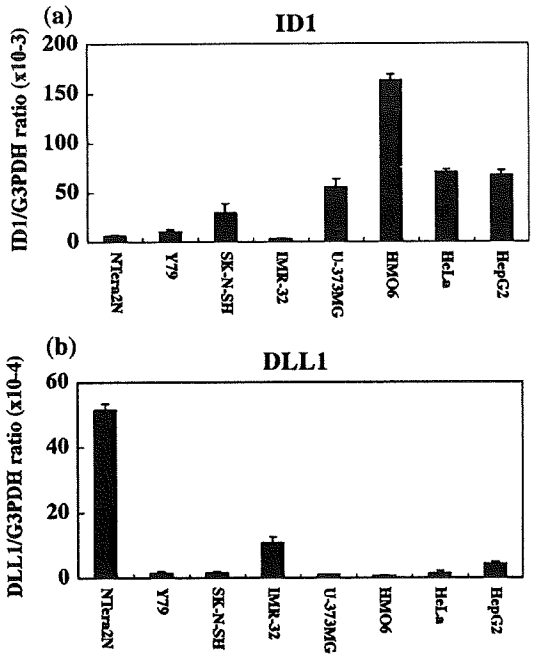

Fig. 4 ID1 and DLL1 expression in various human cell lines. Total RNA of human cell lines, such as NTera2 teratocarcinoma, Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrocytoma, HMO6 microglia, HeLa cervical carcinoma, and HepG2 hepatoblastoma was processed for real-time RT-PCR analysis. The levels of target genes were standardized against the levels of the G3PDH gene. **a** ID1 and **b** DLL1

KeyMolnet indicated two proneural bHLH genes, such as human achaete-scute homolog 1 (HASH1, also known as MASH1 or ASCL) and neurogenin 3 (NGN3, NEUROG3), both of which have an indirect connection with ID1, ID2 and ID3 via HES1, and a T-box gene family member TBX18 as principal regulators of DLL1 expression (Fig. 5). Because microarray analysis indicated that MASH1 is expressed in NPC spheres at much higher levels than NGN3 (data not shown), we confined our attention to a role of MASH1 in the counterbalance between ID and Delta family genes in regulation of gene expression.

Next, we studied the molecular interaction between ID1 and MASH1. By immunoprecipitation analysis of recombinant ID1 and MASH1 proteins coexpressed in HEK293 cells, we identified a direct interaction between ID1 and MASH1 (Fig. 6a, b, lane 2). Then, we cloned two non-overlapping sequences of the human DLL1 promoter containing several E-box sequences, consisting of the region #1 spanning −1,253 and −254 or the region #2 spanning −2,946 and −1,786, in the luciferase reporter vector. Dual luciferase assay indicated that both DLL1 promoter sequences were activated by the expression of MASH1, but this activation was suppressed by the coexpression of ID1 (Fig. 6c, d).

BMP4 Upregulates ID1 and GFAP Expression in Human NPC

Previous studies showed that the serum contains substantial amounts of BMP4 (Kodaira et al. 2006). Because the serum-induced astrocyte differentiation of human NPC was followed by robust upregulation of ID1, we studied the direct effect of BMP4 on expression of ID1 and GFAP in human NPC. When incubated under the serum-free NPC medium, a 72 h-treatment of NPC with 50 ng/ml BMP4 greatly elevated the levels of ID1 and GFAP mRNA expression, suggesting that BMP4 serves as a candidate for astrocyte-inducing factors included in the serum (Fig. 7a, b).

Discussion

We studied the effect of the serum on gene expression profile of cultured human NPC to identify the gene signature of the astrocyte differentiation of human NPC. Following exposure to the serum, human NPC spheres rapidly attached on the plastic surface, and subsequently, adherent cells were differentiated into astrocytes, accompanied by upregulation of GFAP expression, consistent with the previous studies on the rodent NSC and NPC (Chiang et al. 1996; Brunet et al. 2004). The serum elevated the levels of expression of 45 genes in human NPC, including three ID family members ID1, ID2, and ID3, all of which are direct target genes regulated by bone morphogenetic proteins (BMP) (Hollnagel et al. 1999). In contrast, the serum reduced the expression of 23 genes in human NPC, including three Delta-Notch signaling components DLL1, DLL3, and HES5. ID proteins act as a dominant negative regulator of bHLH transcription factors by binding to the ubiquitously expressed bHLH E proteins, such as E2A gene products E12 and E47, or by binding to the cell lineage-restricted bHLH transcription factors (Langlands et al. 1997; Nakashima et al. 2001). By *in silico* molecular network analysis of ID1, ID2, ID3, DLL1, and DLL3 on KeyMolnet, we identified MASH1 as one of important regulators of DLL1 expression. Furthermore, by coimmunoprecipitation analysis, we identified ID1 as a direct binding partner of MASH1. By luciferase assay, we found that activation of DLL1 promoter by MASH1 was counteracted by ID1. Finally, we found that BMP4 elevated the levels of ID1 and GFAP expression in NPC under the serum-free culture conditions. Because the serum contains substantial amounts of BMP4 (Kodaira et al. 2006), our observations raise the possible scenario that the serum factor(s), most probably BMP4, induces astrocyte differentiation by upregulating the expression of ID family genes that repress the proneural bHLH protein-mediated Delta expression in human NPC (Fig. 8).

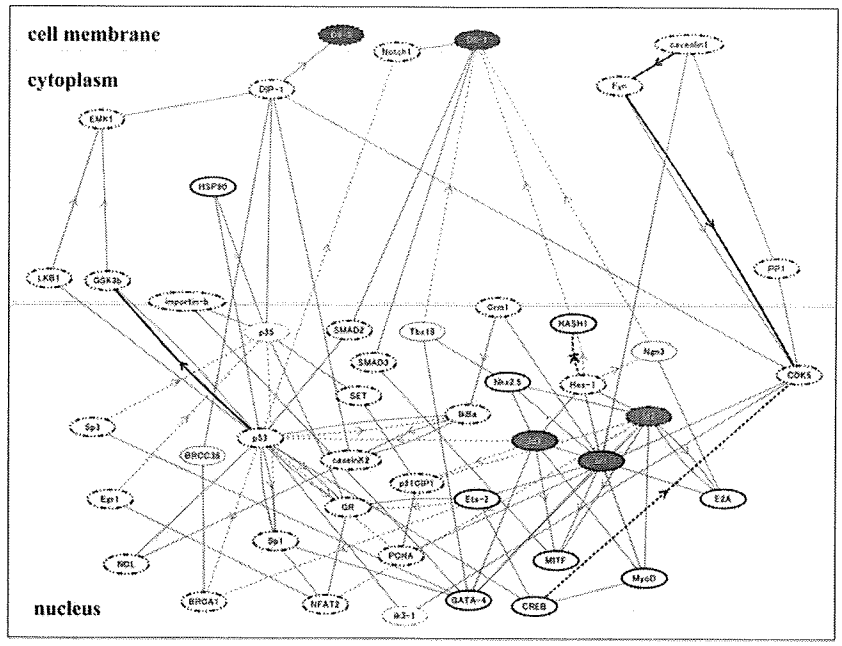

Fig. 5 Molecular network analysis of ID1, ID2, ID3, DLL1, and DLL3. KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database, identified the shortest route connecting the start point molecules of ID1, ID2, and ID3 (red) and the end point molecules DLL1 and DLL3 (blue). The pathway based on the molecules showed a significant relationship with transcriptional regulation by SMAD or CREB and Notch signaling pathway. The molecular network indicated HASH1 (MASH1), neurogenin 3 (NGN3), and TBX18 as principal regulators of DLL1 expression. The molecular relation is shown by solid line with arrow (direct binding or activation), solid line without arrow (complex formation), and dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). Thick lines indicate the core contents, while thin lines indicate the secondary contents of KeyMolnet

The Serum-Induced Astrocyte Differentiation of Human NPC is Characterized by a Counteraction of ID Family Genes on Delta Family Genes

We proposed the hypothesis that ID genes act as a key positive regulator of the serum-induced astrocyte differentiation of human NPC. The following previous observations support this view. The expression of four ID members is transiently elevated in immortalized mouse astrocyte precursor cells during astrocyte differentiation (Andres-Barquin et al. 1997). ID gene expression is rapidly induced in cultured rat astrocytes following stimulation with the serum (Tzeng and de Vellis 1997). Treatment of rodent NPC with BMP4 induces the expression of four ID genes, followed by induction of astrocyte differentiation, while the complex formation of ID4 or ID2 with bHLH proteins OLIG1 and OLIG2 blocks oligodendrocyte lineage commitment (Samanta and Kessler 2004).

ID proteins also act as a negative regulator of neuronal differentiation by preventing premature exit of neuroblasts from the cell cycle (Lyden et al. 1999). Retroviral vector-mediated overexpression of ID1 in the mouse brain in vivo inhibits neurogenesis but promotes astrocytogenesis (Cai et al. 2000). BMP2 induces the expression of ID1 and ID3, which inhibit the transcriptional activity of MASH1 and E47 complex on an E-box-containing promoter, suggesting that ID protein-mediated antagonism of proneural bHLH transcription factors plays a role in inhibition of neuronal differentiation (Nakashima et al. 2001). Combinatorial actions of proneural bHLH and inhibitory HLH factors regulate the timing of differentiation of NPC (Kageyama et al. 2005). ID1 binds not only to E proteins but also to myogenic bHLH transcription factors MYOD and MYF5 with high affinity (Langlands et al. 1997). We found that ID1 is a direct binding partner of neurogenic bHLH transcription factor MASH1. MASH1 deficient mice showed a severe loss of NPC in the subventricular zone of the medial ganglionic eminence, and MASH1, expressed in NPC, regulates neuronal differentiation by inducing the expression of Notch ligands DLL1 and DLL3, resulting in activation of Notch signaling in adjacent cells (Casarosa et al. 1999; Ito et al. 2000). Importantly, Mash1 directly activates the promoter of DLL1 gene (Castro et al. 2006). The activation of Delta-Notch signaling plays a key role in maintenance of NPC in the undifferentiated state (Yoshimatsu et al. 2006).

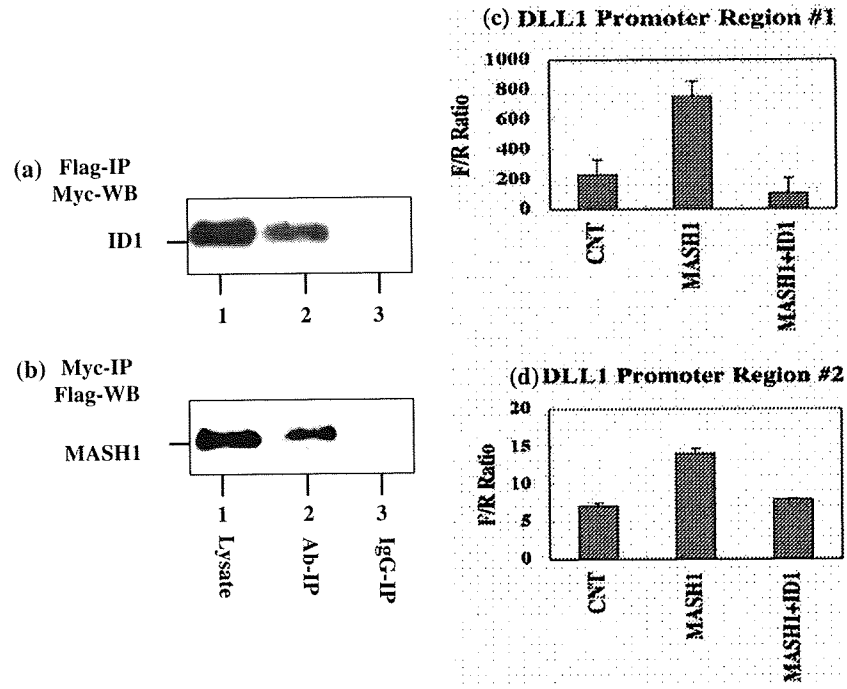

Fig. 6 Activation of the DLL1 promoter by MASH1 was counteracted by ID1. **a**, **b** Coimmunoprecipitation analysis. Recombinant MASH1 protein tagged with Flag and ID1 protein tagged with Myc were coexpressed in HEK293 cells. Immunoprecipitation (IP) followed by Western blotting (WB) was performed by using the antibodies against Flag and Myc. The lanes (1–3) represent (1) input control of cell lysate, (2) IP with anti-Flag or anti-Myc antibody, and (3) IP with normal mouse or rabbit IgG. **c**, **d** Dual luciferase assay. Two non-overlapping regions of the human DLL1 promoter, consisting of the region #1 spanning −1,253 and −254 or the region #2 spanning −2,946 and −1,786, were cloned into the Firefly luciferase reporter vector. It was co-transfected with the Renilla luciferase reporter vector (an internal control) in HEK293 cells, which were introduced with none (CNT), MASH1, or both MASH1 and ID1 expression vectors at 36 h before transfection of the luciferase reporter vectors. At 16 h after transfection of the luciferase reporter vectors, cell lysate was processed for dual luciferase assay. The ratio of Firefly (F)/Renilla (R) luminescence (RLU) is indicated

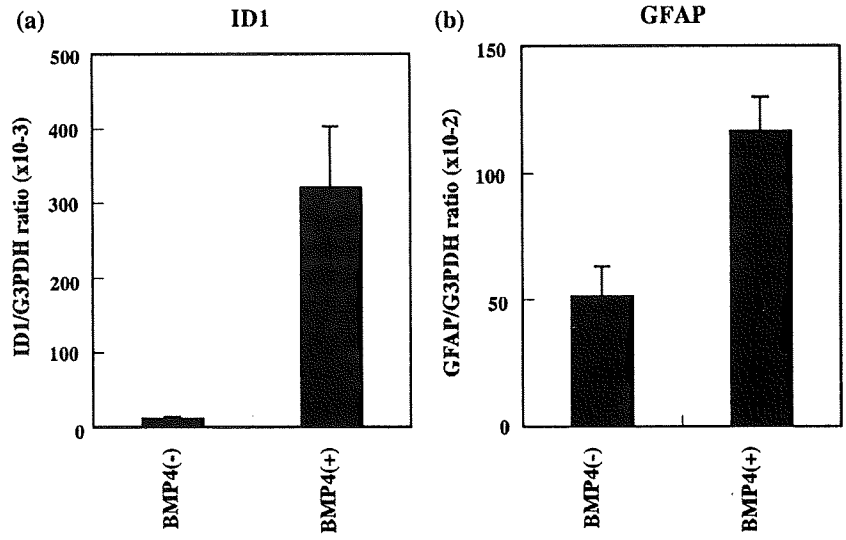

Fig. 7 BMP4 upregulates ID1 and GFAP expression in human NPC. Human NPC were incubated for 72 h in the NPC medium with (+) or without (−) inclusion of 50 ng/ml recombinant human BMP4, and then total cellular RNA was processed for real-time RT-PCR analysis. The levels of target genes were standardized against the levels of the G3PDH gene. **a** ID1 and **b** GFAP

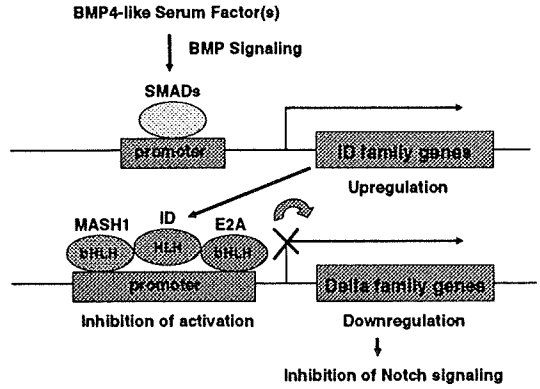

Fig. 8 The serum-induced astrocyte differentiation of human NPC is characterized by a counteraction between ID and Delta family genes. The present observations raise the possible scenario that the serum factor(s), most probably BMP4, induces astrocyte differentiation by upregulating the expression of ID family genes that repress the proneural bHLH protein, probably MASH1-mediated Delta expression in human NPC

The Serum-Induced Astrocyte Differentiation of Human NPC is Accompanied by Upregulation of Astrocyte Function-Related Genes

The serum-induced astrocyte differentiation of human NPC elevated the expression of astrocyte function-related genes (Table 2). Astrocytes express angiotensinogen (AGT) that plays a role in maintenance of the blood–brain barrier (BBB) function (Kakinuma et al. 1998). Astrocytes synthesize cathepsin H (CTSH) that acts as a metabolizing enzyme for neuropeptides and bradykinin (Brguljan et al. 2003). Human astrocytes in culture express complement factor I (CFI) essential for regulating the complement cascade (Gordon et al. 1992). Neuronal and glial progenitor cells secrete meterorin (METRN) that stimulates astrocyte differentiation in culture (Nishino et al. 2004). Calcineurin-dependent calcium signals induce the expression of regulator of calcineurin 1 (RCAN1) in astrocytes, an endogenous calcineurin inhibitor (Canellada et al. 2008).

Reactive astrocytes express connective tissue growth factor (CTGF), a TGF-β1 downstream mediator, involved in glial scar formation (Schwab et al. 2000). Reactive astrocytes express EGFR in response to various insults, and produce transforming growth factor alpha (TGFA) that triggers astrogliosis (Rabchevsky et al. 1998). Reactive astrocytes in Alzheimer disease brains express collectin sub-family member 12 (COLEC12), a member of the scavenger receptor family, which plays a role in amyloid-β clearance (Nakamura et al. 2006). Reactive astrocytes in multiple sclerosis brains express annexin A1 (ANXA1), a calcium-dependent phospholipid-binding protein that acts as an anti-inflammatory mediator (Probst-Cousin et al. 2002). At the site of spinal cord injury, reactive astrocytes produce neurocan (NCAN), a member of the CSPG family, which inhibits axonal regeneration (Jones et al. 2003).

Several serum-responsive genes have implications in astrocyte oncogenesis. FGF binding protein 2 (FGFBP2) is overexpressed in astrocytic tumors (Yamanaka et al. 2006). The expression of regulator of G-protein signaling 4 (RGS4), a negative regulator of G-protein signaling, is elevated in astrocytic tumor cells with a highly migratory capacity (Tatenhorst et al. 2004). Both chitinase 3-like 2 (CHI3L2) and neuromedin B (NMB) are identified as an astrocytoma-associated gene by serial analysis of gene expression (SAGE) profiles (Boon et al. 2004).

The Serum-Induced Astrocyte Differentiation of Human NPC is Accompanied by Downregulation of NPC and Neuronal Function-Related Genes

The serum-induced astrocyte differentiation of human NPC reduced the expression of NPC and neuronal function-related genes (Table 3). Neuronal pentaraxin I (NPTX1) plays a key role in activity-dependent plasticity of excitatory synapses (Xu et al. 2003). Protocadherin 8 (PCDH8) is a neuronal activity-regulated cadherin involved in long-term potentiation in the hippocampus (Yamagata et al. 1999). Spinal cord motor neurons express the ionotropic kainite receptor subunit GRIK1 (GluR5) (Eubanks et al. 1993). Ryanodine receptors RyR1, RyR2, and RyR3 are intracellular calcium release channels expressed in subpopulations of neurons in the human CNS (Martin et al. 1998).

NPC expressing the PDGF α-receptor (PDGFRA) proliferate in response to PDGF-AA associated with induction of c-fos (FOS) expression (Erlandsson et al. 2001). NPC express the transferrin receptor (TFRC, CD71) (Sergent-Tanguy et al. 2006), while oligodendrocyte progenitor cells express NG2 (CSPG4), an integral membrane chondroitin sulfate proteoglycan (Chang et al. 2000). NSC and NPC secrete cystatin C (CST3) into the culture medium, serving as a survival factor (Taupin et al. 2000). Growth arrest-specific 1 (GAS1) induced by Wnt signaling is required for proliferation of progenitors of the cerebellar granule cells and Bergmann glia (Liu et al. 2001). The HMG-box transcription factor Sox4, expressed in neuronal as well as glial progenitors, is downregulated in terminally differentiated neurons or glia (Hoser et al. 2007). Importantly, a recent study by microarray analysis showed that fetal human NPC express PDGFRA, CSPG4, DLL3, GAS1, and SOX4 (Maisel et al. 2007), all of which are downregulated in the serum-treated NPC in the present study.

In summary, we identified 45 serum-upregulated and 23 serum-downregulated genes in human NPC in culture by analysis with a whole human genome-scale microarray. The serum-induced astrocyte differentiation of human NPC

is characterized by a counteraction of ID family genes on Delta family genes.

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特集：疾患の制御―臨床から免疫へ―
総　説

多発性硬化症の病態解析から治療標的の同定へ

大木伸司，山村　隆

Identification of a possible therapeutic target through pathogenic T cell analysis of multiple sclerosis

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summary

Multiple sclerosis is an autoimmune disease affecting the central nervous system (CNS), in which Th17 and Th1 cells are involved. Comprehensive gene expression profiling analysis employing DNA microarray showed that NR4A2, an orphan nuclear receptor, is strongly upregulated in the peripheral blood T cells derived from MS patients. Further analysis revealed that NR4A2 plays a pivotal role for mediating production of inflammatory cytokines from pathogenic T cells. In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, NR4A2 was selectively up-regulated in the CNS-infiltrating T cells and the peripheral blood T cells. Intriguingly, a forced expression of NR4A2 augmented promoter activities of IL-17 and IFN-γ genes, leading to an excessive production of these cytokines by splenic T cells. In contrast, treatment with siRNA specific for NR4A2 resulted in a significant reduction in the production of IL-17 and IFN-γ. Furthermore, treatment with NR4A2-specific siRNA reduced the ability of encephalitogenic T cells to adoptively transfer EAE in recipient mice. These results imply that NR4A2 is an essential transcription factor for triggering the inflammatory cascade in MS/EAE and may serve as a novel therapeutic target of the diseases.

Key words――多発性硬化症，NR4A2，核内受容体，IL-17，Th17 細胞

抄　録

多発性硬化症（Multiple Sclerosis; MS）は中枢神経系の脱髄疾患であり，その本態は自己反応性 T 細胞を含む免疫担当細胞を介した組織障害である．長らく自己免疫疾患に関わる病原性 T 細胞の本体は，IFN-γ 産生性の Th1 細胞と考えられてきたが，最近なって Th1 細胞や Th2 細胞とは機能的に異なる新たなエフェクター細胞 T 集団として，より強力な炎症惹起能を有する IL-17 を産生する Th17 が，強力な自己免疫疾患誘導性を有する病原性 T 細胞集団であることが示された．我々は，自己免疫病態形成に関わる病原性 T 細胞の機能解析を目的として，寛解期 MS 患者由来の末梢血 T 細胞を対象に，DNA マイクロアレイ法を用いた網羅的遺伝子発現解析を施行し，新たな MS 治療標的候補分子として，オーファン核内受容体 NR4A2 を同定した．RNAi 法を用いた T 細胞の NR4A2 発現抑制により，炎症性サイトカイン産生抑制と，実験的自己免疫性脳脊髄炎（Experimental Autoimmune Encephalomyelitis ; EAE）の軽快が認められた．本稿では，NR4A2 をターゲットとした分子標的薬による新規 MS 治療法開発の可能性について紹介する．

はじめに

多発性硬化症（Multiple Sclerosis ; 以下 MS）は，中枢神経系の脱随を主徴とし，Th1 細胞や Th17 細胞などのエフェクター T 細胞をはじめとする免疫担当細胞の機能亢進による組織障害が病態形成に深く関わる典型的な炎症性自己免疫疾患である[1,2]．よって免疫異常制御の観点から MS の病態を理解することは，本疾患の予防や治療への根本的な道を開くことにつながると考えられる．現在臨床で利用されているインターフェロン・ベータ（IFN-β），抗炎症性ステロイドおよび免疫抑制剤などの MS 治療薬や，臨床導入の途上にある薬剤として 4 種のアミノ酸からなるコポリマーであるコパキソン，抗 VLA-4 抗体製剤タイサブリ，およびスフィンゴシン 1 リン酸受容体を標的とした FTY720（Fingolimod）などはいずれも，上記の発想に基づいて見いだされてきたものである．このように近年の著し

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い基礎免疫学の進歩により，慢性炎症を伴う自己免疫疾患の制御薬，あるいは治療薬候補分子が多数見出されてきているが，一方で視神経脊髄型 MS（NMO, Devic 病）では，IFN-β 投与により病態が増悪する例が見られるなど，それぞれの治療薬に対する MS 患者の反応性は症例ごとに大きく異なるという現状がある．このことは，MS の新規治療法を開発ためには，MS 病態の多様性に十分配慮した新たな免疫学的なアプローチが求められていることを意味しており，これは MS 患者サンプルを用いた網羅的解析の中で多様性に関わる項目を適当に相殺し，その中から普遍的な治療標的を探るという手法により克服可能であると考えられる．以上のような観点から我々は，MS の新規治療標的の同定を目的として，DNA マイクロアレイによる MS 患者末梢血 T 細胞の網羅的遺伝子発現解析を行い，健常者に比較して MS 患者 T 細胞で発現が変動する一連の遺伝子群の同定に成功した[3]．そのなかで，MS 患者で最も有意な発現亢進を認めた遺伝子の一つとして同定したオーファン核内受容体 NR4A2 は，MS の動物モデルである実験的自己免疫性脳脊髄炎（Experimental Autoimmune Encephalomyelitis：以下 EAE）マウス由来の中枢神経浸潤 T 細胞でも認められた．T 細胞の NR4A2 発現を変動させると，各種炎症性サイトカインの産生が相関して増減し，NR4A2 特異的 siRNA 処理により，passive EAE モデルにおける EAE の発症が有意に抑制されたことから，NR4A2 が MS などの自己免疫疾患の新規治療ターゲットになりうる可能性が示された[4]．本稿では，MS/EAE の病態形成における NR4A2 の挙動と，炎症性サイトカイン産生制御を介した MS の新規治療法確立の可能性について紹介する．

オーファン核内受容体 NR4A2 とは？

核内受容体は，エストロゲン受容体やグルココルチコイド受容体などを含む分子ファミリーをなしており，ヒトの場合 48 種類の異なる分子が知られている[5,6]．実は本稿の中心となる Th17 細胞と核内受容体との関わりは思いのほか深く，今や Th17 細胞のマーカー分子として知られているレチノイドオーファン受容体 RORγt/NR1F3[7]，リガンド依存性に Th17 細胞機能制御能を有するレチノイン酸受容体 RARα/NR1B1[8]，NF-AT 阻害により IL-17 などの産生制御に関わるプロテインキナーゼ C 基質 EAR2/NR2F6[9] などは，いずれもこのファミリーに含まれる分子である．一方，NR4A ファミリー分子は NR4A1, NR4A2, NR4A3 の 3 種からなり，他の核内受容体と同様，NGFB-I / Nur77（＝NR4A1），Nurr1（＝NR4A2），NOR1（＝NR4A3）などの別名でも知られている[10]．NR4A ファミリー分子は図 1 に示すような様々な生体応答に関わることが示されており，その機能の一部にはファミリー分子間の機能的オーバーラップが認められる．NR4A ファミリー分子のうち NR4A2 の発現部位は比較的中枢神経系に集中しており，なかでも中脳腹側，脳幹や脊髄に強い発現を認める．免疫系においては，T 細胞受容体の架橋や炎症性サイトカインなど刺激により，T 細胞で一過性に発現誘導される immediate early gene として知られている．NR4A ファミリー分子は，複数の機能ドメインからなる構造が，他の核内受容体分子との間で比較的よく保存されている（図 1）．2 つの Zn フィンガーからなる N 末側の DNA 結合ドメイン（DBD）は，受容体間で非常に良く保存されており，標的分子プロモーター内の応答配列に対する特異的結合に関わる．C 末側に位置するリガンド結合ドメイン（LBD）は，各核内受容体分子間での多様性が高く，通常それぞれ異なるリガンドを認識する．一般に核内受容体は，リガンドを結合することで受容体の AF2 ドメインのコンフォメーションが変化し，ヘリックス 12（H12）が活性型の配向をとると，コリプレッサーを遊離してコアクチベーターと会合するようになり転写活性化能を獲得する．一方，リガンドが未知の核内受容体はオーファン受容体と呼ばれ，NR4A ファミリー分子もこの中に含まれる．LBD の構造解析の結果，NR4A2 の LBD はかさ高い芳香環や疎水性の側鎖を持つアミノ酸に覆われており，典型的なリガンド結合ポケットがないことが明らかとなっている[11]．さらに NR4A2 の H12 は，リガンドの存在とは無関係に活性型受容体類似のコンフォメーションをとることが示され，リガンド非依存的に転写活性化能を有するものと考えられている．

NR4A2 の誘導因子と標的分子[10]

NR4A2 は，脂肪酸，プロスタグランジン，カルシウム，増殖因子，ペプチドホルモン，神経伝達分子など様々な因子に応答して速やかに発現が誘導される．これらの刺激はいずれも NF-κB あるいは CREB の活性化を誘導し，NR4A2 遺伝子プロモー

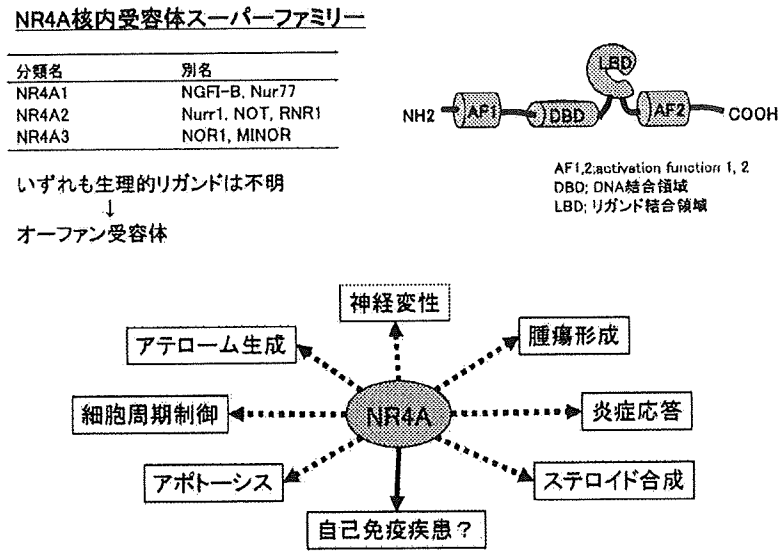

図1 NR4A 核内受容体ファミリー分子

哺乳動物の NR4A 核内受容体ファミリー分子は，NR4A1(NFGI-B/Nur77)，NR4A2(Nurr1)，NR4A3(NOR1) の 3 種の分子からなり，ファミリー分子に共通の構造（AF1/2, DBD, LBD）を有する核内受容体分子である．いずれも生理的なリガンドは不明であるが，図に示すようなさまざまな生体応答に関わることが知られている．免疫系との関連では，遺伝子欠損マウスを用いた解析から，NR4A1 と NR4A3 が胸腺細胞のアポトーシスに関わることが示されており，いわゆる「負の選択」の過程で重要な役割を果たしているものと考えられる．一方，NR4A2 欠損マウスではドパミン産生ニューロンの分化が著しく阻害されるが，胸腺細胞分化は比較的正常に保たれることが示されており，NR4A1/NR4A3 と NR4A2 の機能的な相違をうかがわせる知見であるといえる．

ターの転写活性化領域に結合することで，遺伝子発現を引き起こすと考えられている．一方，発現した NR4A2 分子は，リガンド非依存的に特定の DNA 配列を認識して下流の遺伝子発現を誘導する（図 2）．したがって NR4A2 分子の機能制御は，主に種々の誘導因子による転写誘導レベルで行われていると考えられる．NR4A ファミリー分子の認識配列としては，①(A/T) AAAGGTCA 配列からなる NBRE（NGFI-B response element；単量体あるいは二量体の NR4A 分子が結合），②NBRE 類似の AAAT (G/A) (C/T) CA の逆向き繰り返し配列からなる NurRE（Nur-responsive element；プロオピオメラノコルチン (POMC) プロモーターに存在），③DR5 配列（レチノイド X 受容体（RXR）とのヘテロダイマー形成による）の 3 種の配列が知られている．NR4A2 のターゲットとして最も良く解析されているのが，ドパミン（DA）生成に必須の酵素であるチロシンヒドロキシラーゼ（TH）遺伝子であり，NR4A2 依存的な DA の産生は，TH 遺伝子プロモーターに存在する NBRE を介して誘導される．一方，NR4A2 欠損マウスでは中脳黒質のドパミン産生ニューロンの形成が障害される[12]．さらに家族性パーキンソン病の一部に NR4A2 の遺伝子異常が認められる[13]ことも明らかとなり，TH 発現における NR4A2 の重要性が再認識されている．他にも NR4A2 の標的探索を目指した個別の解析から，Neuropilin-1, vasoactive intestinal peptide (VIP), aldosterone 合成酵素，アロマターゼ，オステオポンチン，オステオカルシンなどがターゲットとして報告されており，NR4A2 は中枢神経機能のみならず，骨代謝の機能制御にも深く関わる可能性が示されている．NR4A2 分子の機能が，さらなる広がりをみせる予感を感じさせる．

免疫系における NR4A ファミリー分子の機能

免疫系における NR4A ファミリー分子の機能に関しては，T 細胞アポトーシス誘導，および胸腺における「負の選択」における NR4A1 分子の機能が，とくに詳細に解析されている[14~17]．すなわち，TCR 刺激によりカルシウム依存性に活性化した MEF2 が NR4A1 の発現を引き起こし，T 細胞アポトーシスを誘導する．おそらくこの経路は，NR4A1 分子と Bcl2 分子との分子間相互作用を介した制御を受ける．そして転写抑制因子 Cabin1 が，mSin3 と HDAC1/2 をリクルートすることにより，あるいは MEF2 と p300 の結合を阻害すること

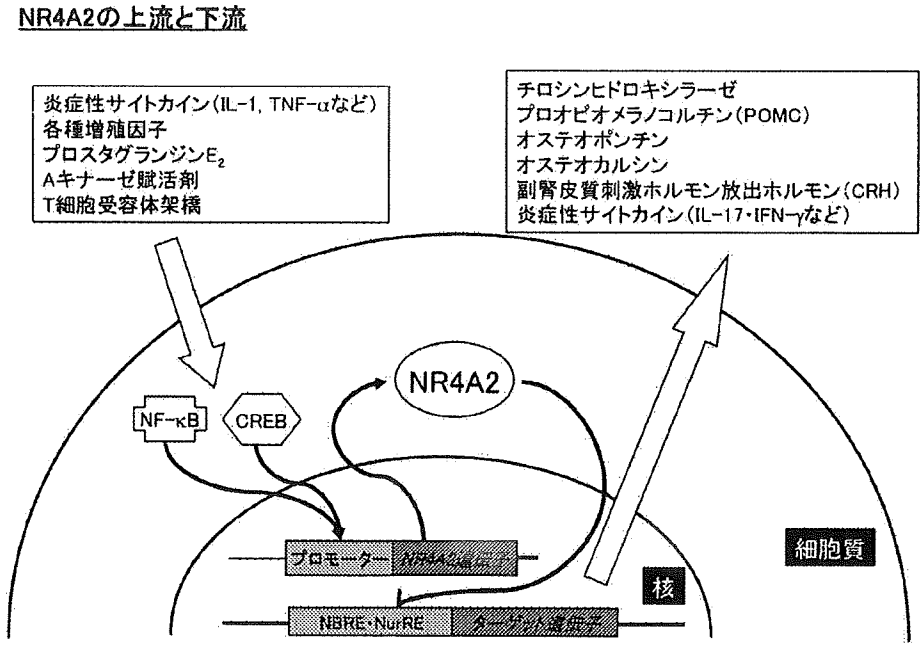

図2　NR4A2の誘導因子とターゲット分子
NR4A2は，脂肪酸，プロスタグランジン，カルシウム，増殖因子，ペプチドホルモン，神経伝達分子など，あるいはストレスや物理刺激に応答して発現が誘導され，いずれもNF-κBあるいはCREBの活性化を介すると考えられている．一方，このようにして発現したNR4A2分子は，多様な会合分子と複合体を形成し，NBRE(NGFI-B response element)，NurRE(Nur-responsive element)，DR5などの特定の配列を認識してターゲット遺伝子の発現を誘導する．これまでにチロシンヒドロキシラーゼを筆頭に，複数のターゲット遺伝子が報告されている（本文参照）．

により，MEF2によるNR4A1の誘導経路を遮断してアポトーシスを抑制すると考えられている[18]．一方，NR4A1欠損マウスの表現系は，胸腺あるいは末梢のT細胞アポトーシスに関して大きな異常はなく，NR4A1が単独でこの経路を制御しているのではないと思われる．おそらく他の分子がNR4A1欠損を補完することにより，強い表現系の発現を抑制していることが予想され，胸腺での発現パターンなどから推測するに，その候補の一つは別のNR4Aファミリー分子NR4A3である．一方，NR4A2欠損マウスでは，中脳のドパミン産生ニューロンの欠損が著しく，胎児は生後すぐに死亡する．このNR4A2欠損マウスの表現系は，NR4A2の機能が他のNR4A1やNR4A3では補完できないことを意味しており，NR4A2が他のNR4Aファミリー分子とは異なる独自の機能を有することを強く示唆している．NR4A2欠損マウスは，生後の長期維持が極めて難しいため，免疫系を含む成体の機能異常に関する解析はほとんどなく，コンディショナル欠損マウスなどを用いた解析が待たれる．

NR4A2と自己免疫疾患

上にも述べたように，多発性硬化症（以下MS）では，Th1細胞やTh17細胞などの炎症性T細胞が脳炎惹起に重要な役割を果たす．CD4陽性ナイーブT細胞は複数の機能的に異なるエフェクターヘルパーT細胞に分化するが，その分化機構は，以前より知られていたTh1細胞，Th2細胞に加えて，Th17細胞[19]と制御性T細胞[20]が発見されたことをきっかけに，格段に複雑になりつつある（図3）．MSの病態形成初期には自己反応性T細胞が決定的な役割を果たすと考えられており，このような病原性T細胞の網羅的な機能解析は，新規治療ターゲットの絞り込みに有効な手段であると思われる．このような観点から我々は，DNAマイクロアレイによるMS患者末梢血T細胞の遺伝子発現解析を通じて，MSで健常者に比較して発現亢進する遺伝子群の同定を試み，その結果，最も有意な発現差異を認めた分子としてNR4A2を同定した[3]．NR4A2分子は核内受容体型の転写因子であるが，T細胞における機能は他のNR4Aファミリー分子とセットで解析されることが多く，個別の機能については不明

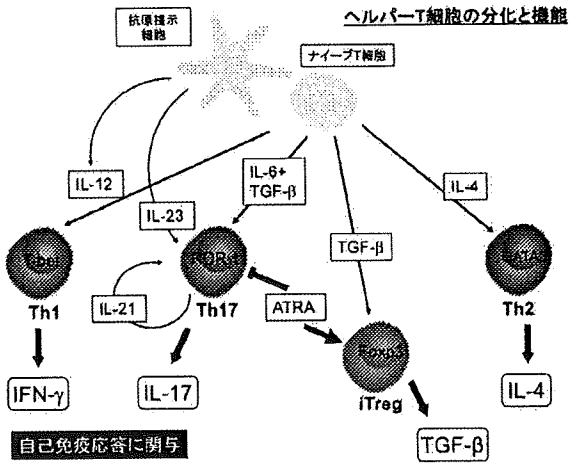

図3　ヘルパーT細胞の分化と機能
ナイーブT細胞が抗原提示細胞場の抗原を認識すると，種々のサイトカイン依存性にそれぞれ機能的に異なるエフェクターT細胞へと分化することが知られている．以前は感染免疫などに関わるTh1細胞と，アレルギー反応などに関わるTh2細胞の二極対立の構図により説明されていたが，近年のTh17細胞と制御性T細胞（Treg細胞）の発見にともない，各エフェクターT細胞の複雑な分化制御機構が次々と明らかになりつつある．自己免疫の観点からは，IFN-γ産生性のTh1細胞と，IL-17産生性のTh17細胞はいずれも自己免疫病態を引き起こす病原性T細胞集団であると考えられており，双方の細胞機能をコントロールできる新たな制御法の確立が重要な課題となっている．

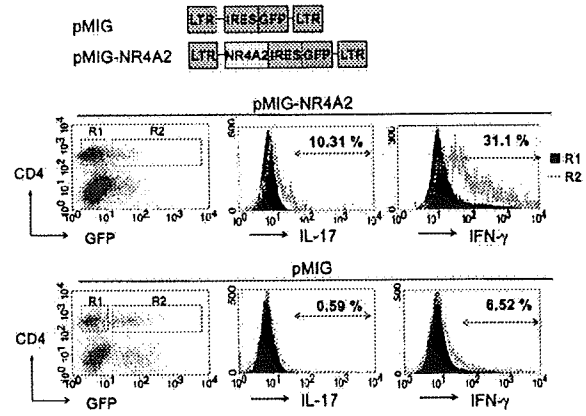

図4　NR4A2を介した炎症性サイトカイン産生増強
T細胞の炎症性サイトカイン産生に対するNR4A2分子の機能を解析するため，NR4A2遺伝子を組み込んだレトロウイルス，あるいはコントロールウイルスを用意し，マウス脾臓CD4陽性T細胞に感染させた．eGFPの発現を指標に感染細胞を同定し，再試激後の炎症性サイトカイン産生を，細胞内サイトカイン染色法を用いて比較した．GFP陰性群（R1）では図の上下でサイトカイン産生レベルに大きな差は認められないが，GFP陽性群（R2）では，コントロール細胞（下段）に比べてNR4A2遺伝子導入細胞（上段）における炎症性サイトカインの産生がいずれも増強した（IL-17；0.59% vs 10.31%，IFN-γ；6.52% vs 31.1%）．

な点が多かったため，MSのマウスモデルである実験的自己免疫性脳脊髄炎（以下EAE）を併用してさらなる病態との関連を解析した[4]．

C57BL/6マウスにMOG_{35-55}ペプチドを免疫することにより誘導したEAEモデルにおいて，脳脊髄（CNS）浸潤細胞，末梢血，所属リンパ節細胞，脾臓細胞から分離したT細胞のNR4A2の発現レベルを，定量PCR法により比較し，CNS浸潤T細胞および末梢血T細胞で，選択的なNR4A2の発現亢進を認めた．さらに再試激後のCNS浸潤T細胞のサイトカイン産生を調べたところ，約30%の細胞がIL-17を産生することが判明し，CNSへの顕著なTh17細胞の集積が認められた．IL-17はMS/EAEの発症に重要な炎症性サイトカインの一種であり，我々はNR4A2とT細胞のIL-17産生との間に何らかの相関がある可能性を考えて，さらに検討を加えた．まずNR4A2の発現亢進が炎症性サイトカイン産生に与える影響を調べるため，IL-17遺伝子，あるいはIFN-γ遺伝子のプロモーター領域を含むレポーター遺伝子を培養細胞に導入して，ルシフェラーゼアッセイを試みた．その結果，NR4A発現プラスミドの添加により，それぞれルシフェラーゼ活性が有意に増強した．さらにNR4A2のcDNAをコードするレトロウイルスを用いて，脾臓T細胞にNR4A2分子を過剰発現させると，TCR刺激後のIL-17およびIFN-γ産生が選択的に亢進した（図4）．以上の結果から，T細胞におけるNR4A2の発現亢進により，炎症性サイトカインの産生が増強することが明らかとなった．次にNR4A2のMS治療ターゲットとしての可能性を探るため，NR4A2の発現を抑制することで，炎症性サイトカインの産生が低下するかどうかを，あらたに設計したNR4A2特異的siRNAを用いて検討した．ヒトおよびマウスのNR4A2遺伝子のDNA配列は非常に良く保存されており，我々が設計したsiRNAはヒトおよびマウスのいずれにも適用可能であったことから，まず健常人の末梢血CD4陽性T細胞をsiRNA処理し，NR4A2の発現を選択的に抑制したときの炎症性サイトカイン産生を調べた．その結果，NR4A2特異的siRNA処理したT細胞では，刺激後のIL-17産生およびIFN-γ産生が，いずれも有意に抑制されていた．次にMS患者の末梢血CD4陽性T細胞を用いて同様の検討を行ったところ，患者T細胞の炎症性サイトカイン産生も，NR4A2特異的siRNA処理により有意に減少することが明らかとなった．さらにsiRNAによる

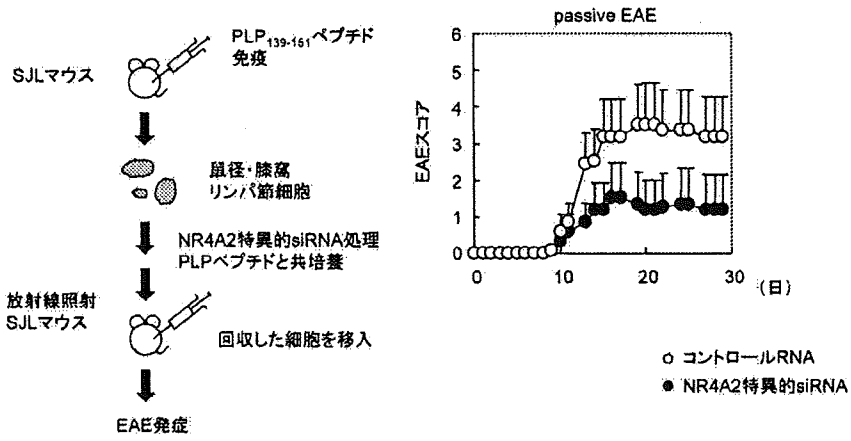

図5　passive EAE における NR4A2 特異的 siRNA の効果

$PLP_{139-151}$ ペプチドを免疫後 10 日目の SJL マウスより，鼠径リンパ節細胞および膝窩リンパ節細胞を分離し，NR4A2 特異的 siRNA あるいはコントロール RNA を遺伝子導入した．さらに $PLP_{139-151}$ ペプチド存在下 in vitro で 3 日間培養した細胞を回収し，放射線照射した未処理 SJL マウスに移入することで，レシピエントマウスに EAE を誘導した．各処理群の EAE スコアを比較した結果，コントロール RNA 処理群で誘導された EAE の臨床スコアに対し，NR4A2 特異的 siRNA 処理群の EAE 臨床スコアは低下し，観察期間を通じて軽度の病態で推移した．NR4A2 特異的 siRNA 処理により，EAE 病態が抑制できることが示された．

NR4A2 の発現抑制が中枢神経系の病態形成に及ぼす効果を，ミエリン抗原（プロテオリピッドプロテイン；PLP）を免疫することで SJL マウスに誘導される病原性 T 細胞を，ナイーブ SJL マウスに移入する passibe EAE モデルを用いて検討した．分離した T 細胞を in vitro で抗原刺激する際に，NR4A2 特異的 siRNA で処理すると，対照 RNA 処理した T 細胞の移入群に比して，移入後の EAE は有意に軽症化することが明らかとなった（図 5）．これらの結果は，NR4A2 の発現あるいは機能制御を介して自己免疫病態が制御できることを示しており，NR4A2 が MS をはじめとする自己免疫疾患の治療ターゲットとなりうる可能性を示すものと考えている．

疾患治療ターゲットとしての NR4A ファミリー分子の可能性

核内受容体の約半数はいまだリガンドが未知のオーファン受容体であり，個々の機能は，その多くが不明のままである．仮に欠損マウスの表現系解析から機能の一端を明らかにしても，その機能を制御できるリガンドの同定なしには，さらなる応用研究への展開は一般には難しい．しかしながら PPAR など一部の核内受容体分子に関しては，代謝制御における重要な生理機能が次々と明らかとなってきている．さらにその結果，高脂血症改善作用を有する PPAR-α の合成リガンドであるフィブラート系化合物や，糖尿病治療作用を有する PPAR-γ の合成リガンドであるチアゾリジン系化合物など，臨床応用上も重要な小分子化合物の同定が次々となされており，核内受容体の新規リガンドの探索は創薬の観点からも非常に重要な研究領域であるといえる．免疫応答制御の分野に限ってみても，種々の核内受容体が免疫系，特に Th17 細胞の制御に深く関わることが明らかとなっている．例えば天然型レチノイン酸および合成 RAR アゴニストが，Th17 細胞分化の抑制と制御性 T 細胞の誘導を介して効果的な自己免疫疾患の抑制作用を示すことなどが明らかとなっており[8,21]，合成 RAR アゴニストを用いた新規自己免疫疾患治療法の開発も，将来有望な研究分野であるといえる．

一方で，上で示した NR4A2 特異的 siRNA を用いた核酸医薬の臨床応用を考えた場合，一般的に siRNA を用いた製剤の臨床応用はいまで限定的であり，RNA 自身の in vivo での安定性に加えて効果的なドラッグデリバリーシステム（DDS）の構築が難しいなど多くの課題を抱えている現状がある．局所的な投与による応用可能性はあるとしても，本 siRNA そのものを用いたアプローチが MS 治療薬としての臨床応用に展開可能かどうかについては今後のさらなる検討が必要である．この他には，小分子化合物を用いた NR4A2 の機能制御が考えられる．NR4A2 のリガンド結合領域は不活性化されていると考えられているが，本来のリガンド結

合領域以外の領域と相互作用することでNR4A2の機能制御能をもつ特異的な低分子化合物をスクリーニングすることにより，効果的なNR4A2阻害剤を探索することができれば，新規自己免疫疾患治療薬のシードが見出される可能性は十分にあると考えられる．例えば，抗癌剤として用いられている6-メルカプトプリン（6-MP）には，NR4A2の活性化能があることが示されており[22]，類縁分子の中に求める小分子化合物が見出される可能性もある．実際にNR4A2ファミリー分子の関与の可能性が示唆されている疾患としては，パーキンソン病，統合失調症，双極性うつ病，動脈硬化症，アルツハイマー病，関節リウマチ，癌などのさまざまな難治性疾患があり，例えば炎症性の関節炎の場合，メトトレキセート処理により滑膜組織におけるNR4A2の発現が低下し，このNR4A2の発現低下に相関して疾患活動性も減少することが示されている[23]．またNR4A2は，THの発現と中脳ドパミン作動性ニューロンの発生に深く関与すること，および一部の家族性パーキンソン病家系にNR4A2変異が見出されたことから，パーキンソン病の治療ターゲットとしての期待も高まっている．実際，パーキンソン病治療薬候補としてNR4A2賦活化小分子化合物の同定がなされており，今後これらの小分子化合物の誘導体を，MSの新規治療薬として転用するアプローチは有望であろうと思われる．さらにもし求める小分子化合物が得られれば，自己免疫誘導生病原性T細胞と，これらが産生するIL-17やIFN-γなどの炎症性サイトカインは，種々の自己免疫疾患への関与が考えられるため，NR4A2をターゲットとした治療アプローチが，MSのみならず他の自己免疫疾患へとさらなる広がりをみせる可能性がある．

生体内恒常性センサーとしてのNR4A2分子の可能性

MSの病態形成に遺伝的背景が関与することは論を待たないが，元々本邦では比較的稀な疾患とされてきたMSの患者数がここ30年間で約20倍に増加していることは，患者を取り巻く生活環境の急激な近代化にともなう環境要因の変化が，MS患者数の増加に関連している可能性を示唆している[24]．我々は以前より，我が国における食生活の欧米化にともなう腸管免疫環境の変調がMSの発症増加と関連する，という仮説の下，MSにおける病原性T細胞の成立における腸管関連リンパ組織の関連を解析してきた．そのなかで，中枢神経疾患であるEAEの感受性が，腸管に集積し腸内細菌叢と密接に関わるMR1拘束性NKT細胞により制御されることを報告し，様々な要因で変化する腸内細菌叢の構成が腸管免疫応答，ひいては自己免疫感受性に及ぼす影響を明らかにした[25]．さらに非吸収性抗生物質を経口投与することで腸内細菌叢を変動させることで，EAE感受性が大きく変動することも見出している[26]．Germ-freeマウスでは腸管免疫系が発達しないことからも，腸内細菌叢が腸管免疫応答制御を介して自己免疫疾患感受性を規定する因子である可能性は高い．一方，このような観点からNR4A2は，T細胞受容体刺激によりT細胞で発現誘導されるのみならず，脂肪酸，プロスタグランジン，カルシウム，増殖因子，ペプチドホルモン，神経伝達分子など様々な生体因子に反応してNF-κB依存性あるいはCREB依存性に発現が誘導される．NR4A2は生体応答をモニターする，極めて鋭敏なセンサー分子として機能しているのである．興味深いことに，ストレス負荷や運動による物理刺激など，一見誘導メカニズムが簡単には想像できないような複雑な刺激に対しても，速やかなNR4A2の発現誘導がおきることが知られている．心的ストレスや過剰な運動とこれに伴う疲労は，MSの重要な再発誘導因子，あるいは増悪因子と考えられており，非本来的なNR4A2の発現誘導が，この過程に何らかのかたちで関与することは十分に考えられる．とくにNR4A2が，元々中枢神経系の機能に関わる分子であることを考えると，ストレスで発現する神経系由来の何らかのNR4A2の誘導因子が，T細胞のNR4A2発現をも同時に誘導してしまうことにより，不要な免疫応答につながる可能性も考えられる．今後さらなる研究が必要な分野であるといえるだろう．

おわりに

MSの病態解明を目的とした研究の過程で我々が新たに見出したオーファン核内受容体NR4A2の免疫系，とくに活性化T細胞の炎症性サイトカイン産生における機能と，引き続く中枢神経系の炎症病態形成における役割について概説した．最近になり，グリア細胞に発現したNR4A2がコリプレッサーと会合することで，炎症によるドパミン産生ニューロン死を抑制する可能性が示された[27]．NR4A2にはさまざまな会合分子が存在するため，