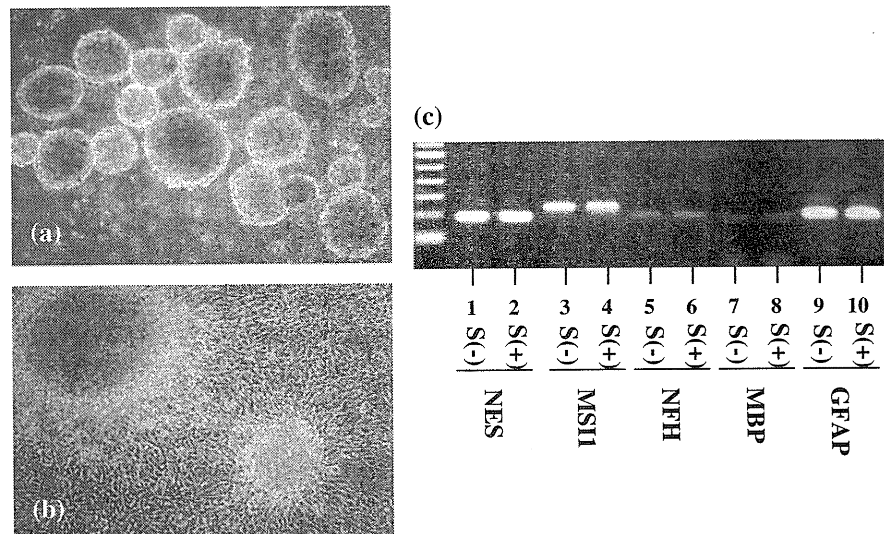

Fig. 1 Human neural progenitor cells (NPC) in culture. **a** Human NPC maintained under the serum-free culture conditions formed free floating growing spheres. **b** Human NPC spheres exposed to 10% FBS rapidly attached on the plastic surface, followed by vigorous outgrowth of a sheet of adherent cells 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

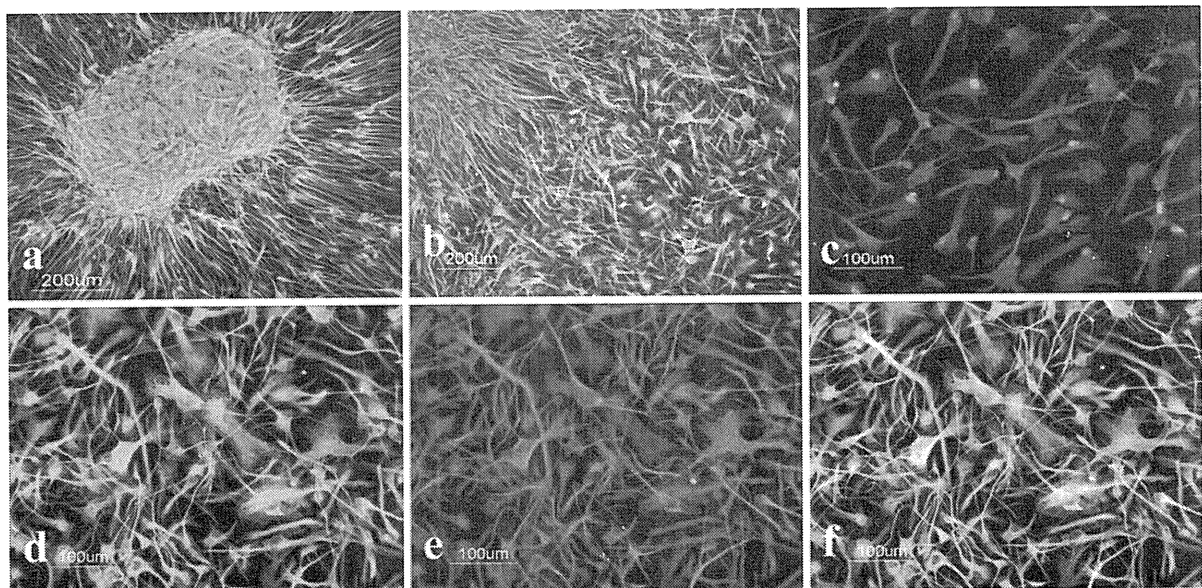

Fig. 2 Nestin, GFAP, and ID1 expression in human NPC in culture. Human NPC spheres attached on poly-L-lysine-coated cover glasses were incubated for 72 h in the NPC medium with (S+) or without (S−) inclusion of 10% FBS, and processed for double-labeling immunocytochemistry for nestin, GFAP, or ID1. **a** 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

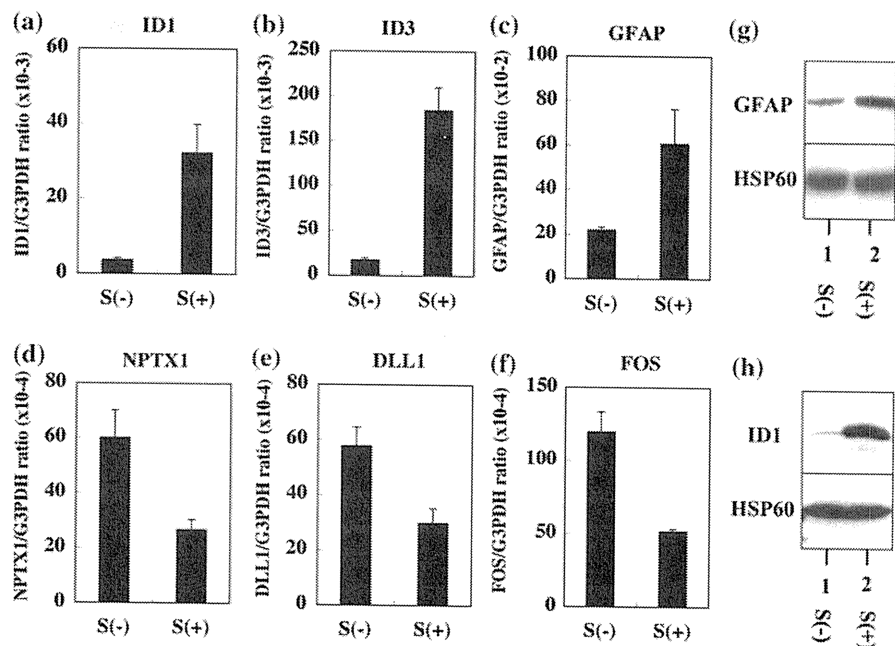

Fig. 3 Validation of microarray data by real-time RT-PCR and western blot analysis. Human NPC spheres were incubated for 72 h in the NPC medium with (S+) or without (S−) inclusion of 10% FBS, and then total cellular RNA or protein extract was processed for real-time RT-PCR and western blot analysis. **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,

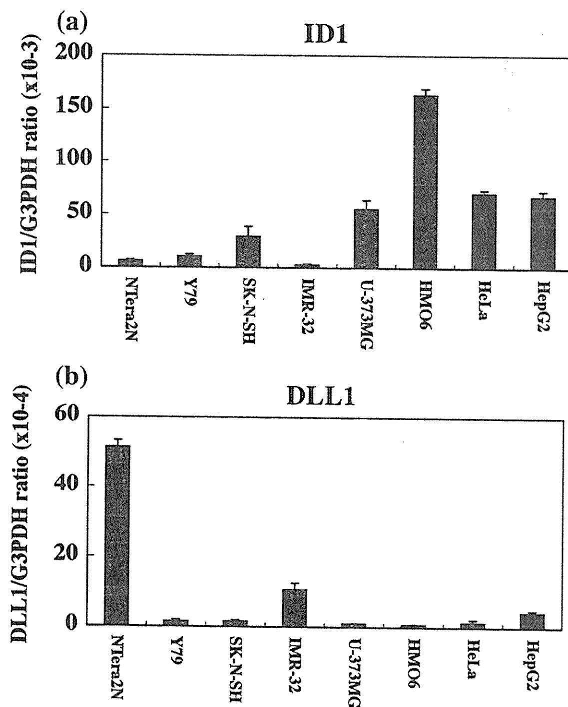

Fig. 4 ID1 and DLL1 expression in various human cell lines. Total RNA of human cell lines, such as NTera2 teratocarcinoma, Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrocytoma, HMO6 microglia, HeLa cervical carcinoma, and HepG2 hepatoblastoma was processed for real-time 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).

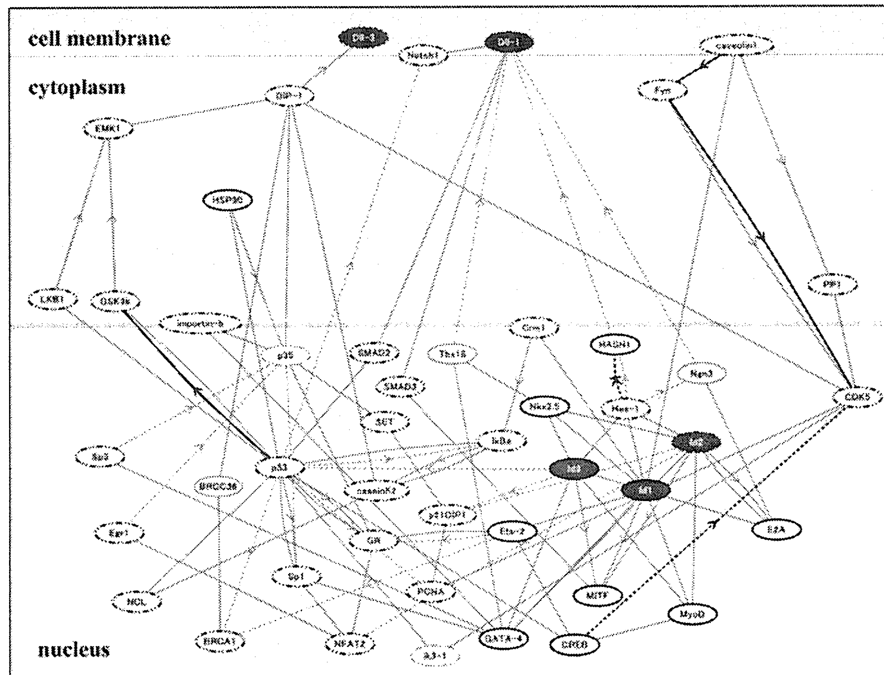

Fig. 5 Molecular network analysis of ID1, ID2, ID3, DLL1, and DLL3. KeyMolnet, a bioinformatics tool for analyzing molecular interaction on the curated knowledge database, identified the shortest route connecting the start point molecules of ID1, ID2, and ID3 (red) and the end point molecules DLL1 and 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).

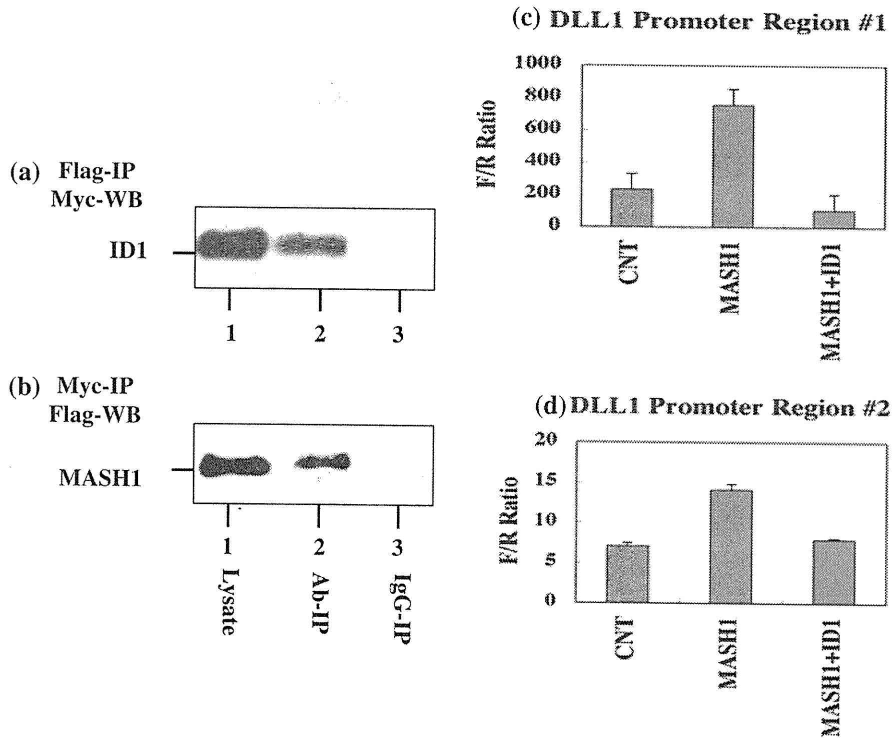

Fig. 6 Activation of the DLL1 promoter by MASH1 was counteracted by ID1. **a, b** Coimmunoprecipitation analysis. Recombinant MASH1 protein tagged with Flag and ID1 protein tagged with Myc were coexpressed in HEK293 cells. Immunoprecipitation (IP) followed by Western blotting (WB) was performed by using 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

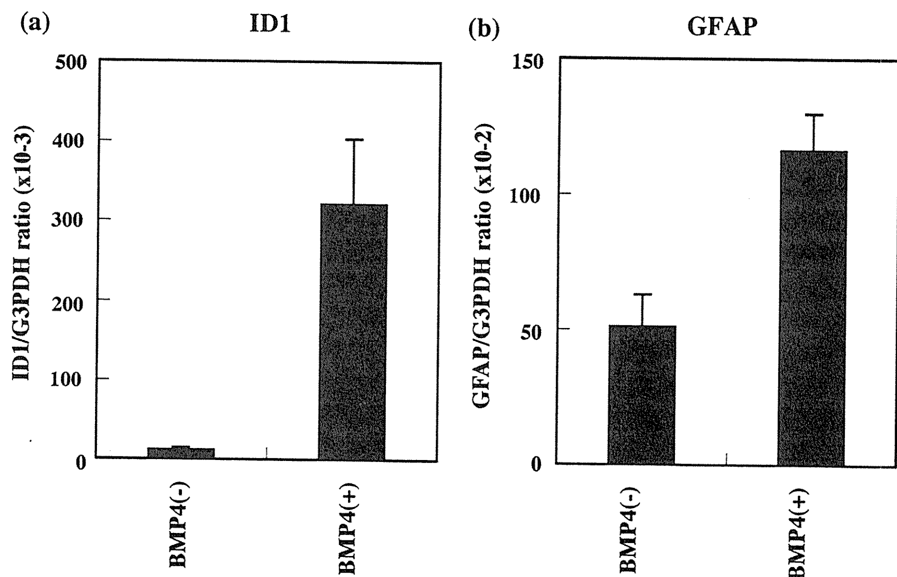

Fig. 7 BMP4 upregulates ID1 and GFAP expression in human NPC. Human NPC were incubated for 72 h in the NPC medium with (+) or without (−) inclusion of 50 ng/ml recombinant human BMP4, and then total cellular RNA was processed for real-time RT-PCR analysis. The levels of target genes were standardized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

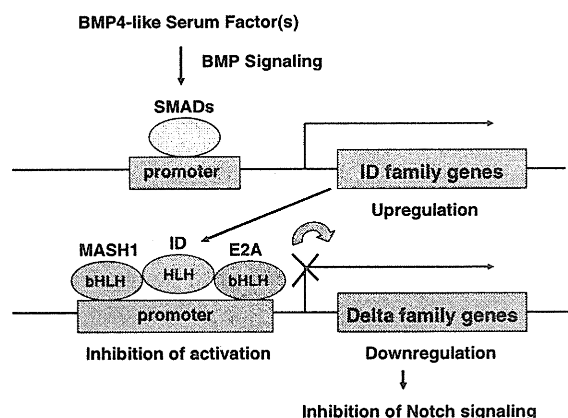

Fig. 8 The serum-induced astrocyte differentiation of human NPC is characterized by a counteraction between ID and Delta family genes. The present observations raise the possible scenario that the serum factor(s), most probably BMP4, induces astrocyte differentiation by upregulating the expression of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

Multiple Sclerosis 2009; **15**: 531–541

Molecular network of the comprehensive multiple sclerosis brain-lesion proteome

JI Satoh[1,2], H Tabunoki[1] and T Yamamura[2]

Background A recent proteomics study of multiple sclerosis (MS) lesion-specific proteome profiling clearly revealed a pivotal role of coagulation cascade proteins in chronic active demyelination. However, among thousands of proteins examined, nearly all of remaining proteins are yet to be characterized in terms of their implications in MS brain-lesion development.
Methods By the systems biology approach using four different pathway analysis tools of bioinformatics, we studied molecular networks and pathways of the proteome dataset of acute plaques, chronic active plaques (CAP), and chronic plaques (CP).
Results The database search on Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein analysis through evolutionary relationships (PANTHER) indicated the relevance of extracellular matrix (ECM)–mediated focal adhesion and integrin signaling to CAP and CP proteome. KeyMolnet disclosed a central role of the complex interaction among diverse cytokine signaling pathways in brain-lesion development at all disease stages, as well as a role of integrin signaling in CAP and CP. Ingenuity pathway analysis (IPA) identified the network constructed with a wide range of ECM components, such as collagen, type I α1, type I α2, type VI α2, type VI α3, fibronectin 1, fibulin 2, laminin α1, vitronectin, and heparan sulfate proteoglycan, as one of the networks highly relevant to CAP proteome.
Conclusions Although four distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin signaling in development of chronic lesions of MS. These *in silico* observations indicate that the selective blockade of the interaction between ECM and integrins in brain lesions *in situ* would be a target for therapeutic intervention in MS. *Multiple Sclerosis* 2009; **15**: 531–541. http://msj.sagepub.com

Key words: extracellular matrix; multiple sclerosis; pathway analysis; proteome; systems biology

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) presenting with relapsing-remitting and progressive clinical courses. An autoimmune process triggered by a complex interplay between genetic and environmental factors may mediate MS, although the causative agents have not yet been identified. Pathologically, MS shows remarkable heterogeneity in inflammatory demyelination, astrogliosis, and axonal degeneration [1]. Even though various drugs are lined up in clinical trials, currently, treatment options with limited efficacies, including interferon-β, glatiramer acetate, and mitoxantrone are available for ordinary clinical practice of MS [2].

The completion of the Human Genome Project in 2003 allows us to systematically characterize the comprehensive disease-associated profiles of the whole human genome [3]. The global analysis of transcriptome, proteome, protein interactome, and metabolome helps us identify disease-specific molecular signatures and biomarkers for diagnosis and prediction of prognosis, and would broaden the spectrum of molecular mechanism-based therapy for MS [4,5]. Actually, the comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes by DNA microarray identified a battery of genes aberrantly regulated in MS, whose role has not been previously predicted during its pathogenesis [6,7]. A recent proteomics study of MS lesion-specific proteome profiling showed

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that overproduction of tissue factor and protein C inhibitor plays a central role in molecular events ongoing in chronic active plaques (CAP) [8]. *In vivo* administration of coagulation cascade inhibitors really reduced the clinical severity in a mouse model of experimental autoimmune encephalomyelitis (EAE), supporting the view that the blockade of the coagulation cascade would be a potential approach for the treatment of MS [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins were left behind to be characterized in terms of their implications in MS brain-lesion development.

Since the global expression analysis of transcriptome and proteome usually produces high-throughput experimental data at a time, it is often difficult to find out the meaningful biological implications of the dataset. Recent advances in systems biology enable us to illustrate the cell-wide map of the complex molecular interactions by using the literature-based knowledgebase of molecular pathways [9,10]. In the scale-free molecular network, targeted disruption of limited numbers of critical components, on which the biologically important molecular connections concentrate, could disturb the whole cellular function by destabilizing the network [11]. From this point of view, the integration of comprehensive transcriptome and proteome data of disease-affected tissues with underlying molecular networks could provide the rational approach not only to characterize disease-relevant pathways but also to achieve the network-based choice of effective drug targets. By using four different pathway analysis tools of bioinformatics, this study was designed to characterize molecular networks and pathways of MS lesion-specific proteome data of Han, *et al.* [8]. Although the analysis by distinct platforms did not lead to fully identical results, they commonly suggested a role of extracellular matrix (ECM) and integrin signaling in chronic lesions of MS. These *in silico* observations indicate that ECM and integrins would be a target candidate for designing therapeutic intervention in MS.

Databases and methods

The dataset of the comprehensive MS brain-lesion proteome

In the original dataset of Han, *et al.* [8], fresh-frozen brain autopsy samples were collected from six MS patients of different clinical subtypes, acute, chronic, progressive, secondary progressive, or chronic progressive, with ages 27–54, and from two age-matched control subjects free of neurological diseases. The postmortem interval of each case ranged from 4 to 24 h. Multiple sclerosis lesions were classified into three distinct categories: acute plaques (AP), CAP (chronic active plaques), or chronic plaques (CP), based on histological evaluation of the disease activity, briefly as follows: AP showed characteristics of acute ongoing inflammation, edema, and active demyelination. CAP was characterized by chronic demyelination with active inflammation at the lesion edges, whereas CP represented chronic inactive demyelination accompanied by profound astrogliosis. Protein samples were prepared from small pieces of brain tissues isolated by laser-captured microdissection, and the tissue pieces were characterized separately by the standard histological examination. The proteins were separated on one-dimensional SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels. Then, the protein bands were dissected and digested in a gel with trypsin, and peptide fragments were processed for mass spectrometric analysis several times to obtain a saturation point. Among 2,574 proteins determined with high confidence, the application of a computational data exploration program named INTERSECT/INTERACT identified 158, 416, and 236 lesion-specific proteins that were detected exclusively in AP, CAP, and CP, respectively. In this study, we tentatively called them as the comprehensive MS brain-lesion proteome dataset.

Conversion of protein IDs into Entrez Gene IDs and KEGG IDs

We converted the protein IDs listed in the dataset described above into the corresponding the National Center for Biotechnology Information (NCBI) Entrez Gene IDs, Gene Symbols, and Kyoto Encyclopedia of Genes and Genomes (KEGG) IDs by searching them on the UniProt knowledgebase (http://www.expasy.org/sprot).

Molecular network analysis

To identify biologically relevant molecular pathways from large-scale proteome data, we have undertaken the systems biology approach. We analyzed them by using four distinct pathway analysis tools endowed with a comprehensive knowledgebase which are as follows: KEGG (http://www.kegg.jp), the protein analysis through evolutionary relationships (PANTHER) classification system (http://www.pantherdb.org), Ingenuity pathways analysis (IPA) (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com), and KeyMolnet (Institute of Medicinal Molecular Design, Tokyo, Japan; http://www.immd.co.jp).

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By importing the list of KEGG IDs, we studied molecular pathways on KEGG, a public database that systematically integrates genomic and chemical information to create the whole biological system *in silico*. KEGG contains manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases [12]. Currently, KEGG contains 90,931 pathways generated from 371 reference pathways. PANTHER, a public database generated by computational algorithms that relate the evolution of protein sequence to the evolution of protein functions and biological roles, provides a structured representation of protein function in the context of biological reaction networks [13]. Currently, PANTHER includes the information on 165 regulatory and metabolic pathways, manually curated by expert biologists. PANTHER visualizes pathway maps with the format compatible with the Systems Biology Markup Language (SBML) standard. By uploading the list of Entrez Gene IDs, PANTHER identifies the genes in terms of over- or under-representation in canonical pathways, followed by statistical evaluation by multiple comparison with a Bonferroni correction.

IPA is a commercial tool built upon a knowledgebase that contains approximately 1,600,000 biological and chemical interactions and functional annotations with scientific evidence. They are collected from more than 300 selected articles, textbooks, and other data sources, manually curated by expert biologists. By uploading the list of Entrez Gene IDs, the network-generation algorithm identifies focused genes integrated in a global molecular network [14]. IPA calculates the score P-value, the statistical significance of association between the genes and the network by the Fisher's exact test.

KeyMolnet is a commercial database, composed of knowledge-based contents on relationships among human genes, molecules, diseases, pathways, and drugs, curated by expert biologists. They are categorized into the core contents that are collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of PubMed database and Human Reference Protein database. By importing the list of Entrez gene ID, KeyMolnet automatically provides corresponding molecules as a node on networks [15]. The "N-points to N-points" network-search algorithm identifies the molecular network constructed by the shortest route connecting the start point molecules and the end point molecules. The generated network was compared side by side with 346 human canonical pathways of the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the most significant contribution to the extracted network. The significance in the similarity between both is scored following the formula, where O = the number of overlapping molecular relations between the extracted network and the canonical pathway, V = the number of molecular relations located in the extracted network, C = the number of molecular relations located in the canonical pathway, T = the number of total molecular relations composed of approximately 90,000 sets, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p))$$
$$\text{Score}(p) = \sum_{x=0}^{\text{Min}(C,V)} f(x)$$
$$f(x) = {}_{C}C_{x} \cdot {}_{T-C}C_{V-x} / {}_{T}C_{V}$$

Results

KEGG and PANTHER searches elucidated a role of ECM-mediated cell adhesion in chronic lesions of MS

First of all, we converted all protein IDs listed in the original database [8] into the corresponding NCBI Entrez Gene IDs, Gene Symbols, and KEGG IDs by searching them on the UniProt knowledgebase. After the removal of unaccepted and redundant IDs, we finally identified 155, 407, and 232 Entrez Gene IDs and KEGG IDs from the AP, CAP, and CP-specific proteome data, respectively. They are listed in Supplementary Tables 1–3*.

When the KEGG IDs of the proteome were uploaded onto the 'Search Objects in Pathway' tool of the KEGG database, the vast majority of AP, CAP, or CP-specific proteins was not mapped on any KEGG human reference pathways (Table 1). However, a battery of CAP-specific proteins were categorized as those located in the pathways linked to focal adhesion (KEGG pathway ID: hsa04510), cell communication (hsa01430), ECM-receptor interaction (hsa04512), purine metabolism (hsa00230), and other biological pathways (not shown). Likewise, a panel of CP-specific proteins was found to be involved in the pathways linked to focal adhesion, regulation of actin cytoskeleton (hsa04810), oxidative phosphorylation (hsa00190), and cell communication (Table 1). These results are derived chiefly from enhanced production and deposition of ECM and receptor components, including collagen, fibronectin, vitronection, integrin, and laminin in CAP and CP lesions. In contrast, relatively small numbers of AP-specific proteins were mapped on the

*Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

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Table 1 The molecular pathway relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KEGG search

Stage	Rank	Functional category (KEGG Pathway ID)	Genes classified
AP	1	Unclassified	123 genes
	2	Oxidative phosphorylation (hsa00190)	NDUFS7, NDUFB9, ATP4A, ATP6V0C
	3	Regulation of actin cytoskeleton (hsa04810)	FGD1, ITGB4, SSH1, ACTA1
CAP	1	Unclassified	281 genes
	2	Focal adhesion (hsa04510)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, MYLK, SHC3, PPP1CA, PARVA, PRKCB1, MYL7, RAC3, SPP1, SRC, THBS1, VTN
	3	Cell communication (hsa01430)	NES, COL1A, COL1A2, COL5A2, COL6A2, COL6A3, KRT78, FN1, GJA1, LAMA1, KRT3, SPP1, THBS1, VTN
	4	ECM-receptor interaction (hsa04512)	COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, LAMA1, HSPG2, SPP1, THBS1, VTN
	5	Purine metabolism (hsa00230)	ADCY5, TYMP, NT5E, PDE2A, PDE3B, PDE4A, PDE4B, PRPS2, GMPS, ENTPD1
CP	1	Unclassified	166 genes
	2	Focal adhesion (hsa04510)	COL4A2, COL6A1, CRK, FYN, ITGA6, LAMB2, LAMC1, PIK3CA, ZYX
	3	Regulation of actin cytoskeleton (hsa04810)	WASF2, BAIAP2, CRK, ITGA6, PIK3CA, TIAM1, MYH14, ARHGEF7
	4	Oxidative phosphorylation (hsa00190)	NDUFB6, NDUFB8, NDUFS5, ATP5I, ATP6V1F
	5	Cell communication (hsa01430)	COL4A2, COL6A1, ITGA6, LAMB2, LAMC1

The list of KEGG IDs of MS brain-lesion proteome was uploaded onto the 'Search Objects in Pathway' tool of the KEGG database. Top 2 for AP and top 4 for CAP and CP of human reference pathways relevant to the proteome data are shown with KEGG pathway IDs and the list of genes classified.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

pathways, such as oxidative phosphorylation and regulation of actin cytoskeleton (Table 1). Thus, the KEGG search suggested that the biological process of ECM and integrin-mediated cell adhesion and communication plays a role in chronic lesions of MS.

When the Entrez Gene IDs of the proteome were imported into the 'Gene Expression Data Analysis' tool of the PANTHER database, the vast majority of AP, CAP, or CP-specific proteins were not mapped on any PANTHER canonical pathways in comparison with a reference set of NCBI human genes (Table 2). However, PANTHER identified a statistically significant relationship between a set of CAP proteins and signaling pathways of chemokines and cytokines, integrin (Figure 1), muscarinic and nicotinic acetylcholine receptors (Table 2). PANTHER suggested an involvement of integrin signaling in CP, but identified no pathways relevant to AP (Table 2). Thus, the PANTHER search indicated that integrin signaling plays a role in both CAP and CP, whereas inflammation mediated by chemokine and cytokine signaling plays a predominant role in CAP.

Table 2 The molecular pathway relevant to MS brain-lesion proteome suggested by PANTHER search

Stage	Rank	Functional category	Number of genes classified	Human reference genes	*P*-value
AP	1	Unclassified	120	22436	6.89E-02 (NS)
CAP	1	Unclassified	321	22436	1,73E-04
	2	Inflammation mediated by chemokine and cytokine signaling pathway	17	315	2,63E-03
	3	Integrin signaling pathway	14	227	3,55E-03
	4	Muscarinic acetylcholine receptor 1 and 3 signaling pathway	7	62	1,17E-02
	5	Nicotinic acetylcholine receptor signaling pathway	8	91	2,03E-02
CP	1	Unclassified	182	22436	9,75E-03
	2	Integrin signaling pathway	9	227	4,33E-02

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing with a reference set of NCBI human genes. The canonical pathways relevant to the proteome data are shown with the number of genes classified and *P*-value evaluated by multiple comparison with a Bonferroni correction.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; and NS, not significant.

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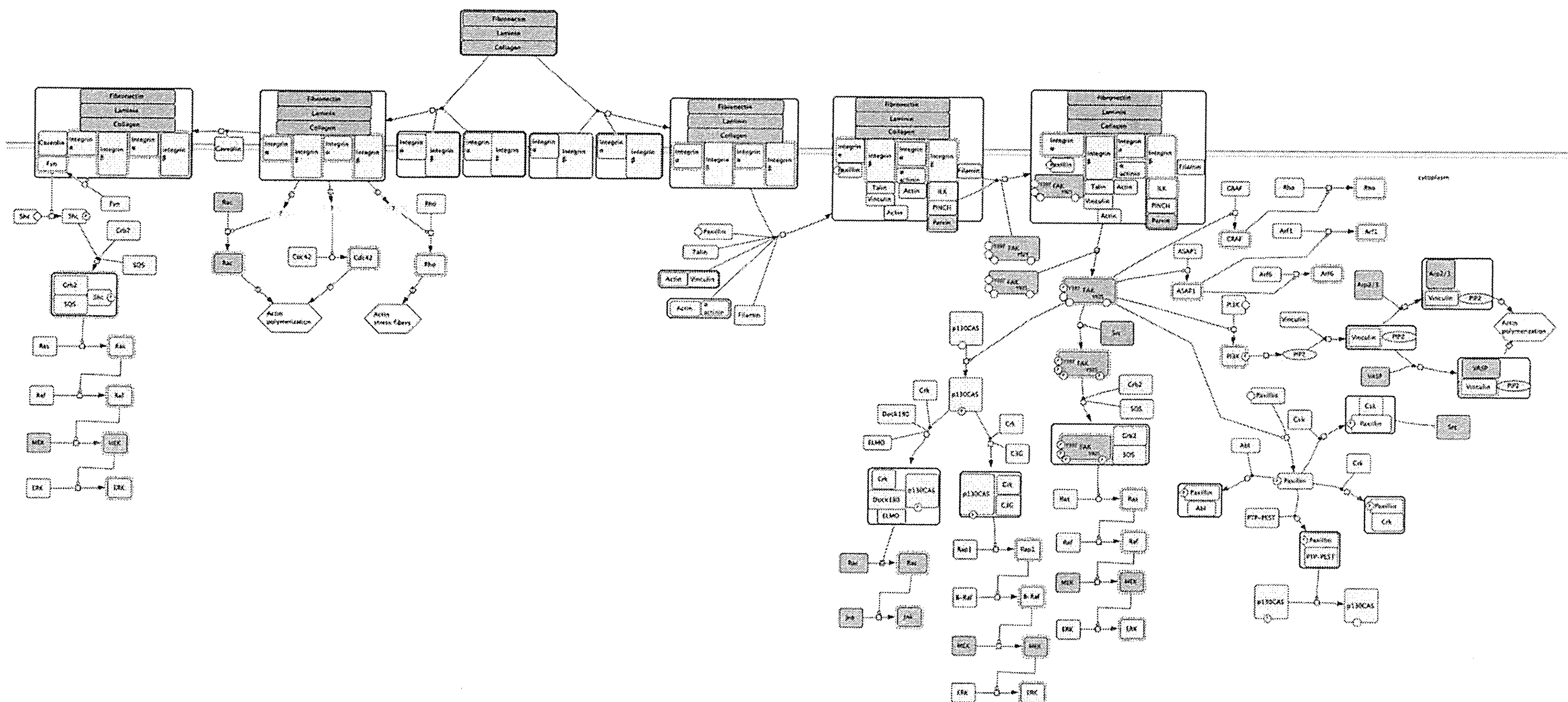

Figure 1 Integrin signaling pathway relevant to CAP proteome suggested by PANTHER. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'Gene Expression Data Analysis' tool of the PANTHER classification system by comparing them with a reference set of NCBI human genes. Integrin signaling pathway was identified as one of canonical pathways statistically relevant to the CAP proteome (Table 2). The pathway is illustrated as the map compatible with the Systems Biology Markup Language (SBML) standard. The molecules colored in pink represent those included in the gene list (Supplementary Table 2). They are composed of fibronectin (Gene symbol: FN1), laminin (LAMA1), collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), Rac (RAC3), MEK (MAP2K4), FAK (PTK2B), parvin (PARVA), Src (SRC), Jnk (MAP2K4), Arp2/3 (ARPC1A), and VASP (ENAH).

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KeyMolnet and IPA searches disclosed a role of the complex interaction of diverse intracellular signaling pathways in brain lesion development of MS

Next, we investigated molecular networks of MS brain proteome by utilizing two different commercial platforms. When the Entrez Gene IDs of the proteome were uploaded onto the "N-points to N-points" search tool of KeyMolnet, it extracted highly complex large-scale molecular networks of the AP, CAP, and CAP-specific proteome (Figure 2). The network of the AP, CAP, or CP proteome is composed of 777, 1,120, or 952 fundamental nodes with 1,892, 2,772, or 2,279 molecular relations, respectively. The statistical evaluation indicated that the top five most relevant molecular networks include IL-4, IL-6, IL-2, and catenin signaling pathways and transcriptional regulation by STAT (signal transducer and activator of transcription) for the AP proteome, PI3K, IL-4, type I IFN, and IL-6 signaling pathways and transcriptional regulation by STAT for the CAP proteome, and IL-4, hepatocyte growth factor (HGF), TCR (T cell receptor), integrin and IL-6 signaling pathways for the CP proteome (Table 3). It is worthy to note that the integrin signaling pathway was ranked as the sixth relevant pathway to the CAP proteome with *P*-value of the score = 2.13E-012. Considerable overlap existed in the results of PANTHER (Table 2) and KeyMolnet (Table 3). The KeyMolnet search disclosed a central role of the complex interaction of diverse cytokine signaling pathways in brain lesion development at all disease stages of MS, and the role of the integrin signaling pathway in both CAP and CP.

When the Entrez Gene IDs of the proteome were imported into the 'Core Analysis' tool of IPA, it highlighted several units of small-scale molecular networks relevant to the proteome data (Table 4). The network most relevant to the AP proteome was linked to the functional category of cellular assembly and organization, cancer, and cellular movement with the score *P*-value = 1.00E-49, where both ERK (extracellular signal-regulated kinase) and Akt (V-akt murine thymoma viral oncogene homolog) act as a hub of the network with highly connected molecular relations (Figure 3A). The network most relevant to the CAP proteome included two categories with the score *P*-value = 1.00E-47. One is the network of dermatological diseases and conditions, connective tissue disorders, and inflammatory disease. This network is constructed with various ECM components, including collagen, type I $\alpha 1$, type I, $\alpha 2$, type VI $\alpha 2$, type VI $\alpha 3$, fibronectin 1, fibulin 2, laminin $\alpha 1$, vitronectin, and heparan sulfate proteoglycan, where ERK acts as a hub (Figure 3B). The other is the network of lipid metabolism, molecular transport, and small molecule biochemistry, where Akt acts as a hub (Figure 3C). The network most relevant to the CP proteome was linked to cell cycle, cell morphology, and cell-to-cell signaling and interaction with the score *P*-value = 1.00E-50, where NF-κB (nuclear factor-kappa B) serves as a hub (Figure 3D). Overall, the biological processes involved in cellular assembly, organization, growth, proliferation, movement, and development are key functional categories shared by AP and CP molecular networks (Table 4). IPA also identified in the canonical pathways relevant to the proteome data. Both calcium signaling and oxidative phosphorylation were categorized as those relevant to AP and CAP proteome, whereas the actin cytoskeleton signaling pathway was considered as the important pathway in both CAP and CP (Table 5). Considerable overlap existed in the results of KEGG (Table 1) and IPA (Table 5).

Discussion

A recent proteomics study of MS lesion-specific proteome profiling clearly showed a pivotal role of coagulation cascade proteins in chronic active demyelination [8]. However, among thousands of proteins this study examined, nearly all of remaining proteins are left behind to be characterized in terms of their implications in MS brain-lesion development. The present study characterized molecular networks and pathways of the proteome data by using four different pathway analysis tools of bioinformatics. Although distinct platforms produced diverse results, they commonly suggested a role of ECM and integrin-mediated signaling as the pathway relevant to chronic lesion of MS. Therefore, these *in silico* observations warrant experimental validation.

In the CNS, ECM proteins provide a microenvironment for neurons and glial cells to maintain the ionic and nutritional homeostasis. They are localized chiefly to the vascular and the astroglial basement membranes and meninges but scarcely found in the brain parenchyma under physiological conditions. ECM proteins interact with integrins, the cell-surface ligands that support a physical link between ECM and cytoskeletal components [16]. Integrins consist of 24 pairs composed of noncovalently linked heterodimeric $\alpha\beta$ subunits. Although the interaction between integrins and ECM proteins is partially redundant, $\beta 1$ integrins are the principal ligand for collagen, fibronectin, and laminin, whereas αv integrins are the primary ligand for vitronectin. Integrins regulate the cytoskeletal rearrangement required for cell growth, movement, proliferation, and differentiation by transducing bidirectional signals in an 'inside-out' and 'outside-in' fashion [16]. Integrins, expressed on

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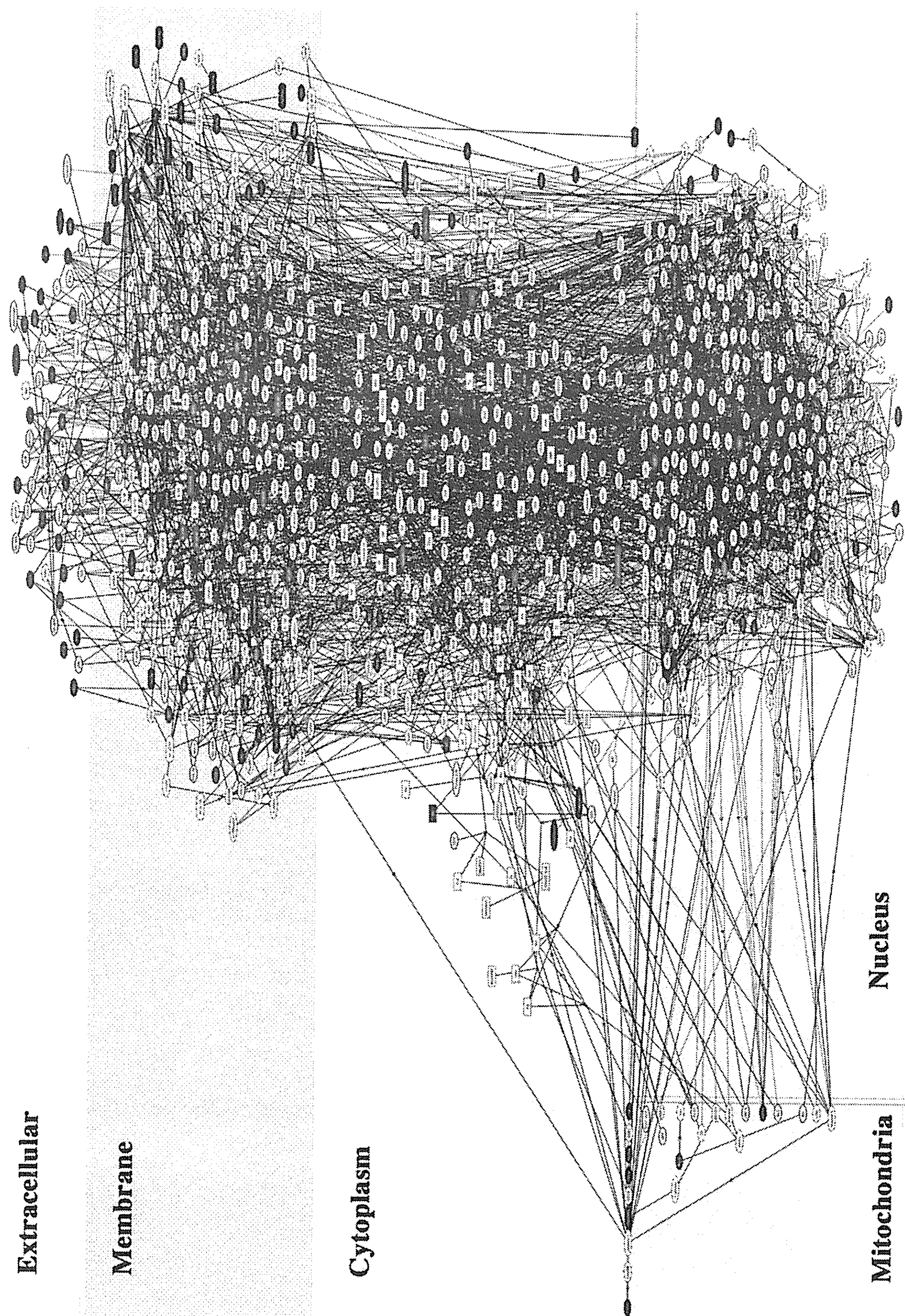

Figure 2 The molecular network of the CAP proteome suggested by KeyMolnet. The list of Entrez Gene IDs of CAP-specific proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. This generated a complex network composed of 1,120 fundamental nodes with 2,772 molecular relations, constructed by the shortest route connecting the start point of 75 MS-linked molecules of the KeyMolnet library (Supplementary Table 4)* and the end point of the CAP-specific proteome. The network is illustrated with respect to subcellular location of molecules. Red nodes represent start point molecules, whereas blue nodes represent end point molecules. Purple nodes express characteristics of both start and end point molecules. White nodes exhibit additional molecules extracted automatically from KeyMolnet core contents to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression). *Supplementary Tables 1–4 are available online at http://msj.sagepub.com/

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Table 3 The molecular network relevant to multiple sclerosis (MS) brain-lesion proteome suggested by KeyMolnet search

Stage	Rank	Functional category	Score	*P*-value
AP	1	IL-4 signaling pathway	42,324	1,794E-13
	2	IL-6 signaling pathway	40,966	4,656E-13
	3	IL-2 signaling pathway	36,684	9,059E-12
	4	Transcriptional regulation by STAT	32,789	1,347E-10
	5	Catenin signaling pathway	32,725	1,408E-10
CAP	1	PI3K signaling pathway	56,937	7,25E-18
	2	IL-4 signaling pathway	46,914	7,541E-15
	3	Transcriptional regulation by STAT	43,694	7,025E-14
	4	IFNα/βsignaling pathway	41,557	3,09E-13
	5	IL-6 signaling pathway	41,274	3,762E-13
CP	1	IL-4 signaling pathway	53,096	1,039E-16
	2	HGF signaling pathway	45,735	1,708E-14
	3	TCRα/βsignaling pathway	43,621	7,39E-14
	4	Integrin signaling pathway	38,501	2,572E-12
	5	IL-6 signaling pathway	38,115	3,359E-12

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'N-points to N-points search' tool of KeyMolnet. The molecular network is constructed by the shortest route connecting the start point of 75 MS-related molecules of the KeyMolnet library (Supplementary Table 4) and the end point of MS lesion–specific proteome. Top 5 networks relevant to the proteome data are shown with the score and *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; CP, chronic plaques; PI3K, phosphoinositide-3-kinase; and HGF, hepatocyte growth factor.

immune cells, act as an adhesion receptor for cell trafficking and serve as a scaffold for immunological synapses. By the KEGG search, we identified focal adhesion, cell communication, and ECM-receptor interaction as molecular pathways most relevant to the CAP proteome. They involve a wide range of ECM components, including collagen (COL1A1, COL1A2, COL5A2, COL6A2, COL6A3), fibronectin (FN1), laminin (LAMA1), vitronectin (VTN), heparan sulfate proteoglycan (HSPG2), thrombospondin (THBS1), parvin (PARVA), and osteopontin (SPP1). Furthermore, we found focal adhesion, regulation of actin cytoskeleton, and cell communication as the pathways involved in CP. They include collagen (COL4A2, COL6A1), laminin (LAMB2, LAMC1), and integrin (ITGA6). The relevance of

Table 4 The molecular network relevant to multiple sclerosis (MS) brain-lesion proteome suggested by IPA search

Stage	Rank	Functional category	The number of genes classified	*P*-value
AP	1	Cellular assembly and organization; cancer; cellular movement	24	1,00E-49
	2	Small molecule biochemistry; molecular transport; cellular assembly and organization	15	1,00E-26
	3	Cellular assembly and organization; cellular function and maintenance; skeletal and muscular system	14	1,00E-24
	4	Cellular development; cellular growth and proliferation; hematological system development and function	13	1,00E-22
	5	Cellular compromise; immune and lymphatic system development and function; hair and skin development and function	12	1,00E-19
CAP	1	Dermatological diseases and conditions; connective tissue disorders; inflammatory disease	29	1,00E-47
	2	Lipid metabolism; molecular transport; small molecule biochemistry	29	1,00E-47
	3	Cardiovascular disease; nephrosis; renal and urological disease	25	1,00E-38
	4	Endocrine system disorders; metabolic disease; renal and urological disease	25	1,00E-38
	5	Skeletal and muscular system development and function; tissue morphology; cardiovascular system development and function	22	1,00E-31
CP	1	Cell cycle; cell morphology; cell–cell signaling and interaction	27	1,00E-50
	2	Tissue morphology; cardiovascular disease; cellular development	24	1,00E-43
	3	Cellular assembly and organization; cell morphology; cellular movement	22	1,00E-38
	4	Cellular assembly and organization; cellular development; cellular growth and proliferation	18	1,00E-29
	5	Cell–cell signaling and interaction, Hematological system development and function; Immune and lymphatic system development and function	15	1,00E-22

The list of Entrez Gene IDs of MS brain-lesion proteome was uploaded onto the 'Core Analysis' tool of IPA. Top five molecular networks relevant to the proteome data are shown with the number of genes classified and the score *P*-value.
Abbreviations: AP, acute plaques; CAP, chronic active plaques; and CP, chronic plaques.

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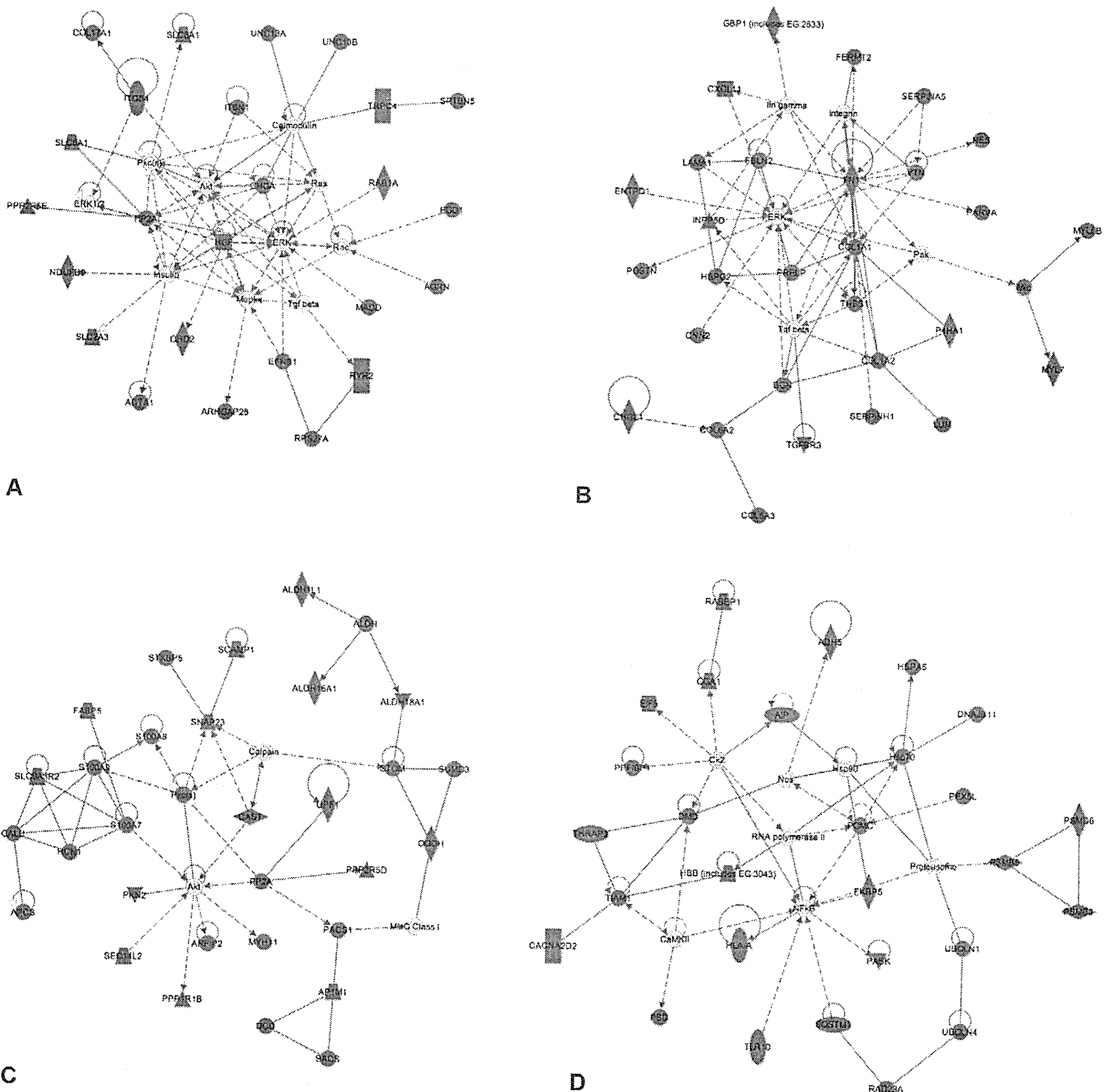

Figure 3 The molecular network of the AP, CAP, and CP proteome suggested by IPA. The list of Entrez Gene IDs of the MS lesion–specific proteome was uploaded onto the 'Core Analysis' tool of Ingenuity pathway analysis (IPA). Molecular networks most relevant to the AP (A), CAP (B and C), or CP (D) proteome are shown. Red nodes represent the molecules included in the gene list (Supplementary Tables 1–3). The molecular network (A) is constructed by 35 nodes, including ACTA1, AGRN, Akt, ARHGAP26, Calmodulin, CHD2, CHGA, COL17A1, EFNB1, ERK, ERK1/2, FGD1, HGF, insulin, ITGB4, ITSN1, MADD, Mapk, NDUFB9, Pkc(s), PP2A, PPP2R5E, RAB1A, Rac, Ras, RPS27A, RYR2, SLC2A3, SLC6A1, SLC8A1, SPTBN5, TGF-β, TRPC4, UNC13A, and UNC13B. The network (B) is constructed by 35 nodes, including BGN, CHI3L1, CNN2, COL1A1, COL1A2, COL6A2, COL6A3, CXCL11, ENTPD1, ERK, FBLN2, FERMT2, FN1, GBP1, HSPG2, IFN-γ, INPP5D, Integrin, LAMA1, LUM, Mlc, MYL7, MYL6B, NES, P4HA1, Pak, PARVA, POSTN, PRELP, SERPINA5, SERPINH1, TGF-β, TGFBR3, THBS1, and VTN. The network (C) is constructed by 35 nodes, including Akt, ALDH, ALDH16A1, ALDH18A1, ALDH1L1, AP1M1, APCS, ARFIP2, Calpain, CALU, CAST, DCD, FABP5, MHC Class I, MYH11, OGDH, PACS1, Pkc(s), PKN2, PP2A, PPP1R1B, PPP2R5D, RCN1, S100A7, S100A8, S100A9, SACS, SCAMP1, SEC14L2, SLC9A3R2, SNAP23, STOM, STXBP5, SUMO3, and UPF1. The network (D) is constructed by 35 nodes, including ADH5, AIP, CACNA2D2, CaMKII, Ck2, DMD, DNAJB11, EIF5, FKBP5, GGA1, HBB, HLA-A, Hsp70, Hsp90, HSPA6, NFkB, Nos, PASK, PEX5L, POMC, PPFIBP1, Proteasome, PSD, PSMB3, PSMB5, PSMD6, RABEP1, RAD23A, RNA polymerase II, SQSTM1, THRAP3, TIAM1, TLR10, UBQLN1, and UBQLN4. The molecular relation is indicated by solid line (direct interaction), dash line (indirect interaction), with filled arrow (acts on), stop (inhibits), stop and filled arrow (inhibits and acts on), and open arrow (translocates to).

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