

vitro and in vivo (Narantuya et al. 2010). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (feeding medium). Human neural and non-neural cell lines other than HMO6 were described elsewhere. LDH release from cultured cells was assessed by using a LDH cytotoxicity detection kit (Takara Bio, Shiga, Japan).

Chemicals

Non-phosphorylated FTY720 (FTY720-non-P; Calbiochem, Darmstadt, Germany) and (*S*)-FTY720 phosphate (FTY720-P; Echelon Biosciences, Salt Lake City, UT, USA) were usually dissolved in dimethyl sulfoxide (DMSO), providing the stock solution at the concentration of 10 mM. For negative controls, the inclusion of DMSO at the concentration of 0.1% v/v (1:1000 dilution) was applied. We found that the solvent alone never induces apoptosis of HMO6 at any incubation time. Sphingosine 1-phosphate (S1P) was obtained from Sigma, St. Louis, MO, USA. SEW2871, a selective S1P1 agonist and W123, a competitive S1P1 antagonist were obtained from Cayman Chemical, Ann Arbor, MI, USA. Suramin, a S1P3/S1P5 inhibitor, Z-DQMD-FMK, a caspase-3 inhibitor, and simvastatin, a HMG-CoA reductase inhibitor were obtained from Calbiochem. Pertussis toxin (PTX), a Gi protein inhibitor, was obtained from Seikagaku Biobusiness, Tokyo, Japan.

RT-PCR Analysis

Total cellular RNA was extracted by using TRIZOL (Invitrogen). RNA treated with DNase I was processed for cDNA synthesis using oligo(dT)₂₀ primers and SuperScript II reverse transcriptase (Invitrogen). Then, cDNA was amplified by PCR using HotStar Taq DNA polymerase (Qiagen, Valencia, CA, USA) and a panel of sense and antisense primer sets following: 5'aagcgtcttacttggtcgtgg3' and 5'tgatctccacccttccagtgcat3' for an 189 bp product of S1P1; 5'ccacagacctgggtgatgttg3' and 5'tccccttaaatgctgctgcc3' for a 200 bp product of S1P2; 5'actttgggtccagagctcttc3' and 5'cattctacgcacaggaaatgtagtg3' for an 193 bp product of S1P3; 5'gttgagtcttgcgtgtggatgg3' and 5'ggtgacatgggaagcccatttg3' for an 183 bp product of S1P4; 5'aggaaatggcatgcgcaaag3' and 5'ctctatggtctccacctcactc3' for a 200 bp product of S1P5; and 5'ccatgttcgtcatgggtgtaacca3' and 5'gccagtagaggcaggatgatgttc3' for a 251 bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene.

For quantitative real-time RT-PCR (qPCR) analysis, cDNA was amplified by PCR in LightCycler ST300

(Roche Diagnostics, Tokyo, Japan) using SYBR Green I and a panel of sense and antisense primer sets with the following: 5'tgatcgttccagaagtggccttg3' and 5'aactgtcgtcctatgttccccacc3' for an 186 bp product of insulin-induced gene 1 (INSIG1) and 5'ctgggggtctccttctatggaag3' and 5'cacgtcatcctccagactgacat3' for an 168 bp product of low density lipoprotein receptor (LDLR). The expression levels of target genes were standardized against the levels of G3PDH, an internal control, detected in corresponding cDNA samples. All the assays were performed in triplicate.

Microarray Analysis

For microarray analysis, total cellular RNA was isolated by using the TRIZOL Plus RNA Purification kit (Invitrogen). The quality of total RNA was evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). One hundred ng of total RNA was processed for cRNA synthesis, fragmentation, and terminal labeling with the GeneChip Whole Transcript Sense Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA). Then, it was processed for hybridization at 45°C for 17 h with Human Gene 1.0 ST Array that contains 28,869 genes (Affymetrix). The arrays were washed in the GeneChip Fluidic Station 450 (Affymetrix), and scanned by the GeneChip Scanner 3000 7G (Affymetrix). The raw data were expressed as CEL files and normalized by the robust multiarray average (RMA) method with the Expression Console software version 1.1 (Affymetrix). The annotation was studied by searching genes on the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (david.abcc.ncifcrf.gov) (da Huang et al. 2009).

Molecular Network Analysis

KeyMolnet is a comprehensive knowledgebase that contains the contents on 123,000 relationships among human genes and proteins, small molecules, diseases, pathways and drugs, regularly updated, and curated by expert biologists (Satoh et al. 2009). By importing the list of Entrez Gene IDs derived from microarray data, KeyMolnet automatically provides corresponding molecules as a node on networks. Among various network-searching algorithms, the "neighboring" network-search algorithm selected one or more molecules as starting points to generate the network of all kinds of molecular interactions around starting molecules, including direct activation/inactivation, transcriptional activation/repression, and the complex formation within the designated number of paths from starting points. The generated network was compared side by side with 430 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, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p)) \quad \text{Score}(p) = \sum_{x=0}^{\text{Min}(C,V)} f(x)$$

$$f(x) = \frac{C \cdot C_x \cdot T - C \cdot C_{V-x}}{T \cdot C_V}$$

Transient Expression of SREBP2

To transiently overexpress sterol regulatory element-binding protein-2 (SREBP2), the gene encoding the N-terminal fragment of SREBP2 spanning amino acid residues 1–484 was amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and a sense and antisense primer set of 5'gcatgggacgacagcggcgagctg3' and 5'tcacagaagaatcgtgagcggctc3', and cloned in the expression vector pEF6 (Invitrogen). The vector was transfected into HMO6 cells by X-tremeGENE HP DNA transfection reagent (Roche Diagnostics). At 24 h after transfection, the cells were processed for western blot analysis. For the control, V5-tagged LacZ cloned in the pEF6 vector was transfected into sister cultures.

Western Blot Analysis

To prepare total protein extract, the cells were homogenized in RIPA buffer supplemented with a cocktail of protease inhibitors (Sigma). The protein extract was centrifuged at 12,000 rpm for 5 min at room temperature (RT). The protein concentration was determined by a Bradford assay kit (BioRad Hercules, CA, USA). The mixture of the supernatant and a 2× Lammeli loading buffer was boiled and separated on SDS-PAGE gels ranging from 8 to 12%. After gel electrophoresis, the protein was transferred onto nitrocellulose membranes, and immunolabeled at RT overnight with rabbit anti-poly-ADP-ribose-polymerase (PARP) antibody (#11835238001; Roche Diagnostics), rabbit anti-cleaved caspase-3 (Asp175) antibody (#9661; Cell Signaling Technology, Danvers, MA, USA), mouse anti-caspase-7 antibody (#9494; Cell Signaling Technology), rabbit anti-caspase-9 antibody (#9502; Cell Signaling Technology), rabbit anti-S1P1 antibody (sc-25489, EDG-1, H-60; Santa Cruz Biotechnology, Santa Cruz, CA), or goat anti-SREBP2 antibody (sc-8151, N-19; Santa Cruz

Biotechnology). Then, the membranes were incubated at RT for 60 min with HRP-conjugated anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by exposing the membranes to a chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA).

In some experiments, the antibodies were stripped by incubating the membranes at 50°C for 30 min in stripping buffer, composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol. Then, the membranes were processed for relabeling with goat anti-heat shock protein HSP60 antibody (sc-1052, N-20; Santa Cruz Biotechnology) used for an internal control of protein loading, followed by incubation with HRP-conjugated anti-goat IgG.

Results

S1P Receptor Expression on Human Microglia Cell Line HMO6

The expression of five S1P receptor mRNAs in a panel of human neural cells and tissues was determined by RT-PCR. All the cells and tissues examined, including the human cerebrum (CBR), fetal astrocytes (AS), neuronal progenitor (NP) cells, NTera2 teratocarcinoma-derived neurons, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astroglioma, and the microglia cell line HMO6, expressed varying levels of S1P1, S1P2, and S1P3 mRNAs, except for Y79 retinoblastoma that did not express S1P1 (Fig. 1a–c, lanes 2–10). In contrast, the levels of G3PDH, a housekeeping gene, were almost constant in the cells and tissues examined (Fig. 1f, lanes 2–10). Although discernible levels of S1P4 and S1P5 mRNAs were identified in the human cerebrum (CBR), both of these mRNAs were almost undetectable in HMO6 (Fig. 1d, e, lanes 2 and 10). No products were amplified when the reverse transcription step is omitted (Fig. 1a–f, lane 1). We verified S1P1 protein expression in HMO6 by western blot (not shown).

Non-Phosphorylated FTY720 Induced Apoptosis of HMO6

A 6 h-exposure of non-phosphorylated FTY720 (FTY720-non-P) induced LDH release from HMO6 cells and cell death in a dose-dependent manner with IC50 of $10.6 \pm 2.0 \mu\text{M}$ (Fig. 2a, c). It is worthy to note that the concentration of FTY720-non-P at lower than $5 \mu\text{M}$ was completely ineffective in inducing cell death of HMO6 (Fig. 2a). Generally, LDH release did not discriminate apoptotic and necrotic cell death. The exposure of FTY720-non-P at a concentration of $10 \mu\text{M}$ mediated the cleavage of PARP in the incubation time longer than 4 h,

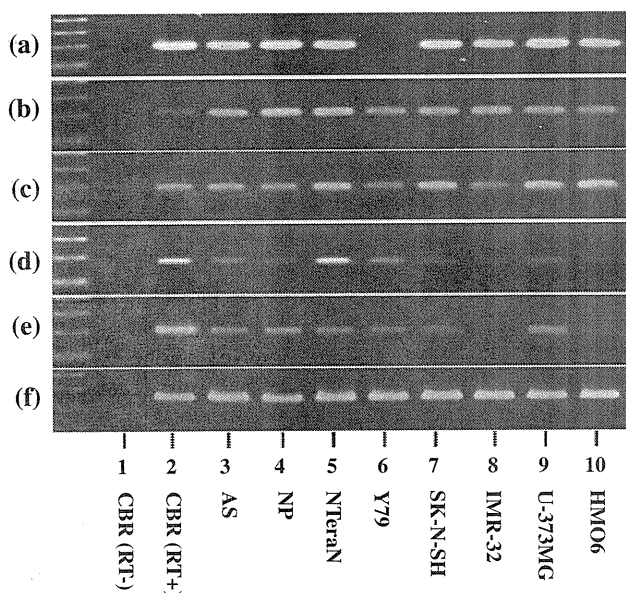


Fig. 1 S1P receptor expression in human neural cell lines. The expression of five S1P receptor mRNAs was studied by RT-PCR. **a** S1P1, **b** S1P2, **c** S1P3, **d** S1P4, **e** S1P5, and **f** G3PDH. The lanes (1–10) represent (1) the human frontal cerebral cortex (CBR) without inclusion of the reverse transcription (RT) step, (2) CBR with inclusion of the RT step, (3) cultured astrocytes (AS), (4) cultured neuronal progenitor (NP) cells, (5) NTera2 teratocarcinoma-derived neurons (NTera2N), (6) Y79 retinoblastoma, (7) SK-N-SH neuroblastoma, (8) IMR-32 neuroblastoma, (9) U-373MG astrocytoma, and (10) HMO6 microglia. The 100 bp ladder marker is shown on the left

indicating that FTY720-non-P induced cell death of HMO6 via apoptosis (Fig. 2d, lanes 7–10).

FTY720-non-P-induced apoptosis of HMO6 was accompanied by the cleavage of caspase-7 and caspase-3 (Fig. 3b, c, lane 2) but not of caspase-9 (Fig. 3e, lane 4), suggesting that the mitochondrial pathway of apoptosis that usually activates caspase-9 did not play a major role. Furthermore, Z-DQMD-FMK, a caspase-3-specific inhibitor, completely blocked FTY720-non-P-induced apoptosis of HMO6 (Fig. 3g, h, lane 10).

FTY720-Induced Apoptosis of HMO6 was Independent of S1P Receptor Binding

Because FTY720, when phosphorylated, binds to S1P1, S1P3, S1P4, and S1P5, all of which are G protein-coupled receptors (GPCR), we utilized Pertussis toxin (PTX), a Gi protein inhibitor, suramin, a S1P3/S1P5 inhibitor, and W123, a S1P1 competitive antagonist to block the ligand-receptor interaction. However, none of these receptor blockers could inhibit FTY720-induced apoptosis of HMO6 (Fig. 4a, lanes 4, 6, 8). Furthermore, SEW2871, a S1P1 selective agonist, and phosphorylated FTY720 (FTY720-P) at a concentration of 10 μ M each did not induce apoptosis of HMO6 during the incubation time of

12 h (Fig. 4c, lanes 11 and 12). In addition, the combined administration of FTY720-P (10 μ M) and FTY720-non-P (10 μ M) did not inhibit apoptosis of HMO6, and treatment with sphingosine-1 phosphate (S1P) (10–50 μ M) did not induce apoptosis of HMO6 (data not shown). These results suggest that FTY720-non-P-induced apoptosis of HMO6 was independent of S1P receptor binding, and both FTY720-P and S1P were incapable of inducing apoptosis of HMO6.

FTY720 Induced SREBP-Responsive Genes

To investigate the molecular mechanism responsible for triggering FTY720-non-P-induced apoptosis of HMO6, we studied the genome-wide gene expression profile by microarray analysis. We identified 30 genes with an over 2-fold increase in HMO6 cells treated for 2 h with 10 μ M FTY720-non-P versus those exposed to the vehicle (DMSO) (Table 1). Among them, the DAVID program categorized seven genes as a group of the genes associated with steroid and/or sterol metabolism (Table 1). None of apoptosis initiator and executor genes were induced in HMO6 cells at 2 h after initiation of the treatment. Upregulated expression of INSIG1 and LDLR in FTY720-non-P-treated HMO6 cells was validated by qPCR analysis (Fig. 5a, b).

Next, we imported the list of Entrez Gene IDs of the 30 genes upregulated in FTY720-non-P-treated HMO6 cells into KeyMolnet, a tool for analyzing molecular interactions on the comprehensive knowledgebase. KeyMolnet generated the molecular network, presenting with the most significant relationship with transcriptional regulation by sterol regulatory element-binding protein (SREBP). (the score = 69.719 with the P -value = 1.029E–21) (Fig. 5c). These results suggest that in HMO6 cells, FTY720-non-P activates SREBP proteins, either SREBP1 or SREBP2, belonging to the bHLH-Zip transcription factor family that promotes the synthesis of enzymes involved in cholesterol and fatty acid biosynthesis. To exclude a direct effect of vehicle (DMSO), in which FTY720-non-P was dissolved, on gene expression, we performed an additional set of microarray experiment by exposing HMO6 cells to FTY720-non-P dissolved in ethanol. We again identified the similar gene expression profile composed of upregulation of key SREBP-target genes, regardless of the solvent (See Table 1 in Electronic Supplementary Material).

SREBP2 is primarily involved in cholesterol synthesis, while SREBP1 chiefly regulates fatty acid synthesis (Sato 2010). INSIG1 identified by microarray analysis encodes an ER protein that plays a pivotal role in regulating intracellular cholesterol levels by interacting with SREBP cleavage-activating protein (SCAP) having the sterol-sensing domain activated by reduced cellular cholesterol levels. Thereafter, we have focused on SREBP2 expression

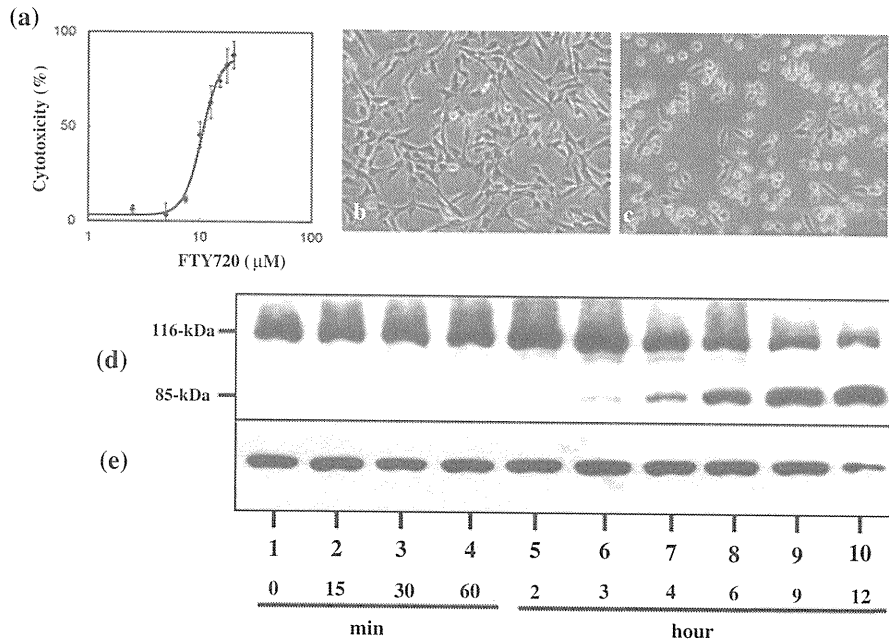


Fig. 2 Non-phosphorylated FTY720 induced apoptosis of HMO6 cells. HMO6 cells were exposed for various time periods to varying concentrations of non-phosphorylated FTY720 (FTY720-non-P). **a** LDH release assay, **b** the phase contrast photomicrograph of the cells exposed for 6 h to vehicle (DMSO), **c** the phase contrast photomicrograph of the cells exposed for 6 h to 10 μM FTY720-non-P,

d western blot of PARP (an 116-kDa uncleaved form and an 85-kDa cleaved form), and **e** western blot of HSP60, an internal control of protein loading. The lanes (1–10) represent (1) untreated HMO6 cells, and HMO6 cells treated for (2) 15 min, (3) 30 min, (4) 1 h, (5) 2 h, (6) 3 h, (7) 4 h, (8) 6 h, (9) 9 h, and (10) 12 h with 10 μM FTY720-non-P

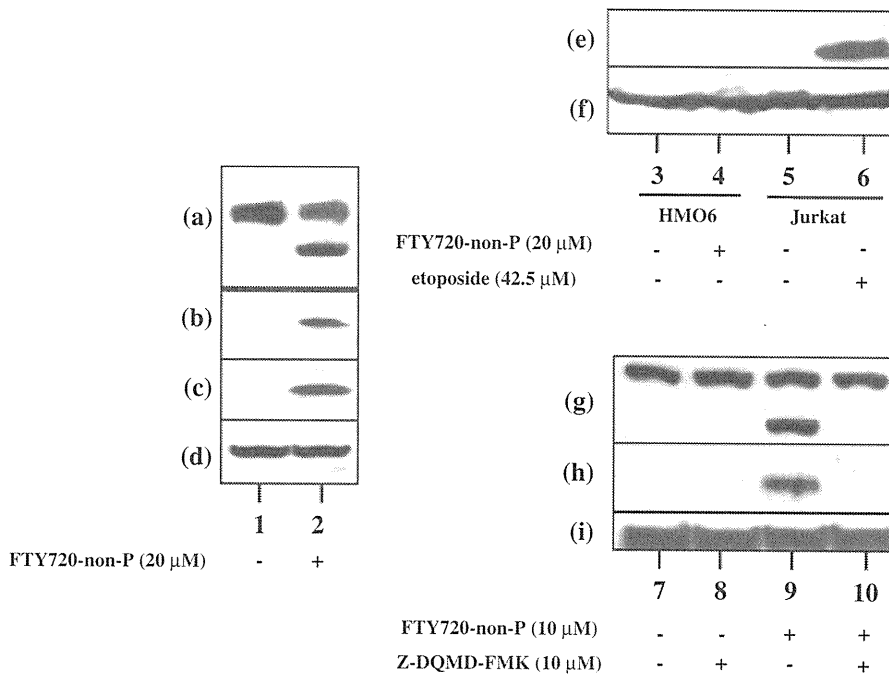


Fig. 3 FTY720-induced apoptosis of HMO6 was accompanied by activation of caspases 3 and 7. HMO6 cells were exposed for various time periods to varying concentrations of FTY720-non-P. For the positive control of caspase-9 activation, Jurkat cells were exposed to etoposide, an apoptosis-inducing agent. **a–i** indicate western blot of **a**, **g** PARP, **b** caspase-7 (a 20-kDa cleaved form), **c**, **h** caspase-3 (a 19-kDa cleaved form), **e** caspase-9 (a 37-kDa cleaved form), and **d**, **f**,

i HSP60, an internal control of protein loading. The lanes (1–10) indicate HMO6 cells treated with (1, 3) vehicle (DMSO) and (2, 4) 20 μM FTY720-non-P for 9 h, and Jurkat cells treated with (5) vehicle (DMSO) and (6) 42.5 μM etoposide for 6 h, and (7) untreated HMO6 cells, and HMO6 cells treated with (8) 10 μM Z-DQMD-FMK, (9) 10 μM FTY720-non-P, and (10) a combination of 10 μM Z-DQMD-FMK and 10 μM FTY720-non-P for 12 h

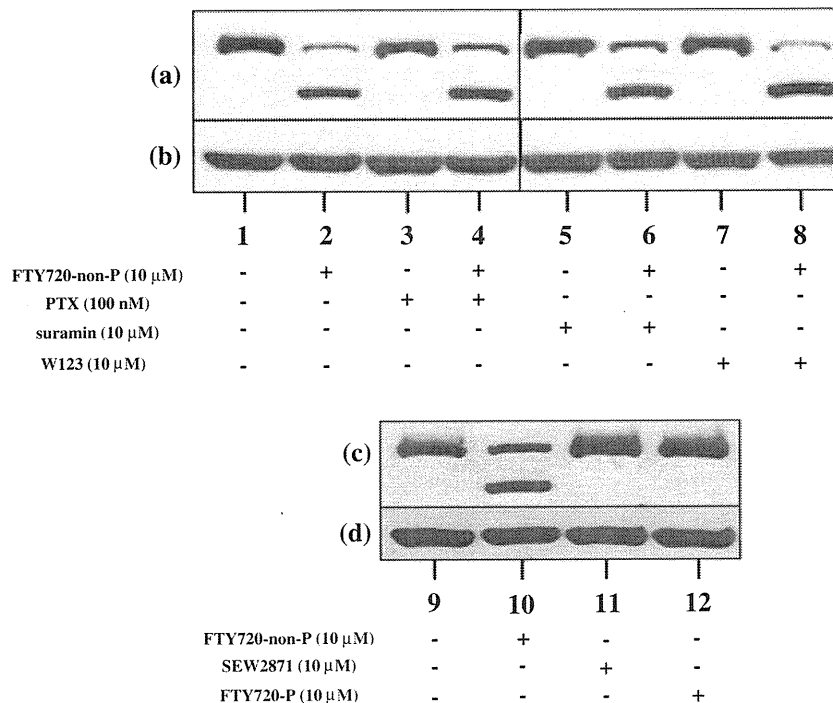


Fig. 4 FTY720-induced apoptosis of HMO6 was independent of S1P receptor binding. HMO6 cells were exposed for 12 h to 10 μM FTY720-non-P with or without inclusion of various S1P receptor agonists and antagonists. Pretreatment started at 30 min before exposure to FTY720-non-P. **a–d** indicate western blot of **a**, **c** PARP and **b**, **d** HSP60, an internal control of protein loading. The lanes (1–12) indicate (1, 9) untreated HMO6 cells, and HMO6 cells treated with (2, 10) 10 μM FTY720-non-P exposure alone, (3) 100 nM

pertussis toxin (PTX) pretreatment alone, (4) 100 nM PTX pretreatment and 10 μM FTY720-non-P exposure, (5) 10 μM suramin pretreatment alone, (6) 10 μM suramin pretreatment and 10 μM FTY720-non-P exposure, (7) 10 μM W123 pretreatment alone, (8) 10 μM W123 pretreatment and 10 μM FTY720-non-P exposure, and HMO6 cells treated with a 12 h-exposure to (11) 10 μM SEW2871 or (12) 10 μM phosphorylated FTY720 (FTY720-P)

in HMO6 cells. The N-terminal fragment of SREBP2 is cleaved, dimerized, and translocated to the nucleus in response to the activating stimuli (Sato 2010). We identified the cleaved form of SREBP2 in HMO6 cells following an 1 h-exposure to FTY720-non-P ranging from 10 to 20 μM or by treatment with 3 μM simvastatin, a HMG-CoA reductase inhibitor capable of activating SREBP2 (Fig. 6a, lanes 2–4). Neither FTY720-non-P nor simvastatin alone at a concentration of 5 μM each induced apoptosis of HMO6 (Fig. 2a; Fig. 6b, lanes 7, 8). In contrast, a 12 h-pretreatment with 5 μM simvastatin enhanced FTY720-non-P-induced apoptosis of HMO6 cells, suggesting a proapoptotic effect mediated by SREBP2 activation following simvastatin treatment (Fig. 6b, lane 10).

A recent study showed that statins activate SREBP2, which positively controls the expression of caspase-7, resulting in induction of apoptosis of human gastric cancer cells (Gibot et al. 2009). When the N-terminal fragment of SREBP2 was overexpressed in HMO6 cells, the cleavage of PARP and caspase-3 was greatly enhanced, compared with the cells with overexpression of LacZ (Fig. 6c, f, lane 12), while the levels of procaspase-7 and cleaved caspase-7 were unaltered (Fig. 6g, lane 12).

Discussion

The present study revealed that non-phosphorylated FTY720 (FTY720-non-P) induced apoptosis of human microglia HM06 in a time- and dose-dependent manner with IC50 of 10.6 ± 2.0 μM. The apoptosis was inhibited by Z-DQMD-FMK, a caspase-3 inhibitor, but not by Pertussis toxin, a Gi protein inhibitor, suramin, a S1P3/S1P5 inhibitor, or W123, a S1P1 competitive antagonist, although HMO6 expressed S1P1, S1P2, and S1P3. Furthermore, both phosphorylated FTY720 (FTY720-P) and SEW2871, S1P1 selective agonists, did not induce apoptosis of HMO6. These observations suggest that FTY720-non-P-induced apoptosis of HMO6 cells is independent of S1P receptor binding.

Supporting these observations, FTY720, serving as a potential anti-cancer agent, induces apoptosis of various human cancer cell lines derived from liver, kidney, pancreas, and breast, multiple myeloma and leukemia cells, which is often mediated by S1P receptor-independent mechanisms (Matsuoka et al. 2003; Lee et al. 2004; Liu et al. 2010; Nagaoka et al. 2008). The concentrations required to induce apoptosis of tumor cells in vitro are about

Table 1 Upregulated genes in HMO6 following treatment with FTY720-non-P

Rank	Fold change	Entrez gene ID	Gene symbol	Gene name
1	5.75	25774	GSTTP1	Glutathione S-transferase theta pseudogene 1
2	3.16	150527	LOC150527	Hypothetical LOC150527
3	2.78	728380	RPL7P26	Ribosomal protein L7 pseudogene 26
4	2.74	3638	<u>INSIG1</u>	Insulin induced gene 1
5	2.72	158160	HSD17B7P2	Hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2
6	2.70	3157	<u>HMGCS1</u>	3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble)
7	2.68	346007	EYS	Eyes shut homolog (Drosophila)
8	2.48	26834	RNU4-2	RNA, U4 small nuclear 2
9	2.46	163720	CYP4Z2P	Cytochrome P450, family 4, subfamily Z, polypeptide 2 pseudogene
10	2.44	54541	DDIT4	DNA-damage-inducible transcript 4
11	2.41	6351	CCL4	Chemokine (C-C motif) ligand 4
12	2.39	3949	<u>LDLR</u>	Low density lipoprotein receptor
13	2.37	6307	<u>SC4MOL</u>	Sterol-C4-methyl oxidase-like
14	2.34	286359	LOC286359	Hypothetical LOC286359
15	2.30	391003	PRAMEF18	PRAME family member 18
16	2.29	23175	LPIN1	Lipin 1
17	2.25	54897	CASZ1	Castor zinc finger 1
18	2.16	3156	<u>HMGCR</u>	3-hydroxy-3-methylglutaryl-coenzyme A reductase
19	2.16	196335	OR56B4	Olfactory receptor, family 56, subfamily B, member 4
20	2.10	3283	<u>HSD3B1</u>	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
21	2.09	8553	BHLHE40	Basic helix-loop-helix family, member e40
22	2.07	10517	FBXW10	F-box and WD repeat domain containing 10
23	2.06	256892	OR51F1	Olfactory receptor, family 51, subfamily F, member 1
24	2.05	4598	<u>MVK</u>	Mevalonate kinase
25	2.04	196074	METT5D1	Methyltransferase 5 domain containing 1
26	2.04	901	CCNG2	Cyclin G2
27	2.03	439927	C1orf180	Chromosome 1 open reading frame 180
28	2.01	10551	AGR2	Anterior gradient homolog 2 (<i>Xenopus laevis</i>)
29	2.01	91074	ANKRD30A	Ankyrin repeat domain 30A
30	2.00	1831	TSC22D3	TSC22 domain family, member 3

HMO6 cells were exposed to non-phosphorylated FTY720 (10 μ M) or vehicle (DMSO) for 2 h. The genome-wide transcriptome was studied on Human Gene 1.0 ST array. The genes with an over 2-fold increase in FTY720-non-P-treated HMO6 cells are listed. The genes associated with steroid and/or sterol metabolism annotated by the DAVID program are underlined

two orders of magnitude greater than the blood concentration in the clinical setting, i.e., 5.4 ng/ml in plasma (Brinkmann et al. 2001, 2010). FTY720 has a half-life of approximately 10 days in vivo, and is cleared predominantly by a metabolic pathway requiring cytochrome P450 4F2 (CYP4F2) (Jin et al. 2011). The enzymatic activity of CYP4F2 is inhibited by certain drugs like ketoconazole, and the gene encoding CYP4F2 has a variety of single nucleotide polymorphisms (SNPs) (www.ncbi.nlm.nih.gov/snp). Therefore, in poor metabolizers of FTY720 receiving a CYP4F2 inhibitor, if they exist, the blood concentration of FTY720 could increase up to the range of toxic levels.

FTY720-non-P goes through the plasma membrane without requirement of the receptor binding, and targets

directly key intracellular enzymes involved in sphingolipid metabolism, such as sphingosine kinases, phospholipase A2, and S1P lyase (Bandhuvula et al. 2005). FTY720 also inhibits ceramide synthases, resulting in a decrease in cellular levels of ceramide, dihydroceramide, shingosine, and S1P, and an increase in dihydroshingosine and dihydroshingosine-1-phosphate, all of which alter the endogenous balance between survival and apoptotic signals (Berdyshev et al. 2009). FTY720-non-P promotes phosphorylation of 14-3-3zeta on Ser58 that disrupts 14-3-3 dimer formation, resulting in releasing proapoptotic mediators (Woodcock et al. 2010). FTY720, phosphorylated by SPHK2 located inside the plasma membrane, is transported outside the cells via the S1P transporter named spinstar

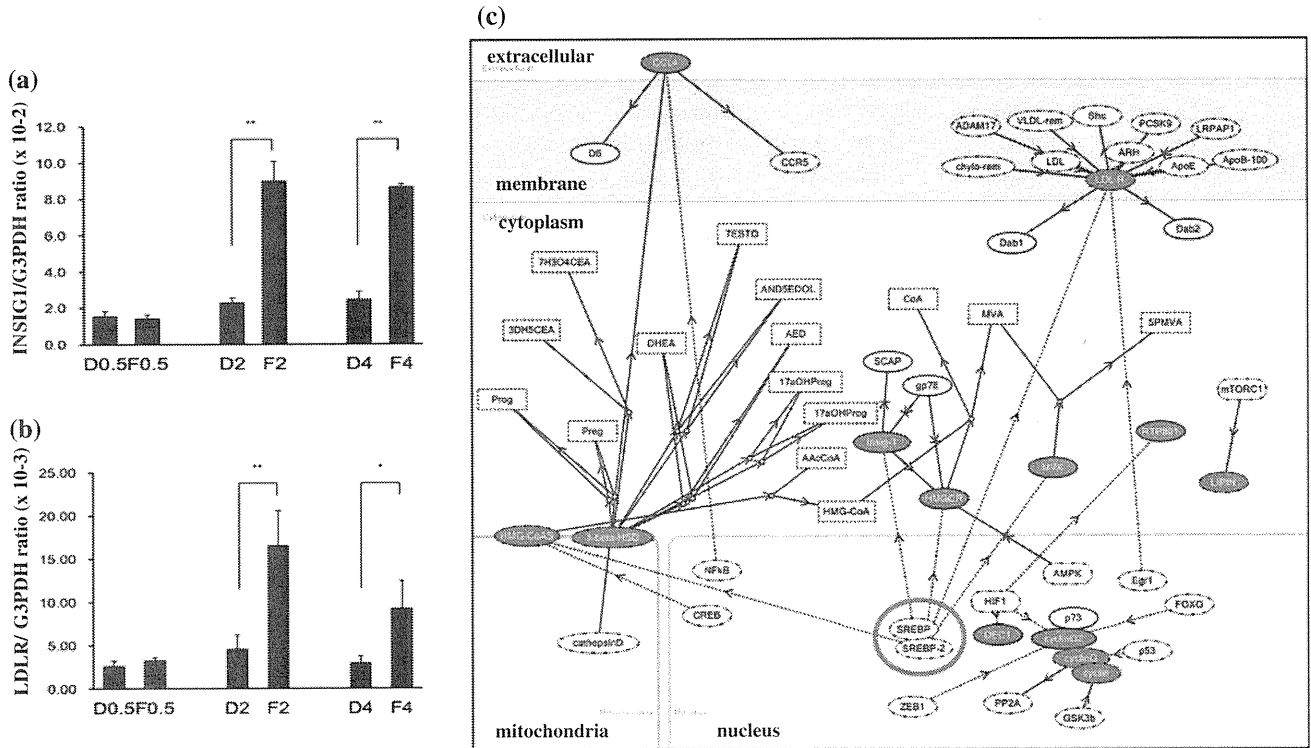


Fig. 5 FTY720 Induced SREBP-Responsive Genes. HMO6 cells were exposed for 2 h to 10 μ M FTY720-non-P or vehicle (DMSO). Then, total RNA was processed for the genome-wide gene expression profiling on a microarray, followed by molecular network analysis by KeyMolnet and validation by qPCR. We identified 30 upregulated genes in FTY720-non-P-treated HMO6 cells (Table 1). **a–c** indicate qPCR of **a** INSIG1 and **b** LDLR, and **c** molecular network of FTY720-non-P-induced genes. Abbreviations: D, vehicle (DMSO); F, FTY720-non-P; 0.5, 30 min; 2, 2 h; and 4, 4 h. In **c**, red filled nodes

represent FTY720-non-P-induced genes, whereas white open nodes exhibit additional nodes extracted automatically from the core contents of KeyMolnet 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). The transcription factor SREBP (SREBP2) is highlighted by a red thick circle

homolog 2 (SPNS2), and then the phosphorylated FTY720 binds to S1P receptors expressed on the surface of the plasma membrane (Hisano et al. 2011).

Being consistent with our observations, FTY720-non-P but not FTY720-P induces apoptosis of human breast and colon cancers (Nagaoka et al. 2008). FTY720 inhibits cytosolic phospholipase A2 (cPLA₂) in a manner independent of S1P receptor binding (Payne et al. 2007). FTY720-non-P but not FTY720-P inhibits PKC activation, which is associated with cell-surface expression of S1P1 (Sensken and Gräler 2010). Furthermore, FTY720-P counteracts FTY720-non-P-induced apoptosis of human fibroblasts by activating Bcl-2 (Potteck et al. 2010).

Several previous studies showed that FTY720-induced apoptosis is often accompanied by activation of a series of caspases (Wang et al. 1999). We found activation of both caspase-3 and caspase-7 during FTY720-non-P-induced apoptosis of HMO6. Furthermore, FTY720-induced apoptosis also involves various mechanisms, such as dephosphorylation of protein kinase B (Akt) (Matsuoka et al. 2003; Lee et al. 2004), deregulation of mitogen-activated

protein kinases (MAPKs), focal adhesion kinase (FAK), and Rho-GTPase (Matsuda et al. 1999; Sonoda et al. 2001), and activation of protein phosphatase 2A (PP2A) (Liu et al. 2010). Here, we for the first time showed that FTY720-non-P-induced apoptosis of HMO6 cells is positively regulated by the SREBP2-dependent signaling pathway.

A recent study showed that statins induce apoptosis of human gastric cancer cells by activating SREBP1 and SREBP2, both of which transcriptionally upregulate caspase-7 (Gibot et al. 2009). Statin-dependent apoptosis is prevented by replenishment of mevalonate, the immediate product by the HMG-CoA reductase activity (Xia et al. 2001). A previous study showed that activation of caspase-3 releases SREBP proteins from ER membrane in a proteolytic reaction distinct from the sterol-regulated cleavage, resulting in nuclear transport of SREBP and transcriptional activation of sterol-regulatory genes (Higgins and Ioannou 2001). However, during FTY720-non-P-induced apoptosis of HMO6 cells, we identified activation of SREBP2 as early as at 1 h after initiation of the treatment, which is long before detection of the PARP cleavage, suggesting that

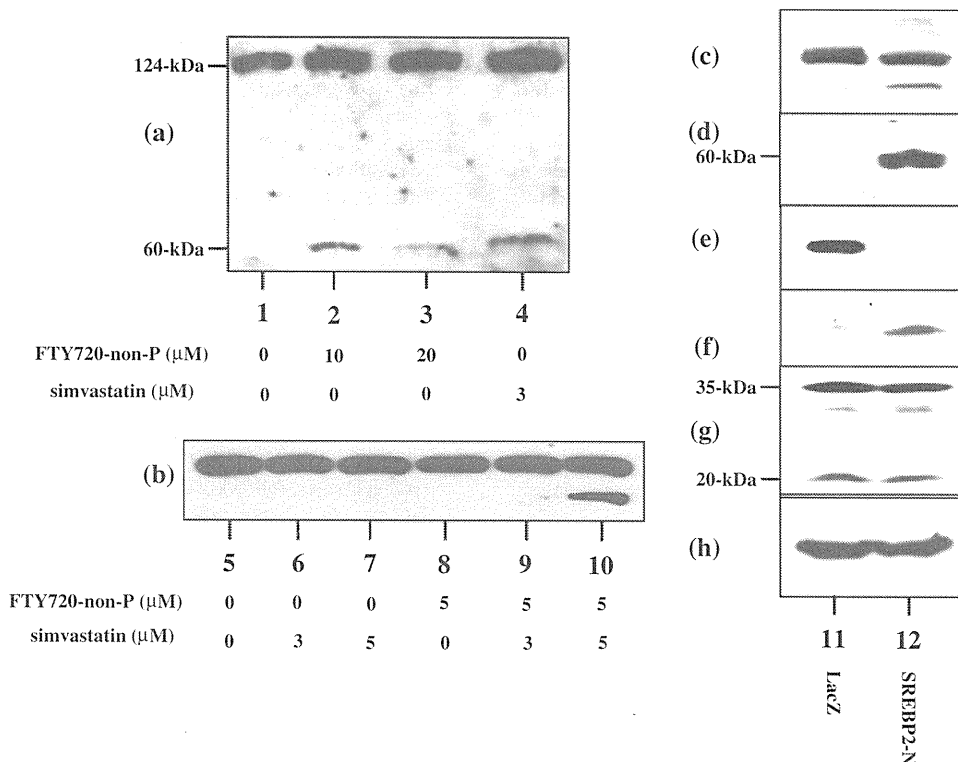


Fig. 6 Activation of SREBP2 by FTY720-non-P in HMO6 cells. HMO6 cells were exposed to FTY720-non-P or simvastatin, or transfected with the vector expressing the N-terminal fragment of SREBP2 (SREBP2-N) or LacZ. **a–h** indicate western blot of **a, d** SREBP2, **b, c** PARP, **e** V5, **f** cleaved caspase-3, **g** caspase-7 (a 35-kDa proform and a 20-kDa cleaved form), and **h** HSP60. The lanes **1–12** represent (**1, 5**) untreated HMO6 cells, and HMO6 cells treated for 1 h with (**2**) 10 μM FTY720-non-P, (**3**) 20 μM FTY720-non-P, (**4**) 3 μM simvastatin, and HMO6 cells pretreated for simvastatin

starting at 12 h before a 6 h-exposure to FTY720-non-P, whose conditions are composed of (**6**) 3 μM simvastatin pretreatment alone, (**7**) 5 μM simvastatin pretreatment alone, (**8**) no simvastatin pretreatment and 5 μM FTY720-non-P exposure, (**9**) 3 μM simvastatin pretreatment and 5 μM FTY720-non-P exposure, and (**10**) 5 μM simvastatin pretreatment and 5 μM FTY720-non-P exposure, and HMO6 cells with overexpression of (**11**) V5-tagged LacZ or (**12**) SREBP2-N

SREBP activation is not a secondary phenomenon following caspase-3 activation. Furthermore, we found that activation of SREBP2 by overexpression of the N-terminal fragment of SREBP2 in HMO6 cells enhances the cleavage of PARP and caspase-3 in the absence of FTY720. Moreover, we found that pretreatment with simvastatin enhanced FTY720-non-P-induced apoptosis of HMO6 cells. Statins activate SREBP2 and induce apoptosis of various cells (Xia et al. 2001; Gibot et al. 2009). All of these observations suggest that the SREBP2-dependent signaling pathway is intrinsically proapoptotic, when it is aberrantly regulated.

A recent study showed that FTY720 inhibits intracellular transport of cholesterol to ER in human macrophages, being independent of S1P1 binding, indicating that FTY720-non-P certainly affects the cellular cholesterol processing (Blom et al. 2010). Importantly, cholesterol interacts specifically with sphingosine in human intestinal epithelial cells under physiological conditions (Garmy et al. 2005). S1P is intracellularly generated by sphingosine kinases SPHK1 and SPHK2 from sphingosine, a breakdown product of the cell membrane constituent sphingomyelin (Chi 2011). S1P and

its synthetic analog FTY720-P share S1P1, S1P3, S1P4, and S1P5 expressed on the plasma membrane. All of these observations propose a possible scenario that excessive amounts of intracellular FTY720-non-P disturb the complex metabolic network of cholesterol and sphingolipids, resulting in activation of the SREBP2-dependent proapoptotic signaling pathway.

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References

- Bandhuvula P, Tam YY, Oskouian B, Saba JD (2005) The immune modulator FTY720 inhibits sphingosine-1-phosphate lyase activity. *J Biol Chem* 280:33697–33700

- Berdyshev EV, Gorshkova I, Skobeleva A, Bittman R, Lu X, Dudek SM, Mirzapojazova T, Garcia JG, Natarajan V (2009) FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J Biol Chem* 284:5467–5477
- Blom T, Bäck N, Mutka AL, Bittman R, Li Z, de Lera A, Kovanen PT, Diczfalusy U, Ikonen E (2010) FTY720 stimulates 27-hydroxycholesterol production and confers atheroprotective effects in human primary macrophages. *Circ Res* 106:720–729
- Brinkmann V, Wilt C, Kristofic C, Nikolova Z, Hof RP, Chen S, Albert R, Cottens S (2001) FTY720: dissection of membrane receptor-operated, stereospecific effects on cell migration from receptor-independent antiproliferative and apoptotic effects. *Transplant Proc* 33:3078–3080
- Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov* 9:883–897
- Chi H (2011) Sphingosine-1-phosphate and immune regulation: trafficking and beyond. *Trends Pharmacol Sci* 32:16–24
- Choi JW, Gardell SE, Herr DR, Rivera R, Lee CW, Noguchi K, Teo ST, Yung YC, Lu M, Kennedy G, Chun J (2011) FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. *Proc Natl Acad Sci USA* 108:751–756
- Coelho RP, Payne SG, Bittman R, Spiegel S, Sato-Bigbee C (2007) The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. *J Pharmacol Exp Ther* 323:626–635
- da Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57
- Dev KK, Mullershausen F, Mattes H, Kuhn RR, Bilbe G, Hoyer D, Mir A (2008) Brain sphingosine-1-phosphate receptors: implication for FTY720 in the treatment of multiple sclerosis. *Pharmacol Ther* 117:77–93
- Durafourt BA, Lambert C, Johnson TA, Blain M, Bar-Or A, Antel JP (2011) Differential responses of human microglia and blood-derived myeloid cells to FTY720. *J Neuroimmunol* 230:10–16
- Garmy N, Taïeb N, Yahi N, Fantini J (2005) Interaction of cholesterol with sphingosine: physicochemical characterization and impact on intestinal absorption. *J Lipid Res* 46:36–45
- Gibot L, Follet J, Metges JP, Auvray P, Simon B, Corcos L, Le Jossic-Corcos C (2009) Human caspase 7 is positively controlled by SREBP-1 and SREBP-2. *Biochem J* 420:473–483
- Higgins ME, Ioannou YA (2001) Apoptosis-induced release of mature sterol regulatory element-binding proteins activates sterol-responsive genes. *J Lipid Res* 42:1939–1946
- Hisano Y, Kobayashi N, Kawahara A, Yamaguchi A, Nishi T (2011) The sphingosine 1-phosphate transporter, SPNS2, functions as a transporter of the phosphorylated form of the immunomodulating agent FTY720. *J Biol Chem* 286:1758–1766
- Jack C, Ruffini F, Bar-Or A, Antel JP (2005) Microglia and multiple sclerosis. *J Neurosci Res* 81:363–373
- Jin Y, Zollinger M, Borell H, Zimmerlin A, Patten CJ (2011) CYP4F enzymes are responsible for the elimination of fingolimod (FTY720), a novel treatment of relapsing multiple sclerosis. *Drug Metab Dispos* 39:191–198
- Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH, Haas T, Korn AA, Karlsson G, Radue EW, FTY720 D2201 Study Group (2006) Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 355:1124–1140
- Lee TK, Man K, Ho JW, Sun CK, Ng KT, Wang XH, Wong YC, Ng IO, Xu R, Fan ST (2004) FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation. *Carcinogenesis* 25:2397–2405
- Liu Q, Alinari L, Chen CS, Yan F, Dalton JT, Lapalombella R, Zhang X, Mani R, Lin T, Byrd JC, Baiocchi RA, Muthusamy N (2010) FTY720 shows promising in vitro and in vivo preclinical activity by downmodulating cyclin D1 and phospho-Akt in mantle cell lymphoma. *Clin Cancer Res* 16:3182–3192
- Matsuda S, Minowa A, Suzuki S, Koyasu S (1999) Differential activation of c-Jun NH2-terminal kinase and p38 pathways during FTY720-induced apoptosis of T lymphocytes that is suppressed by the extracellular signal-regulated kinase pathway. *J Immunol* 162:3321–3326
- Matsuoka Y, Nagahara Y, Ikekita M, Shinomiya T (2003) A novel immunosuppressive agent FTY720 induced Akt dephosphorylation in leukemia cells. *Br J Pharmacol* 138:1303–1312
- Miron VE, Jung CG, Kim HJ, Kennedy TE, Soliven B, Antel JP (2008) FTY720 modulates human oligodendrocyte progenitor process extension and survival. *Ann Neurol* 63:61–71
- Miron VE, Ludwin SK, Darlington PJ, Jarjour AA, Soliven B, Kennedy TE, Antel JP (2010) Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. *Am J Pathol* 176:2682–2694
- Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, Seuwen K (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol* 5:428–434
- Nagai A, Nakagawa E, Hatori K, Choi HB, McLarnon JG, Lee MA, Kim SU (2001) Generation and characterization of immortalized human microglial cell lines: expression of cytokines and chemokines. *Neurobiol Dis* 8:1057–1068
- Nagaoka Y, Otsuki K, Fujita T, Uesato S (2008) Effects of phosphorylation of immunomodulatory agent FTY720 (fingolimod) on antiproliferative activity against breast and colon cancer cells. *Biol Pharm Bull* 31:1177–1181
- Narantuya D, Nagai A, Sheikh AM, Masuda J, Kobayashi S, Yamaguchi S, Kim SU (2010) Human microglia transplanted in rat focal ischemia brain induce neuroprotection and behavioral improvement. *PLoS One* 5:e11746
- Payne SG, Oskertizian CA, Griffiths R, Subramanian P, Barbour SE, Chalfant CE, Milstien S, Spiegel S (2007) The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors. *Blood* 109:1077–1085
- Potteck H, Nieuwenhuis B, Lüth A, van der Giet M, Kleuser B (2010) Phosphorylation of the immunomodulator FTY720 inhibits programmed cell death of fibroblasts via the S1P3 receptor subtype and Bcl-2 activation. *Cell Physiol Biochem* 26:67–78
- Sato R (2010) Sterol metabolism and SREBP activation. *Arch Biochem Biophys* 501:177–181
- Satoh J, Tabunoki H, Arima K (2009) Molecular network analysis suggests aberrant CREB-mediated gene regulation in the Alzheimer disease hippocampus. *Dis Markers* 27:239–252
- Sensken SC, Gräler MH (2010) Down-regulation of S1P1 receptor surface expression by protein kinase C inhibition. *J Biol Chem* 285:6298–6307
- Sonoda Y, Yamamoto D, Sakurai S, Hasegawa M, Aizu-Yokota E, Momoi T, Kasahara T (2001) FTY720, a novel immunosuppressive agent, induces apoptosis in human glioma cells. *Biochem Biophys Res Commun* 281:282–288
- Van Doorn R, Van Horssen J, Verzijl D, Witte M, Ronken E, Van Het Hof B, Lakeman K, Dijkstra CD, Van Der Valk P, Reijerkerk A, Alewijnse AE, Peters SL, De Vries HE (2010) Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. *Glia* 58:1465–1476
- Wang JD, Takahara S, Nonomura N, Ichimaru N, Toki K, Azuma H, Matsumiya K, Okuyama A, Suzuki S (1999) Early induction of apoptosis in androgen-independent prostate cancer cell line by FTY720 requires caspase-3 activation. *Prostate* 40:50–55

- Wei Y, Yemisci M, Kim HH, Yung LM, Shin HK, Hwang SK, Guo S, Qin T, Alsharif N, Brinkmann V, Liao JK, Lo EH, Waeber C (2011) Fingolimod provides long-term protection in rodent models of cerebral ischemia. *Ann Neurol* 69:119–129
- Woodcock JM, Ma Y, Coolen C, Pham D, Jones C, Lopez AF, Pitson SM (2010) Sphingosine and FTY720 directly bind pro-survival 14-3-3 proteins to regulate their function. *Cell Signal* 22: 1291–1299
- Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD, Penn LZ (2001) Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. *Leukemia* 15:1398–1407
- Zhang Z, Zhang Z, Fauser U, Artelt M, Burnet M, Schliesener HJ (2007) FTY720 attenuates accumulation of EMAP-II+ and MHC-II+ monocytes in early lesions of rat traumatic brain injury. *J Cell Mol Med* 11:307–314

RESEARCH

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Comprehensive analysis of human microRNA target networks

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Abstract

Background: MicroRNAs (miRNAs) mediate posttranscriptional regulation of protein-coding genes by binding to the 3' untranslated region of target mRNAs, leading to translational inhibition, mRNA destabilization or degradation, depending on the degree of sequence complementarity. In general, a single miRNA concurrently downregulates hundreds of target mRNAs. Thus, miRNAs play a key role in fine-tuning of diverse cellular functions, such as development, differentiation, proliferation, apoptosis and metabolism. However, it remains to be fully elucidated whether a set of miRNA target genes regulated by an individual miRNA in the whole human microRNAome generally constitute the biological network of functionally-associated molecules or simply reflect a random set of functionally-independent genes.

Methods: The complete set of human miRNAs was downloaded from miRBase Release 16. We explored target genes of individual miRNA by using the Diana-microT 3.0 target prediction program, and selected the genes with the miTG score ≥ 20 as the set of highly reliable targets. Then, Entrez Gene IDs of miRNA target genes were uploaded onto KeyMolnet, a tool for analyzing molecular interactions on the comprehensive knowledgebase by the neighboring network-search algorithm. The generated network, compared side by side with human canonical networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events, enabled us to identify the canonical network with the most significant relevance to the extracted network.

Results: Among 1,223 human miRNAs examined, Diana-microT 3.0 predicted reliable targets from 273 miRNAs. Among them, KeyMolnet successfully extracted molecular networks from 232 miRNAs. The most relevant pathway is transcriptional regulation by transcription factors RB/E2F, the disease is adult T cell lymphoma/leukemia, and the pathological event is cancer.

Conclusion: The predicted targets derived from approximately 20% of all human miRNAs constructed biologically meaningful molecular networks, supporting the view that a set of miRNA targets regulated by a single miRNA generally constitute the biological network of functionally-associated molecules in human cells.

Introduction

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs conserved through the evolution. They mediate posttranscriptional regulation of protein-coding genes by binding to the 3' untranslated region (3'UTR) of target mRNAs, leading to translational inhibition, mRNA destabilization or degradation, depending on the degree of sequence complementarity [1]. During the biogenesis of miRNAs, the primary miRNAs (pri-miRNAs) are transcribed from the intra- and inter-genetic regions of the genome by RNA polymerase II, followed by processing by the RNase III enzyme Droscha into pre-miRNAs. After nuclear export, they are cleaved by the RNase III enzyme Dicer into mature miRNAs consisting of approximately 22 nucleotides. Finally, a single-stranded miRNA is loaded onto the RNA-induced silencing complex (RISC), where the seed sequence located at positions 2 to 8 from the 5' end of the miRNA plays a pivotal role in recognition of the target mRNA [2]. At present, more than one thousand of human miRNAs are registered in miRBase Release 16 <http://www.mirbase.org>. The 3'UTR of a single mRNA is often targeted by several different miRNAs, while a single miRNA concurrently reduces the production of hundreds of target proteins [3]. Consequently, the whole miRNA system (microRNAome) regulate greater than 60% of all protein-coding genes in a human cell [4]. By targeting multiple transcripts and affecting expression of numerous proteins, miRNAs play a key role in fine-tuning of diverse cellular functions, such as development, differentiation, proliferation, apoptosis and metabolism. Therefore, aberrant regulation of miRNA expression is deeply involved in pathological events that mediate cancers [5] and neurodegenerative disorders [6].

Recent advances in systems biology have made major breakthroughs by illustrating the cell-wide map of complex molecular interactions with the aid of the literature-based knowledgebase of molecular pathways [7]. The logically arranged molecular networks construct the whole system characterized by robustness, which maintains the proper function of the system in the face of genetic and environmental perturbations [8]. In the scale-free molecular network, targeted disruption of limited numbers of critical components designated hubs, on which the biologically important molecular interactions concentrate, efficiently disturbs the whole cellular function by destabilizing the network [9]. Therefore, the identification of the hub in the molecular network constructed by target genes of a particular miRNA helps us to understand biological and pathological roles of individual miRNAs. Recently, Hsu et al. studied the human microRNA-regulated protein-protein interaction (PPI) network by utilizing the Human Protein Reference Database (HPRD) and the miRNA target prediction program TargetScan [10]. They found that an individual miRNA often targets the hub gene of the PPI network, although they did not attempt to characterize relevant pathways, diseases, and pathological events regulated by miRNA target genes.

At present, the question remains to be fully elucidated whether a set of miRNA target genes regulated by an individual miRNA in the whole human microRNAome generally constitute the biological network of functionally-associated molecules or simply reflect a random set of functionally-independent genes. To address this question, we attempted to characterize molecular networks of target genes of all human miRNAs by using KeyMolnet, a bioinformatics tool for analyzing molecular interactions on the comprehensive knowledgebase.

Materials and methods

MicroRNA Target Prediction

The complete list of 1,223 human miRNAs was downloaded from miRBase Release 16 <http://www.mirbase.org>. We searched the target genes of individual miRNA on the Diana-microT 3.0 target prediction program (diana.cslab.ece.ntua.gr/microT), which was selected because of the highest ratio of correctly predicted targets over other prediction tools [11]. Diana-microT 3.0 calculates the miRNA-targeted gene (miTG) score that reflects the weighted sum of the scores of all conserved and non-conserved miRNA recognition elements (MRE) on the 3'UTR of the target mRNA. The miTG score correlates well with fold changes in suppression of protein expression [11]. To optimize the parameter of miRNA-target interaction, we considered the target genes with a cutoff of the miTG score equal to or larger than 20 as the highly reliable targets, because we found that the targets with the miTG score < 20 exhibited the significantly lower precision score, an indicator of correctness in predicted interactions [11], compared with those having the score ≥ 20 ($p = 2.78E-08$ by Mann-Whitney's U-test).

Molecular Network Analysis

Ensembl Gene IDs of target genes retrieved by Diana-microT 3.0 were converted into the corresponding Entrez Gene IDs by using the DAVID Bioinformatics Resources 6.7 program <http://david.abcc.ncifcrf.gov>[12], where non-annotated IDs were deleted. Then, Entrez Gene IDs of miRNA target genes were uploaded onto KeyMolnet.

KeyMolnet is a tool for analyzing molecular interactions on the literature-based knowledgebase that contains the contents on 123,000 molecular relationships among human genes and proteins, small molecules, diseases, pathways and drugs, established by the Institute of Medicinal Molecular Design (IMMD) (Tokyo, Japan) [13-15]. The core contents are collected from selected review articles and textbooks with the highest reliability, regularly updated and carefully curated by a team of expert biologists. KeyMolnet contains a panel of human canonical networks constructed by core contents in the KeyMolnet library. They represent the gold standard of the networks, composed of 430 pathways, 885 diseases, and 208 pathological events. Detailed information on all the contents is available from IMMD <http://www.immd.co.jp/en/keymolnet/index.html> upon request.

We utilized the neighboring network-search algorithm that selects the set of miRNA target genes as starting points to generate the network around starting points within one path, composed of all kinds of molecular interactions, including direct activation/inactivation, transcriptional activation/repression, and the complex formation. By uploading the list of Entrez Gene IDs onto KeyMolnet, it automatically provides corresponding molecules and a minimum set of intervening molecules as a node on networks. The generated network was compared side by side with human canonical networks described above. The algorithm that counts the number of overlapping molecules and/or molecular relations between the extracted network and the canonical network identifies the canonical network showing the most statistically significant contribution to the extracted network. This algorithm is essentially based on that of the GO::TermFinder [16]. The significance in the similarity between the extracted network and the canonical network is scored following the formula, where O = the number of overlapping molecules and molecular relations for the pathway or overlapping molecules alone for the disease and the pathological event between the extracted network and the canonical network, V = the number of molecules

and/or molecular relations located in the extracted network, C = the number of molecules and/or molecular relations located in the canonical network, T = the number of total molecules and/or molecular relations of KeyMolnet, currently composed of approximately 15,700 molecules and 123,000 molecular relations, and the x = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score}(p)) \quad \text{Score}(p) = \sum_{x=0}^{\min(C,V)} f(x) \quad f(x) = \frac{C^x \cdot T - C^x \cdot T^x}{T^x \cdot C^x} \quad (1)$$

Results

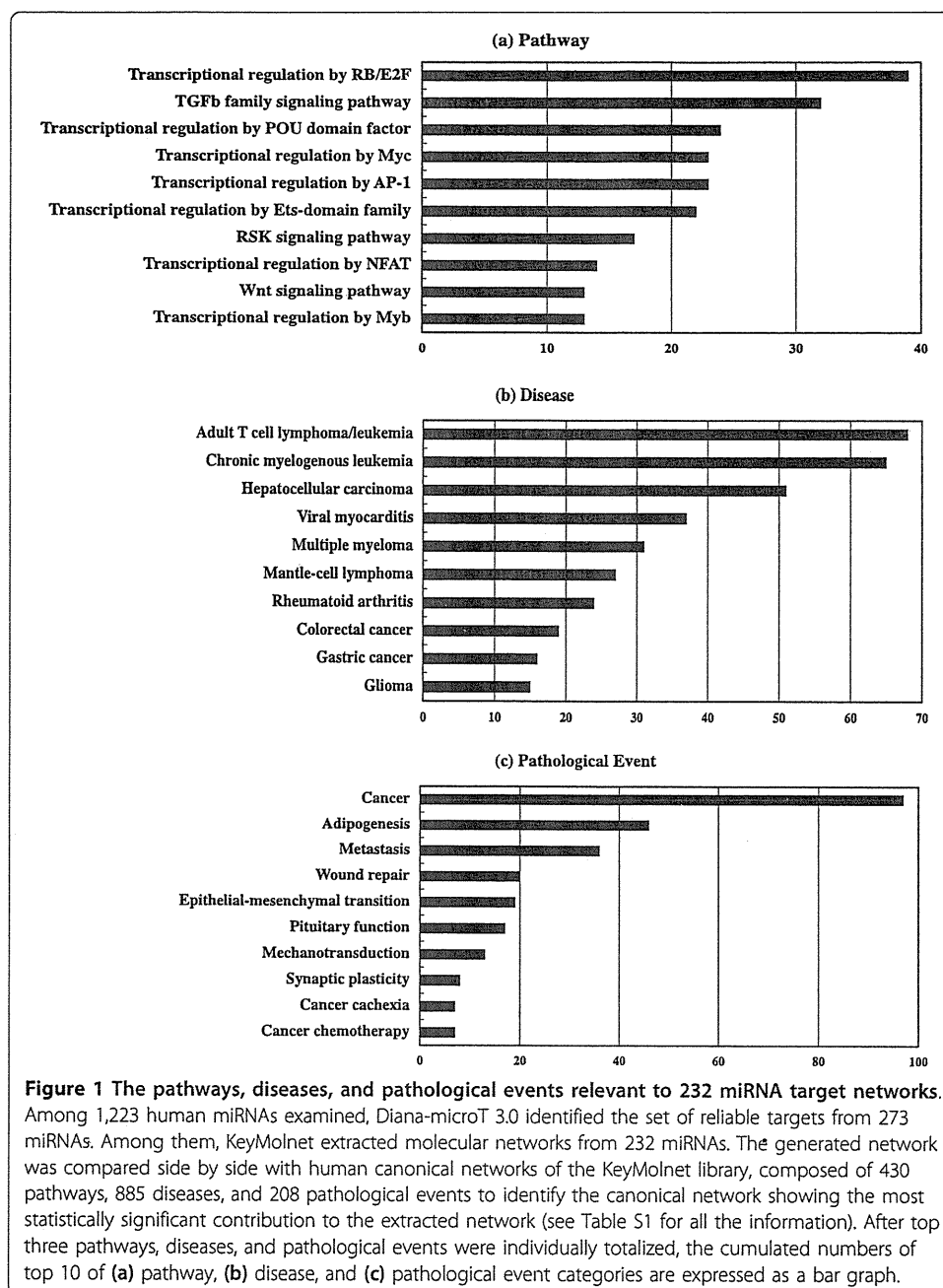
Molecular Network of MicroRNA Target Genes

Among 1,223 human miRNAs examined, Diana-microT 3.0 predicted the targets from 532 miRNAs (43.5%). Among the 532 miRNAs, 273 miRNAs contained a set of highly reliable targets showing the miTG score ≥ 20 . Among 273 miRNAs having reliable targets, KeyMolnet successfully extracted molecular networks from 232 miRNAs. They are comprised of 19% of total human miRNAs (microRNAome). Then, the generated network was compared side by side with human canonical networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events. We found that not all 232 miRNAs contained entire categories of canonical networks because several miRNAs comprised relatively small numbers of targets. See Additional file 1 for all the information on 232 miRNAs and their target networks. When top three pathways, diseases, and pathological events were individually totalized, the most relevant pathway is 'transcriptional regulation by RB/E2F' ($n = 39$; 6.8% of total), followed by 'TGF-beta family signaling pathway' ($n = 32$; 5.6%) and 'transcriptional regulation by POU domain factor' ($n = 24$; 4.2%), the most relevant disease is 'adult T cell lymphoma/leukemia' ($n = 68$; 12.1%), followed by 'chronic myelogenous leukemia' ($n = 65$; 11.5%) and 'hepatocellular carcinoma' ($n = 51$; 9.1%), and the most relevant pathological event is 'cancer' ($n = 97$; 24.7%), followed by 'adipogenesis' ($n = 46$; 11.7%) and 'metastasis' ($n = 36$; 9.2%) (Figure 1 and Additional file 1).

Next, we identified the large-scale miRNA target networks by uploading targets greater than 100 per individual miRNA onto KeyMolnet (Table 1). Fifty-two miRNAs that construct such a large-scale miRNA target network include let-7, miR-9, 17, 19, 20, 26, 27, 29, 30, 32, 92, 93, 96, 98, 101, 106b, 124, 137, 147, 153, 218, 372, 429, 495, 506, 519, 520, 603, and their closely-related family members. The targets of these miRNAs established highly complex molecular networks, in which the pathways of 'transcriptional regulation by RB/E2F', 'transcriptional regulation by Ets-domain family', and 'transcriptional regulation by p53', the diseases of 'chronic myelogenous leukemia' and 'viral myocarditis', and the pathological event of 'cancer' were notably accumulated (Table 1). Importantly, distinct members belonging to the same miRNA family, for example, five miR-30 family members ranging from miR-30a to miR-30e constructed a virtually identical molecular network (Table 1).

Biological Implications of MicroRNA Target Networks

As described above, the present observations indicated that a set of miRNA target genes regulated by an individual miRNA generally constitute the biological network of



functionally-associated molecules in human cells. Therefore, it is highly important to obtain deeper insights into biological implications of miRNA target networks.

The protooncogene *c-myc* is a key transcription factor for normal development of hematopoietic cells. A recent study showed that miR-15a targets *c-myc*, while *c-myc* binds to the promoter of miR-15a, providing an autoregulatory feedback loop in human hematopoietic cells [17]. Consistent with this study, we found ‘transcriptional regulation by myb’ as the most relevant pathway to the miR-15a target network (the score = 602; the score p-value = 7.39E-182) (Figure 2 and Additional file 1). These observations propose a scenario that miR-15a synchronously downregulates both *c-myc* itself and downstream genes transcriptionally regulated by *c-myc*, resulting in

Table 1 The large-scale human microRNA target networks

MicroRNA	Number of Targets	Molecules in KeyMolnet Networks	Top Pathway	Score	p-Value	Top Disease	Score	p-Value	Top Pathological Event	Score	p-Value
hsa-let-7a	244	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-let-7b	242	1016	Transcriptional regulation by p53	594	1.83E-179	Viral myocarditis	113	9.32E-35	Cancer	206	7.66E-63
hsa-let-7c	243	1020	Transcriptional regulation by p53	593	2.49E-179	Viral myocarditis	113	1.11E-34	Cancer	206	1.10E-62
hsa-let-7d	145	885	Transcriptional regulation by RB/E2F	836	2.18E-252	Chronic myelogenous leukemia	72	1.95E-22	Cancer	130	9.68E-40
hsa-let-7e	236	1111	Transcriptional regulation by p53	575	8.90E-174	Viral myocarditis	116	1.20E-35	Cancer	175	1.86E-53
hsa-let-7f	244	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-let-7g	245	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-let-7i	245	1022	Transcriptional regulation by p53	593	2.69E-179	Viral myocarditis	113	1.21E-34	Cancer	206	1.31E-62
hsa-miR-9	352	1115	Transcriptional regulation by PPARα	340	5.28E-103	Hepatocellular carcinoma	72	1.69E-22	Cancer	171	3.50E-52
hsa-miR-17	195	961	Transcriptional regulation by RB/E2F	971	3.27E-293	Chronic myelogenous leukemia	92	2.83E-28	Cancer	181	3.58E-55
hsa-miR-19a	226	1094	Transcriptional regulation by RB/E2F	760	2.10E-229	Chronic myelogenous leukemia	113	1.26E-34	Cancer	253	7.04E-77
hsa-miR-19b	225	1094	Transcriptional regulation by RB/E2F	760	2.10E-229	Chronic myelogenous leukemia	113	1.26E-34	Cancer	253	7.04E-77
hsa-miR-20a	165	1038	Transcriptional regulation by RB/E2F	856	1.64E-258	Chronic myelogenous leukemia	87	6.09E-27	Cancer	85	3.33E-26
hsa-miR-20b	198	981	Transcriptional regulation by RB/E2F	962	2.35E-290	Chronic myelogenous leukemia	98	3.39E-30	Cancer	183	6.98E-56
hsa-miR-26a	148	672	Transcriptional regulation by RB/E2F	919	1.76E-277	Chronic myelogenous leukemia	107	6.15E-33	Cancer	181	3.20E-55
hsa-miR-26b	148	672	Transcriptional regulation by RB/E2F	919	1.76E-277	Chronic myelogenous leukemia	107	6.15E-33	Cancer	181	3.20E-55
hsa-miR-27a	229	1192	Transcriptional regulation by CREB	1022	2.23E-308	Chronic myelogenous leukemia	95	1.96E-29	Cancer	194	3.05E-59
hsa-miR-27b	261	1337	Transcriptional regulation by CREB	1022	2.23E-308	Chronic myelogenous leukemia	94	4.51E-29	Cancer	211	4.11E-64
hsa-miR-29a	119	543	Transcriptional regulation by Ets-domain family	430	4.36E-130	Glioma	85	3.46E-26	Cancer	139	1.41E-42

Table 1 The large-scale human microRNA target networks (Continued)

hsa-miR-29b	118	578	Transcriptional regulation by Ets-domain family	422	1.15E-127	Glioma	82	1.55E-25	Cancer	146	1.44E-44
hsa-miR-29c	118	543	Transcriptional regulation by Ets-domain family	430	4.36E-130	Glioma	85	3.46E-26	Cancer	139	1.41E-42
hsa-miR-30a	455	1494	Transcriptional regulation by RB/E2F	777	9.43E-235	Chronic myelogenous leukemia	86	1.11E-26	Cancer	195	2.39E-59
hsa-miR-30b	455	1480	Transcriptional regulation by RB/E2F	781	1.08E-235	Chronic myelogenous leukemia	87	7.01E-27	Cancer	188	1.92E-57
hsa-miR-30c	454	1495	Transcriptional regulation by RB/E2F	778	6.13E-235	Chronic myelogenous leukemia	86	1.15E-26	Cancer	191	3.63E-58
hsa-miR-30d	452	1491	Transcriptional regulation by RB/E2F	778	7.28E-235	Chronic myelogenous leukemia	86	1.01E-26	Cancer	195	1.96E-59
hsa-miR-30e	455	1481	Transcriptional regulation by RB/E2F	780	1.29E-235	Chronic myelogenous leukemia	87	7.25E-27	Cancer	188	2.05E-57
hsa-miR-32	261	905	Transcriptional regulation by RB/E2F	842	2.74E-254	Gastric cancer	80	8.85E-25	Cancer	157	4.19E-48
hsa-miR-92a	219	642	Transcriptional regulation by MEF2	335	1.51E-101	Viral myocarditis	59	1.62E-18	Epithelial-mesenchymal transition	83	7.76E-26
hsa-miR-92b	258	701	Transcriptional regulation by MEF2	328	1.59E-99	Viral myocarditis	60	1.23E-18	Cancer	94	3.97E-29
hsa-miR-93	195	958	Transcriptional regulation by RB/E2F	972	2.37E-293	Chronic myelogenous leukemia	92	2.47E-28	Cancer	181	2.77E-55
hsa-miR-96	142	688	Transcriptional regulation by Ets-domain family	407	3.42E-123	Viral myocarditis	36	1.06E-11	Cancer	106	1.37E-32
hsa-miR-98	162	671	Transcriptional regulation by Myb	549	4.73E-166	Viral myocarditis	85	2.66E-26	Cancer	126	1.42E-38
hsa-miR-101	188	806	Transcriptional regulation by AP-1	492	1.10E-148	Hepatocellular carcinoma	70	6.40E-22	Cancer	127	4.26E-39
hsa-miR-106b	164	1028	Transcriptional regulation by RB/E2F	854	7.21E-258	Chronic myelogenous leukemia	87	5.48E-27	Cancer	85	2.93E-26
hsa-miR-124	285	1346	Transcriptional regulation by RB/E2F	756	3.57E-228	Chronic myelogenous leukemia	83	9.34E-26	Cancer	185	1.90E-56
hsa-miR-137	288	941	Transcriptional regulation by MITF family	339	1.19E-102	Adult T cell lymphoma/leukemia	66	1.30E-20	Cancer	179	1.00E-54
hsa-miR-147	199	867	Transcriptional regulation by RB/E2F	805	4.06E-243	Chronic myelogenous leukemia	113	6.60E-35	Cancer	132	2.57E-40
hsa-miR-153	154	1019	Transcriptional regulation by Myb	507	2.35E-153	Multiple myeloma	60	6.44E-19	Cancer	174	4.31E-53

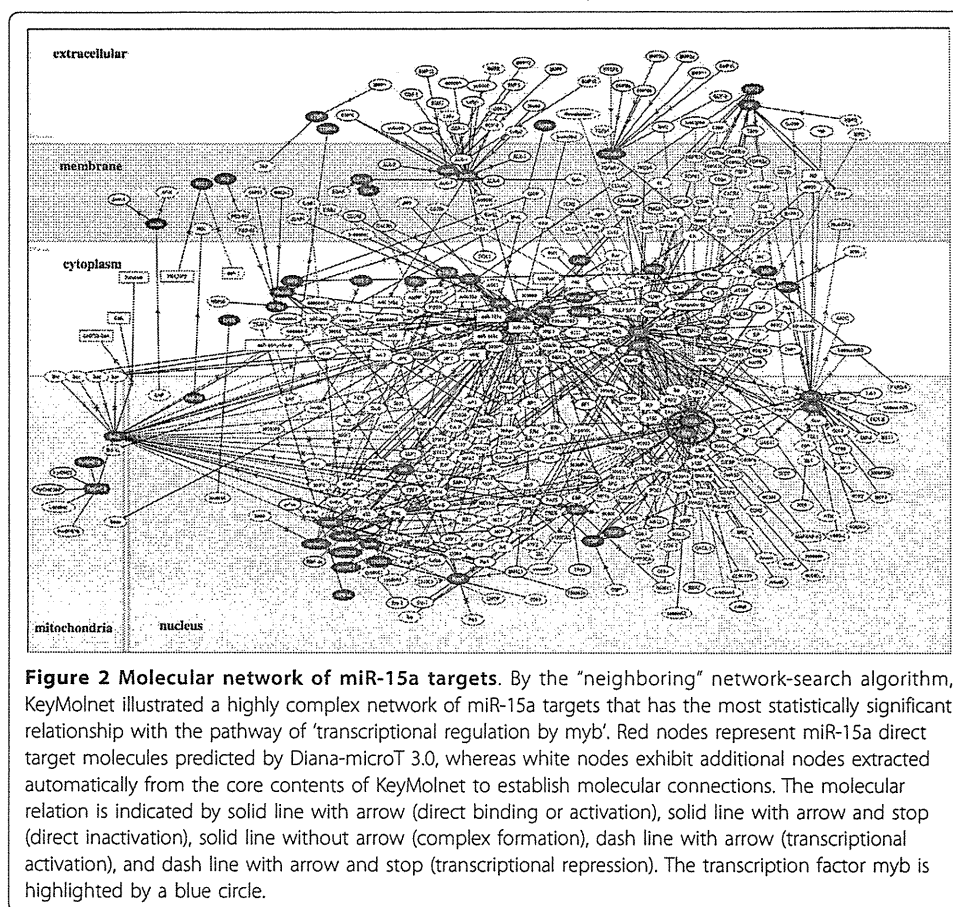
Table 1 The large-scale human microRNA target networks (Continued)

hsa-miR-218	155	830	Transcriptional regulation by AP-1	344	2.28E-104	Hepatocellular carcinoma	69	1.63E-21	Cancer	136	1.52E-41
hsa-miR-372	101	562	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	85	1.90E-26	Cancer	144	2.75E-44
hsa-miR-429	123	634	Transcriptional regulation by RB/E2F	918	2.45E-277	Chronic myelogenous leukemia	76	1.71E-23	Cancer	130	5.28E-40
hsa-miR-495	156	601	Transcriptional regulation by Ets-domain family	431	2.14E-130	Rheumatoid arthritis	77	5.90E-24	Adipogenesis	79	1.32E-24
hsa-miR-506	394	1536	Transcriptional regulation by Ets-domain family	317	4.69E-96	Viral myocarditis	99	1.73E-30	Cancer	172	1.43E-52
hsa-miR-519a	281	1256	Transcriptional regulation by RB/E2F	811	5.32E-245	Chronic myelogenous leukemia	106	1.34E-32	Cancer	220	8.03E-67
hsa-miR-519b-3p	281	1256	Transcriptional regulation by RB/E2F	811	5.32E-245	Chronic myelogenous leukemia	106	1.34E-32	Cancer	220	8.03E-67
hsa-miR-519c-3p	281	1256	Transcriptional regulation by RB/E2F	811	5.32E-245	Chronic myelogenous leukemia	106	1.34E-32	Cancer	220	8.03E-67
hsa-miR-520a-3p	184	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-520b	182	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-520c-3p	182	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	93	9.28E-29	Cancer	145	1.77E-44
hsa-miR-520d-3p	183	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-520e	184	690	Transcriptional regulation by RB/E2F	1022	2.23E-308	Chronic myelogenous leukemia	94	6.95E-29	Cancer	146	1.12E-44
hsa-miR-603	252	1150	Transcriptional regulation by Ets-domain family	344	3.26E-104	Multiple myeloma	84	4.36E-26	Cancer	161	4.24E-49

Among 1,223 human miRNAs examined, Diana-microT 3.0 predicted reliable targets from 273 miRNAs. Among them, KeyMolnet extracted molecular networks from 232 miRNAs. The generated network was compared side by side with human canonical networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events. The canonical pathways, diseases, and pathological events with the most statistically significant contribution to the extracted network are shown. The table contains only the large-scale miRNA target networks generated by importing targets greater than 100 per individual miRNA into KeyMolnet. See Additional file 1 for all the information on 232 miRNAs and their target networks.

efficient inactivation of the whole molecular network governed by the hub gene *c-myb*. These results suggest a collaborative regulation of gene expression at both transcriptional and posttranscriptional levels that involve coordinated regulation by miRNAs and transcription factors.

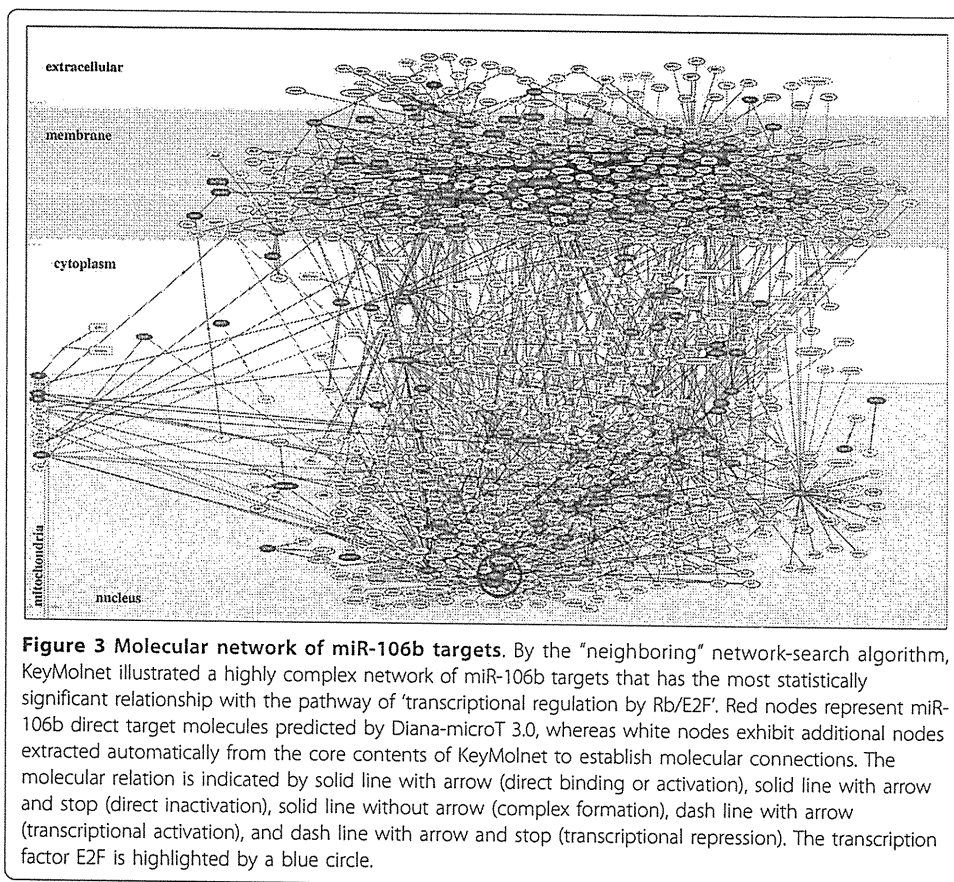
The retinoblastoma protein Rb/E2F pathway acts as a gatekeeper for G1/S transition in the cell cycle. The Rb/E2F-regulated G1 checkpoint control is often disrupted in cancer cells. A recent study showed that miR-106b is directly involved in posttranscriptional regulation of E2F1 [18]. E2F1 activates transcription of miR-106b, while miR-



106b targets E2F1, serving as a miRNA-directed negative feedback loop in gastric cancer cells [18]. Supporting these findings, we identified ‘transcriptional regulation by Rb/E2F’ as the most relevant pathway to the miR-106b target network (the score = 854; the score p-value = 7.21E-258) (Figure 3, Table 1 and Additional file 1). The relationship between miR-106b and Rb/E2F would provide another example of coordinated regulation of gene expression by miRNAs and transcription factors.

We found ‘transcriptional regulation by p53’ as the most relevant pathway to the target network of all let-7 family members except for let-7d (Table 1). It is worthy to note that the tumor suppressor p53 regulates the expression of components of the miRNA-processing machinery, such as Drosha, DGCR8, Dicer, and TARBP2, all of which have p53-responsive elements in their promoters [19]. Furthermore, Dicer and TARBP2, along with p53, serve as a target of the let-7 family miRNAs, suggesting a close link between p53 and let-7 in miRNA biogenesis [19]. The expression of let-7 family members was greatly reduced in certain cancer cells [20].

The microphthalmia associated transcription factor (MITF), a basic helix-loop-helix zipper (bHLH-Zip) transcription factor, acts as not only a master regulator of melanocyte differentiation but also an oncogene promoting survival of melanoma. Recent studies indicate that MITF is a direct target of both miR-137 and miR-148b [21,22]. Again, we identified ‘transcriptional regulation by MITF family’ as the most relevant pathway to both miR-137 (the score = 339; the score p-value = 1.19E-102) and miR-



148b (the score = 40; the score p-value = 3.91E-142) target networks (Table 1 and Additional file 1).

Cellular responsiveness to glucocorticoids (GCs) is regulated by the delicate balance of the glucocorticoid receptor (GR) protein, GR coactivators and corepressors, GR splice variants and isoforms, and regulators of GR retrograde transport to the nucleus. A recent study showed that miR-18a targets the GR protein, and thereby inhibits GR-mediated biological events in neuronal cells [23]. Consistent with this, we found ‘transcriptional regulation by GR’ as the most relevant pathway to the miR-18a target network (the score = 1022; the score p-value = 2.23E-308) (Additional file 1).

Zinc finger transcription factors ZEB1 and ZEB2 act as a transcriptional repressor of E-cadherin. A recent study showed that the expression of miR-200b, which targets both ZEB1 and ZEB2, was downregulated in the cells that undergo TGF-beta-induced epithelial to mesenchymal transition (EMT), and was lost in invasive breast cancer cells [24]. We identified ‘transcriptional regulation by ZEB’ as the third-rank significant pathway (the score = 155; the score p-value = 1.88E-47) and ‘EMT’ as the third-rank significant pathological event relevant to the miR-200b target network (the score = 61; the score p-value = 4.15E-19) (Additional file 1).

Discussion

In general, a single miRNA concurrently downregulates hundreds of target mRNAs by binding to the corresponding 3’UTR of mRNA via either perfect or imperfect sequence complementarity [3]. Such fuzzy mRNA-miRNA interactions result in the redundancy