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Screening of cell death genes with a mammalian genome-wide RNAi library

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We report the construction and application of a mammalian genome-wide RNAi library. The oligodeoxynucleotides encoding ~200,000 shRNA sequences that targeted 47,400 human transcripts were inserted into a lentivirus vector pFIV-H1-puro, and a pool of pseudovirus particles with a complexity of ~200,000 were used to infect target cells. From the cells surviving apoptogenic Fas stimulation, four candidate shRNA sequences were obtained that provided resistance to Fas-induced cell death, including two shRNAs for caspase-8, an shRNA for Bid, and an shRNA for Fas. The reconstructed shRNAs with these sequences were shown to reduce expression of the respective gene products and increase survival after Fas stimulation. When similar selection was performed for tunicamycin-induced apoptosis, no shRNA strongly inhibiting tunicamycin-induced cell death was isolated, although a few reconstructed shRNAs led to a slight increase of survival. Thus, this genome-wide shRNA library proved useful for selection of genes that are involved in cell death, but some limitation was also revealed.

Keywords: apoptosis/genome-wide/library/RNAi/shRNA.

Abbreviations: BH3, Bcl-2 homology 3; DISC, death-inducing signalling complex; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; pAb, polyclonal antibody; PI, propidium iodide; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; TM, tunicamycin; UPR, unfolded protein response.

Forward genetics is one of the powerful approaches to identifying components involved in biological functions, as demonstrated in lower organisms such as nematodes and flies (*1*, *2*). Genome-wide loss-of-function genetic modifications have generally been achieved by chemical mutagenesis or insertion of transposons or viruses into chromosomal DNA, which sometimes alter the phenotype of the organism. Although reverse genetics to modify specific genes of interest is a common technique for studying gene function in higher eukaryotes, the authentic genetic modifications used in forward genetics (particularly those achieved with chemical modifications) are difficult to apply, because identification of the genes responsible for the phenotype requires an enormous amount of time and effort, as demonstrated in the identification of genes for various familial diseases (*3*).

Instead of authentic genetic modifications, the RNA interference (RNAi) technique can be a powerful method for suppressing the expression of specific genes in a genome-wide manner. RNAi was first recognized as a gene silencing process that specifically targeted viral RNA in plants (*4*, *5*), and was later found to be a mechanism conserved in a wide variety of eukaryotic organisms (*6*–*10*). It has since been widely used for sequence-specific silencing of target genes to analyze their functions in various organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals. Short double-stranded RNAs with part of the sequence of the target mRNA, called small interfering RNAs (siRNAs), are incorporated into the RNA-induced silencing complex (RISC), after which the assembled siRNA/RISC recognizes and cleaves the target mRNA (*11*). Short hairpin RNA (shRNA) consisting of a double-stranded RNA stem and an RNA loop also acts like siRNA after processing (*12*). Therefore, introduction of a mixture of siRNAs/shRNAs targeting all transcripts should be equivalent to genome-wide loss-of-function genetic modification. It would be useful to construct a genome-wide RNAi library that contains siRNAs targeting almost all transcripts, because it would allow convenient screening for genes associated with various biological phenomena, including cell death, proliferation and differentiation.

Apoptosis is one of the mechanisms of cell death, and it has a crucial role in various biological events, including development, tissue homeostasis and removal of unwanted cells (*13*). Apoptosis is induced by various stimuli, including stimulation of the Fas death receptor, endoplasmic reticulum (ER) stress, chemotherapy drugs and reactive oxygen species (*14*).

Apoptotic signals triggered by Fas stimulation are transmitted by two pathways depending on the type of cells (*15*). In type I cells, Fas stimulation activates caspase-8 by rapid formation of the death-inducing signalling complex (DISC) (*16*), followed by activation of caspase-3, and activation of these caspases as well as apoptotic death are not blocked by overexpression of Bcl-2, an anti-apoptotic protein (*17*, *18*). In type II cells, DISC formation is less prominent and signals are transmitted to the mitochondria, probably via Bid (*19*), a Bcl-2 homology 3 (BH3)-only protein in Bcl-2 family, so that Fas-mediated apoptosis is inhibited by Bcl-2 overexpression. Once signals are transmitted to the mitochondria, cytochrome *c* is released and binds to Apaf-1 to activate caspase-9, and consequently caspase-3 (*20*–*22*). In both cell types, activated caspase-3 cleaves various cellular substrates to produce apoptotic characteristics.

The ER is the site for the synthesis and folding of secreted, membrane-bound and some organelle-targeted proteins. Disturbance of ER function, induced by tunicamycin (TM) or thapsigargin, results in the accumulation and aggregation of unfolded proteins (*23*), and this process is termed 'ER stress'. Although various protective responses are activated to promote survival in the face of ER stress, including the unfolded protein response (UPR) and ER-associated degradation (ERAD) (*24*–*28*), persistent protein aggregation will lead to stress that induces cell death. Cell death induced by ER stress is being extensively studied at present because it has been implicated in the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease (*29*–*32*) and Parkinson's disease (*30*, *33*, *34*). Several genes have been reported to be involved in ER stress-induced cell death, including caspase-12 (*31*), calpain (*35*), Bim (*36*), ASK1 (*37*), JNK (*37*), PERK (*38*) and CHOP (*39*), but the precise mechanisms have still been not elucidated.

We constructed a genome-wide RNAi library by utilizing a lentivirus vector system. We then applied the library to screen for genes involved in Fas-induced cell death and TM-induced cell death, and found the library very useful for detecting genes that play a role in cell death mediated by the relatively simple mechanisms.

Materials and Methods

Cells, antibodies and reagents

D98/AH2 cells (*40*), which are descendants of HeLa cells (*41*), and their derivatives were maintained in RPMI-1640 medium containing 10% foetal bovine serum at 37°C with 5% CO_2. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum at 37°C with 10% CO_2. D98/AH2 cells were transfected with pCAGGS-human Bcl-2 (*42*) to generate D98-HB2 cells. Vector transfected cells (D98-V cells) were also generated in a similar way. The antibodies used were agonistic anti-Fas mouse monoclonal antibody (mAb) (clone CH-11: MBL, Japan), anti-caspase-8 mouse mAb (clone 5D3, MBL), anti-Bid goat polyclonal antibody (pAb) (R&D), anti-Bim rabbit pAb (BD PharMingen), anti-JNK mouse mAb (MAB17761, R&D), anti-CHOP mouse mAb (MA1-250 (9C8), Affinity BioReagents), anti-PERK rabbit pAb (H300, SantaCruz), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mouse mAb (MAB374, CHEMICON), anti-Apaf-1 rat mAb (MAB3505, CHEMICON), horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-rat IgG antibodies (Cell Signaling), HRP-conjugated anti-goat IgG antibody (SantaCruz) and fluorescein isothiocyanate (FITC)-conjugated anti-Fas antibody (clone UB2: MBL). TM and propidium iodide (PI) were obtained from Wako (Japan). FITC-conjugated Annexin V was purchased from BioVision. z-VAD-fmk was from Peptide Institute (Japan). Necrostatin-1 (Nec-1) and Hoechst33342 were from Sigma. Enhanced Chemiluminescence Plus (ECL Plus) Western Blotting Detection reagents were purchased from GE Healthcare. The plasmids pFIV-H1-puro and pPack were products from System Bioscience.

Construction of the shRNA Library

From each capture probe sequence (25-mer) of the Affymetrix GeneChip Human Genome U133 plus 2.0, targeting more than 47,000 transcripts, we generated three sequences by extending two nucleotides at the 5′-end, one nucleotide at both the 5′- and 3′-ends or two nucleotides at the 3′-end (depending on the genomic sequence) to obtain a 27-mer sequence. Thus, 33 sequences were obtained from the 11 capture probes for each target transcript. The siRNAs were examined by using SBI's proprietary algorithm in silico, and three to five siRNAs satisfying our criteria were selected for each target transcript. The criteria included a GC content in the range of 40–50%, fewer than five consecutive G nucleotides, and fewer than four consecutive A or U nucleotides. Each shRNA was designed to have a 27-mer stem with 2-3 GT mismatches in the sense region and a 12-nucleotide loop CUUCCUGUCAGA. In total, 216,568 shRNAs were designed. Oligonucleotides containing the shRNA sequences together with 5′- and 3′-flanking sequences were purchased from Atactic Technologies. The shRNA oligonucleotides were amplified by PCR. Multiple shRNA fragments were pooled and inserted into the pFIV-H1-puro cloning vector at the BamHI and EcoRI sites by multiplex ligation. Ligation products were transformed into TransforMax EC100 electrocompetent cells (Epicentre). The library thus created (pFIV-H1-puro 50k shRNA library) is available from System Bioscience (SBI) as GeneNet^{TM} siRNA Libraries.

Virus preparation and infection of D98/AH2 cells

To prepare pseudoviral particles from the above library, HEK293T cells (9×10^7) were transfected with pFIV-H1-puro 50k shRNA library plasmids (45 μg in total) and plasmids pPack (90 μg in total) using Lipfectamine2000 (Invitrogen). After incubation for 4 h, the culture medium was replaced with fresh medium, and the cells were incubated for additional 48 h. Then the medium containing pseudoviral particles from the lentiviral shRNA library were collected, filtered, and used to infect D98/AH2 cells (1.4×10^7 cells × three pools) in the presence of 4 μg/ml polybrene. Infected cells were selected by incubation with 1 μg/ml puromycin for 4 days, and three pools of the infectants were separately maintained throughout. More than 80% of cells were infected with the pseudovirus, judging from the survival after puromycin treatment (data not shown).

Concentration of cells with resistance to apoptotic stimuli

From each of the three pools of D98/AH2 cells infected with the pFIV-H1-puro 50k shRNA library obtained as described above, 2×10^7 cells were plated in 15 cm dishes (2×10^6/dish). After incubation for 24 h, cells were treated with 1 μg/ml agonistic anti-Fas-antibody for 10 h or with 2 μg/ml TM for 42 h. Cells without any stimulation were also prepared similarly. Then all of the cells were harvested, washed with fresh medium to remove residual drugs, and plated into new dishes. After incubation for 10 days, the surviving cells were harvested.

Identification of candidate shRNA sequences

Total RNA was purified from untreated cells or survivors (1×10^7) recovered from each of the three pools by using TRIzol (Invitrogen) according to the manufacturer's instructions. The antisense portions of shRNA regions in the recovered RNAs were amplified by reverse transcription followed by two-step PCR according to the SBI GeneNet^{TM} Library Manual. The second nested PCR was performed using 5′-phosphate nested reverse primer and nested forward biotinylated primer. The biotinylated shRNA probes thus obtained were treated with exonuclease lambda (Epicentre) to remove the non-biotinylated sense strand. Hybridization onto an Affymetrix

GeneChip Human Genome U133 plus 2.0 was carried out with ~7.5 μg of biotinylated siRNA probes according to the manufacturer's standard protocols and using the recommended reagents.

Data analysis

From raw microarray intensity data, probes represented in the pFIV-H1-puro 50k shRNA library were extracted by using Data Analysis Software and further analysis was performed with GeneSpring GX Software (version 7.3.1). Per-chip normalization was performed by dividing the signal of each gene by the median intensity for the chip, for each of the six arrays within each cell line. For each probe, the ratios of the hybridization intensities of treated cells relative to control cells were computed within each pool. Overrepresented probes were selected when the ratio of intensities between treated cells and control cells showed a difference of more than 2-fold for anti-Fas antibody and 12-fold for TM in all pools. Then the overrepresented probes were analyzed by Welch's *t*-test. The Benjamini and Hochberg procedure (*43*) was used to control the false discovery rate. Probes satisfying the criterion of $P < 0.05$ were used for further analysis.

Reconstruction of shRNAs bearing pFIV-H1-puro

Oligonucleotides containing a sequence complementary to the primer sequence as described below, the BamHI site, the sense sequence of the target shRNA, the loop sequence, the antisense sequence, the RNA Polymerase I terminator and the EcoRI site were purchased from Gene Design (Japan). Sense strands were synthesized by using LA Taq polymerase (Takara, Japan) with a primer (AGTTCTGTATGAGACCACTT). After treatment with BamHI and EcoRI, the resulting double-stranded DNA fragments that encoded the respective shRNAs were inserted into a pFIV-H1-puro cloning vector by using a DNA ligation kit version 2.1 (Takara, Japan). The sense sequences of the shRNAs selected in this study are shown in Tables I and II. Control constructs were synthesized similarly, and the sense sequences were as follows: Apaf-1 (GTGGAGAGTTCCTTAGGAA) and random (negative control) (ACGTCGTGCGCGTGATAACGCGATAAT).

Transfection of cells with siRNAs

The following siRNAs were produced by B-Bridge International, Inc: human apaf-1 siRNA, 5′-GGAUAAUGGUGGCAGCAA A-3′; human Bim siRNA, 5′-CCGAGAAGGUAGACAAUUG-3′; human TRAF2, GGACCAAGCUGGAAGCCAA; human JNK, G GUAAUAGAUGCAUCUAAA; human CHOP, GGAGGAAGA CCAAGGGAGA; human PERK, GGUAAAAAGCAGUGGGA UU and negative control siRNA, 5′-UUUUCCGAACGUGUCA CGU-3′. The siRNA to human Ask1 were purchased from QIAGEN (catalog no. is SI02224026). Cells (1×10^6) were transfected twice on alternate days with 5 μg of siRNA by electroporation using Amaxa system (Amaxa).

Assay of apoptosis

D98/AH2 cells and their derivatives were plated in 6-well dishes (2×10^5 cells/well). After 24 h, cells were treated with 1 μg/ml of an agonistic anti-Fas-antibody for 10 h or with 2 μg/ml of TM for 42 h. Then the cells were harvested and fixed in 4% formaldehyde/PBS for 24 h. Fixed cells were stained with 10 μM Hoechst33342 in the dark for 5 min and were observed under a fluorescence microscope (Carl Zeiss, AXIO). For quantitative analysis, all cells were harvested after treatment with 2 μg/ml TM for 48 h and stained with PI, after which the fluorescence intensity was measured on a FACS Canto II flow cytometer (BD Biosciences). Data were expressed as the mean ± SD of three independent experiments, and *P*-values were calculated by Student's *t*-test.

Survival assay

D98/AH2 cells and their derivatives were treated with anti-Fas-antibody or TM under the indicated conditions, and then were harvested and washed to remove residual apoptosis inducers, after which cell numbers were measured by using a cell counter (Beckman Coulter). Serially diluted cells were seeded into 24-well dishes. After incubation for several days, the number of surviving colonies was counted under a microscope. At least 100 colonies were counted and data were expressed as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test.

Western blot analysis

Cells were collected and suspended in buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 0.1% CHAPS and 10% sucrose) containing protease inhibitors (Complete Mini: Roche). Then the cells were lysed by adding Triton-X100 at a final concentration of 1%. After clarification by centrifugation, the protein concentration of the lysates was measured by using DC Protein Assay Reagent (BioRad). Total protein from the lysates was separated by 15% SDS–PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). Then immunoblot analysis was performed with the respective primary antibodies and an HRP-conjugated secondary antibody using Enhanced Chemiluminescence (ECL) Western Blotting Detection reagents (GE Healthcare).

Table I. Target sequences and genes of shRNA probes selected by screening for Fas-induced cell death.

Target sequence	Genbank ID	Target gene	shRNA ID
GATACACAGGACAGTTCACAGCAAATT	AU152583	NM_014021.2|SSX2IP	SSX2IP
GTGAATAACTGTGTTTCCTACCGAAAC	g4502582	NM_001228.3|CASP8	CASP8-1
ATGGATTTATCATCACCTCAAACGAGA	g4502582	NM_001228.3|CASP8	CASP8-2
GGGACAGCATGGACCGTAGCATCCCTC	g13543452	NM_197966.1|BID	BID
TAACACGTTCCAACACAGCAGTAGTGG	U00955	NM_020920.1|CHD8	CHD8
ACCAAGGTTCTCATGAATCTCCAACCT	Z70519	NM_000043.3|FAS	FAS

Table II. Target sequences and genes of shRNA probes selected by screening for TM-induced cell death.

Target sequence	Genbank ID	Target gene	shRNA ID
TCACCTTCGCAGATTCCAAGTTCCAGG	AA639585	NM_024327.1| ZNF576	ZNF576
GATTCTTCACCAGGGTTATCACACGTA	AF102850	NM_013338.3| ALG5	ALG5
CTTGAATAAATCTTAGTCTCTGTTTCT	AI022702	EST	EST-1
GAAGGCCACCTGAAGGGTGGACAGGCA	AI129346	NM_021075.3| NDUFV3	NDUFV3
GATGGTGCAGGTGCTTTCTATTATTTA	AU146087	EST	EST-2
GTGCAGGTGCTTTCTATTATTTAATAG	AU146087	EST	EST-3
CTGCTGCCCTGACTGGGATGAGGGTGA	AV661533	NM_182775.1| ALS2CL	ALS2CL
ACCCTATCTGCCTGTGAGGACTGGGCA	AW134997	EST	EST-4
CCCTGGGATGAGTGCACACTGGGATGG	BC040315	EST	EST-5
ATCTACACTCACATGATATTCTTATTC	BF696304	NM_032832.3| LRP11	LRP11
CAGAACTTACTGCTTAGTCTTTGTACT	BG386322	EST	EST-6
TTTAATTGCATACCATTCTCTTCACAG	N74222	NM_153028.1| ZNF75A	ZNF75A

Flow cytometry

Cells were collected and incubated with FITC-conjugated anti-Fas antibody for 5 min, after which the fluorescence intensity was measured by using a FACS Canto II flow cytometer.

Results

Library construction

It was considered useful to construct a loss-of-function genetic screening system in genome-wide manner for identification of the components involved in cellular signalling pathways. To achieve this goal, we aimed to establish a genome-wide knockdown library by utilizing the RNAi technique with a lentivirus vector system. Since the efficiency for reducing target mRNA levels varies among different siRNAs and the existence of single nucleotide polymorphisms may impair the activity of RNAi, we considered that several siRNA sequences targeting each transcript should be included in the library. We also intended to include siRNA sequences with a higher efficiency in the library. We found that the principal siRNA prediction rules and the rules for short hybridization probes were similar in the nucleotide length, GC content and exclusion of consecutive nucleotides (see Supplementary Table 1). Supported by these findings, the capture probe sequences of the Affymetrix GeneChip Human Genome U133 plus 2.0 (targeting >47,000 transcripts) were adopted to construct a genome-wide RNAi library. This strategy allowed us to use the GeneChip to identify candidate siRNA sequences of interest during library screening experiments, as described below.

We used 27-mer siRNAs instead of the common 19-mer siRNAs because it has been shown that a longer has a higher knockdown potency (*44*). The procedure to construct genome-wide RNAi library was illustrated in Supplementary Fig. 1. For each of the >47,000 target transcripts on the Affymetrix GeneChip Human Genome U133 plus 2.0, three to five siRNA sequences satisfying our criteria were selected as described in 'Materials and methods' section. Each shRNA was designed as a 27-mer stem with two to three GU mismatches in the sense region and a 12-nucleotide loop (CUUCCUGUCAGA). A total of 216,568 shRNAs were designed.

Oligonucleotides containing shRNA sequences together with their 5′- and 3′-flanking sequences were amplified by PCR, and inserted into the pFIV-H1-puro cloning vector between the BamHI and EcoRI sites. The resultant library plasmids were used to transfect HEK293T cells with the packaging plasmids pPack to generate pseudoviral particles from the lentiviral shRNA library for infection of the target cells.

Characteristics of Fas-induced death of D98/AH2 cells

Using the new genome-wide RNAi library, we attempted to detect genes involved in Fas-induced or TM-induced cell death. Among several cell lines, cell line D98/AH2 (derived from HeLa) was the most suitable, because these cells survived apoptosis-inducing treatment if the apoptotic signalling pathways were blocked, as described below.

When vector-transfected D98/AH2 cells (D98-V cells) were treated with agonistic anti-Fas antibody, chromatin condensation and nuclear fragmentation, which are characteristic features of apoptosis, were observed (Fig. 1A). These morphological changes were suppressed by overexpression of Bcl-2, an anti-poptotic protein (Fig. 1A). Thus, anti-Fas antibody treatment of D98/AH2 cells induced typical apoptosis and these were type II cells for Fas-induced apoptosis.

It was reported that Fas stimulation induced necrotic cell death under the condition where the activity of caspase-8 was inhibited in some cell lines (*45–47*). The cell death is called necroptosis (*47*) or programmed necrosis (*48*, *49*) and is inhibited by necrostatin-1 (Nec-1) (*47*) through inhibition of RIP1 kinase (*50*). It was also reported that cell death was not induced by treatment of HeLa cells with anti-Fas antibody in the presence of an inhibitor of caspases, z-VAD-fmk (*45*). To confirm that apoptosis is the major mechanism in Fas-mediated death of D98/AH2 cells, which

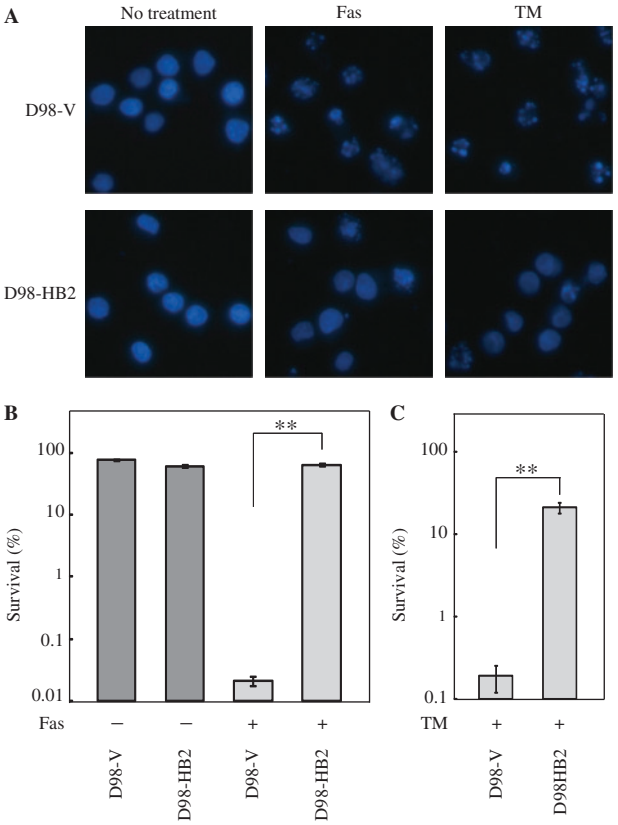

Fig. 1 Fas- and TM-induced apoptosis and inhibition by Bcl-2. (A) D98-V and D98-HB2 cells were treated with or without anti-Fas antibody (1 μg/ml) for 10 h or TM (2 μg/ml) for 42 h, and nuclear morphology was assessed as described in 'Materials and methods' section. (B) D98-V and D98-HB2 cells were treated with or without anti-Fas antibody (1 μg/ml) for 10 h and colony-forming activity was assessed as described in 'Materials and methods' section. Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $^{**}P<0.001$. (C) D98-V and D98-HB2 cells were treated with TM (2 μg/ml) for 42 h and colony-forming activity was assessed as described in 'Materials and methods' section. Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $^{**}P<0.001$.

are descendants of HeLa cells, we treated D98/AH2 cells with anti-Fas antibody in the presence of z-VAD-fmk. As shown in Fig. 2A, cell death induced by Fas stimulation was strongly inhibited by the presence of z-VAD-fmk even after incubation for 48 h, and necrostatin-1 did not affect the cell death. Under a similar condition, when Jurkat cells were incubated in the presence of z-VAD-fmk with anti-Fas antibody, cell death was inhibited partially by z-VAD-fmk, and the cell death observed in the presence of z-VAD-fmk was efficiently inhibited by necrostatin-1 (Supplementary Fig. 2). Thus, both apoptotic cell death and necrotic cell death of Jurkat cells were induced by Fas stimulation, but D98/AH2 cells did not undergo necrosis by Fas-stimulation even in the absence of caspase-8 in our cell death system.

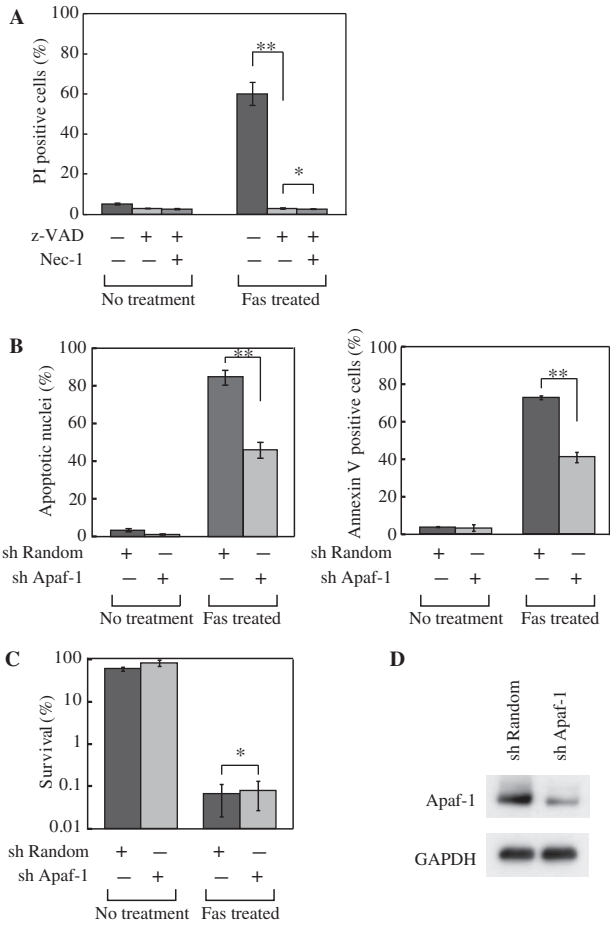

Fig. 2 Characteristics of Fas-induced cell death of D98/AH2 cells. (A) D98/AH2 cells were treated with or without anti-Fas antibody (1 μg/ml) for 48 h in the presence or absence of z-VAD-fmk (100 μM) and Nec-1 (20 μM), and the extent of cell death was measured using flow cytometer after staining with PI as described in 'Materials and methods' section. Data are shown as mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $^{**}P<0.001$, $^{*}P>0.2$. (B) D98/AH2 cells were stably infected with pseudoviruses carrying shRNAs targeting Apaf-1 gene or with control pseudoviruses (Random). Infectants were treated with anti-Fas antibody (1 μg/ml) for 10 h, and nuclear morphology (left) and staining with Annexin V (right) were assessed as described in 'Materials and methods' section. Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $^{**}P<0.001$. (C) Infectants obtained as described in (B) were treated with anti-Fas antibody (1 μg/ml) for 10 h and colony-forming activity was assessed as described in 'Materials and methods' section. Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $^{*}P>0.7$. (D) Total protein (15 μg) from infectants obtained as described in (B) was examined for expression of Apaf-1 and GAPDH (loading control) by western blotting as described in 'Materials and methods' section.

We next examined whether blocking the apoptotic signalling pathways could preserve the viability of the cells under apoptotic conditions. D98-V cells or Bcl-2-expressing D98-HB2 cells were treated with anti-Fas antibody and then were harvested, washed and seeded into new dishes. About 70% of untreated D98-V or D98-HB2 cells formed colonies under these conditions (Fig. 1B). After treatment with anti-Fas-antibody for 10 h, <0.1% of D98-V cells survived, but D98-HB2 cells showed almost the same viability as untreated cells according to the clonogenic assay (Fig. 1B). These results indicated that when the apoptotic signalling pathways were shut off by overexpression of Bcl-2, D98/AH2 cells could survive despite treatment with anti-Fas antibody.

Apaf-1 functions downstream of the mitochondria in the intrinsic apoptotic signalling to activate caspase-9 and caspase-3 (*20–22*). When Apaf-1 was silenced by shRNA in D98/AH2 cells (Fig. 2D), apoptotic morphological changes of nuclei and apoptotic phosphatidyl serine exposure (Fig. 2B) were reduced, indicating that Apaf-1 was involved in Fas-mediated apoptosis of D98/AH2 cells. However, when cells with silencing of Apaf-1 were treated with anti-Fas antibody, and were seeded similarly, they did not show significant improvement of survival (Fig. 2C). Therefore, when shRNAs that decreased molecules acting upstream of the mitochondria in the signalling pathways involved in Fas-induced cell death are introduced into D98/AH2 cells, these cells would remain viable and be recovered after cell-death-inducing treatments.

Isolation of candidate genes for Fas-induced cell death

We first applied the pFIV-H1-puro 50k shRNA library constructed in this study to screen for genes that were responsible for Fas-induced death of D98/AH2 cells. We created three independent pools of 2×10^7 D98/AH2 cells infected with the pFIV-H1-puro 50k shRNA library, and each pool was separately incubated with anti-Fas antibody (1 μg/ml) for 10 h. Then cells were collected separately from each pool, washed and seeded into new dishes. Additional incubation for 10 days allowed the survivors to grow, and then the survived cells were recovered. Through these processes, the copy numbers of the shRNA sequences in the cells escaped the Fas-induced cell death specifically increased in the cell pools, so that these sequences could be detected by microarray analysis using the Affimetrix GeneChip.

Total RNA was prepared from these cells, and the shRNA sequences concentrated after treatment were detected by using the Affimetrix GeneChip as described in 'Materials and methods' section. First, the signal intensities of probes in untreated sample of one pool well correlated with those in the other pool (Fig. 3A), suggesting that the copy numbers of each shRNA in the different infectant pools were similar and that the complexity of shRNA sequences in the library was

maintained during virus preparation and virus infection. After anti-Fas antibody treatment, several probes showed an increase of hybridization intensity (Fig. 3B, Table III), indicating that the cells bearing these sequences were concentrated by our treatment. These sequences could include those that mediated resistance to Fas treatment, as well as sequences from cells that escaped Fas stimulation and sequences from cells that acquired resistance to Fas through mechanisms other than RNAi. Among them, the sequences that provided resistance to Fas treatment should show an increased hybridization intensity in all three independent screening experiments, while the others should not necessarily. Using these criteria, we identified six candidate siRNA sequences that showed a >2-fold increase of intensity in all three independent screening experiments (Table I). There were two siRNA sequences for caspase-8, one for Bid and one for Fas, which are molecules that are known to be involved in the regulation of Fas-induced apoptosis (*19*, *51–53*). The other two siRNAs targeted SSX2IP and CHD8, which have not been reported to be involved in Fas-induced apoptosis.

Characteristics of siRNA sequences obtained by screening for Fas-induced cell death

To evaluate the effectiveness of the siRNA sequences obtained by screening, we reconstructed virus-producing plasmids for the six candidates (Table I) and a negative control with a random sequence. D98/AH2 cells were infected by pseudoviruses produced from the reconstructed plasmids, and viability after anti-Fas antibody treatment and the efficiency of silencing the target genes were examined. Among the six candidates, shRNAs targeting caspase-8, Bid and Fas increased the survival of infected cells after anti-Fas antibody treatment (Fig. 4A), but those targeting SSX2IP and CHD8 did not affect cell survival. Thus, SSX2IP and CHD8 seem irrelevant to Fas-induced apoptosis, and were probably obtained accidentally. When expression of caspase-8 and Bid was examined by western blotting, we found that the siRNA sequences targeting caspase-8 and Bid effectively silenced the respective genes (Fig. 4B). Measurement of Fas expression by flow cytometry showed that the shRNAs targeting Fas also effectively reduced the

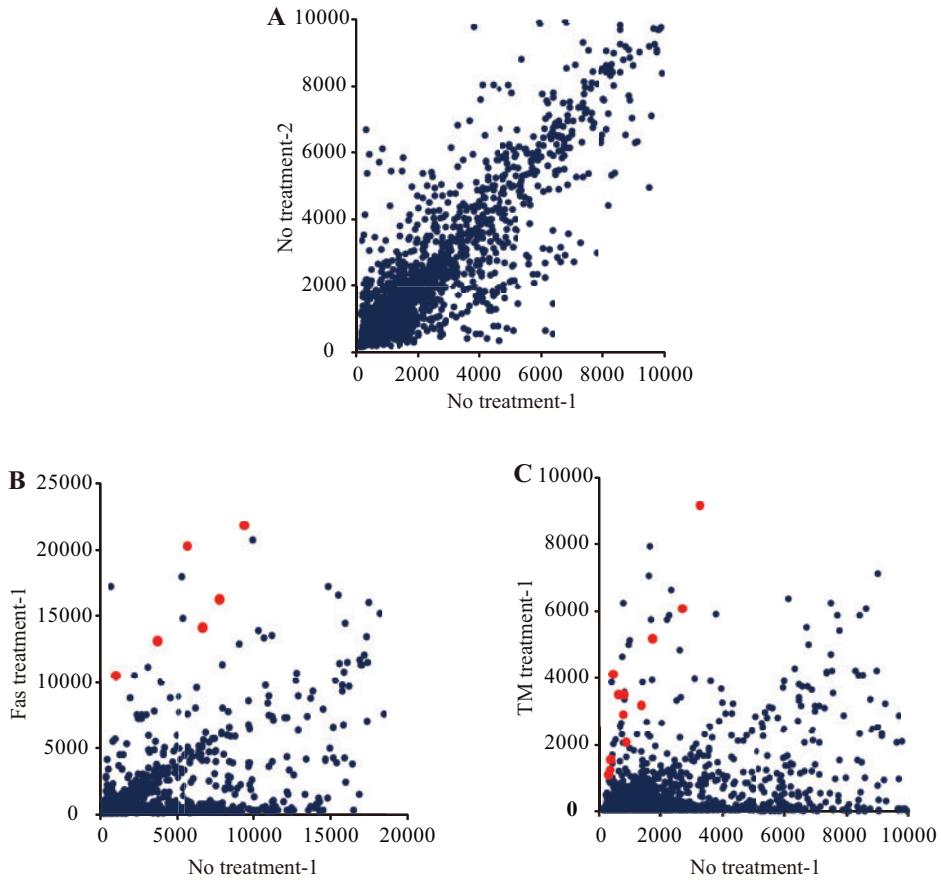

Fig. 3 Hybridization intensity of shRNA probes from control cells and treated cells. The hybridization intensity of 10,000 randomly selected probes on the Affimetrix GeneChip is shown. The raw data before normalization are presented. (A) The signal intensity of each probe obtained from an untreated sample of pool-1 was plotted against that of pool-2. (B) The signal intensity of each probe obtained from an untreated sample of pool-1 plotted against that from a Fas-treated sample of pool-1. Most probes showed a reduction of signal intensity after Fas treatment, suggesting that the cells carrying these sequences were lost or decreased by the treatment. Probes selected for this study are shown by red dots. Their hybridization intensity increased, indicating that the cells carrying these sequences were concentrated by the treatment. Several sequences indicated by blue dots also showed an increase of hybridization intensity in pool 1, but there was no increase of intensity in either or both of the experiments using pools 2 or 3 (data not shown), while the probes marked by red dots showed an increase of intensity in all three independent experiments. (C) The signal intensity of each probe obtained from an untreated sample of pool-1 plotted against that from a TM-treated sample of pool-1. The probes selected in this study similarly show an increase of hybridization intensity.

Table III. Signal intensities of shRNA probes selected by screening for Fas-induced cell death.

shRNA	Gene	N1	N2	N3	F1	F2	F3
SSX2IP	SSX2IP	5640	5881	4099	20300	25622	11420
CASP8-1	CASP8	7805	7846	3604	16198	15379	18868
CASP8-2	CASP8	1092	1252	1317	10575	15126	10362
BID	BID	9380	13908	13381	21848	36467	32259
CHD8	CHD8	6700	5988	5072	14117	15082	12965
FAS	FAS	3752	3358	3029	13124	18626	17509

The signal intensities obtained by hybridization on an Affimetrix GeneChip are listed for each shRNA probe selected by screening for Fas-induced cell death. N1, N2 and N3: signals obtained from untreated samples of infectant pools #1, #2 and #3, respectively. F1, F2 and F3: signals obtained from anti-Fas antibody-treated samples of infectant pools #1, #2 and #3, respectively. Raw values before normalization are presented.

level of Fas expression (Fig. 4C–E). The silencing efficiency of the two shRNAs targeting caspase-8 was different (Fig. 4B), and was well correlated with cell viability after anti-Fas antibody treatment (Fig. 4A). Thus, the genome-wide RNAi library constructed in this study was proven to be a useful screening method for genes involved in Fas-induced cell death and therefore should also be useful when screening genes involved in various other biological mechanisms.

Characteristics of the TM-induced death of D98/AH2 cells

We next attempted to apply the genome-wide shRNA library to screen for genes that were responsible for TM-induced cell death. First we characterized the

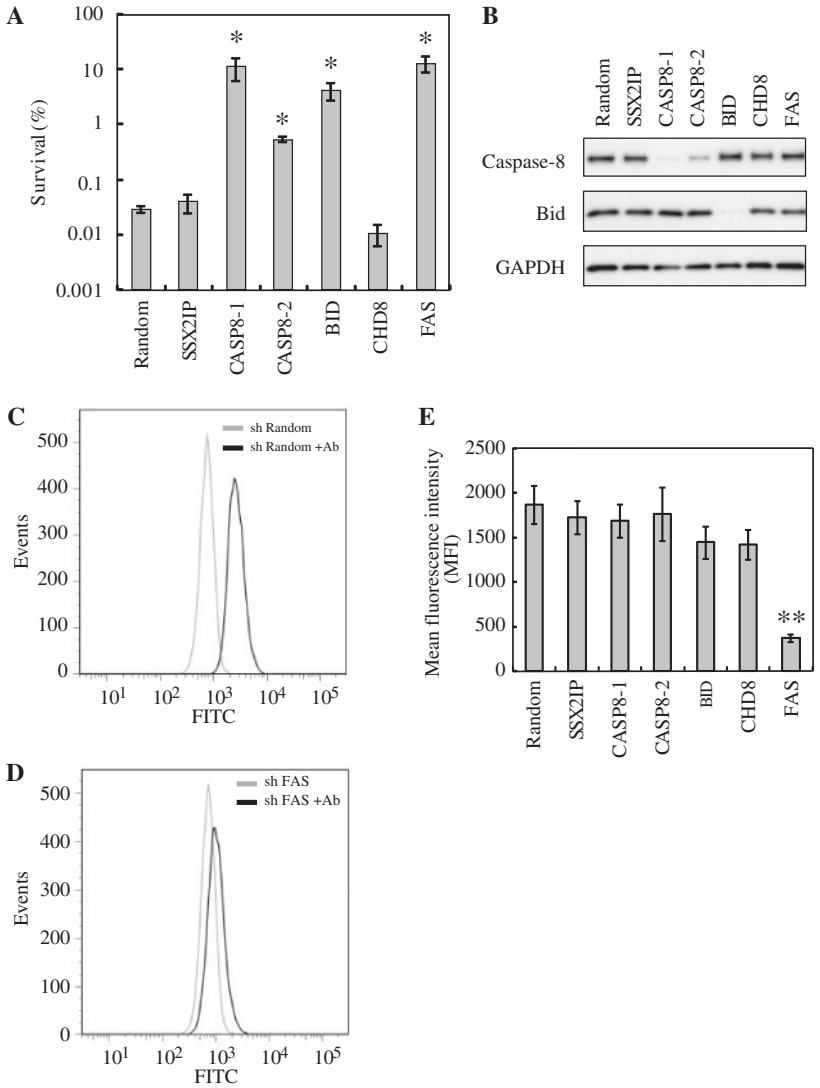

Fig. 4 Effect of candidate shRNAs on Fas-induced apoptosis. (**A**) D98/AH2 cells were stably infected with pseudoviruses carrying reconstructed shRNAs targeting the indicated candidate genes. Infectants were treated with anti-Fas antibody (1 μg/ml) for 10 h and colony-forming activity was assessed as described in 'Materials and methods' section. Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $*P<0.02$. The other *P*-values were all >0.1. (B) Total protein (15 μg) from infectants obtained as described in (A) was examined for expression of Caspase-8, Bid and GAPDH (loading control) by western blotting as described in 'Materials and methods' section. (C and D) Expression of Fas in D98/AH2 cells stably infected with control shRNA (C) or a reconstructed plasmid expressing shRNA that targeted Fas (D) was examined by flow cytometry using FITC-anti-Fas antibody as described in 'Materials and methods' section. The *y*-axis indicates the number of events detected. The difference between the mean fluorescence intensity (*x*-axis) of cells stained with FITC-anti-Fas antibody and that of cells without staining indicates the expression of Fas. (E) Values are the mean fluorescent intensity (MFI), calculated as described in (C and D) were shown for expression of Fas in the infectants obtained as described in (A). Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $**P<0.001$. The other *P*-values were all >0.1.

death of D98/AH2 cells induced by TM. When vector-transfected D98/AH2 cells (D98-V cells) were treated with TM, chromatin condensation and nuclear fragmentation were observed, similarly to the cell death induced by Fas-stimulation (Fig. 1A). These morphological changes were suppressed by overexpression of Bcl-2 (Fig. 1A). Thus, TM treatment of D98/AH2 cells induced typical apoptosis.

Several genes were reported to be involved in ER stress-induced apoptosis (*31*, *35–39*). To examine whether these genes were involved in TM-induced death of D98/AH2 cells, we transfected siRNAs to Apaf-1, Ask1, JNK, PERK, CHOP and Bim transiently to D98/AH2 cells, and treated with TM for 48 h. Each siRNA efficiently reduced the level of the respective target protein (Fig. 5B and C). CHOP and Bim were induced by ER stress (*36*, *54*), and the induced CHOP and Bim were also silenced by the respective siRNA (Fig. 5B and C). Under these conditions, cell death as assessed by PI staining (Fig. 5A) and apoptosis as assessed by Annexin V staining (Supplementary Fig. 3) induced by treatment with TM were inhibited by siRNA to Apaf-1, but not by other siRNAs (Fig. 5A, Supplementary Fig. 3). Thus, cell death induced by treatment with TM of D98/AH2 cells was Apaf-1 dependent, Bcl-2-inhibitable apoptosis. However, Ask1, JNK, PERK, CHOP and Bim were not involved in the cell death, or simple

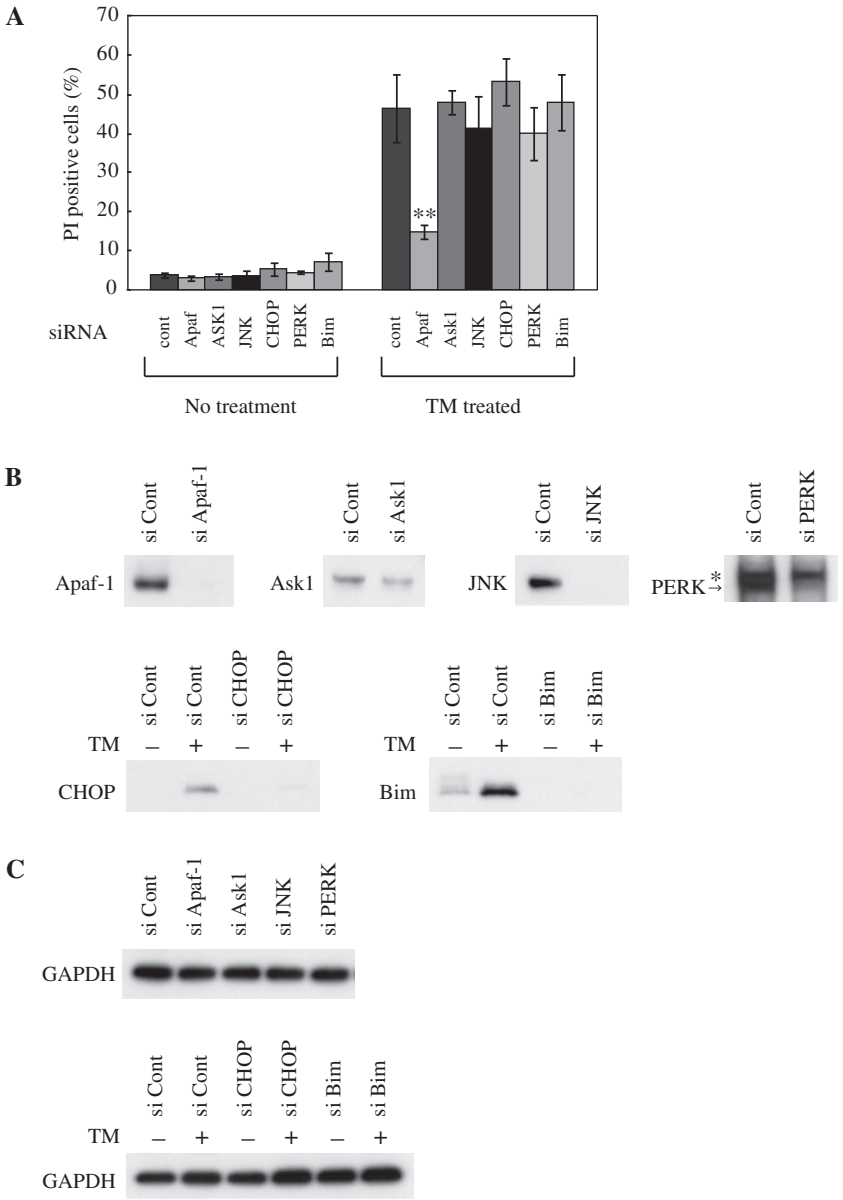

Fig. 5 Characteristics of TM-induced cell death of D98/AH2 cells. (A) D98/AH2 cells transfected with siRNA against Apaf-1, Ask1, JNK, CHOP, PERK and Bim were treated with TM (2 μg/ml) for 48 h and the extent of cell death was measured using flow cytometer after staining with PI. Data are shown as mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $^{**}P < 0.001$. The *P*-values of others are more than 0.2. (B and C) Total protein (15 μg) from transfectants obtained as described in (A) was examined for expression of Apaf-1 Ask1, JNK, PERK, CHOP, Bim (B) and GAPDH (C: loading control) by western blotting as described in 'Materials and methods' section. Asterisks shows non-specific band. For CHOP and Bim, samples from cells treated with TM for 24 h were also examined, because these proteins were induced by ER stress.

knockdown of these genes were not enough to prevent the cell death.

We next examined whether blocking the apoptotic signalling pathways could preserve the viability of the cells treated with TM. After treatment with TM for 42 h, D98-V cells or D98-HB2 cells were assessed for clonogenic activity as performed for Fas-induced apoptosis described above. Only ~0.2% of D98-V cells survived, and D98-HB2 cells showed a dramatic increase of colony formation (Fig. 1C), although with lesser efficiency than observed after anti-Fas antibody treatment (Fig. 1B). These results indicated that shutting off the apoptotic signalling pathways by overexpression of Bcl-2 could elevate the colony-forming activity of D98/AH2 cells after TM treatment. Therefore, the introduction of shRNAs that silenced the genes involved in the signalling pathways for the TM-induced cell death could possibly confer higher viability to the cells after TM treatment.

Isolation of candidate genes for TM-induced cell death

We also attempted to isolate genes involved in TM-induced cell death by using the same pFIV-H1-puro 50k shRNA library as that employed for Fas-induced cell death. Three independent pools of 2×10^7 D98/AH2 cells infected with the shRNA library were treated with TM (2 μg/ml) for 42 h, and the siRNA sequences concentrated by this treatment were detected in the same manner as when screening for genes related to Fas-induced cell death (Fig. 3C, Table IV). We identified 12 candidate siRNA sequences that showed a more than 12-fold increase in all three independent screening experiments (Table II). Note that we selected shRNAs that showed 12-fold increase in GeneChip intensities in TM-stimulated cell death, while 2-fold increase in Fas-stimulated cell death. The average of the signal intensity for TM-screening was lower than that for Fas-screening in our experiments (compare Fig. 3B and C), probably because of the difference in the varieties and amounts of shRNAs recovered, so normalized values in TM-screening were calculated higher, resulting in higher cut-off line for TM treatment.

Characteristics of siRNA sequences obtained by screening for TM-induced cell death

To evaluate the effectiveness of the siRNA sequences obtained through screening for TM resistance, we reconstructed virus-producing plasmids for the 12 candidates. D98/AH2 cells were infected with pseudoviruses produced by the reconstructed plasmids and extent of apoptosis was assessed by PI staining after incubation for 48h (Fig. 6A). Cell viability was also examined by replating cells that were treated with TM for 24 h (Fig. 6B), instead of 42 h in selection, because we expected that shorter incubation with TM would provide cells with less stimuli, and thus result in more obvious difference in the clonogenic assay. As shown in Fig. 6A, shRNA for Apaf-1 reduced the extent of apoptosis, to the extent similar to that with siRNA to Apaf-1 (Fig. 5A). The shRNA for ALG5 (1 of 12 shRNAs obtained by screening) slightly suppressed TM-induced apoptosis, but the other shRNAs

Table IV. Signal intensities of shRNA probes selected by screening for TM-induced cell death.

shRNA	Gene	N1	N2	N3	T1	T2	T3
ZNF576	ZNF576	784	693	435	2903	2539	1869
ALG5	ALG5	3244	467	2127	9098	6717	5929
EST-1	EST	346	341	281	1056	2544	1580
NDUFV3	NDUFV3	834	784	649	3482	2271	3703
EST-2	EST	659	1081	663	3482	4746	3328
EST-3	EST	370	502	302	1240	1800	1513
ALS2CL	ALS2CL	2720	2743	1869	6061	6501	7329
EST-4	EST	1360	1464	1006	3154	3793	4555
EST-5	EST	885	898	677	2044	2073	2293
LRP11	LRP11	486	768	863	4107	2356	4226
EST-6	EST	1755	1993	844	5124	6888	7172
ZNF75A	ZNF75A	420	295	257	1517	1556	1787

The signal intensities obtained by hybridization on an Affimetrix GeneChip are listed for each shRNA probe selected by screening for tunicamycin-induced cell death. N1, N2 and N3: signals obtained from untreated samples of infectant pools #1, #2 and #3, respectively. T1, T2 and T3: signals obtained from tunicamycin-treated samples of infectant pools #1, #2 and #3, respectively. Raw values before normalization are presented.

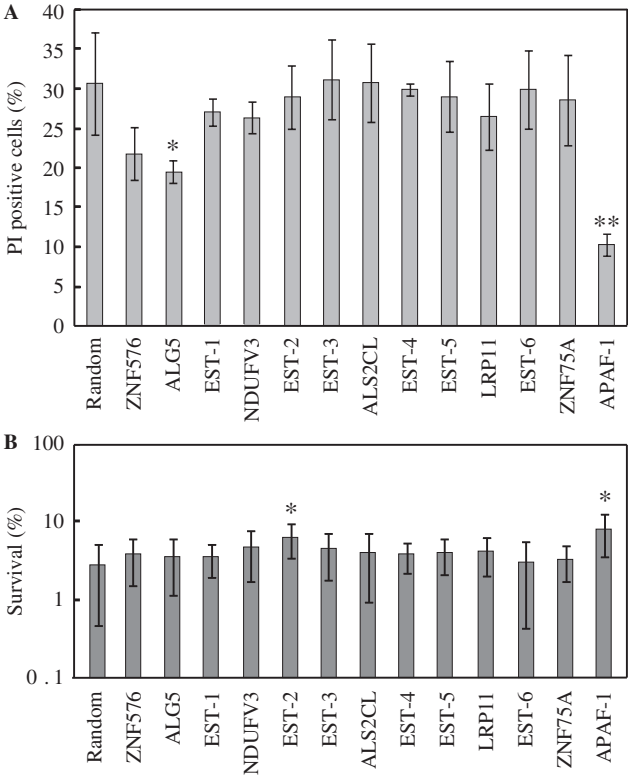

Fig. 6 Effect of candidate shRNAs on TM-induced cell death. (A) D98/AH2 cells were stably infected with pseudoviruses carrying reconstructed shRNAs targeting the candidate genes. Infectants were treated with TM (2 μg/ml) for 48 h and apoptosis was measured as described in 'Materials and methods' section. Data are shown as the mean ± SD from three independent experiments. *P*-values were calculated by Student's *t*-test. $*P<0.05$, $**P<0.01$. The other *P*-values were all >0.1. (B) Infectants shown in A were treated with TM (2 μg/ml) for 24 h and colony-forming activity was assessed as described in 'Materials and methods' section. Data are shown as the mean ± SD from six independent experiments. *P*-values were calculated by Student's *t*-test. $*P<0.05$. The other *P*-values were all >0.2.

showed no statistically significant effect. When D98/AH2 cells carrying these shRNA constructs were replated after TM treatment, cell lines carrying shRNAs to EST-2 and Apaf-1 exhibited slightly better survival (Fig. 6B), although the improvement was much less than that noted in our study of Fas resistance. The other shRNAs had no statistically significant effect. These results indicated that, although the candidates were concentrated by the screening for TM resistance, inactivation of each gene alone did not provide cells with strong protection against TM-induced cell death.

Discussion

A loss-of-function genetic screening system in genome-wide manner is one of the powerful approaches for identifying components involved in various biological functions. Among several loss-of-function genetic modifications, the RNAi technique is a powerful method for suppressing the expression of specific genes. Therefore, introduction of a mixture of siRNAs/shRNAs targeting all transcripts in cells is a useful method to generate genome-wide loss-of-function genetic modification with an advantage of gene tagging. In this article, we described the construction of genome-wide shRNA library in a pooled format, and its application to screen genes involved in cell death mechanisms.

There were two approaches to construct genome-wide shRNA library; one is in an arrayed format and the other is in a pooled format. The arrayed format strategy requires the development of a huge collection of shRNA constructs for all individual genes. Because shRNAs are arrayed in multiwell plates, a major advantage of an arrayed format is that shRNA constructs of interest could be easily identified through its location within the screening plate. There are currently two commercially available genome-wide shRNA libraries in an arrayed format, which cover 28,000 and 16,000 genes, respectively. Successful use of these arrayed libraries requires special high throughput facilities and techniques, so it is rather difficult for individual researchers to perform RNAi screen with them.

On the other hand, the pooled format strategy can be achieved by bulk cloning of synthesized oligonucleotides. After a library of shRNA constructs is transduced into cells, cells exhibiting the desired phenotypic changes can be isolated by appropriate screening. shRNA sequences of interest can then be recovered by PCR and identified by sequence analysis or microarray hybridization. Because only a single shRNA is expressed per cell in most cases, the pooled format should be desirable for analysis of a simple, not multiple, signalling pathway leading to a screening event.

We constructed our mammalian genome-wide RNAi library in a pooled format using lentiviral vector, mainly because of the lower cost for its construction and the easier handling of the product. Based on the similarity of the prediction rules for efficient siRNA sequences to the selection rules for short probes for GeneChip (Supplementary Table 1), we adopted the capture probe sequences of the Affymetrix GeneChip Human Genome U133 plus 2.0 for siRNA sequences in the library (Supplementary Fig. 1), allowing us to use the commercially available array to identify candidate shRNA sequences that were concentrated during library screening experiments. Thus, our shRNA library targets 47,400 human transcripts, with a complexity of ~200,000. The library is currently available from System Bioscience (SBI) as GeneNet^{TM} siRNA Libraries. The similar library has been successfully used for screening experiments to identify cellular proteins that contribute to HIV-1 replication (*55*).

We applied the library to screen for genes involved in two typical cell death mechanisms; Fas-induced cell death and TM-induced cell death. Because apoptotic signals triggered by Fas stimulation are transmitted through two pathways (type I and type II) depending on cell types (*15*), we first examined whether D98/AH2 cells are type I or type II. We found that typical apoptosis induced by Fas stimulation was blocked by overexpression of Bcl-2 in D98/AH2 cells, suggesting that D98/AH2 cells are type II. Therefore, we speculate that Fas stimulation of D98/AH2 cells induces the assembly of FADD and pro-caspase-8 to Fas receptor, and then the activated caspase-8 cleaves Bid, which transmits apoptotic signal to the mitochondria. From the mitochondria that received the signal from Bid, cytochrome *c* is released and binds to Apaf-1 to activate caspase-9, and consequently caspase-3.

Although silencing Apaf-1 or overexpression of Bcl-2 could prevent apoptosis of D98/AH2 induced by Fas-stimulation, the colony forming activity was recovered only by overexpression of Bcl-2 (Figs 1B and 2C). This is probably because prevention of signalling pathways of Fas-induced apoptosis at upstream of the mitochondria can restore viability, but not at downstream of the mitochondria. Therefore, genes involved in the upstream steps of the mitochondria are expected to be isolated for Fas-induced apoptotic cell death, when shRNAs are isolated from survived cells after incubation for 10 days during screening procedures. To pick up genes involved in the downstream steps of the mitochondria, it should be necessary to pool non-apoptotic cells just after Fas-treatment using, for instance, a cell sorter.

For screening genes involved in apoptotic cell death, we performed the identical selection with three independent pools of cells transfected with the shRNA library. If screening pressure is very strong enough to generate several clones to isolate, it should be better to treat all the cells carrying shRNA-producing constructs at once, and pick up the surviving colonies for identification of the shRNA sequences in the clones. However, most of the screening systems using cell death-inducing treatments allow from 0.01 to 1% of cells to survive after the treatments. These survivals should include cells bearing shRNAs that affected the cell death signalling pathways, as well as cells that escaped cell death stimulation and cells that acquired resistance through mechanisms other than RNAi. The shRNAs found in latter two cases are unrelated to the

cell death signalling pathways. Therefore, if we pick up all surviving colonies in such a case, we need to analyze too many clones for the sequences of shRNA, although most of them are irrelevant. To avoid these problems, we took an advantage of using shRNA sequences derived from Affymetrix GeneChip. Although the concentrated sequences included lots of irrelevant probes, we should be able to eliminate most of them by performing multiple independent selections, because the irrelevant clones do not necessarily survive in all independent selections. On the other hand, shRNAs that cause survival during death-inducing treatments should be concentrated after the treatment in all independent selections. We, therefore, carried out three independent selections, and picked up several sequences that were concentrated in all the three selection pools.

We identified caspase-8, Bid and Fas as the genes involved in the Fas-induced apoptosis pathway through the screening. The results were consistent with our expectation that genes involved in the upstream steps of the mitochondria would be isolated for Fas-induced apoptotic cell death, and we believe that this fact demonstrates the usefulness of the library we have made. The silencing of these genes did not recover the survival completely after Fas stimulation (Fig. 4A). Especially the recovery by shRNA: CASP8-2 was not so efficient. One of the possible reasons is that we cannot abolish the function of the target genes completely by shRNAs. The results in Fig. 4A and B showed good correlation between the efficiency to prevent cell death and the efficiency to reduce caspase-8, suggesting that the library we made in this study is useful even when the silencing efficiency is not so high. Furthermore, our successful isolation of shRNA sequence with partial silencing activity indicates that the screening system with our shRNA library is quite powerful to select the essential genes that participate in some biological functions.

We failed to identify FADD, another factor in DISC, in the current screening for Fas-induced cell death. We do not think that this is because the library is not saturated. Supplementary Table 2 shows the raw data for signal intensities of shRNA probes for FADD obtained by hybridization on an Affymetrix GeneChip. The results shown in this table indicate that all four shRNA sequences for FADD are included in the library. Therefore, the failure to identify shRNA probe for FADD was not because the library was not 'saturated' on the sequence basis. Although the FADD shRNA probes #1 to #3 decreased their copy numbers during Fas-selection, the copy number of the probe #4 seemed to be maintained. Unfortunately, the probe #4 was omitted in our screening, probably because the extent of the signals recovered was lower than the criteria that we set for positive signal. Although the precise reasons for the low efficiency for accumulation of shRNA sequences for FADD are not known, we speculate that the efficiency of silencing of these shRNA sequences was relatively low. This situation could be predicted, because, as described above the shRNA sequences included in the library were designed *in silico*, so all the shRNA sequences were not necessarily highly efficient in silencing. Furthermore, the efficiency of silencing could be different depending on cell types. Therefore, for the genome-wide shRNA library in a pooled format, we believe it is important to show that it is saturated on the basis of the sequences. Even though the library is saturated on the basis of the sequences, it is certainly possible that the library is not functionally saturated.

We also picked up other genes, SSX2IP and CHD8, but they did not provide improved survival for cells treated with death-inducing stimuli (Fig. 4A). Although we performed three independent selections and picked up clones that were recovered in all three independent pools, we still have certain chance to pick up irrelevant clones.

There are several reports showing that Fas stimulation induces necrotic cell death, so called necroptosis (*47*) or programmed necrosis (*48*, *49*), under the condition where the activity of caspase-8 is depressed in some cell lines. We found that D98/AH2 cells, descendants of HeLa cells, did not undergo necrosis by Fas-stimulation in the presence of z-VAD-fmk (Fig. 2A), consistent with the previous report (*45*). Thus, apoptosis is the major cell death mechanisms in HeLa cells by Fas-stimulation, and they do not undergo necrosis even in the absence of caspase-8 in our cell death system. The activation of the single cell death signalling pathway is probably the reason why we could successfully identify the genes responsible for Fas-induced cell death.

We also applied the genome-wide shRNA library to identify genes involved in TM-induced cell death. Although we identified a few clones that provided statistically significant levels of survival after TM treatment, the improvement was much less than that noted in our study of Fas resistance. One possible reason could be that multiple signalling pathways are involved in TM-induced cell death, so silencing one of the pathways only partially inhibits cell death. Consistent with this speculation, silencing of Apaf-1 was effective for reducing TM-induced apoptosis, as assessed by PI staining (Fig. 6A), but it only provided a similar improvement of clonogenic survival to that observed with the 12 candidates (Fig. 6B). Similarly, while the viability of Bcl-2-overexpressing D98/AH2 cells after anti-Fas antibody treatment was almost the same as that of untreated cells, viability was lower after TM treatment (Fig. 1B and C), suggesting that a form of cell death that was not regulated by Bcl-2 and Apaf-1 was also involved in TM-induced cell death.

Several genes were reported to be involved in ER stress-induced apoptosis (*31*, *35–39*). Among them, we reported that silencing of caspase-4, a human authologue of mouse caspase-12, did not prevent ER stress-induced apoptosis of D98/AH2 cells that we used here (*32*). Calpain family consists of more than 14 members, and silencing of calpain activity by shRNA should be difficult. In this study, we silenced other genes, and found that their silencing did not prevent TM-induced apoptosis of D98/AH2 cells (Fig. 5). The involvement of these genes in ER stress-induced apoptosis is controversial, probably because

their involvement could be cell line-specific or some of these genes could function simultaneously. Because no responsible genes for TM-induced apoptosis of D98/AH2 cells were reported, and silencing of several genes, including genes we isolated in this study, did not solely prevent TM-induced apoptosis of D98/AH2 cells, multiple pathways seem to be activated in TM-induced cell death in our system. Because infection of shRNA-producing virus can mostly silence one gene, it should be difficult to identify shRNAs that effectively suppress cell death involving multiple pathways. In this sense, the failure to identify shRNAs that effectively suppressed TM-induced cell death does not necessarily mean the uselessness of the library, but means that screening with shRNA library may not be successfully applicable to some biological events such as the TM-induced apoptosis, in which multiple signalling pathways are simultaneously activated.

In both Fas-induced cell death and TM-induced cell death, shRNA to Apaf-1 inhibited apoptotic manifestations, but it did not provide clonogenic survival. One possible explanation is that even if the apoptotic signalling pathways are interrupted at Apaf-1 step, the mitochondria are still damaged, and cells cannot survive any more. The other possibility is that, as described for FADD shRNA above, shRNAs to Apaf-1 included in this library do not silence the target efficiently enough to affect the survival. The Supplementary Table 3 shows the raw data for signal intensities of shRNA probes for Apaf-1 obtained by hybridization on an Affimetrix GeneChip before and after selection with Fas stimulation and TM stimulation. We again found all 12 shRNA sequences for Apaf-1 included in the library. This also indicates that shRNA to Apaf-1 is saturated on the basis of sequence. Therefore, the failure to identify shRNA probe for Apaf-1 might be caused by the absence of the shRNA sequences with silencing efficiency enough to increase the survival of cells after cell death-inducing treatments, or by the inability of silencing of Apaf-1 to support clonogenicity.

In summary, we constructed a genome-wide RNAi library based on a lentivirus system and showed it useful for identifying genes involved in biological processes using Fas-induced cell death as a model. Our strategy to perform multiple independent selections followed by analysis with the GeneChip enables us to apply our RNAi library to select genes of interest by appropriate screening even in case of relatively low screening pressure. We also recognize some limitation with viral shRNA library that shRNA library may not be successfully applied when multiple signalling pathways lead to a screening event, because infection of shRNA-producing virus can mostly silence one gene. Certainly this limitation is not specific for our library. We believe that our library is a powerful tool for screening genes involved in various biological processes.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

Takayuki Mizutani and Kayoko Yamada belong to B-Bridge International Inc., which has the right to sell the products of System Biosciences, Inc. in Japan.

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