Genome-Scale MicroRNA and Small Interfering RNA Screens Identify Small RNA Modulators of TRAIL-Induced Apoptosis Pathway

Dmitriy Ovcharenko,[1,2] Kevin Kelnar,[1] Charles Johnson,[1] Nan Leng,[1] and David Brown[1]

[1]Asuragen Inc., and [2]Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin, Texas

Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) binds to death receptors 4/5 and selectively induces caspase-dependent apoptosis. The RNA interference screening approach has led to the discovery and characterization of several TRAIL pathway components in human cells. Here, libraries of synthetic small interfering RNA (siRNA) and microRNAs (miRNA) were used to probe the TRAIL pathway. In addition to known genes, siRNAs targeting *CDK4, PTGS1, ALG2, CLCN3, IRAK4*, and *MAP3K8* altered TRAIL-induced caspase-3 activation responses. Introduction of the miRNAs *let-7c, mir-10a, mir-144, mir-150, mir-155*, and *mir-193* also affected the activation of the caspase cascade. Putative targets of these endogenous miRNAs included genes encoding death receptors, caspases, and other apoptosis-related genes. Among the novel genes revealed in the screen, *CDK4* was selected for further characterization. *CDK4* was the only member of the cyclin-dependent kinase gene family that bore a unique function in apoptotic signal transduction. [Cancer Res 2007;67(22):10782–8]

Introduction

Apoptosis, a form of cell suicide, is responsible for the removal of unwanted or supernumerary cells during development and in adult homeostasis (1–3). Members of the proinflammatory cytokine tumor necrosis factor (TNF) family play an important role in multiple cellular mechanisms, including cell proliferation, differentiation, septic shock, necrosis, and apoptosis (4, 5). Like other TNF family members, TNF-related apoptosis-inducing ligand (TRAIL, Apo-2L) is capable of activating intrinsic caspase-dependent apoptotic machinery (6–8). The TRAIL signaling pathway is of particular interest in cancer therapeutics because it has been shown to be active in the selective natural killer cell–mediated control of tumors (9). TRAIL acts through the death receptors DR4 and DR5 and induces apoptosis via the formation of a death-inducing signaling complex consisting of FADD and activated procaspase-8 (10). The activated caspase-8 can proceed to initiate apoptosis through at least two cell type–specific downstream signaling pathways. An expanded knowledge of the complexity of this signaling pathway would contribute to a more complete mechanism of TRAIL action and may help identify new avenues for therapeutic manipulation of TRAIL signaling.

Many apoptotic pathway components, including those involved in TNF-induced apoptosis, have been identified using gene silencing methods (11, 12). The recent development of unbiased functional genomics approaches using small interfering RNA (siRNA) libraries (13–17) has enabled high-throughput experimentation. These approaches have uncovered multiple novel genes involved TRAIL-induced apoptosis *in vitro* (13, 18). It is conceivable, however, that cell type–specific genes may also be involved in TRAIL signaling, so these pathways need to be explored over a number of cells types and their developmental and proliferative states. Additionally, non–protein-coding gene-regulatory elements may also influence these signaling pathways, and screening for these is also highly amenable to high-throughput methods. Consequently, discovery-based investigations into TRAIL signaling are still capable of yielding new and interesting hypotheses about the actions and regulation of this pathway.

The influence of regulatory noncoding RNA molecules on apoptotic cell signaling has not been extensively explored, but increasing evidence points to a role for microRNAs (miRNA) in the control of intrinsic developmental and proliferative cell programs and ligand-induced cell signaling (19–21). miRNAs are newly identified, highly conserved small RNA molecules up to 22 nucleotides in length, which are encoded in plant and animal genomes (22). miRNAs regulate the gene expression by binding to the 3′-untranslated regions (3′-UTR) of specific mRNAs. A single miRNA can regulate anywhere from a few genes to hundreds of genes, and because over 200 miRNA genes are present in higher eukaryotes, this gene-regulatory miRNA network impacts diverse cellular functions (23–25).

To further characterize the genes and gene networks regulating apoptosis in mammalian cells, MDA-MB-453 breast cancer cells were transfected with more than 17,000 unique siRNAs and nearly 200 synthetic miRNAs. Screening of phenotypic defects in these cells revealed that up to 2% of siRNAs and more than 20% of miRNAs significantly affected TRAIL-induced caspase-3 activation. These results uncovered novel regulators of TRAIL-induced apoptotic signaling and point to a role for miRNA in apoptosis regulation.

Materials and Methods

Cell culture. Human hepatocarcinoma cells [HepG2; American Type Culture Collection (ATCC)], human lung epithelial carcinoma cells (A549; ATCC), and human cervical adenocarcinoma cells (HeLa; ATCC) were cultivated in DMEM containing 10% fetal bovine serum (FBS; Invitrogen) and maintained under a humidified atmosphere of 5% CO_2 at 37°C. Cells were split 1:8 at 90% confluence and transfected between passages 10 and 18. Human breast cancer cells (MDA-MB-453; ATCC) were grown in Leibovitz's L-15 media supplemented with 10% FBS (Invitrogen) and maintained under a humidified atmosphere at 37°C, without additional CO_2. Cells were split 1:6 at 90% confluence and transfected between passages 24 and 28.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Dmitriy Ovcharenko, Institute for Cellular and Molecular Biology, University of Texas at Austin, 1 University Station A4800, Austin, TX 78712. Phone: 512-350-0302; Fax: 512-651-0601; E-mail: ovcharenko@mail.utexas.edu.

doi:10.1158/0008-5472.CAN-07-1484

Oligonucleotide synthesis. Single-stranded RNA oligonucleotides were synthesized and high-performance liquid chromatography purified (>90% purity as analyzed by mass spectrometry). Single-stranded RNA oligonucleotides were annealed to generate the double-stranded siRNAs and miRNA precursor molecules that were used for transfection. Annealed oligonucleotides were analyzed by nondenaturing PAGE (Ambion).

siRNA and miRNA controls. Silencer negative control siRNA 1, 2, and 3 (Ambion) were employed as non-silencing siRNA controls. Pre-miR negative control miRNA 1 and 2 (Ambion) were employed as random sequence precursor miRNA controls. All data were normalized to the mean value from three non-silencing siRNAs or from two random sequence miRNAs.

Chemical transfection. In preparation for transfection, cells were harvested by incubation with 0.05% trypsin-EDTA/PBS (Invitrogen) for 5 min at 37°C, and trypsin was inactivated by the addition of serum-containing growth medium. Cell viability was assessed by trypan blue (Invitrogen) exclusion, and the cell suspension was stored at 37°C in polypropylene tubes for no longer than 1 h until transfection.

The large-scale RNAi screen was done in 384-well black plates (BD Biosciences) with an automatic liquid handling system (Evolution P3, Perkin-Elmer). Transfection complexes containing 30 nmol/L siRNA and 0.3 μL siPORT NeoFX transfection reagent (Ambion) were allowed to form in OptiMEM serum-free media (Invitrogen), in a total volume of 18 μL, for 20 min. MDA-MB-453 cells (5,500 cells in 60 μL of complete growth medium per well) were then overlaid onto transfection complexes, and plates were sealed with free-gas exchange lids (Axygen) to prevent water evaporation. A total of 17,280 siRNAs targeting 5,760 genes were transfected in duplicate. Cells were treated at 48 h post-transfection with 100 ng/mL TRAIL (Sigma).

Secondary siRNA and miRNA screens were done in 96-well plates with MDA-MB-453 or HeLa cells (10,000 cells in 120 μL of complete growth medium per well). For the preparation of transfection complexes, 30 nmol/L of siRNA or miRNA was combined with 0.3 μL siPORT NeoFX transfection reagent (Ambion) and incubated in a total volume of 30 μL (in OptiMEM) for 20 min. Transfections were done in triplicate. Cells were treated at 48 h post-transfection with 100 to 200 ng/mL TRAIL.

Caspase-3/7 assay. Sixteen hours after TRAIL (Sigma) treatment, cells were assayed for caspase-3/7 activity assay. Caspase lysis/activity buffer (80 μL per well) contained 30 μmol/L fluorescent AC-DEVD-AFC substrate (Bachem), 0.5% NP40, and 0.3 nmol/L EDTA. 7-Amino-4-trifluoromethyl-coumarin (AFC) fluorescence was measured (excitation: 400 nm; emission: 505 nm) in black plates using a Spectramax Gemini XS Microplate Spectrofluorometer (Molecular Devices). All measurements were background corrected. For assay of caspase-3/7 activity as part of secondary screens, the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) was employed according to the manufacturer's recommended protocol.

miRNA target prediction method. Both miRBase Targets v4.0 and miRAID targets prediction software (Asuragen) were employed to predict miRNA/mRNA pairs. The former uses the miRBase algorithm to identify potential binding sites for a given miRNA in genomic sequences (26). The latter uses a technique based on PicTar (27). In brief, the miRAID algorithm parses sets of 3-UTR sequences into overlapping 30 nucleotide segments. Each of these segments is then tested for target sites based on the following strict criteria. First, a minimum of seven out of eight consecutive nucleotides must exhibit Watson-Crick complementarity to the 5′ miRNA "seed" sequence (nucleotides 1–7, 2–8). Moreover, the seven nucleotide region must have been conserved across multiple alignments of five genomes (i.e., human, chimpanzee, mouse, rat, and dog), which were extracted from multiple alignments of 16 vertebrate genomes with the human genome (UCSC release hg18). Second, the optimal free energy (mfe) of the miRNA/mRNA duplexes, calculated using RNAhybrid 2.1 (with the −s3utr_human option for vertebrate sequences), could not exceed −16 kcal/mol. The final rank of miRNA/mRNA pairs was based on multiple predicted target sites, optimal free energy, and multi-genome alignment conservation. The predicted miRNA/mRNA pairs were cross-checked with siRNA screen hits and genes known to be involved in the TRAIL signaling pathway. The overlapping results between computational prediction methods and combined siRNA screen data are reported in Supplementary Table S1.

Real-time reverse transcription-PCR analysis. Total RNA from siRNA-transfected cells was isolated using the MagMAX96 Total RNA Isolation Kit (Ambion). Purified, DNase-treated RNA was reverse transcribed with random decamers using the RETROscript Kit (Ambion). Gene expression levels were determined by real-time PCR on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) using SuperTaq reagents (Ambion) and SYBR Green (Molecular Probes). *GAPDH* data were collected using a human primer set (forward: 5′-GAAGGTGAAGGTCGGAGT-3′; reverse: 5′-GAAGATGGTGATGGGATTTC-3′). As an additional control, 18S rRNA was amplified (forward: 5′-TTGACTCAACACGGGAAACCT-3′, reverse: 5′-AGAAAGAGCTATCAATCTGTCAATCCT-3′) to adjust for well-to-well variances in the amount of starting template. All corrected values were normalized to those obtained for non-silencing siRNA-transfected samples.

Statistical analysis. Comparisons of group means (versus negative controls) were done using Welch's t tests. Values of $P < 0.01$ were accepted as significant.

Results

Evaluation of siRNA library screening efficiency. Chemical transfection reagents can be used to transiently transfect siRNAs into immortalized cell lines to reduce the expression of specific genes. We have previously developed and characterized methods to enable large-scale siRNA delivery via reverse transfection (17). In this method, cell suspensions are added to wells already containing siRNA/transfection reagent mixtures. To accommodate screening with a large number of siRNAs and miRNAs, MDA-MB-453 cells were reverse transfected in 384-well plates. Real-time reverse transcription-PCR analysis of target mRNA expression at 48 h post-transfection revealed that well-characterized siRNAs targeting *GAPDH, p53*, and *JAK1* (30 nmol/L) provided at least 90% reduction in specific mRNA expression with a coefficient of variation of 15% (data not shown). These results confirm the efficiency and reproducibility of this delivery method.

Identification of genes that differentially influence TRAIL-induced apoptosis via siRNA library screening. To identify potential modulators of TRAIL-induced apoptosis, 17,280 siRNAs targeting 5,760 individual human genes (three distinct siRNAs per target gene) as well as positive and negative control siRNAs were transfected into breast cancer MDA-MB-453 cells. siRNAs targeting single genes were eliminated from the analysis because they likely represent off-target effects by the siRNAs (28). Forty-eight hours post-transfection, the cells were treated with 100 ng/mL TRAIL for 16 h. Cells were then selected based on their susceptibility to apoptosis, as measured by caspase-3 activity (29). TRAIL-induced caspase-3 activity was markedly altered by 264 different siRNAs (Fig. 1). In all cases, caspase-3 activity was induced at least 5-fold or reduced at least 3-fold.

A secondary screen was done to validate alterations in the caspase-3 phenotype exhibited by two or more siRNAs per gene in the large-scale RNAi analysis. Table 1 summarizes the effects of selected siRNA transfections on resting and TRAIL-induced caspase-3 activity in MDA-MB-453 cells. The data are represented as the mean of three individual siRNAs, normalized to a negative control. The siRNA-induced effects were compared with the negative controls using Welch's t tests. Fold change was calculated and assigned a screen score value, which represents the statistical likelihood of different siRNAs targeting the same mRNA to result in a similar phenotypic change. The siRNAs that were shown to affect TRAIL-induced apoptosis targeted multiple genes that have previously been shown to be involved in the TRAIL pathway (i.e., *caspase-3, caspase-8, BID, DR4, DR5, GSK3A, PRKCD, STK6*, and *BUB1B*) and that have been identified in other RNAi screens

(13, 18). We also identified other genes including *CDK4, PTGS1, ALG2, CLCN3, IRAK4*, and *MAP3K8* that have not been previously linked with TRAIL-induced apoptosis. Some siRNA silenced genes, including *PAK1*, exhibited phenotypes that were different from literature reports (18). This was likely due to the different cell lines used. For example, other groups have done siRNA studies in cervical adenocarcinoma HeLa cells.

Gene silencing affects baseline and TRAIL-induced caspase-3 activation. Because some siRNAs may alter cellular phenotypes via pathways that are independent of death receptor 4/5, we assessed caspase-3 activity before and after TRAIL treatment. In all cases, comparisons were made to non-silencing siRNA-transfected controls (Table 1). Gene silencing of *CDK4, BID, BUB1B, ALG2, CFLAR, CDK2, caspase-2, IRAK4, SGEF*, and *CLCN3* altered caspase activity both before and after TRAIL treatment. *BCL2* and *GSK3A* siRNAs exerted their effects primarily before TRAIL-induced caspase activation. The remainder of siRNAs affected caspase-3 activation only in the presence of TRAIL, suggesting that these target genes contributed either directly or indirectly to TRAIL-induced activation of caspase cascades.

Identification of natural small RNAs regulating apoptosis via synthetic miRNA screening. miRNAs are naturally occurring small RNAs that regulate gene expression primarily at the translational level (23, 24). miRNAs have important regulatory functions in development, apoptosis, and metabolism (30, 31). Although a few miRNAs seem to impact apoptosis (32, 33), it is unclear if small RNAs may regulate apoptosis through death-receptor signal transduction. To test this possibility, MDA-MB-453 cells were transected with 187 individual synthetic miRNAs representing the majority of the human miRNAs that were known to exist at the time. Thirty-four of these miRNAs led to a differential caspase-3 activation phenotype (Table 2). To distinguish miRNAs that directly affect the TRAIL pathway from those that affect an unrelated pathway(s), hits from this TRAIL screen were compared with results from previous screens, in which we identified miRNAs exerting effects on caspase-3 activity independently of TRAIL (Table 3). Several miRNAs altered caspase activity in a TRAIL-independent manner. These included *mir-10a, mir-28, mir-196a*, and *mir-337,* which induced caspase-3 activity, and *mir-96, mir-145, mir-150, mir-155*, and *mir-188*, which blocked caspase-3 activation.

Next, we attempted to identify plausible interactions between the potential targets of miRNAs that significantly altered TRAIL-induced apoptosis and the genes that were hits in the siRNA screen. miRNA targets were predicted using both Asuragen miRAID and miRBase mRNA target prediction approaches. As shown in Supplementary Table S1, many of the genes that yielded phenotypes when down-regulated in the TRAIL screen were predicted to be regulated by one or more of the miRNAs that were positive screens. A total of 31 (out of 53) predicted miRNA targets correlated with experimentally observed siRNA phenotypes. Thus, these targets may represent primary miRNA gene targets.

We also used the miRBase Target database (Wellcome Trust Sanger Institute) to identify potential interactions between the miRNA and siRNA screen hits. Two miRNAs, *mir-144* and *mir-182*, were predicted to directly target caspase-3. Although the activation of caspase-3 is typically achieved via proteolytic cleavage of procaspase-3, the transfection of these miRNAs 2 days before TRAIL treatment is likely sufficient to deplete the levels of procaspase-3 readily available for activation. This is supported by the observation that a siRNA targeting caspase-3 potently reduces the caspase-3 activity (Fig. 2). *Mir-182* was also predicted to target *FADD*, a key adaptor molecule mediating death receptor–activated signaling. *Mir-96* and *mir-182* have highly homologous 5′-seed sequences (*mir-96*, UUUGGCACUAGCACAUUUUUGC; *mir-182*, UUUGGCAAUGGUAGAACUCACA), suggesting that *mir-96* may also target caspase-3 and *FADD. Mir-7* was predicted to target *BAD*, an inhibitor of the TRAIL pathway, and *Let-7c* was predicted to target *RAS* and *FASLG*. Putative miRNA target genes and their roles in apoptosis and cell cycle progression are depicted schematically in Fig. 3.

To identify a potential link between siRNA and miRNA screen results, bioinformatic analysis was done to predict the siRNA targeting *CDK4*. Unfortunately, we were unable to identify and validate the *CDK4* gene sequence as a primary target of any miRNAs

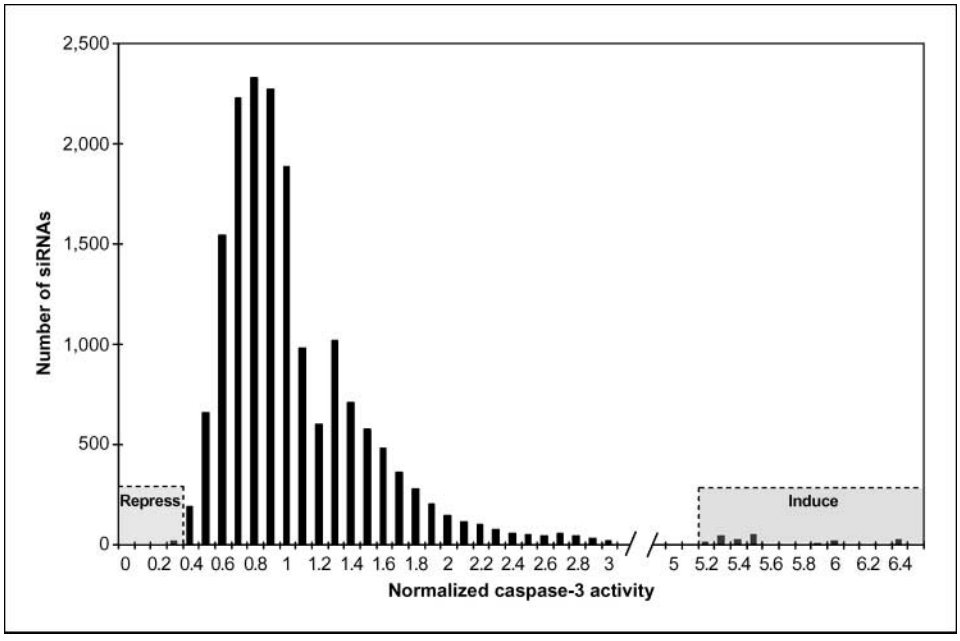

Figure 1. Screening for siRNAs that differentially affect TRAIL-induced apoptosis. Human breast cancer MDA-MB-453 cells were transfected with 17,280 siRNAs targeting 5,760 individual human genes. Following transfection, cells were exposed to TRAIL, and caspase-3 activity was measured. Data were normalized to a negative control (mean value of three separate non-silencing siRNAs).

Table 1. Caspase-3 activation before and after TRAIL (100 ng/mL) treatment

Accession number	Gene symbol	Full gene name	Screen score	Fold change	*P*
Activity before TRAIL treatment					
NM_000075	*CDK4*	Cyclin-dependent kinase 4	8.30	0.80	0.00239
NM_001196	*BID*	BH3 interacting domain death agonist	8.21	0.74	0.00886
NM_001211	*BUB1B*	BUB1 budding uninhibited by benzimidazoles 1	8.20	1.38	0.00257
NM_000633	*BCL2*	B-cell chronic lymphocytic leukemia/lymphoma 2	6.07	1.37	0.00946
NM_033087	*ALG2*	Asparagine-linked glycosylation 2 homologue	5.52	1.26	0.02065
NM_003879	*CFLAR*	CASP8 and FADD-like apoptosis regulator	4.23	1.11	0.02103
NM_052827	*CDK2*	Cyclin-dependent kinase 2	4.10	1.19	0.01832
NM_032983	*CASP2*	Caspase 2	3.17	0.70	0.01746
NM_019884	*GSK3A*	Glycogen synthase kinase 3α	3.01	0.75	0.02967
NM_016123	*IRAK4*	Interleukin-1 receptor–associated kinase 4	2.89	0.82	0.04947
NM_015595	*SGEF*	Src homology 3 domain–containing guanine nucleotide exchange factor	2.85	0.88	0.04721
NM_001829	*CLCN3*	Chloride channel 3	2.79	0.88	0.04702
Activity after TRAIL (100 ng/mL) treatment					
NM_004346	*Caspase-3*	Caspase 3, apoptosis-related cysteine protease	23.04	0.44	4.3E−05
NM_000075	*CDK4*	Cyclin-dependent kinase 4	21.45	0.37	0.00032
NM_033355	*Caspase-8*	Caspase 8, apoptosis-related cysteine protease	20.54	0.41	0.00020
NM_003879	*CFLAR*	CASP8 and FADD-like apoptosis regulator	16.96	3.68	0.00993
NM_003844	*TNFRSF10A*	TNF receptor superfamily, member 10a	11.89	0.63	0.00287
NM_001829	*CLCN3*	Chloride channel 3	10.5	0.70	0.00063
NM_016123	*IRAK4*	Interleukin-1 receptor–associated kinase 4	9.24	0.61	0.00381
NM_002577	*PAK2*	p21 (CDKN1A)-activated kinase 2	9.18	0.62	0.00385
NM_005204	*MAP3K8*	Mitogen-activated protein kinase kinase kinase 8	9.10	0.57	0.00552
NM_002576	*PAK1*	p21/Cdc42/Rac1-activated kinase 1	8.95	0.62	0.00387
NM_001261	*CDK9*	Cyclin-dependent kinase 9	8.02	0.65	0.00532
NM_033497	*HK1*	Hexokinase 1	7.97	0.65	0.00552
NM_145319	*MAP3K6*	Mitogen-activated protein kinase kinase kinase 6	7.95	0.61	0.00787
NM_007170	*TESK2*	Testis-specific kinase 2	7.90	1.40	0.00362
NM_052827	*CDK2*	Cyclin-dependent kinase 2	7.11	0.66	0.00920
NM_005922	*MAP3K4*	Mitogen-activated protein kinase kinase kinase 4	7.08	0.63	0.01117
NM_006908	*RAC1*	ras-related C3 botulinum toxin substrate 1	6.79	0.60	0.01725
NM_000962	*PTGS1*	Prostaglandin-endoperoxide synthase 1	6.31	2.67	0.09447
NM_212539	*PRKCD*	Protein kinase Cδ	6.26	0.62	0.02087
NM_033087	*ALG2*	Asparagine-linked glycosylation 2 homologue	6.09	2.26	0.06788
NM_004735	*LRRFIP1*	leucine-rich repeat (in FLII) interacting protein 1	5.88	0.78	0.00994
NM_001196	*BID*	BH3-interacting domain death agonist	5.49	0.65	0.02836
NM_032983	*Caspase-2*	Caspase 2, apoptosis-related cysteine protease	5.46	0.65	0.02829
NM_000875	*IGF1R*	Insulin-like growth factor 1 receptor	5.44	0.59	0.04020
NM_138957	*MAPK1*	Mitogen-activated protein kinase 1	5.14	1.47	0.03066
NM_001211	*BUB1B*	BUB1 budding uninhibited by benzimidazoles 1	5.13	1.48	0.03095
NM_003842	*TNFRSF10B*	TNF receptor superfamily, member 10b	4.96	0.69	0.02933
NM_015595	*SGEF*	Src homology 3 domain–containing guanine nucleotide exchange factor	4.01	0.68	0.06595
NM_020476	*ANK1*	Ankyrin 1, erythrocytic	3.46	1.84	0.15250
NM_002610	*PDK1*	Pyruvate dehydrogenase kinase, isoenzyme 1	2.84	0.69	0.14060
NM_002737	*PRKCA*	Protein kinase Cα	2.44	1.23	0.04913
NM_002184	*IL6ST*	Interleukin 6 signal transducer	2.39	1.26	0.04864
NM_198436	*STK6*	Serine/threonine kinase 6	2.14	1.60	0.04818
NM_003258	*TK1*	Thymidine kinase 1, soluble	1.98	1.55	0.04996

known at the time. Nevertheless, we found that overexpression of *mir-216* resulted in a marked down-regulation of *CDK4* (data not shown), suggesting that *CDK4* is an indirect target of *mir-216*.

Involvement of CDK4 in TRAIL-mediated caspase-3 activation. siRNAs targeting *CDK2, CDK4*, and *CDK9* siRNAs revealed potential interrelationships between cell cycle regulation and apoptosis signal transduction pathways. Indeed, siRNAs targeting *CDK4* were some of the most potent in suppressing caspase-3 activation. Therefore, we characterized the role of each gene family member by silencing the expression of *CDK1/CDC2, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, CDK8, CDK9* (with three siRNAs per gene) before TRAIL exposure (Fig. 2). As was observed in the initial screen, *CDK2, CDK4*, and *CDK9* siRNAs reduced caspase-3 activity in treated cells, with *CDK4* reducing caspase-3 activity by ~75%. siRNAs targeting the other cyclin-dependent kinases had little effect on caspase activation, suggesting that there are direct ties

between *CDK2, CDK4*, and *CDK9* and the caspase cascade. Interestingly, silencing of *CDK6*, which not only shares a 71% amino acid sequence identity with CDK4, but also has the same cell cycle regulatory function (34), did not result in repression of apoptotic signaling. This result suggests that *CDK4* may have an apoptosis-specific signaling role. In fact, *CDK4* was recently found to be essential for the maintenance of breast tumor cell proliferation, whereas *CDK6* did not impact initiation and growth of tumors (35).

Discussion

The TRAIL-induced apoptosis pathway has been extensively studied due to its widespread functions in normal and disease physiology and its apparent ability to selectively target cancer cells. Here, we have expanded the list of TRAIL pathway participants to include both new signaling proteins and also non-translated RNA molecules. Our results indicate that 36 genes, including previously identified pathway components, and 34 miRNAs participate directly or indirectly in the regulation of the pathway. Interestingly, the expression of many of the genes known to regulate apoptosis seems to be regulated by the miRNAs identified in this apoptosis screen.

Table 2. The effect of miRNA overexpression on TRAIL-induced caspase-3 activation

Accession number	Identification	Fold change	*P*
MI0000266	hsa-mir-10a	2.02	9.6E−07
MI0000487	hsa-mir-193	2.00	3.9E−06
MI0000064	hsa-let-7c	1.75	1.2E−06
MI0000103	hsa-mir-101	1.72	4.3E−06
MI0000825	hsa-mir-345	1.68	5.4E−05
MI0000238	hsa-mir-196a	1.68	1.2E−05
MI0000806	hsa-mir-337	1.66	1.3E−05
MI0000086	hsa-mir-28	1.66	0.00467
MI0000263	hsa-mir-7	1.65	3.8E−06
MI0003130	hsa-mir-202	1.57	0.00088
MI0000294	hsa-mir-218	1.51	0.00033
MI0000776	hsa-mir-368	1.50	2.5E−05
MI0000463	hsa-mir-153	1.47	2.8E−05
MI0000239	hsa-mir-197	1.45	0.00197
MI0000779	hsa-mir-371	1.42	7.3E−05
MI0000814	hsa-mir-338	0.88	0.02462
MI0000284	hsa-mir-204	0.84	0.02660
MI0000458	hsa-mir-142	0.80	0.00196
MI0000777	hsa-mir-369	0.79	0.01205
MI0000082	hsa-mir-25	0.79	0.00466
MI0000822	hsa-mir-133b	0.75	0.00065
MI0000484	hsa-mir-188	0.75	0.00095
MI0000262	hsa-mir-147	0.75	0.00698
MI0000809	hsa-mir-151	0.74	0.00050
MI0000481	hsa-mir-184	0.74	0.02107
MI0000098	hsa-mir-96	0.73	0.01103
MI0000292	hsa-mir-216	0.73	0.00124
MI0000461	hsa-mir-145	0.71	0.00010
MI0000272	hsa-mir-182	0.69	0.00190
MI0000080	hsa-mir-24	0.69	0.00058
MI0000462	hsa-mir-152	0.67	8.0E−05
MI0000479	hsa-mir-150	0.66	0.00028
MI0000681	hsa-mir-155	0.65	0.00029
MI0000460	hsa-mir-144	0.60	8.1E−05

Table 3. The effect of miRNA overexpression on baseline caspase-3 activity

Accession number	ID	Cell type
miRNAs increasing caspase-3 activity (versus negative control miRNA)		
MI0000060	hsa-let-7a	A549, BJ
MI0000063	hsa-let-7b	Jurkat
MI0000651	hsa-mir-1	BJ, Jurkat
MI0000266	hsa-mir-10a	A549
MI0000267	hsa-mir-10b	Jurkat
MI0000069	hsa-mir-15a	BJ
MI0000070	hsa-mir-16	BJ
MI0000079	hsa-mir-23a	A549
MI0000439	hsa-mir-23b	A549, HeLa
MI0000086	hsa-mir-28	Jurkat
MI0000089	hsa-mir-31	HeLa
MI0000442	hsa-mir-122	Jurkat
MI0000289	hsa-mir-181a	A549
MI0000238	hsa-mir-196a	A549
MI0000490	hsa-mir-206	BJ
MI0000290	hsa-mir-214	A549, HeLa
MI0000812	hsa-mir-331	A549
MI0000806	hsa-mir-337	BJ
MI0002464	hsa-mir-412	A549, HeLa
miRNAs decreasing caspase-3 activity (versus negative control miRNA)		
MI0000083	hsa-mir-26a	BJ, Jurkat
MI0000098	hsa-mir-96	A549, HeLa
MI0000100	hsa-mir-98	Jurkat
MI0000111	hsa-mir-105	A549, HeLa
MI0000471	hsa-mir-126	A549, Jurkat, HeLa
MI0000454	hsa-mir-137	A549, HeLa
MI0000461	hsa-mir-145	BJ
MI0000479	hsa-mir-150	BJ
MI0000681	hsa-mir-155	Jurkat
MI0000484	hsa-mir-188	BJ

Among the more interesting genes that were revealed to modulate apoptosis was *CDK4*, a cyclin-dependent kinase that is capable of inducing G_1 arrest in response to the DNA damage sensor, p53. It is well established that CDK4 phosphorylates key regulatory substrates including retinoblastoma protein (pRb) to trigger G_1-S cell cycle progression. *CDK4* mutation and up-regulated expression are found in human tumors (36, 37). CDK4/CDK6 knock-out mice show normal organogenesis, and proliferation of most cell types is not inhibited, suggesting that alternative mechanisms can bypass the CDK-mediated cell cycle events (38, 39). In line with these findings, *CDK4* kinase inhibitors may be ineffective as cancer therapeutics (40). In our study, down-regulation of *CDK4* resulted in G_1-S phase arrest and a failure to undergo apoptosis. Silencing of *CDK6*, which shares >70% of sequence homology with *CDK4* and is believed to exert essential cell cycle function in $CDK4^{-/-}$ null mice, did not result in the repression of caspase-3 activation. This suggests that *CDK4* modulates apoptosis independently of its cell cycle function. Moreover, among the CDK family members, *CDK4* seems to be unique in its ability to regulate apoptosis.

The finding that miRNAs may affect TRAIL-induced apoptotic pathways significantly enhances the complexity of ligand-induced apoptosis. miRNAs enhancing caspase-3 activation can exert their action via targeting inhibitors of TRAIL pathway (mir-7 targeting BAD), or affecting other genes (let-7c targeting RAS and FASLG),

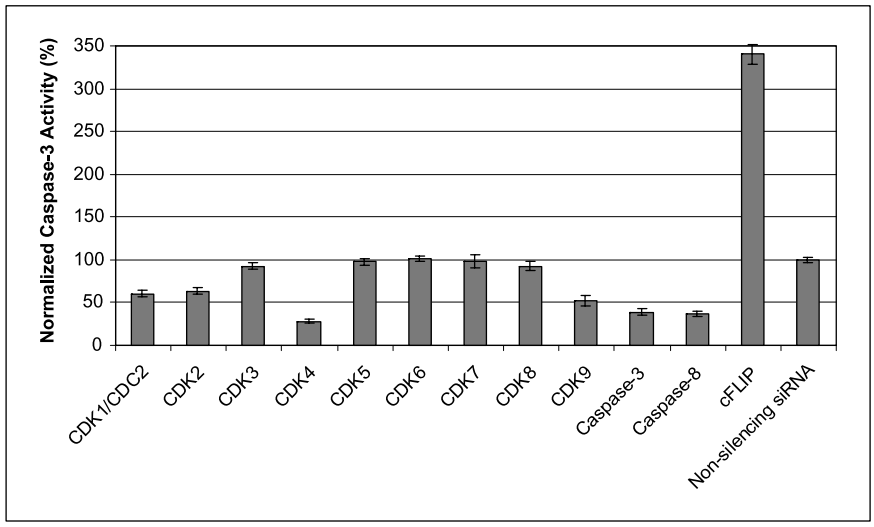

Figure 2. Role of individual *CDK* family members in TRAIL-induced apoptosis. Three separate siRNAs targeting *CDK1/CDC2, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, CDK8*, or *CDK9* were transfected in MDA-MB-453 cells. Following transfection, cells were exposed to TRAIL, and caspase-3 activity was measured. Data are normalized to a negative control (mean value of three separate non-silencing siRNAs) and are expressed as mean ± SD of three transfections.

therefore amplifying apoptosis induction. The collection of miRNAs that affect both TRAIL-induced and TRAIL-independent apoptosis include several small RNAs known to be differentially expressed in human cancers. *Mir-15a* and *mir-16* are frequently down-regulated or deleted in B-cell chronic lymphocytic leukemia (32). Both miRNAs are capable of inducing apoptosis by negatively regulating *BCL2* at the post-transcriptional level (41). Accordingly, overexpression of *mir-15a* and *mir-16* in a leukemic cell line model potently induces apoptosis (32). We observed a similar phenotype of these miRNAs in TRAIL-independent apoptosis in human skin fibroblast BJ cells.

One group of miRNAs—*mir-143, mir-145*, and *let-7*—are all down-regulated in tumors from patients with several different cancers (25, 42, 43). It is worth contemplating whether dysregulation of one or more of these miRNAs may allow cells to bypass apoptotic pathways. Although overexpression of the *let-7* family has been shown to induce apoptosis in multiple cell systems, it was extremely interesting that increased levels of *miR-145* decreased the capacity of cells to respond to TRAIL. *Mir-145* seems to be down-regulated in many cancer cells, providing a potential explanation as to why cancer cells respond more readily to TRAIL than untransformed cells.

A recent study showed that a transgenic mouse line overexpressing *mir-155* developed lymphoproliferative disease and malignancy (44). Consistent with the ability of *mir-155* overexpression to induce neoplastic disease, we found that *mir-155* was one of the most potent miRNA suppressing apoptosis in MDA-MB-453 cells and T-cell leukemia Jurkat cells. This apoptosis-inhibiting effect of *mir-155* is supported by the observation that *mir-155* is overexpressed in breast, lung, and colon cancer tumors (44). The finding that overexpression of *mir-145, mir-216, mir-182*, and *mir-96* miRNA reduced caspase-3 activation (Table 2) is particularly

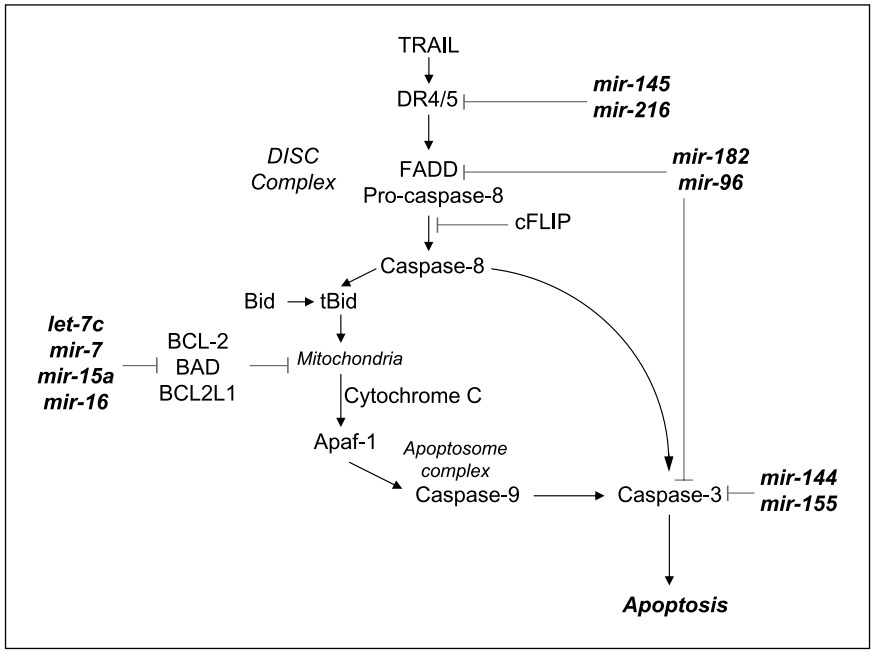

Figure 3. Schematic showing the roles of putative miRNA target genes in TRAIL-induced apoptosis pathway.

interesting in light of the fact that these miRNAs were predicted to interact with *DR4/5* and *FADD*, both of which lie upstream of caspase-3 activation (Fig. 3). On the other hand, transfection of miRNAs (i.e., *let-7, mir-7, mir-15a*, and *mir-16*) that were predicted to interact with *BCL2, BAD, BCL2L1* elicited an increase in caspase-3 activity. This finding strongly suggests that *let-7, mir-7, mir-15a*, and *mir-16* do in fact target those genes. Most importantly, they point to a role for miRNA in apoptosis regulation.

Acknowledgments

Received 4/24/2007; revised 8/15/2007; accepted 9/21/2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank L. Ford, C. Erickson, and J. Richburg for helpful discussions. We thank A. Ellington, T. Pappas, and K. Cole-Edwards for manuscript review. We are grateful to C. Trudell and C. Nannamore for providing siRNA libraries. We thank O. Ovcharenko for help with graphics.

References

1. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell 1997;88:347–54.
2. Miller LJ, Marx J. Apoptosis. Science 1998;281:1301.
3. Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. Nat Rev Cancer 2004;4:592–603.
4. Rothe J, Gehr G, Loetscher H, Lesslauer W. Tumor necrosis factor receptors: structure and function. Immunol Res 1992;11:81–90.
5. Tracey KJ, Cerami A. Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol 1993;9:317–43.
6. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305–8.
7. Yagita H, Takeda K, Hayakawa Y, Smyth MJ, Okumura K. TRAIL and its receptors as targets for cancer therapy. Cancer Sci 2004;95:777–83.
8. Pan G, O'Rourke K, Chinnaiyan AM, et al. The receptor for the cytotoxic ligand TRAIL. Science 1997;276:111–3.
9. Takeda K, Smyth MJ, Cretney E, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001;7:94–100.
10. Chaudhary PM, Eby M, Jasmin A, Bookwalter A, Murray J, Hood L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-κB pathway. Immunity 1997;7:821–30.
11. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806–11.
12. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494–8.
13. Ren YG, Wagner KW, Knee DA, Aza-Blanc P, Nasoff M, Deveraux QL. Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. Mol Biol Cell 2004;15:5064–74.
14. Silva J, Chang K, Hannon GJ, Rivas FV. RNA-interference–based functional genomics in mammalian cells: reverse genetics coming of age. Oncogene 2004;23:8401–9.
15. Sachse C, Echeverri CJ. Oncology studies using siRNA libraries: the dawn of RNAi-based genomics. Oncogene 2004;23:8384–91.
16. Xin H, Bernal A, Amato FA, et al. High-throughput siRNA-based functional target validation. J Biomol Screen 2004;9:286–93.
17. Ovcharenko D, Jarvis R, Hunicke-Smith S, Kelnar K, Brown D. High-throughput RNAi screening *in vitro*: from cell lines to primary cells. RNA 2005;11:985–93.
18. Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, Cooke MP. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. Mol Cell 2003;12:627–37.
19. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–69.
20. Thompson BJ, Cohen SM. The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in *Drosophila*. Cell 2006;126:767–74.
21. Kulshreshtha R, Ferracin M, Wojcik SE, et al. A microRNA signature of hypoxia. Mol Cell Biol 2007;27:1859–67.
22. Du T, Zamore PD. MicroPrimer: the biogenesis and function of microRNA. Development 2005;132:4645–52.
23. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843–54.
24. Reinhart BJ, Slack FJ, Basson M, et al. The 21 nucleotide let-7 RNA regulates *C. elegans* developmental timing. Nature 2000;403:901–6.
25. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. Cell 2005;120:635–47.
26. Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. RNA 2004;10:1507–17.
27. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet 2005;37:495–500.
28. Sledz CA, Williams BR. RNA interference and double-stranded-RNA–activated pathways. Biochem Soc Trans 2004;32:952–6.
29. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59:734–41.
30. Banerjee D, Slack F. Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. Bioessays 2002;24:119–29.
31. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
32. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A 2005;102:13944–9.
33. Xu P, Guo M, Hay BA. MicroRNAs and the regulation of cell death. Trends Genet 2004;20:617–24.
34. Meyerson M, Enders GH, Wu CL, et al. A family of human cdc2-related protein kinases. EMBO J 1992;11:2909–17.
35. Yu Q, Sicinska E, Geng Y, et al. Requirement for CDK4 kinase function in breast cancer. Cancer Cell 2006;9:23–32.
36. Hall M, Peters G. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Adv Cancer Res 1996;68:67–108.
37. Carnero A. Targeting the cell cycle for cancer therapy. Br J Cancer 2002;87:129–33.
38. Tsutsui T, Hesabi B, Moons DS, et al. Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. Mol Cell Biol 1999;19:7011–9.
39. Malumbres M, Sotillo R, Santamaria D, et al. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell 2004;118:493–504.
40. Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 2006;24:1770–83.
41. Calin GA, Croce CM. Genomics of chronic lymphocytic leukemia microRNAs as new players with clinical significance. Semin Oncol 2006;33:167–73.
42. Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003;1:882–91.
43. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004;64:3753–6.
44. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eu-miR155 transgenic mice. Proc Natl Acad Sci U S A 2006;103:7024–29.