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FAPP2 Gene Downregulation Increases Tumor Cell Sensitivity to Fas-Induced Apoptosis

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Abstract

The gene for phosphatidylinositol-4-phosphate adaptor-2 (FAPP2) encodes a cytoplasmic lipid transferase with a plekstrin homology domain that has been implicated in vesicle maturation and transport from trans-Golgi to the plasma membrane. The introduction of ribozymes targeting the FAPP2 gene in colon carcinoma cells induced their apoptosis in the presence of Fas agonistic antibody. Furthermore, by quantitative PCR we showed that a siRNA specific to FAPP2, but not a randomized siRNA control, reduced FAPP2 gene expression in tumor cells. Transfection of FAPP2 siRNA into human tumor cells then incubated with FasL resulted in reduction of viable cell numbers. Also, FAPP2 siRNA transfected glioma and breast tumor cells showed significant increases in apoptosis upon incubation with soluble FasL, but the apoptosis did not necessarily correlate with increased Fas expression. These data demonstrate a previously unknown role for FAPP2 in conferring resistance to apoptosis and indicate that FAPP2 may be a target for cancer therapy.

Keywords

ribozyme; astrocytomas; apoptosis; FAPP2; apoptosis; Fas ligand; siRNA; colon carcinoma; glioma; breast tumor

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Introduction

Apoptosis is a distinctive form of cell death. It occurs in normal and pathological processes and can be induced by a number of stimuli. Fas (CD95) and Fas ligand (FasL) are members of the TNF death receptor/ligand family. FasL binding to Fas-expressing cells can trigger their apoptosis. A significant number of cancer cell types including colon, breast and brain coexpress Fas and FasL yet are resistant to apoptosis induced by this death receptor/ligand pair [1; 2; 3; 4].

We used an inverse functional genomics approach for gene discovery, based on the use of a randomized hairpin ribozyme gene library, to identify a number of novel target genes that when downregulated could sensitize Fas-resistant tumor cells to Fas-induced apoptosis [3]. The library of ribozymes was stably introduced by retroviral transduction into cancer cells that were normally resistant to apoptosis. Selected ribozymes that reproducibly conferred a proapoptotic phenotype were exploited to identify the gene(s) involved in this alteration. Specifically, the binding site of the hairpin ribozyme, containing 16 nucleotides of unique sequence, was used to query the NCBI nucleotide sequence database by BLAST search and identify the corresponding gene. Following this protocol we identified the FAPP2 adaptor protein gene [3], previously unassociated with proapoptotic function, as one that may play a role in the apoptotic pathway.

In this report we describe the overexpression of the FAPP2 gene in tumor cells, and the down regulation of the FAPP2 gene by either target validation ribozymes or by a specific FAPP2 siRNA. Previous reports showed that apoptotic stimuli, such as low concentration actinomycin D (actD), can mediate Fas/FasL-induced apoptosis in tumor cells exhibiting resistance to Fas-induced apoptosis [1; 4; 5]. We selected a panel of Fas/FasL-resistant tumor cells for testing apoptosis-inducing activity of a siRNA targeting the FAPP2 gene by initially testing actD sensitization to FasL induced apoptosis. In these cell lines the FAPP2 siRNA imparted cell sensitization to Fas/FasL mediated apoptosis, thereby indicating that FAPP2 may be an effective therapeutic target for tumors.

Materials and methods

Cell Lines and Tissues

Human glioma cell lines, T98G, U-87MG, U-251MG U-373MG, and 10-08-MG, the metastatic breast cell line, MDA-MB-231-1833 (1833) [6], and DLD1 colon carcinoma cells were cultured in Dulbecco′s modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were chosen for these experiments because they displayed resistance to FasL induced (10 to 150 ng/ml) or Fas agonistic CH11 antibody induced (160 ng/ml) cell death, but gained sensitivity to apoptosis when also placed in the presence of low concentration actinomycin D (actD, 0.02 to 0.1 μg/ml, Alexis), as similarly determined [7]. Freshly-resected human brain specimens, collected under IRB-approved guidelines, were obtained from adult patients undergoing lobectomies for seizures.

Ribozymes, siRNA and primers

Templates for the target validation hairpin ribozymes were synthesized by IDT containing the restriction enzyme sites Bam HI and Mlu I. RzFAPP-1: sense 5′-AATAAAGGATCCATTTCACAAGAAGCCAACCAGAGAAACACACGTTGTGGTATATT ACCTGGTACGCGTAACAAT-3′; antisense 5′-ATTGTTACGCGTACCAGGTAATATACCACAACGTGTGTTTCTCTGGTTGGCTTCTTGT GAAATGGATCCTTTATT-3′; RzFAPP-5: sense 5′-AATAAAGGATCCTTAGATTTAGAAACTTACCAGAGAAACACACGTTGTGGTATATTA CCTGGTACGCGTAACAAT-3′; antisense 5′-ATTGTTACGCGTACCAGGTAATATACCACAACGTGTGTTTCTCTGGTAAGTTTCTAA ATCTAAGGATCCTTTATT. The disabled ribozyme (dRz) has a three nucleotide change that is underlined in the following sequence: dRz sense 5′-AATAAAGGATCCTTAGATTTAGAAACTTACCAGAGCGTCACACGTTGTGGTATATTA CCTGGTACGCGTAACAAT-3′; antisense 5′-ATTGTTACGCGTACCAGGTAATATACCACAACGTGTGACGCTCTGGTAAGTTTCTAA ATCTAAGGATCCTTTATT-3″. Templates were annealed in 10 mM Tris buffer (pH 8.0) and 25 mM NaCl by heating to 90°C for 10 min, then slowly cooling to room temperature. Templates were digested with Bam HI and Mlu I (New England Biolabs) and ligated into the LHPM vector [8]. The siRNAs targeting the FAPP2 gene were designed using the siRNA target finder web site at AMBION.com. Potential target sites of these siRNAs were subjected to a homology search as previously described [7]. siRNA targeting FAPP2, and as controls, randomized siRNA and siRNA targeting luciferase (luc) were synthesized, purified, and annealed in phosphate buffered saline (PBS, Ambion) [7]. siRNA sequences with chemical modifications follow: lower case letters indicate 2′-*O*-methyl modification at that position. S indicates phosphorothioate linkage. dT indicates deoxythymidine. FAPP2 siRNA: sense 5′-GAuGGAucuuGuuGGAAAuusu -3′; antisense 5′-AUUUCcAAcAAGAUCcAUCUsU-3′; randomized control siRNA: sense 5′-GuAGuAGuAGuAGuAGuAAusU-3′; antisense 5′-UuACuACuACuACuACuACUsU-3′; luc siRNA: sense 5′-cuuAcGcuGAGuAcuucGAdTsdT - 3′; antisense 5′ - UCGAAGuACUcAGCGuAAGdTsdT - 3′. The primers for quantitative real time-polymerase chain reaction (qPCR) were synthesized by IDT. The FAPP2 primer set: Fwd 5′ACATCAGGATCCGATTGAGA3′; Rev 5′ATGCACCTTCTGGATGTGTT3′; a GAPDH primer set was obtained from RealTime Primers: Fwd 5′ GAGTCAACGGATTTGGTCGT 3′; Rev 5′ TTGATTTTGGAGGGATCTCG 3′.

Transfection of ribozyme plasmids and siRNA

For plasmid transfection, DLD1 cells were seeded at 2 x 10^6 cells/well in DMEM with 10% FBS in 6-well plates the day prior to transfection. The next day the medium was aspirated and replaced with serum-free DMEM. Lipofectamine/ribozyme plasmid complexes were formed in Opti-MEM (Invitrogen) by adding 10 μg DNA to 4 μl Lipofectamine/well according to the manufacturer protocol. The DNA/lipid complexes were added to the appropriate wells of the 6-well plate and incubated for 6 hr at 37°C. An equal volume of DMEM with 10% FBS was added and the plates were incubated overnight at 37°C. At 48 hr

post-transfection, stable cell lines were selected by the addition of 400 μg/ml of G418 (active fraction, Sigma).

For transfection of siRNA, cells were seeded at 2.5 x 10^4 cells/well in DMEM with 10% FBS in 24-well plates the day prior to transfection. Oligofectamine/siRNA complexes were formed in serum-free DMEM by adding siRNA (50 nM) to 2 μl of Oligofectamine (Invitrogen) per well. Complexes were formed at 25°C for 10 min and then added to the cells in serum-free DMEM (50 μl per well). Cells were incubated for 18 hr at 37°C. The transfection medium was aspirated and replaced with DMEM with 10% FBS.

Quantitative PCR for FAPP2 mRNA

RNA was isolated from nontransfected glioma cells, or siRNA-transfected cells at 48 hr post-transfection, using the Absolutely RNA kit (Stratagene) according to the manufacturer protocol. The RNA was reverse transcribed into cDNA using the iScript cDNA kit (BioRad). The FAPP2 mRNA levels were quantitated by the SYBR green method on the iQ5 ribocycler (BioRad) in a 96-well format. Standard curves were obtained for FAPP2 and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with template dilutions from 1:50 to 1:6750. Triplicate samples were quantified when run at template dilutions of 1:200 along with minus RT and minus template controls. Amplification was continued for 40 cycles as follows: 94°C-10s, 55°C-15s, 65°C-30s.

Flow cytometry for Fas expression

Cells were harvested with PBS containing 2 mM EDTA, washed and stained with a FITC-conjugated monoclonal antibody specific to Fas (CD95, DX2, BD Pharmingen, San Diego, CA). The stained cells were washed, resuspended and analyzed on an LSR II flow cytometer and the mean fluorescence intensities (MFI) of Fas were determined using BD-DIVA software.

Cell death ELISA

DLD-1 cells were transfected and treated with CH-11 Fas agonistic antibody as described earlier. Cells in the 24-well plate were trypsinized, pelleted by centrifugation at 2000 g.min, washed with PBS and counted on a hemocytometer. Two hundred cells were transferred into each of triplicate wells and lysed according to the manufacturer protocol, then 50 μl were placed into the wells of a cell death ELISA plate. The ELISA, a onestep sandwich immunoassay that uses anti-histone and anti-DNA to detect nucleosomes, was run according to the manufacturer protocol (Roche). The absorbances at 405 nm read from wells containing substrate alone were used as blanks, and the fold differences in absorbances from the experimental versus control wells in which total nucleosome accumulation occurred was determined. Experiments were performed twice.

MTT assays for viable cell number

Control or siRNA transfected cells were plated in DMEM with 10% FBS in 24-well plates for 48 hr post-transfection. FasL (SuperFasLigand, Alexis) was added and the cells were incubated at 37°C for 18 hr. The medium was aspirated and replaced with serum-free DMEM. MTT assay was done as previously described [7]. The data were presented as the

percentage reduction in viable cell numbers of the treated cells from those in untreated control wells, calculated as 100 - [(A570-A650 sample/A570-A650NT) × 100]. Duplicate samples were analyzed and experiments were performed at least twice.

Acridine orange/ethidium bromide staining to morphologically identify apoptotic cells by fluorescence microscopy

Cells were seeded and transfected as described for the MTT assay. FasL was added 48 hr post-transfection and the cells incubated for 18 hr. Cells were stained with Acridine orange/ethidium bromide (32 μl, Sigma Aldrich) at 100 μg/ml in PBS as previously described [7]. The percentages of apoptotic cells were determined by counting the apoptotic cells from a total of 400 cells (i.e., 200 cells counted in each of two high fields).

Statistical calculations

Data were analyzed by 2-way ANOVA using the Prism statistics package (Graph Pad Software Inc). Bonferroni post-tests were done to compare treatment groups and to determine statistical significance. The p values are indicated in the figures by asterisk number: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Validation of the FAPP2 gene target by transfection of ribozyme expression vectors

The FAPP2 gene was identified as one of several potential therapeutic targets after phenotypically screening the DLD1 colon carcinoma cell line transfected with a proapoptotic ribozyme library in the presence of Fas agonistic antibody. In brief, ribozymes RzFAPP-1 and RzFAPP-5 were designed, along with a disabled version of RFAPP-5 (dRz) to use as a negative control, and cloned into ribozyme expression vectors. DLD1 cells were transfected with each of the three vectors and stably-transfected cell populations were selected with G418. The transfected cells were exposed to the CH-11 Fas agonistic antibody before assessing for apoptosis by a cell death ELISA that quantitates DNA fragmentation. Both target validation ribozymes seemingly conferred sensitivity to the Fas agonistic antibody relative to those transfected with the control dRz. Quantitation of DNA fragmentation in cells transfected with RFAPP-1 and RFAPP-5 was 1.5 + 0.21 and 2.1 + 0.23 fold greater than that of dRz transfected cells. A Bonferroni post-test comparing RzFAPP-1 or -5 with the dRz control group showed statistical significance was obtained with RzFAPP-5 (* $p<0.05$).

FAPP2 mRNA is slightly overexpressed in glial tumor cells compared to normal brain

Two different normal brain (NB) cell specimens and three different glioma cell lines, 10-08-MG, U-251MG, and U-373MG were analyzed for FAPP2 mRNA expression (Fig. 1). Samples were normalized to the expression of β-actin by densitometric readings and then the fold change in expression for each glioma was compared to the average reading for normal brain. The fold changes for 10-08-MG, U-251MG, and U-373MG were 1.5, 1.4, and 1.2, respectively. Thus, the FAPP2 mRNA is slightly overexpressed in glioma cells relative to normal brain.

FAPP2 siRNA decreases FAPP2 expression and increases sensitivity to FasL-mediated apoptosis in several tumor types

We designed a randomized siRNA and one specific to FAPP2, then examined the ability of the transfected siRNA at reducing FAPP2 mRNA expression in tumor cells compared to control nontransfected cells. After transfection of the randomized siRNA into glioma cell lines, U-251MG, U-373MG and U-87MG, the expression of FAPP2 mRNA was reduced 10 to 20%, whereas if they were transfected with the FAPP2 siRNA the expression of FAPP2 was reduced 50 to 80% (Fig. 2).

Selection of FasL-resistant tumor cell lines

As mentioned earlier, the panel of tumor cell lines chosen for this work had to be resistant to FasL-induced apoptosis, or actD induced apoptosis, but exhibit sensitivity when in the presence of both [7]. Five glioma cell lines, T98G, U-87MG, U-251MG, U-373MG, and 10-08-MG, as well as the breast carcinoma metastatic subline 1833 met these criteria. The concentration of FasL for each particular cell line (listed in the legend to Fig. 3) that resulted in > 30% killing in the presence of actD was chosen for use in subsequent experiments that tested sensitivity to FasL-induced apoptosis when FAPP2 gene expression was reduced with siRNA.

Downregulation of FAPP2 sensitizes FasL-resistant tumor cell lines to apoptosis

MTT assays were performed with the glioma cell lines T98G, U-87MG, U-251MG, U-373MG, and 10-08-MG (Fig. 3a), and the metastatic breast carcinoma cell line 1833 (Fig. 3b). The cells were either treated with FasL, transfected with randomized or FAPP2 siRNA, or both treated and transfected. Compared to the respective untreated, nontransfected cells, only a 5–10% reduction in viable cell number occurred upon their exposure to FasL, indicating that this treatment had little cytoreductive effect. Likewise, transfection of each cell type with randomized siRNA, with or without FasL treatment, was not effective (<10%) in reducing cell numbers. Downregulation of FAPP2 by siRNA transfection was also largely uneventful for all cells with the exception of 10-08-MG cells where viable cell numbers were reduced approximately 20%. However, when the cells were transfected with FAPP2 siRNA and exposed to FasL, significant reductions in viable cell numbers (18–32%) were observed (*$p<0.05$, **$p<0.01$, ***$p<0.001$) compared to the most relevant of controls, randomized siRNA transfected tumor cells exposed to FasL (Fig. 3).

Three gliomas and the 1833 breast carcinoma cell line were assessed for apoptosis by the acridine orange/ethidium bromide morphologic assay. The control and experimental groups were similar to those used for the MTT assays, except we incorporated the use of a siRNA targeting luciferase, offering validation with a different control that could be used for imaging studies later. Representative fluorescent photomicrographs of 1833 cells transfected with luciferase siRNA and treated with FasL show low basal levels of apoptotic cells (Fig. 4a) that can be compared to the higher numbers of apoptotic cells observed in those transfected with FAPP2 siRNA and treated with FasL (Fig. 4b). The FAPP2 siRNA transfected 1833 cells exposed to FasL demonstrate representative apoptotic cells (bright green) that appear as smaller cells with condensed nuclei (small arrow), membrane blebbing (arrowhead), and fragmented nuclei (large arrow).

The percentages of apoptotic cells from the morphologic assay were determined by counting the number of condensed or disintegrated nuclei for 200 cells in each of two different high power fields (Fig. 4c). The apoptotic percentages for all of the cell lines transfected with siRNA to FAPP2 and exposed to FasL were significantly higher (***$p<0.001$) compared to the counterpart control cells transfected with luc siRNA and treated with FasL, with U-87MG glioma cells exhibiting the most dramatic effect.

Sensitization to Fas-induced apoptosis by FAPP2 downregulation does not correlate with increased Fas expression

Transfection with FAPP2 siRNA resulted in no to small increases in Fas expression compared to luc siRNA transfected or nontransfected tumor cells. For instance, Fas increased 1.1 to 1.2 fold in the U-87MG, U-251MG, and T98G cell lines, whereas there was no change in the U-373MG and 1833 cell lines (Table 1). Transfection with control luc siRNA caused an up- or down-regulation of Fas expression compared to nontransfected cells. These data suggest that the changes in Fas expression following downregulation of FAPP2 by siRNA and incubation with FasL are cell line specific. Furthermore, the inconsistent induction of Fas expression in cells with downregulated FAPP2 suggests other mechanisms are operational for engendering apoptosis than mere upregulation of Fas itself.

Discussion

Previously, we reported screening a retroviral ribozyme library for proapoptotic genes that would activate FasL-induced apoptosis in tumor cells that exhibited resistance to Fas/FasL-induced apoptosis [3]. In a manner similar to how we identified a role for the PATZ1 gene in conferring sensitivity of glioma cells to apoptotic stimuli [7], here we report the FAPP2 gene (also known as PLEKHA8) was identified to have similar effects. The protein product of the FAPP2 gene contains a plekstrin homology (PH) domain; it binds phosphatidylinositol-4-phosphate (PtdIns(4)P) and localizes the protein to the Golgi complex [9; 10; 11; 12]. The PH domain also facilitates interaction of FAPP2 with ADP-ribosylation factor, which along with PtdIns(4)P binding, allows FAPP2 to control the transport of cargo from the Golgi to the cell surface [10; 13; 14]. The FAPP2 protein additionally contains a domain found in glycolipid transport proteins and functions in the synthesis of sphingolipids [9; 15]. FAPP2 reversibly transports glucosylceramide from the ER to the Golgi and facilitates the production of ceramides in the cell [9; 16]. Although FAPP2 has not previously been functionally associated with FasL-induced apoptosis or any other part of the known apoptotic pathways, ceramides are sensory signaling molecules known to be involved in apoptotic pathways [17; 18].

We have uniquely observed that the FAPP2 gene, when down-regulated, promotes death receptor induced apoptosis in tumor cells. The gene has never been associated with having a role in apoptosis. Further, we describe the *in vitro* efficacy of a siRNA directed against this novel target gene that when transfected into tumor cells, exhibited anti-tumor effects including the activation of apoptosis by FasL or by Fas agonistic antibodies, or anti-proliferative responses. Since downregulation of FAPP2 sensitizes cells to Fas-induced apoptosis even in the absence of increased Fas expression, it is likely that FAPP2 is

contributing to an as yet undescribed, compensatory pathway that results in apoptotic induction in the presence of FasL. Deciphering how the FAPP2 gene functions in a pathway to confer Fas resistance to tumor cells should increase our understanding of how tumor cells become resistant to death receptor triggered apoptosis [19]. This may be especially relevant for breast cancer as the FAPP2 gene has been tentatively identified serologically as a potential breast cancer antigen [20]. Continuing the evaluation of this siRNA, as well as siRNAs against other novel genes for activating death receptor mediated apoptosis [3; 19; 21] may result in new therapeutic approaches for cancer.

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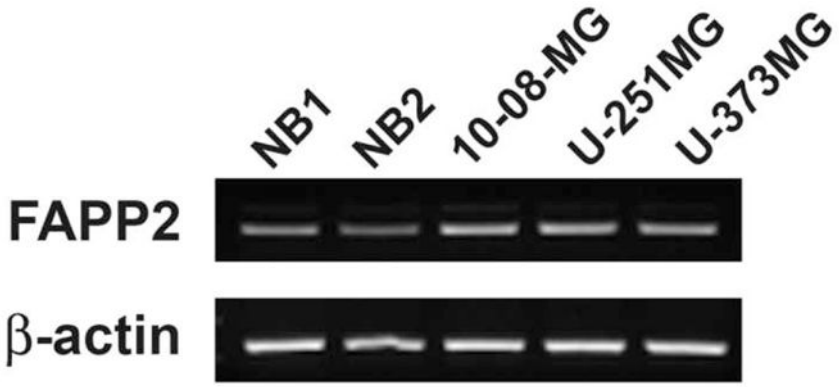

Figure 1.

FAPP2 mRNA levels are shown for extracts from two different normal brain (NB) specimens (lanes 1, 2) and three gliomas (10-08-MG, U-251MG, U-373MG in lanes 3–5, respectively). The β actin loading controls are at the bottom.

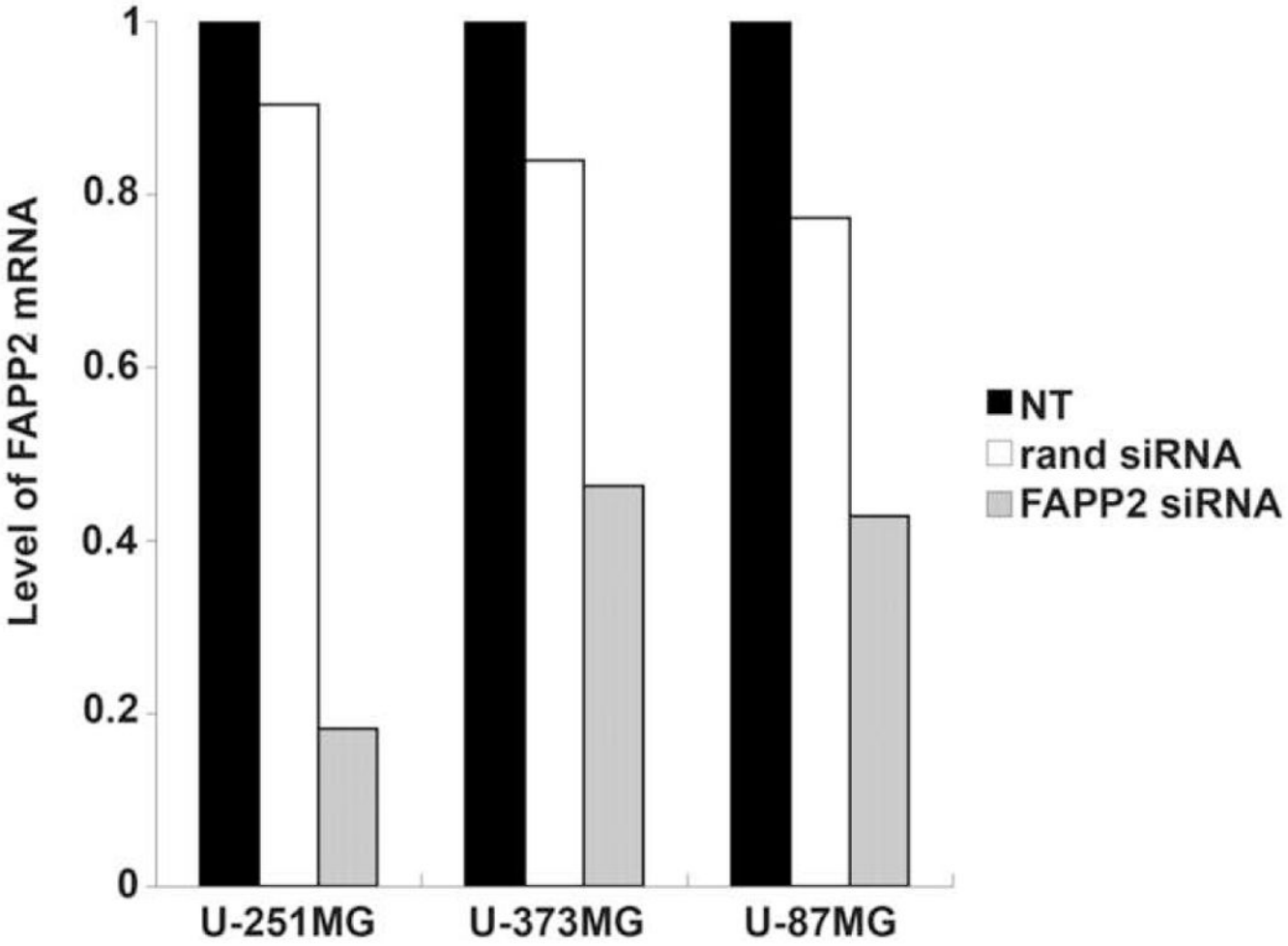

Figure 2.

RT-PCR analysis of FAPP2 gene knockdown in glioma cells after transfection with siRNA to FAPP2. All data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) prior to being compared for mRNA levels. With the expression level of the nontransfected glioma cells (NT) set at 1, the relative levels of FAPP2 in those cells transfected with siRNA to the randomized sequence (rand), or with siRNA to the FAPP2 gene are shown. Real time PCR was performed using gene specific primers and SYBR green detection on an iQ5 ribocycler. The levels were determined by numerical readings taken from the ribocycler.

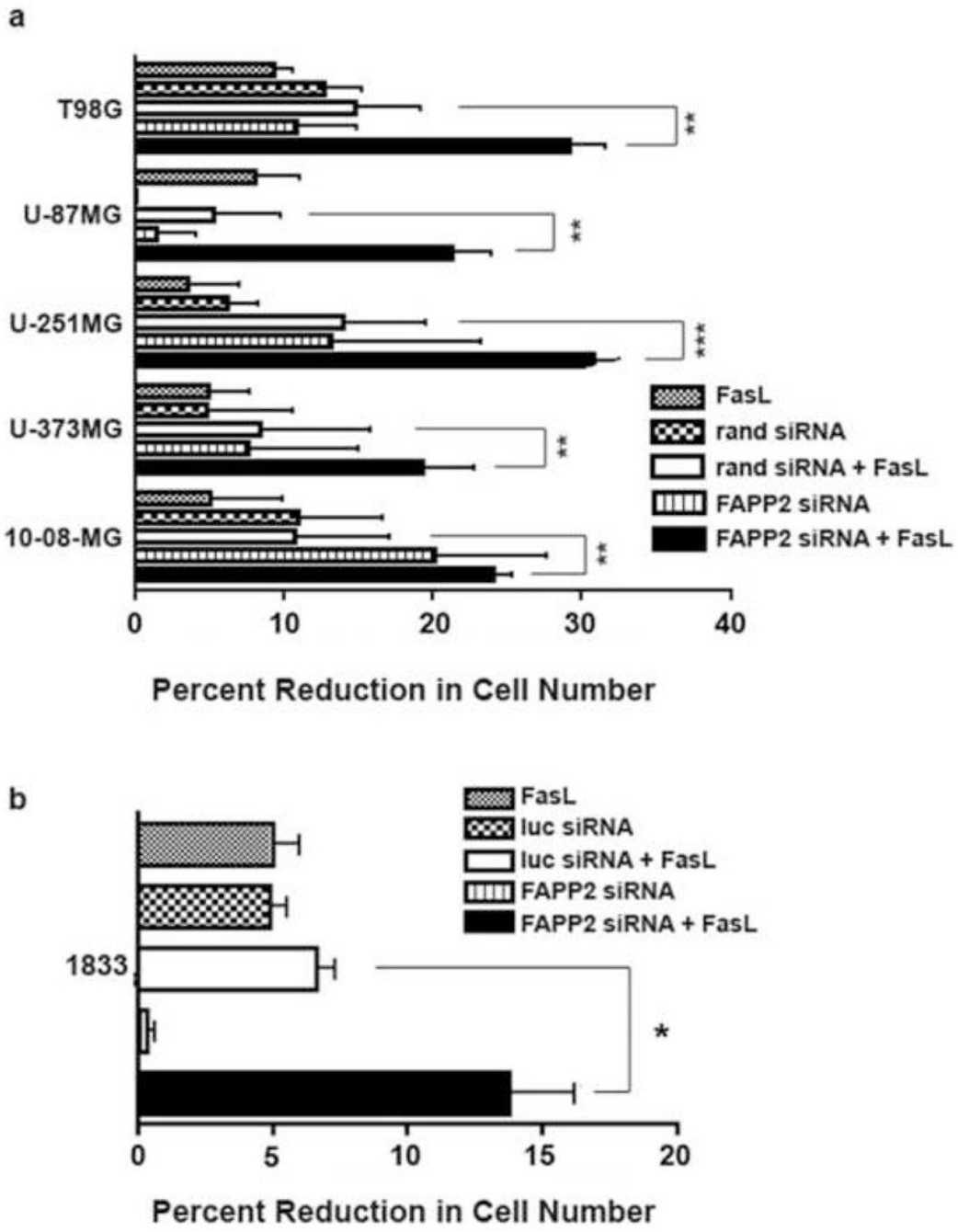

Figure 3.

Reduction in viable cell number following downregulation of FAPP2 in human tumor cell lines exposed to FasL. Cells were seeded at 2.5 x 10^4 cells/well in a 24-well plate the day prior to transfection. Transfections with Oligofectamine were performed with siRNA at a concentration of 50 nM. At 48 hr post-transfection, FasL at the concentrations listed: T98G, 10 ng/ml; U-87MG, 10 ng/ml; U-251MG, 50 ng/ml; U-373MG, 50 ng/ml; 10-08-MG, 150 ng/ml; 1833 50 ng/ml, was added and incubated overnight at 37°C. Viable cell numbers were estimated by the MTT assay. The control wells contained: 1) nontransfected cells + FasL, 2) cells transfected with a randomized siRNA sequence (rand), 3) cells transfected with rand siRNA + FasL, 4) cells transfected with FAPP2 siRNA, or 5) cells transfected with FAPP2 siRNA + FasL. Representative data showing reductions in a)

glioma or b) 1833 breast carcinoma cell numbers ± SE. Data were analyzed by 2 way-ANOVA and Bonferroni post-tests to determine statistical significance. The p values are: *$p<0.05$, ** $p<0.01$; *** $p<0.001$.

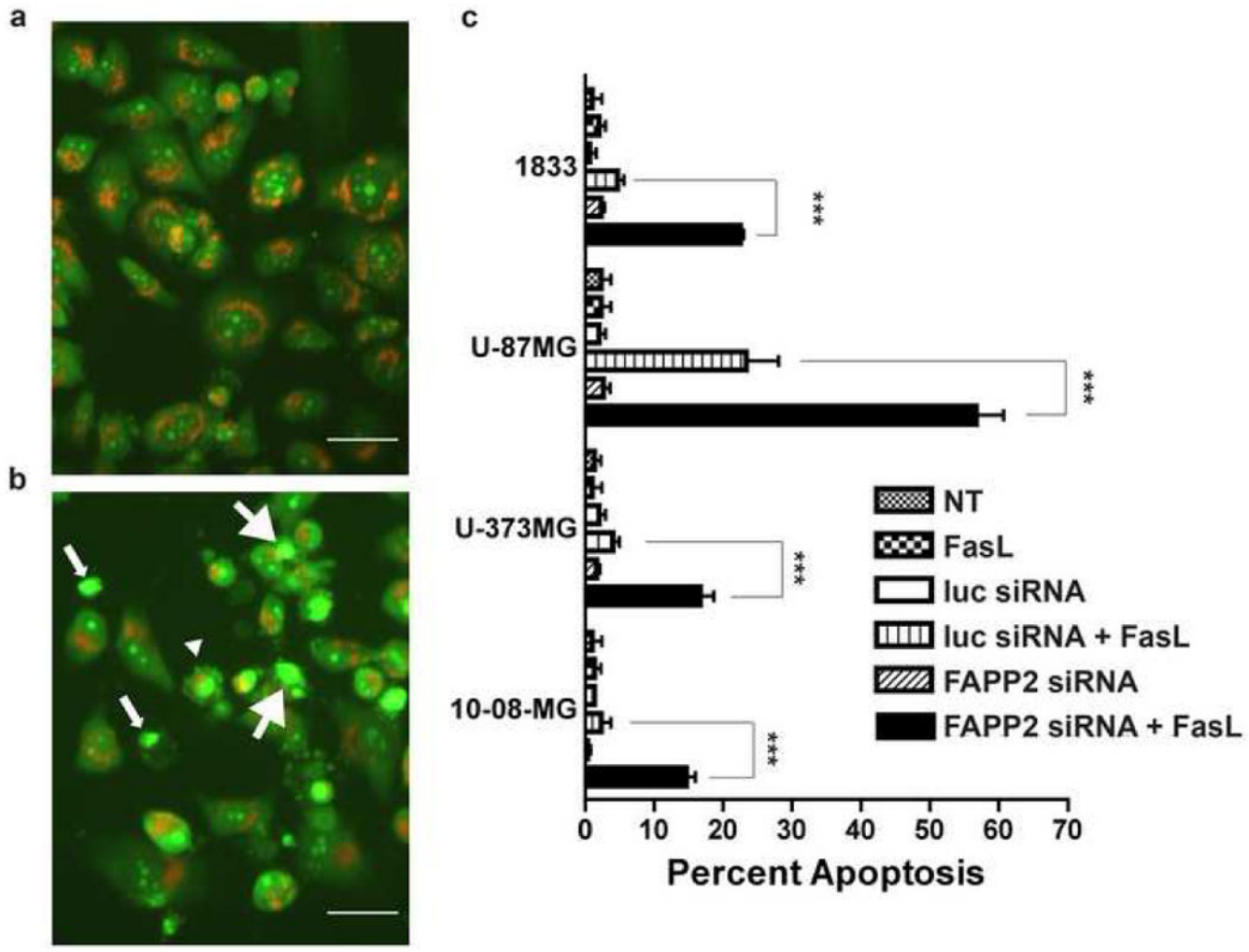

Figure 4.

Fluorescence microscopy of 1833 breast carcinoma cells after undergoing a morphologic assay for apoptosis by acridine orange/ethidium bromide staining. a) 1833 cells transfected with control luciferase (luc) siRNA + FasL, b) 1833 cells transfected with FAPP2 siRNA + FasL. Bright green breast tumor cells are seen with morphologic changes indicative of apoptosis: nuclear fragmentation (large arrows), membrane blebbing (small arrowhead), and small cells with condensed nuclei (small arrows). Bars = 50 μm c) Percentages of apoptotic glioma or 1833 breast tumor cells quantified from the morphologic assays. Bars are shown for nontransfected (NT), siRNA-transfected glioma cells (luc or FAPP2), treated or untreated with FasL. Apoptotic cells were counted from a total of 400 cells. The percentages of apoptotic cells ± SE are shown from duplicate experiments. Statistical significance by Bonferroni post-tests (***$p<0.001$) was demonstrated between the most relevant control, luc siRNA + FasL, and the experimental FAPP2 siRNA + FasL.

Table 1

Expression of Fas by nontransfected and siRNA-transfected cell lines

Cell Lines	Nontransfected	luc siRNA	FAPP2 siRNA
1833	26.0±1.4	19.6±1.5***	20.6±0.6
U-87MG	73.0±1.4	73.6±1.1	82.6±2.1***
U-373MG	22.5±0.7	21.3±0.6	21.0±1.0
U-251MG	49.5±2.1	57.3±3.5***	70.0±1.0***
T98G	48.0±0.0	55.0±0.3***	63.6±1.5***

*** The differences in MFI at $p<0.001$ by 2-way ANOVA.