Thorough validation of siRNA-induced cell death phenotypes defines new anti-apoptotic protein

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ABSTRACT

Loss-of-function by means of RNA interference in cultured human cells enables rapid pathway dissection on a genome-scale. Improved siRNA design and key validation protocols are required to eliminate falsely identified phenotypes resulting from potential off-target consequences. Here, we demonstrate a validation strategy involving several steps for verifying cell death phenotypes revealed during loss-of-function screening. First, from a set of 45 novel human genes we identified gene candidates that, when silenced, induce apoptosis in cultured HeLa cells. For those candidates, we performed more extensive validation with multiple effective siRNAs. In addition, we designed rescue experiments involving candidate genes delivered exogenously and containing silent mutations in the siRNA target regions. Rescue of the observed knockdown phenotype demonstrated an original and more stringent validation of the siRNA's selectivity and the phenotype specificity for the target gene. As a result, our data reveals an anti-apoptotic function for novel human breast adenocarcinoma marker BC-2, adding new depth to BC-2′s description as a putative tumor marker involved in cancer related pathways.

INTRODUCTION

Rapid gene silencing through RNA interference (RNAi) has accelerated the genome-wide study of gene function with unparalleled speed by offering a simpler alternative to previously established reverse genetic approaches. Large-scale RNAi has facilitated the search for genes required for diverse biological processes enabling stepwise dissection of specific signaling pathways. Recently, large-scale RNAi in the form of short interfering RNAs (siRNA) has been successfully employed in human cell lines to screen for regulators of TRAIL-induced apoptosis (1), to link specific kinase subfamilies involved in regulating clathrin-mediated endocytosis (2) and to identify genes essential for cell division (3).

However, reports that siRNAs may induce 'off-target' fluctuations in the expression of genes not related to their cognate mRNAs (4–6) have generated skepticism over the specificity of siRNA in mammalian cells. The somewhat limited specificity implied by off-target results emphasizes not only the need for careful siRNA design but also the critical interpretation with which RNAi-derived phenotypes must be regarded. Phenotypic observations generated by specific siRNAs designed according to the most stringent guidelines must be confirmed experimentally by channeling data through a series of key validation steps. In this report, we performed RNAi in human HeLa cells targeting a set of novel genes in medium-scale assays screening for phenotypes influencing cell viability and specifically apoptosis. In addition, we included a combination of validation steps that allowed us to identify with confidence among our collection a new anti-apoptotic protein essential for cell viability.

MATERIALS AND METHODS

Gene cloning, oligonucleotide templates for siRNAs

Detailed methods for cloning the novel genes included in the present study have been described previously (7,8). The 21 nt cDNA templates used for siRNA production were designed using Ambion siRNA-finding software, according to the criteria described previously (9). These siRNA templates are compatible for use with the Silencer™ siRNA Construction Kit (Ambion). In the preliminary siRNA design, three cDNA templates for siRNA production were selected for each gene and were generated according to the manufacturer's recommendations. Following the preliminary cell proliferation and apoptosis screening assays, gene candidates were selected for which a second effective siRNA was generated by the method described above. The first and second siRNAs were tranfected in parallel in order to compare gene knockdown efficiency. siRNA templates are shown in Supplementary Table 2. Sense and antisense templates were synthesized by Thermo Electron

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GmbH. A non-specific siRNA was included as negative control (Dharmacon Inc).

Cell culture and siRNA transfection

Cultured HeLa cells were transfected with plasmid DNA or siRNA as described (10) with some modifications. Briefly, human HeLa cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were passed regularly to maintain robust growth. Before 24 h of transfection, cells were trypsinized, collected and diluted with fresh medium either with or without antibiotics depending on whether transfections were performed with HiPerFECT (Qiagen) or Lipofectamine 2000 (Invitrogen), respectively. Initial transfections for identifying effective siRNAs were carried out in 24-well cell culture plates, while cell apoptosis assays were carried out in 96-well cell culture microplates (COSTAR3603, CORNING). Each sample was assayed in triplicate. siRNAs were used at a final concentration of 20 nM in all experiments.

Generation of point mutations

For rescue experiments, mutations were generated using the QuikChange II Site-Directed Mutagenesis Kit (STRATAGENE), according to the manufacturer's protocol. To fully abolish the siRNA effect, two nucleic acids in the siRNA-targeted regions were mutated.

Immunofluorescence microscopy

Cell fixation and immunostaining were performed according to previously described methods (7). Cells were stained with rabbit anti-cleaved Caspase 3 (Cell Signaling Technology) diluted 1:100 subsequently detected with donkey anti-rabbit conjugated with Cy3 diluted 1:1200 (Jackson Laboratories).

Cell apoptosis assays

Caspase-3/7 activities were measured using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega) according to the manufacturer's protocol. HeLa cells were plated in triplicate in 96-well cell culture plates (COSTAR3603, CORNING). After 48 h siRNA transfection, cells were lysed with lysis buffer containing caspase substrate Z-DEVD-R100, and incubated at room temperature until being analyzed. Assays were measured by detection with a fluorescence microplate reader (LABVISION system, BMG Labtechnologies), and the fluorescence was measured at an excitation/emission wavelength of 485/535 nm. Results are presented as the mean ± the standard deviation of the triplicates.

Western blotting, RNA isolation and RT–PCR analysis

Total RNA from siRNA transfected cell lines was prepared according to the methods described previously (8). Gene knockdown efficiency by the specific siRNAs was assessed both from protein levels by western blot and from mRNA levels by RT–PCR for every gene in our collection according to methods also described previously (8).

RESULTS AND DISCUSSION

The objectives of our study were to explore loss-of-function for a set of novel human genes using RNAi in cultured HeLa cells and to establish an efficient and reliable validation strategy for identifying anti-apoptotic proteins. The 45 human genes reported here (Supplementary Table 1) were selected on the basis of previously described criteria for the purpose of employing various cell-based screening methods for gaining insight into function (7,8). Briefly, the selected human genes encode proteins for which little or no functional data is available as determined by limited descriptions from public resources such as NCBI; hence they are referred to as novel. Additionally, the genes share at least one common domain between several eukaryotic genomes, including fly and worm, a property that denotes a conserved function.

Loss-of-function screening identifies candidates influencing caspase activity

In order to gain a basic insight into the function of the novel genes, we first aimed to identify genes that influence cell viability by carrying out assays measuring apoptosis. Effective siRNAs for each gene were identified according to previously described methods (8). siRNA efficiency was evaluated from both the transcript level of the target gene and the corresponding protein level of an exogenously added gene product. Only the siRNAs that are able to effectively reduce expression of both the corresponding mRNAs and the ectopically expressed gene products were considered for our functional assays (Supplementary Table 2, Supplementary Figure 1). HeLa cells were then transfected with the selected 45 siRNAs in 96-well plates and assays were performed using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) monitoring the activation of caspases 3 and 7, indicators of apoptosis, by fluorescence detection. We observed that siRNA-mediated inactivation of two candidate genes (RefSeq Accession nos NM_014453 and NM_025238 or genes BC-2 and BTBD1 isoform 1, respectively) triggered a significant increase in caspase 3/7 activity within 48 h of siRNA transfection (Figure 1A).

Importantly, recent reports have demonstrated a possible risk for siRNA-induced off-target consequences (4–6). These observations have challenged the idea that siRNAs are able to induce silencing exclusively for their target gene. That is to say, the cognate gene function may have little to do with the observed phenotype. It has been suggested that some off-target effects arise from unintended sequence homology with other transcripts containing as few as 15 or less contiguous bases in common. Therefore, if using only one siRNA per gene, the reliability of any observed phenotype would be questionable and difficult to interpret, a factor that strongly compelled us to pursue a secondary selection of effective siRNAs for the identified candidates. Additional siRNAs selected against BC-2 and BTBD1 demonstrated RNA and protein silencing efficiency comparable to that of the first siRNAs tested when transfected into HeLa cells in parallel (Figure 1B). In order to validate the phenotypic hits, we repeated the Apo-ONE Homogeneous Caspase-3/7 Assay with both effective siRNAs identified for the two candidate genes (Table 1). BC-2 reduction by the second siRNA strongly activated caspase 3/7 in HeLa cells (Figure 2E), a finding that
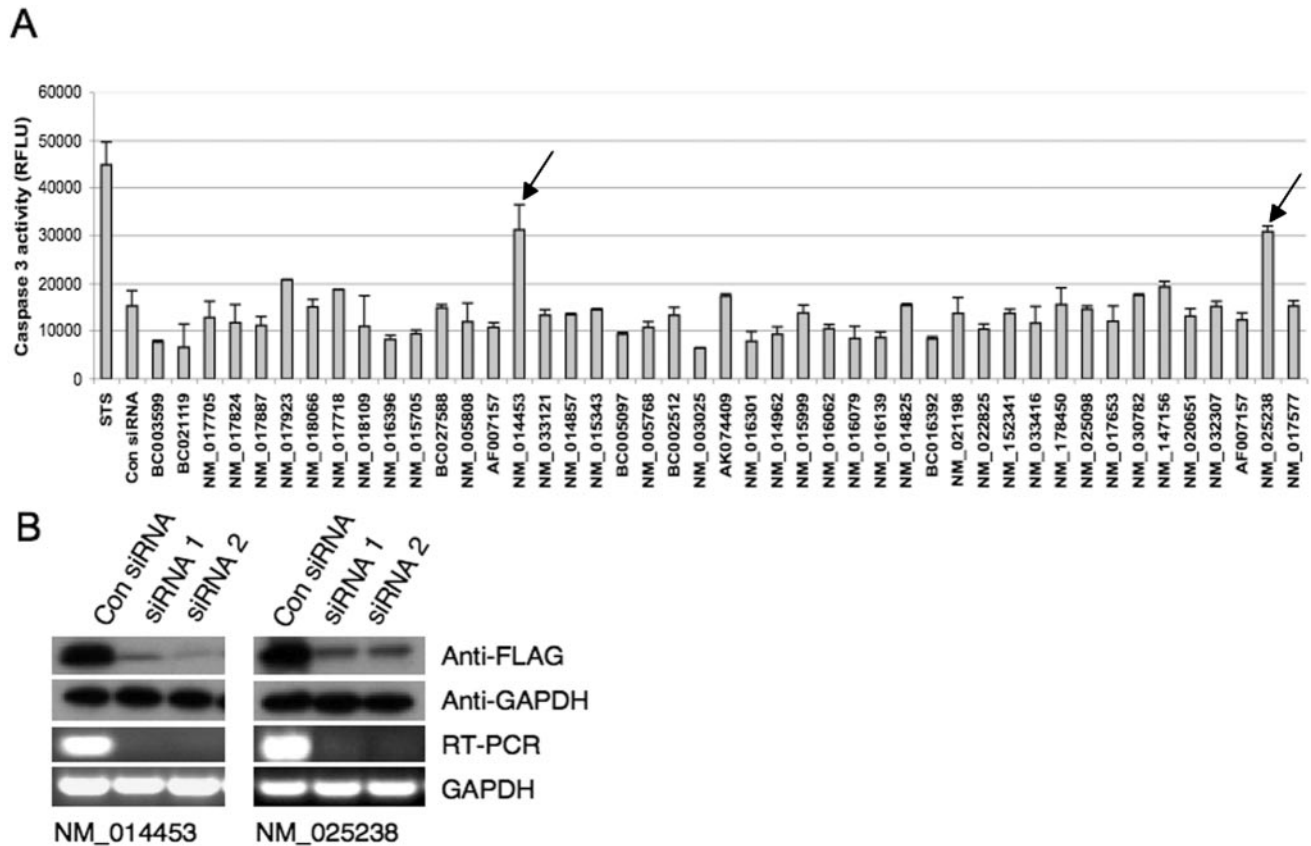
Figure 1. Activation of caspase 3 characterizes siRNA-induced cell apoptosis. (**A**) HeLa cells were transfected in parallel with 45 gene-specific siRNAs and a non-specific control siRNA. Refseq and GenBank nucleotide accession numbers are indicated for each gene. Error bars represent the standard deviation of mean values. Two candidate genes were isolated for further validation studies (indicated by arrows). Two effective siRNAs were designed for each candidate and their efficiency was evaluated in two ways. (**B**) First, exogenous levels of 3xFLAG fusion proteins expressing the genes of interest were monitored in co-transfection experiments with corresponding siRNAs. Fusion protein levels were detected by anti-FLAG. Second, endogenous gene levels of the targeted mRNA by RT–PCR.

Table 1. Candidate genes identified and corresponding siRNA target sequences

Refseq accession nos	Symbol	Description	siRNA 1	siRNA 2
NM_014453	BC-2	Putative breast adenocarcinmarker	AAGGCCAGATGGATGCTGTTC	AAGGAGGAGATGATGAATGAT
NM_025238	BTBD1	BTB (POZ) domain containing Isoform 1	AAAGTAGTGCATGAGACACCT	AATAGATTCCAGCAAGTAGAA
Control siRNA DNA template			AACAGTCGCGTTTGCGACTGG	

verified the specificity of both BC-2 siRNAs and corroborated the phenotypic consequence of the gene targeting experiments. However, the second siRNA targeting BTBD1 did not induce equivalent levels of caspase 3/7 activity in HeLa cells compared to the first siRNA, suggesting that the initially chosen BTBD1 siRNA gave rise to an off-target or toxic effect not linked to the function of this gene (Supplementary Figure 2C).

Downstream assays support caspase activation as a consequence of BC-2 reduction

In order to confirm the findings obtained by the Apo-ONE assay, we performed additional experiments monitoring the effects of BC-2 inactivation. Immunofluorescence microscopy with an antibody against cleaved-caspase 3, the active form of the protein, reiterated the Apo-ONE assay results showing a marked increase in detection of this marker in cells transfected with the two separate siRNAs against BC-2 (Figure 2A). Activation of apoptosis was also confirmed by western blot with an antibody against poly-ADP-ribose polymerase (PARP) which is cleaved downstream of caspase 3 at a later stage of apoptosis (Figure 2B).

Induction of apoptosis through loss of BC-2 can be rescued

Rescue experiments represent a more stringent way in which the selectivity and the specificity of siRNA knockdown may be verified. This strategy is particularly interesting both from the pharmaceutical point of view where, for many genes, the RNAi-knockdown can only be measured from enzymatic or metabolic activity, and especially for addressing those genes for which only one siRNA is available.
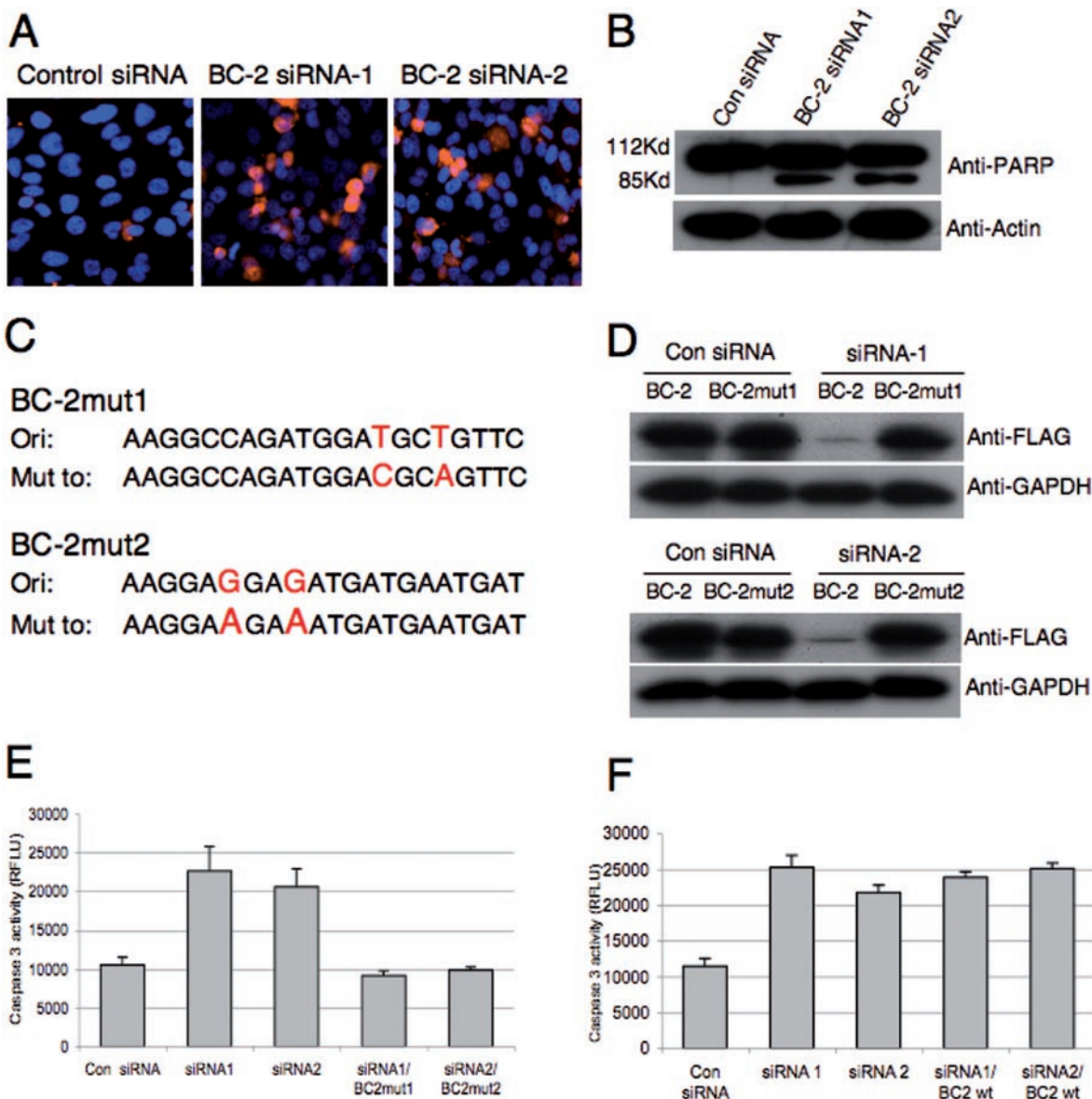
Figure 2. BC-2 reduction promotes apoptosis. BC-2 siRNA-induced apoptosis was detected by immunofluorescence in HeLa cells stained with rabbit anti-cleaved Caspase 3 (**A**). PARP cleavage was also detected by western blot using anti-PARP (**B**). Two different 3xFLAG-tagged BC-2 mutants (BC-2mut1 and BC-2mut2) were generated by site-directed mutagenesis with two silent point mutations each corresponding to siRNA targeted regions (**C**). In parallel, plasmids containing the original 3xFLAG-tagged BC-2 or the mutated versions were co-transfected into HeLa cells with siRNA1, siRNA2 or with control siRNA. Point mutations fully abolished siRNA effects on the exogenous BC-2 fusion protein (**D**). Rescue experiments were performed by co-transfection with BC-2 siRNAs (siRNA1 and siRNA2) and a plasmid construct expressing either the mutated 3xFLAG-tagged BC-2 (BC-2mut1 and BC-2mut2) (**E**) or the 3xFLAG-tagged wild-type BC-2 (BC-2wt) (**F**). After 48 h, the cultured cells were assayed for Caspase 3/7 activity. Error bars represent the standard deviation of mean values. The mutated BC-2 proteins prevent cells from BC-2 siRNA-induced apoptosis (E). BC-2wt does not rescue siRNA-induced cell death (F).

To further validate both the selectivity of the siRNAs for the BC-2 gene and the specificity of the phenotype, we performed a rescue experiment employing two expression constructs containing the entire coding region of the BC-2 gene fused to an N-terminal 3xFLAG sequence. The two constructs, one for each siRNA, were designed to contain silent point mutations by substituting the third base position of two codons each within the respective regions targeted by the two siRNAs (Figure 2C). We demonstrate that the two point mutations are sufficient to fully abolish any siRNA knockdown of the expression level of the mutant BC-2 protein, further indicating that the siRNAs are highly selective for the endogenous BC-2 gene (Figure 2D). We further demonstrate that both of the exogenous BC-2 mutant constructs successfully rescued HeLa cells from apoptosis when co-transfected with the corresponding siRNAs, suggesting that this phenotype is directly linked to an anti-apoptotic BC-2 function (Figure 2E). Rescue experiments were repeated using a plasmid containing the wild-type BC-2 gene (BC-2 wt). As expected, wild-type BC-2 does not rescue apoptotic cell-death induced by the BC-2 siRNAs, as expression of the non-mutated protein, both endogenous and overexpressed, is successfully abrogated

by both siRNAs (Figure 2F). During the process of identification of effective siRNAs for our novel genes, we observed that as little as 1 nM siRNA could sufficiently silence exogenously overexpressed genes. By performing the rescue experiments for both effective siRNAs, we determined that the activation of caspase 3 was specifically elicited by loss of the BC-2 gene. Furthermore, the discriminate silencing of the non-mutated BC-2, but not the mutated BC-2, supports the idea that siRNA signaling requires near perfect homology with the target transcript.

The putative breast adenocarcinoma 2 gene is also described as CHMP2A or chromatin modifying protein 2A; charged multivesicular body forming protein 2A and contains an ESCRT-III domain that is associated with endosome to lysosome protein sorting and transport. However, it is clear that BC-2′s function may lie beyond that which has been described for members of the ESCRT-III complex (11). Interestingly, BC-2′s closest relative among the CHMP family of proteins, CHMP1, has been reported to be a nuclear matrix protein possibly involved in gene silencing and cell cycle progression (12). Based on our previous finding that overexpressed BC-2 localizes to the nucleus both diffusely and in discrete foci (13), BC-2 may participate in nuclear events leading to transcriptional repression of genes necessary for apoptosis. The discovery of this new anti-apoptotic protein may be of particular importance considering that the aberrant expression of such proteins has been shown to contribute to tumor development. The mis-regulation of anti-apoptotic genes can confer resistance to cell death programs normally executed in response to stress, DNA damage or insults to proper chromosome segregation (14). This ability to avert death directly contributes to cancer progression. The identification of anti-apoptotic genes, such as BC-2 will provide new candidates for cancer therapies (15).

siRNA induced apoptosis cannot be rescued by BTBD1

Although both siRNAs directed against BTBD1 showed comparable silencing efficiency (Figure 1B), the two siRNAs did not induce similar levels of caspase activity (Supplementary Figure 2C). Because of the discrepancy between the two BTBD1 siRNAs in the level of caspase activity induced, we sought further validation of this phenotype by employing rescue experiments similar to those performed for BC-2 (Supplementary Figure 2). Expressing the BTBD1mut1 construct did not prevent the HeLa cell apoptosis induced by the initial BTBD1 siRNA, suggesting that this siRNA induces an off-target or toxic effect. In this case, the rescue experiment was a necessary additional measure for resolving the conflicting results provided by two independent siRNAs against BTBD1.

Concluding remarks

In conjunction with our aim to dissect function for our genes of interest, we have demonstrated a series of essential steps toward the validation of siRNA-induced phenotypic hits. Furthermore, the validation strategy introduced here has enabled the identification of a novel anti-apoptotic gene that may uncover an important link in mechanisms leading to cancer development.

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