Long-Term BRCA1 Down-Regulation by Small Hairpin RNAs Targeting the 3' Untranslated Region

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Mutations in the BRCA1 gene are responsible for the majority of hereditary breast/ovarian cancers. The functional significance of many mutations/splicing variants identified during the screening of high-risk individuals is difficult to predict due to the lack of *in vitro* functional tests correlating sequence variants with a risk of cancer development. RNA interference is a promising tool in analyzing functional properties of BRCA1 mutations. Here we designed and functionally analyzed shRNAs directed to 3'-UTR of BRCA1 mRNA that may be used to knock-down expression of endogenous BRCA1. Using retroviral infection, we achieved long-term down-regulation of BRCA1 in a cell-type specific manner. We propose that 3'-UTR-directed shRNAs, coupled with up-regulation of exogenous mutated BRCA1 variants, may constitute a versatile system for the functional analysis of BRCA1 gene alterations.

Keywords: BRCA1, Retroviral infection, RNAi, shRNA, UTR

Tumor suppressor BRCA1 (Breast Cancer 1, OMIM #113705) is ubiquitously expressed multifunctional phosphoprotein participating in the regulation of DNA damage repair [1], chromatin modifications [2], cell cycle control [3, 4], apoptosis [5], transcription [4], and centrosome function [6, 7]. 1,863 amino acids long BRCA1 protein contains two conserved domains. The N-terminal RING (Really Interesting New Gene) domain possesses an E3 ubiquitin ligase activity when complexed with another RING domain-containing protein, BARD1 (BRCA1-Associated RING Domain 1) [8]. C-terminally located BRCT (BRCA1 Carboxyl-Terminal) domain mediates phosphopeptide-specific protein-protein binding [9]. RING domain-mediated ubiquitination and BRCT-mediated interaction with various partner proteins are responsible for diverse BRCA1 functions.

The BRCA1 gene was originally cloned as one of the genes that conferred genetic predisposition to early-onset breast and ovarian cancers [10]. Mutations in BRCA1 gene are responsible for the majority of hereditary breast and ovarian cancers [11]. Thousands of nonsense and missense mutations and sequence variants were identified and are collected in the BIC (Breast Information Core) database (http://research.nhgri.nih.gov/bic/). Functional significance of mutations identified in high-risk individuals or families with recurrent breast and/or ovarian cancers is predicted based on the segregation with the disease within a particular family. Besides many disease-causing mutations, numerous missense mutations, unclassified sequence variants, splicing variants and gene polymorphisms were reported. However, the functional significance of these alterations is difficult to predict because *in vitro* functional tests exactly correlating any mutation/splicing variant with a corresponding risk of breast/ovarian cancer development are currently unavailable [12].

RNA interference (RNAi) is a phylogenetically conserved mechanism of double-stranded RNA (dsRNA)-mediated mRNA silencing [13]. RNAi can be triggered either by small interfering RNAs (siRNAs; derived from exogenous dsRNA) or by endogenously produced ~ 21-22 bp non-coding RNAs, called microRNAs (miRNAs). In humans, miRNAs are transcribed as stem-loop containing precursors (pri-pre-miRNAs) mainly by RNA Polymerase II, but also by RNA Polymerase III. These precursors are sequentially processed by RNase III-like endonucleases Drosha and Dicer, respectively, giving rise

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to mature miRNAs. Mature siRNAs and miRNAs exert their RNAi function as a part of RISC (RNA-Induced Silencing Complex) or miRNP complex, respectively. Detailed mechanisms of miRNA/siRNA biogenesis, dsRNA-induced silencing complex (RISC) assembly and mechanisms of miRNA/siRNA function were currently reviewed [13-16].

RNAi can be triggered *in vitro* either by exogenous application of siRNAs or by intracellular expression of small hairpin RNAs (shRNA) from transfected plasmids. SiRNAs are 19-bp long, synthetic dsRNA molecules bearing 2-bp 3' overhangs. These are transiently transfected to target cells and enter RNAi machinery at the stage of the RISC formation (i.e. by-passing the Drosha- and Dicer-mediated cleavage steps). Effect of transiently transfected siRNAs is short, lasting only a couple of days in proliferating cells, generally not longer than a week [17, 18]. On the contrary, shRNAs are expressed from plasmid as precursor molecules similar to endogenous pre-miRNAs. These precursors are processed by Drosha and Dicer and enter the RISC silencing complex. The expression of shRNA is frequently driven by RNA polymerase II-based [19] or RNA polymerase III-based [20] promoters. ShRNA expression cassettes cloned into retroviruses enable infection of hard-to-transfect cell lines and primary cultures [21-23].

There are several limitations using RNAi. The most serious are potential off-target effects mediated by each particular siRNA/shRNA [24, 25]. Careful design of siRNA sequences can by-pass, but does not completely eliminate, these non-specific effects [24]. To avoid misinterpretation of results, basic rules for RNAi experiments were formulated [26, 27]. However, the ultimate control for any RNAi experiment remains a rescue expression of the target gene (ideally within the physiological range) in a form refractory to siRNA/shRNA inhibition [26].

Variety of siRNAs/shRNAs targeting the ORF (open-reading frame) region of BRCA1 mRNA were published. Construction of BRCA1 gene resistant to RNAi requires an introduction of silent mutation(s) in the target sequence. For each siRNA/shRNA targeting the ORF region of BRCA1, its necessary to construct corresponding RNAi-resistant form. This may be very laborious and time-consuming process. However, even the introduction of "silent" mutation(s) can affect the protein structure and function [28]. Targeting the 3'-UTR region of BRCA1 mRNA circumvents the need of specific RNAi-resistant BRCA1 construct. For all 3'-UTR-directed siRNAs/shRNAs, wtBRCA1 ORF sequence can be used as an general "RNAi-resistant" control, avoiding thus the introduction of any mutation. Moreover, 3'-UTR directed siRNAs/shRNAs target all BRCA1 mRNA variants present in the cell, including sequence variants and alternatively spliced mRNAs.

In this study, we described and functionally evaluated retroviral-delivered shRNAs targeting 3'-UTR sequence of human BRCA1 mRNA. RNAi targeting the 3'-UTR is advantageous in the terms of targeting BRCA1 mRNA regardless of the potential existence of sequence variants (missense and nonsense mutations, alternative splicing, insertions, and deletions). Retroviral-mediated, stable, long-lasting RNAi is suitable for evaluating BRCA1 function even in hard-to-transfect cells, including primary cell cultures isolated from tissues of cancer patients or BRCA1 mutation carriers.

Materials and Methods

*Cell culture***.** MCF-7, MDA-MB-231 and HeLa cells were obtained from American Type Culture Collection (ATCC). Cell were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. Culture medium for MCF-7 cells was further supplemented with 0.01 mg/ml bovine insulin (Gibco). NIH3T3 cells were cultured in DMEM + 10% Calf Serum (Colorado Serum Company).

Infection. Infection of human cell lines was performed using Phoenix amphotropic packaging cell line. Briefly, shRNA-containing retroviruses were transfected into Phoenix cells by standard calcium phosphate method. Beginning at 24 h after transfection, viral supernatants from Phoenix cells were collected every 12 h, pooled, filtered through 0.45 μm PVDF membrane (Millipore), supplemented with 8 μg/ml polybrene (Sigma-Aldrich) and used to infect target cells in the logarithmic phase of growth. Four infections were performed in total. Selection by puromycin (Sigma-Aldrich) was started 24 h after the last infection.

Growth curves. Cells were seeded in 6-well plates at density $2x10^4$ (MCF-7 and MDA-MB-231) or $1.2x10^4$ (HeLa) cells per well. Cells were cultured in media containing puromycin and the culture media were changed at the day 3. Staining of cells was performed at day 0, 2, 4 and 6 (day 0 is the first day after plating the cells). Cells were washed in PBS, fixed in 10% formaldehyde for 30 minutes, and stained by 0.1% crystal violet (Sigma-Aldrich) solution in 10% ethanol for at least 30 minutes. Stained cells were extensively rinsed with water. The dye was extracted with 10% acetic acid and the absorbance of solution was measured at λ = 590 nm. Staining an empty well without cells was used as a control to zero the instrument. If needed samples were diluted with water. Data were plotted as a relative absorbance ratio to the day 0.

Construction of shRNA plasmids. For each shRNA to be cloned into pSUPER.retro.puro plasmid [20], two complementary oligonucleotides were synthesized (sequences available on request), annealed and cloned into pSUPER.retro.puro plasmid according to the manufacturer's protocol. Positive clones were checked by sequencing (ABI Prism 310, Applied Biosystems) in both directions.

For each shRNA to be cloned into LMP plasmid [29], an 97-bp oligonucleotide was synthesized (sequences available on request) and used as a template in PCR reaction with following primers: miR-30-Fwd: 5'- CAGAAGGCTCGAGAA GGTATATTGCTGTTGACAGTGAGCG and miR-30_Rev:

5'- TGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG. Specific 138-bp PCR product was gel-purified (Zymoclean Gel DNA Recovery Kit, ZymoResearch) and cloned into LMP plasmid. Positive clones were verified by sequencing.

All shRNAs used in this study are numbered according to the position of the first nucleotide of shRNA's target sequence in BRCA1 reference mRNA (GenBank accession number NM_007294.2).

Reporter assay. pGL4.10-SV40_3UTR reporter plasmid containing human BRCA1 3'-UTR sequence was constructed in two steps. First, SV40 promoter from phRL-SV40 plasmid (Promega) was transferred into promoter-less pGL4.10[Luc2] plasmid (Promega). Next, BRCA1 3'-UTR (NM_007294; region 5820-7102) was PCR-amplified from HeLa cells and cloned into Xba I site of pGL4.10-SV40. Final construct was verified by sequencing.

NIH3T3 cells were plated 16 h prior to the transfection ($1.5x10^5$ cells per well in 6-well plates). A 2 ng portion of pGL4.10-SV40_3UTR reporter plasmid was co-transfected with 50 ng – 1.5 μg of particular shRNA-expressing plasmid using FuGENE 6 (Roche). Where appropriate, pBluescript plasmid DNA was added to equal the total amount of DNA per well. pGL4.10-SV40 reporter plasmid without BRCA1 3'-UTR and irrelevant shRNAs targeting mouse CCAAT/Enhancer Binding Protein γ (C/EBPγ) gene were used as negative controls. Culture media were changed 24 h after transfection. At 48 h after transfection, the cells were lysed in Passive lysis buffer (Promega) and analyzed using the Luciferase assay system (Promega). Luciferase values were normalized to protein levels (Bio-Rad Protein Assay).

Transient transfections. Cells were seeded in 6-well plates at density $1.5x10^5$ (MCF-7 and MDA-MB-231) or $1x10^5$ (HeLa) cells per well 24 h before the transfection. Transient transfections were performed using FuGENE 6 (Roche), Lipofectamine 2000 (Invitrogen) or Metafectene (Cambio) transfection reagents according to manufactures' protocols. Media were changed 24 h after the transfection and cells were collected and analyzed 48 h post-transfection.

RNA isolation and quantitative real-time PCR. Total RNA was isolated by RNA Blue kit (Top-Bio). Reverse transcription was performed by SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. A 1 μl aliquot of prepared cDNA was used as a template for quantitative real-time PCR (qRT-PCR). qRT-PCR was performed on LightCycler 2.0 System (Roche) using Light Cycler Fast Start DNA Master SYBR Green I kit (Roche). BRCA1-specific primers F1 (5'-AGAGTGTCCCATCTGTCTGGAGTTG) and R1 (5'-GGACACTGTGAAGGCCCTTTCTTC) targeting BRCA1 coding sequence (mRNA: 185-304 bp) were used for qRT-PCR. Reactions were cycled 50 times at 95°C for 10 s, 70°C for 10 s and 72°C for 10 s. Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD) were analyzed from the same cDNA at amplification conditions identical to those described for BRCA1. qRT-PCR results were analyzed by LightCycler software (Roche) and values of crossing points (CPs) and amplification efficiencies were evaluated for each reaction. Statistical significance of changes in BRCA1 mRNA levels relative to housekeeping genes was calculated by pair wise fixed reallocation randomization test using the REST-2005 software [30].

Flow cytometry. Cells were harvested by Trypsin/EDTA, washed with PBS and re-suspended in PBS to the final concentration 10^6 cells per ml. Cells were fixed in 1% formaldehyde for 15 minutes, and the fixation was stopped by adding glycine to the final concentration 125 mM. After washing with PBS, cells were fixed in 70% cold ethanol O.N. at – 20 °C. Cells were stained with anti-BRCA1 antibody (D-20; Santa-Cruz) followed by anti-rabbit AlexaFluor 488-conjugated secondary antibody (Molecular Dynamics). All antibodies were diluted in PBS containing 1% BSA and 0.1% Triton X-100. In controls, the primary antibody was omitted or pre-incubated with specific blocking peptide (sc-641P; Santa-Cruz).

For cell cycle analysis, cell were collected by Trypsin/EDTA and fixed in 70% cold ethanol O.N. at – 20 °C. Cells were washed 3 times in PBS and finally resuspended in 500 μl PBS containing 20 μg/ml RNase A (Roche) and 50 μg/ml propidium iodide (Sigma-Aldrich). Cells were incubated 30 minutes at room temperature and analyzed on FACSort flow cytometer (Becton–Dickinson). Flow cytometry data were processed by the WinMDI 2.8 software.

Results and Discussion

Design of shRNAs. All shRNA used in this study were designed to target the 3'-UTR of human BRCA1 mRNA. Sh_5890, sh_6073 and sh_6095 were designed manually according to accepted rules [31-33]. Sh_6069 and sh_6252 were predicted by BIOPREDsi siRNA-predicting algorithm [34]. All these shRNAs were cloned into pSUPER.retro.puro plasmid, where the expression is under the control of RNA polymerase III-driven H1 promoter [20].

During the progression of our study, RNA polymerase II-driven shRNA expression plasmids based on the endogenous miR-30 were described [29]. We constructed additional sh_6335, sh_6867 and sh_6965 in miR-30-based LMP retroviral plasmid. All target shRNA sequences were evaluated and are listed in the RNAi Codex database [35]. Because BIOPREDsi as the best potential target predicted the sh_6965, we cloned the sh_6965 to pSUPER.retro.puro plasmid as well to have the same shRNA sequence in both types of plasmids.

Sequences sh_6069, sh_6252, sh_6335 and sh_6965 were also predicted by recently published siExplorer algorithm implementing some new rules [36]. Although, the rules for siRNA/shRNA prediction are far from to be definitive and 100% effective [32, 33], the majority of our shRNAs fulfill recently accepted guidelines.

shRNA are capable to down-regulate BRCA1 expression in reporter system. To test shRNA function, we used luciferase-

based reporter system in transiently transfected murine NIH3T3 cells. We co-transfect pGL4.10-SV40_3UTR reporter plasmid together with shRNA-expressing plasmids and monitored the effect of human BRCA1 3'-UTR on luciferase activity. The luciferase expression was inhibited by all shRNA constructs tested (Fig. 1) in a concentration dependent manner. Inhibition > 90% was observed at concentrations 50 ng and 1.5 μg for pSUPER.retro.puro and LMP plasmid, respectively (data not shown). Observed shRNA-mediated inhibition of luciferase signal was specific for BRCA1 3'-UTR, since luciferase signal from pGL4.10-SV40 reporter plasmid was not affected (data not shown). Irrelevant shRNA constructs targeting mouse CCAAT/Enhancer Binding Protein γ (C/EBP γ) were used as negative controls (Fig. 1). At concentrations higher than 200 ng, luciferase activity was also inhibited by these control shRNAs, but in far less extend (~10%, ~25% and ~40% inhibition at 200 ng, 500 ng and 1.5 μg, respectively; data not shown). This inhibition may be mediated by a non-specific, miRNA-like translation inhibition rather than expected siRNA-like mRNA cleavage mechanism [16].

The performance of shRNAs cloned into pSUPER.retro.puro plasmid was overall better than those cloned into LMP plasmid (Fig. 1). The difference between plasmids was still apparent at 1.5 μg, the highest concentration tested, where LMP plasmid inhibited luciferase activity by ~90% (data not shown). We expect the majority of luciferase activity detected in the assay originate from the beginning of the experiment before the luciferase mRNA is inhibited by RNAi. This means that the action of LMP-derived shRNAs is delayed compared to pSUPER-derived shRNAs. This variation may be caused by different expression levels achieved from Pol III (human H1; [20]) or Pol II (viral LTR; [19]) promoters used in pSUPER.retro.puro and LMP plasmid, respectively. The differences in the efficiency of shRNA-precursors' processing and loading into the RISC complex may play role as well.

We conclude that all shRNAs are proficient in down-regulating reporter luciferase expression in BRCA1 3'-UTR-dependent manner and that the pSUPER.retro.puro plasmid is more potent than the LMP plasmid in this assay system.

Transient shRNA-mediated BRCA1 down-regulation. To verify shRNAs function on the protein level in more physiological settings, we transiently transfected HeLa cells with shRNA constructs and looked at endogenous BRCA1 protein levels using flow cytometry. The BRCA1 protein levels were down-regulated by all shRNAs except sh_6095 (Fig. 2 and data not shown). We also tested co-transfection of two shRNA-expressing plasmids, but the resulting down-regulation was not superior compared to individual shRNAs (data not shown). This is consistent with published observations that the effect of perfectly complementary siRNAs is not additive, whereas miRNAs can function in a combinatorial way [37].

Observed down-regulation was only moderate compared to results obtained in a reporter system. One reason for moderate BRCA1 down-regulation can be low transfection efficiency in HeLa cells. To confirm this, we transfected HeLa cells by pEGFP-C1 plasmid and monitor EGFP expression by flow cytometry. Typically, less than 25% of HeLa cells were EGFP-positive independently of transfection reagent used (data not shown). Low transfection efficiency was also confirmed by the selection of transiently transfected cells by puromycin. Thus, overall moderate BRCA1 protein down-regulation observed in HeLa cells is in part due to low percentage of shRNA-expressing cells.

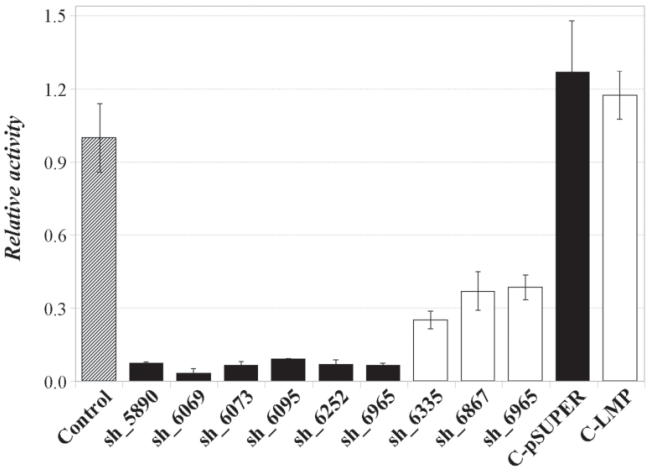

Figure 1. Luciferase Reporter Assay. NIH3T3 cells were transiently transfected with 2 ng of pGL4.10-SV40_3UTR reporter construct either alone or together with 50 ng shRNA-expressing plasmids. Reporter activity was normalized to protein levels and the value for reporter construct alone was set to 100%. Data are plotted as relative activity ± SD (average of 3 independent transfections) and represent the typical experiment. Section lined bar: control (transfection of reporter plasmid alone); black bars: pSUPER.retro.puro plasmid; empty bars: LMP plasmid. C-pSUPER and C-LMP are irrelevant control shRNAs targeting mouse CCAAT/Enhancer Binding Protein γ (C/EBPγ) expressed from pSUPER.retro.puro and LMP plasmid, respectively.

Another possibility for moderate BRCA1 down-regulation may be insufficient shRNAs' expression at the time of analysis, i.e. 48 h post-transfection. This is, however, not probable since shRNAs were working well in the reporter system at the same time point (Fig. 1). Moderate down-regulation can be seen when the target protein has long half-life or exists in specific compartments (e.g. preferential nuclear localization of BRCA1 compared to cytoplasmic action of RNAi). Here, sufficiently long time is necessary for depleting the protein from all stores. However, longer post-transfection intervals were not tested in our assay system because the levels of gene expression induced by transient transfection are decreasing rapidly from 48 h post-transfection as assessed by EGFP expression analysis (data not shown).

We conclude that all shRNAs except the sh_6095 are able to down-regulate endogenous BRCA1 expression in HeLa cells, despite with different potency and that to achieve a complete BRCA1 down-regulation, long-lasting shRNA expression may be needed.

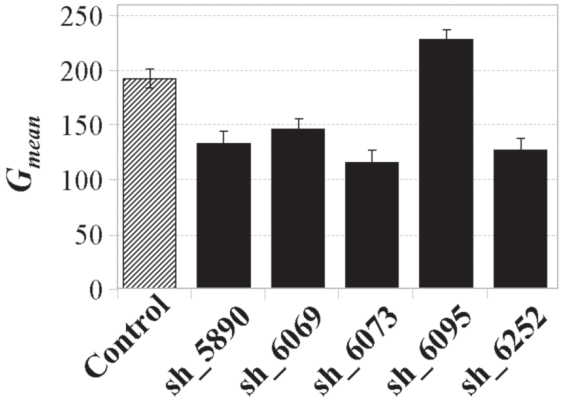

Figure 2. BRCA1 down-regulation in HeLa cells. HeLa cells were transiently transfected with shRNA-expressing pSUPER.retro.puro plasmids. BRCA1 expression was measured by flow cytometry 48 h post transfection. Intensity is expressed as G_{mean} ± CV (Coefficient of variation). Data from a representative experiment are shown.

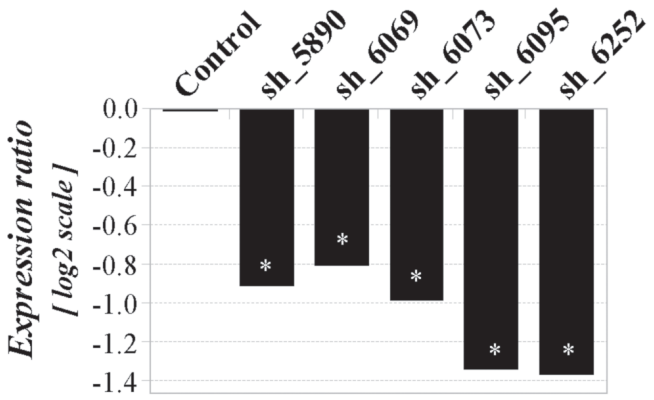

Figure 3. Down-regulation of BRCA1 mRNA levels in MCF-7 cells. Quantitative real-time PCR (qRT-PCR) analysis of BRCA1 mRNA expression in MCF-7 cells. Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD) were used as internal controls. qRT-PCR results were evaluated by REST-2005 software and changes in BRCA1 mRNA expression levels relative to housekeeping genes were calculated and normalized to $\log_2$ scale based on the efficiencies of PCR reactions. BRCA1 relative expression in control MCF-7 cells (treated with empty pSUPER.retro.puro plasmid) is equal to 0. Statistical significance of changes in BRCA1 mRNA levels was calculated by pair wise fixed reallocation randomization test using the REST-2005 software and p values (marked by *) are p=0.001. Data from a typical experiment are presented.

Long-term BRCA1 down-regulation by shRNAs. To establish long-lasting BRCA1 down-regulation in culture cell lines, we advantaged of viral infections, which provide more uniform expression in target cells compared to transient transfections. We infected human breast cancer cell line MCF-7 at a low MOI (Multiple of Infection) to accomplish even more uniform shRNA expression (theoretically at low MOI each cell is infected only by single retroviral particle). After selection, the population of surviving cells was analyzed for changes in BRCA1 mRNA levels by qRT-PCR. The levels of BRCA1 mRNA were significantly down-regulated by all shRNA tested (Fig. 3 and data not shown) by the factor of ~ 2 ($p<0.001$). BRCA1 down-regulation was cell line-specific, since no consistent effect of shRNAs was present in MDA-MB-231 and HeLa cells (data not shown). Interestingly, sh_6095 showed significant BRCA1 mRNA up-regulation in MDA-MB-231 and HeLa cells. This effect was confirmed by qRT-PCR from independently prepared cDNAs. Although, siRNA/shRNAs are supposed to silence homologous sequences, Li et al. observed long-lasting, sequence-specific induction of target genes by siRNAs directed to promoters of E-cadherin, $p21^{WAF1/CIP1}$ and VEGF [38]. Argounate 2 (Ago2) protein and the 5' end of siRNA ("seed" sequence) were critical for observed activation, which is reminiscent of microRNA action [13, 16] and siRNA-mediated transcriptional silencing [39, 40]. Moreover, this effect was siRNA- and cell type-specific similarly as in our case. Recently, Vasudevan and Steitz [41] observed activation of TNFα mRNA translation in serum starved HEK293 and monocytic THP-1 cells mediated by AU-rich sequence in the 3'-UTR of TNFα mRNA. This activation was absolutely dependent on Ago2 and fragile-X-mental-retardation protein 1 (FXR1) proteins [40]. It is not known whether observed Ago2-mediated translation activation was dependent on miRNA(s) or not. However, it can be speculated that such a context-dependent activation effect is more general. We cannot exclude other possibilities such an increase in BRCA1 mRNA stability mediated by the sh_6095. Exact conditions that may be responsible for observed cell line-specific "stimulatory" effect in our system are under investigation.

We conclude that shRNAs delivered to target cells via infection are able to down-regulate endogenous BRCA1 mRNA levels in a cell type-specific manner.

Functional Effect of Long-term BRCA1 down-regulation. We investigated the influence of BRCA1 down-regulation on proliferation of MCF-7 cells. All shRNAs tested significantly reduced the proliferation rate of MCF-7 cells (Fig. 4). This effect was cell line specific, since the proliferation rate was reduced only marginally in HeLa and MDA-MB-231 cells (data not shown). BRCA1 regulates the cell cycle through mediating the effects of checkpoint kinases (ATM, ATR, Chk1, Chk2) and was implicated in the regulation of S, G_1 as well as G_2/M checkpoints [3]. BRCA1 down-regulation may attenuate correct checkpoint function and together with delay in DNA damage repair may slow-down the cell cycle progression. Cell cycle analysis by propidium iodide staining revealed only slight increase of cells in G_0 phase (~ 5%) with no indication of apoptosis in MCF-7 and HeLa cells (data not shown). However, even such small changes in cell cycle may cause differences in proliferation rate over the period of 6 days as we assayed.

What is the basis of a cell line-specific effect? One possibility is that the inhibitory effect of shRNAs on cell

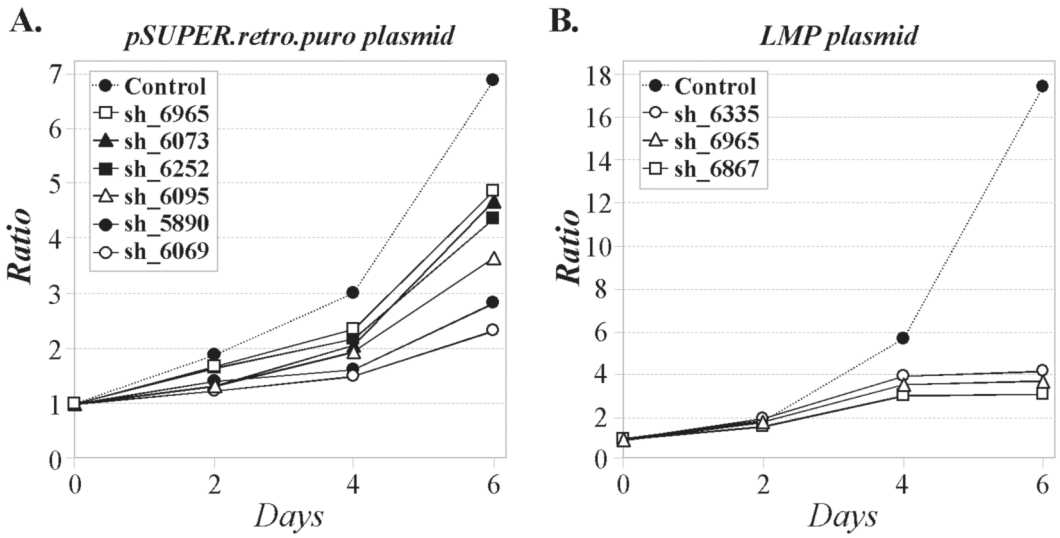

Figure 4. Functional effect of BRCA1 down-regulation on proliferation of MCF-7 cells. MCF-7 cells were infected with control (empty retrovirus) or shRNA-expressing retroviruses, drug selected and used for growth assays. Cell proliferation was monitored over a 6-day period. Each value was normalized to the cell number at day 0. A representative example is shown from at least two independent experiments performed in triplicates. [A] Infection with shRNAs expressed from pSUPER.retro.puro plasmid. Dotted line: control; filled circle: sh_5890; open circle: sh_6069; filled triangle: sh_6073; open triangle: sh_6095; filled square: sh_6252; open square: sh_6965. [B] Infection with shRNAs expressed from LMP plasmid. Dotted line: control; circle: sh_6335; square: sh_6867; triangle: sh_6965.

proliferation corresponds to the magnitude of BRCA1 mRNA down-regulation. MCF-7 cells express relatively high levels of endogenous BRCA1 compared to other cells lines [42] indicating potential important role of BRCA1 in this particular cell line. Expression levels of BRCA1 and its functional importance may prerequisite the final shRNAs' action.

Cell type-specific effect on proliferation rate may be due to intrinsic differences between the cell lines used. MCF-7 and MDA-MB-231 cells are derived from breast adenocarcinomas, whereas HeLa cells are derived from cervical adenocarcinoma. Mutations in BRCA1 are known to predispose to breast and ovarian cancers but having no effect on cervical cancer [11]. Thus, the lack of shRNAs' action in HeLa cells may be related to tissue-specific functions of BRCA1. As opposed to MCF-7 cells, MDA-MB-231 cells lack endogenous estrogen receptor and express non-functional, mutated form of p53 tumor suppressor containing missense G>A mutation in exon 8 (pR280K). The role of hormonal exposure and especially estrogen receptors was anticipated in tissue-specific action of BRCA1 [43]. The tumor suppressor p53 plays a key role in coordinating responses to stress factors including DNA damage [44, 45] where BRCA1 plays an important regulatory role [1]. Thus, inhibition of BRCA1 expression by shRNAs may delay DNA damage repair and this may signal to activate p53 followed by cell cycle arrest or ultimately by apoptosis. Such an effect may be more apparent in cells expressing wt p53 with preserved checkpoints regulation (e.g. MCF-7 cells). It will be interesting to follow up cells for more passages and monitor the accumulation of DNA defects.

Finally, the possibility of negative selection against cells with highly down-regulated BRCA1 expression cannot be ruled out. Such a negative selection may act in cell type-specific and/or p53-specific manner. If negative pressure toward BRCA1 expression is considered, only cells with low BRCA1 down-regulation (and thus mitigate effect on proliferation rate) due to e.g. epigenetic silencing of inserted retrovirus will preferentially survive the selection. To rule out the possibility of negative selection, conditional expression of shRNAs should be implemented [29].

Nevertheless, our shRNAs are able to down-regulate BRCA1 expression. BRCA1 down-regulation has cell type-specific functional consequences *in vitro*. Exact mechanisms involved are under investigation.

Versatile system for BRCA1 functional studies. RNAi is a broadly used method. To avoid misinterpretation of results and to follow up the formulated standards for RNAi [26, 27], rescue experiments by RNAi-resistant variant of gene of interest have to be implemented. Up to date, more than 50 papers were published using siRNAs directed to BRCA1 ORF. For each siRNA/shRNA targeting the ORF region, silent mutation(s) have to be introduced in the siRNA's target sequence, which is laborious and time-consuming process. Designing siRNAs/shRNAs to target the 3'-UTR region circumvents the necessity to construct RNAi-resistant gene for each of these shRNAs. In this case, wild-type form of gene can be used, since transgenes are usually expressed without their UTR sequences. Moreover, siRNAs/shRNAs targeting 3'-UTR will down-regulate all sequence variants, including

alternatively spliced forms, which may not be the case for ORF-targeted shRNAs.

Up to date, only one siRNA targeting 3'-UTR of BRCA1 mRNA was described and used in transient transfection experiments [6, 7]. Our goal was to design shRNAs targeting BRCA1 3'-UTR region, which can be used universally for long-term BRCA1 down-regulation. We designed 8 shRNAs in two different retroviruses and demonstrated that all shRNAs are able to down-regulate BRCA1 expression irrespective of retrovirus used. Observed shRNAs' effects were cell-type specific, similarly as described by others [38].

Approaches combining RNAi with rescue up-regulation were reported recently [46, 47]. The system described by Hölzel et al. [46] is based on co-transfection of two episomal vectors, one expressing shRNA and another one expressing RNAi-resistant variant of target gene. On the other hand, Ma et al. [47] used one plasmid expressing both shRNA and tetracycline-inducible RNAi-resistant variant of target gene. Both systems require transfection of target cells and are thus not suitable for use with hard-to-transfect cells. Moreover, if long-term down-regulation is considered, the laborious and time-consuming process of selection and subsequent permanent clones isolation is necessary.

Retrovirally expressed shRNA, as those described in this paper, ensure stable, long-lasting RNA interference effect suitable for evaluating BRCA1 function even in hard-to-transfect cells, including primary cell cultures isolated from tissues of cancer patients or BRCA1 mutation carriers. However, to create really universal and versatile system devoid of any shRNA-based or negative selection-based non-specific effects, conditional expression of shRNA [23, 29, 48] should be implemented.

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