Identification of *Id4* as a regulator of *BRCA1* expression by using a ribozyme-library-based inverse genomics approach

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Expression of the breast and ovarian cancer susceptibility gene *BRCA1* is down-regulated in sporadic breast and ovarian cancer cases. Therefore, the identification of genes involved in the regulation of *BRCA1* expression might lead to new insights into the pathogenesis and treatment of these tumors. In the present study, an ''inverse genomics'' approach based on a randomized ribozyme gene library was applied to identify cellular genes regulating *BRCA1* expression. A ribozyme gene library with randomized target recognition sequences was introduced into human ovarian cancer-derived cells stably expressing a selectable marker [enhanced green fluorescence protein (EGFP)] under the control of the *BRCA1* promoter. Cells in which *BRCA1* expression was up-regulated by particular ribozymes were selected through their concomitant increase in EGFP expression. The cellular target gene of one ribozyme was identified to be the dominant negative transcriptional regulator *Id4*. Modulation of *Id4* expression resulted in inversely regulated expression of *BRCA1*. In addition, increase in *Id4* expression was associated with the ability of cells to exhibit anchorage-independent growth, demonstrating the biological relevance of this gene. Our data suggest that *Id4* is a crucial gene regulating *BRCA1* expression and might therefore be important for the *BRCA1* regulatory pathway involved in the pathogenesis of sporadic breast and ovarian cancer.

BRCA1 has been linked to the genetic susceptibility of a majority of familial breast and ovarian cancers (1, 2). Its dominant nature as a tumor suppressor gene has been supported by the finding of frequent loss of heterozygosity (LOH) of the wild-type allele in breast tumors from mutation carriers (1, 3, 4). Although mutational loss of function of *BRCA1* does not seem to contribute to sporadic cancers (5–7), *BRCA1* mRNA levels are reduced or undetectable in sporadic breast and ovarian carcinomas and breast cancer cell lines (8–11). CpG methylation of the promoter may contribute to this decreased expression (12, 13), but is unlikely to be the sole mechanism (14). Thus, the regulatory pathway leading to the reduction of *BRCA1* expression in sporadic breast cancer remains unclear. The identification of candidate genes in this pathway can potentially lead to the development of new therapeutic options.

Here, we report a ribozyme-library-based ''inverse genomics'' approach to identify cellular regulators of *BRCA1* expression. Ribozymes are catalytic RNA molecules that bind to defined RNA targets based on sequence complementarity, and enzymatically cleave these RNA targets. Typically, hairpin ribozymes cleave upstream of a GUC triplet in the target sequence, thereby inhibiting the expression of the corresponding gene (15–17). A library of hairpin ribozymes with randomized substrate binding sequences could potentially cleave any RNA substrate containing a GUC. Expression of the ribozyme library in cells and subsequent selection for a given phenotype enables the identification of single ribozymes responsible for this particular phenotype. The target recognition sequence of ribozymes that

reproducibly confer phenotypic changes is used to identify the corresponding target gene(s). We have recently used this approach to identify cellular genes involved in Hepatitis C virus translation mediated by its internal ribosome entry site (18). In the present application, ribozyme-library-transduced cells were selected for altered *BRCA1* promoter-driven reporter gene expression. By using this approach, we identified the dominant negative transcriptional regulator *Id4* as a crucial gene involved in *BRCA1* regulation, and provide further insights into the biological properties of *Id4* protein in the context of *BRCA1* expression.

Materials and Methods

Construction of the Ribozyme Library Vector. The Moloney retroviral genome based vector pLHPM-RzLib was constructed to contain a ribozyme (Rz) expression cassette with eight random nucleotides in helix 1 and four random nucleotides in helix 2 driven by the tRNA^{val} promoter as described (18). To demonstrate the randomness of the Rz library insert, 50 individual bacterial colonies were analyzed by standard sequencing techniques using primer NL6H6 (5′-CTGACTCCATCGAGC-CAGTGTAGAG-3′). Additionally, *in vitro* cleavages of several short RNA transcripts by a comparable ribozyme library confirmed the randomness (data not shown). An unrelated control ribozyme, directed against the negative strand of the 5′UTR of the Hepatitis C Virus (''CNR3'') was cloned into pLHPM and used as a control ribozyme.

Reporter Vector Construction. To construct a *BRCA1* promoter-driven enhanced green fluorescence protein (EGFP) reporter vector, a 2.9-kb fragment was amplified from a pGL2 vector containing a 3.8-kb genomic *BRCA1 Pst*I-fragment (19) using primers BA-5Pst (5′-ATCTTTCTGCAGCTGCTGGCCCGG-3′) and BA-3Age (5′-GTGTAAACCGGTAACGCGAAGAG-CAGATA-3′) and cloned into pEGFP-1 (CLONTECH). Vector pCMV-EGFP, with a CMV promoter driving EGFP gene ex-

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pression, was used as a control vector. The hygromycin reporter vector was constructed by inserting the hygro/HSV-Tk fusion gene from pCMV-HyTk into pcDNA3.1(−) (Invitrogen), generating the control vector pc-HyTk (18). The *BRCA1*-driven HyTk plasmid pBR-HyTK was obtained by replacing the CMV promoter with a 2.9-kb genomic *BRCA1* fragment (*Bgl*II–*Sap* I) containing the promoter region.

Construction and Characterization of Reporter Cell Clones. PA-1 cells were transfected with pBR-EGFP or pCMV-EGFP by using a standard calcium phosphate transfection method. SK-BR-3 cells were transfected with pBR-EGFP, pCMV-EGFP, pc-HyTk, and pBR-HyTk; T47-D cells were transfected with pBR-EGFP and pCMV-EGFP by using Lipofectamine Plus (GIBCO/BRL) according to the manufacturer's instructions. Following selection with G418 (GIBCO/BRL), single colonies were functionally analyzed by flow cytometry analyzing EGFP expression (EGFP reporter cells), or characterization for their resistance against hygromycin B (hygromycin reporter cells). The integrity of the *BRCA1* promoter/EGFP cassette in SK-BR-3 cells transfected with pBR-EGFP was confirmed by PCR amplification. Clones from SK-BR-3 cells transfected with pBR-HyTk were tested for the integrity of the *BRCA1* promoter/HyTk cassette by genomic southern blot.

Viral Vector Production. Retroviral particles were produced by triple transfection of the following plasmids: (*i*) pLHPM-Rz (containing control Rz or individual Rzs) or pLHPM-RzLib (containing the Rz library), (*ii*) gag-pol, and (*iii*) VSV-G (vesicular stomatitis virus G-protein) into CF2 cells. Plasmids gag-pol and VSV-G for retroviral production were a kind gift of Ted Friedman (University of California San Diego). Cells were transfected with *Trans*IT-LT1 (Panvera, Madison, WI). Retroviral particles were collected every 24 h beginning on day two after addition of fresh media continuing until day five. Viral titers of G418-resistant polyclonal cell populations were estimated by using a standard titration assay performed on HT1080 cells.

Retroviral Ribozyme Library Vector or Control Vector Transduction and Fluorescence Activated Cell Sorting (FACS). An individual clone of PA-1-based *BRCA1* promoter driven EGFP reporter cells (P8EGBR3) was used for transduction with the retroviral ribozyme library or control vector. A total of 1.9×10^7 cells were transduced with retroviral ribozyme library vector and 7.4×10^6 cells with control vector at an multiplicity of infection of 1 and subsequently selected for stably transduced cells in 0.3 μg/ml Puromycin (Sigma). Following selection, cells were analyzed for their EGFP expression by flow cytometry and subsequently sorted for the highest 10% (sort 1) or the highest 3% (subsequent sorts) of the population.

Ribozyme Rescue by PCR and Ribozyme Sequence Analysis. Genomic DNA was isolated from enriched (sorted) cells and the ribozyme genes were amplified by PCR and recloned into pLHPM to generate new retroviral particles. Individual colonies were analyzed for their ribozyme sequences.

Soft-Agar Growth. Dishes were prepared with an underlayer of 0.6% Agar (Difco) in Iscove media (GIBCO/BRL) containing 10% FCS (HyClone). Cells were plated at 6×10^4 or 9×10^4 cells per well (9.4 cm²) in Iscove media containing 0.4% Agar and 7.8% FCS and overlaid with an upper layer of 0.6% Agar in Iscove media containing 10% FCS. Cells were refed twice per week with regular growth media containing 10% FCS, which was added onto the upper layer.

Construction of *Id4* Expression Vectors. The *Id4* cDNA sequence was amplified by using primers *ID4*-3 (5′-TCCGAAGGGAGT-GACTAGGACACCC-3′) and *ID4*-7 (5′-TTCTGCTCTTC-CCCCTCCCTCTCTA-3′). The amplified product was cloned with the retroviral expression vector pLPCX (CLONTECH) in sense- or antisense-orientation.

Construction of Target Validation Ribozymes. Hairpin ribozyme cleavage sites were identified within the sequence of the candidate target gene according to target recognition sequence requirements (xxxxN*G*TCxxxxxxx; x representing bases complementary to helices 1 & 2): GCGCN*G*TCCAGGTGTG ["validation" ribozyme (VRz)1], GACCN*G*TCCAGCCGCG (VRz2). Ribozymes were constructed by annealing of overlapping ribozyme specific oligonucleotides (20). Double-stranded DNA was cloned into pLHPM and retroviral particles were produced.

RNA Analysis. Total RNA was extracted from cultures grown to 80% confluency and Northern blot analysis was performed as described elsewhere (18). For quantitative real-time reverse transcription–PCR (RT-PCR) analysis, the TaqMan technology (7700 Sequence Detector, Perkin–Elmer) was applied according to the manufacturer's instructions for one-tube RT-PCR by using the standard curve method. Primers and probes (IDT) were chosen with the help of PRIMER EXPRESS Software (Perkin–Elmer)—sequences are available on request. Probes were labeled with 5′-FAM (reporter) and 3′-TAMRA (quencher). RNAs were quantified photometrically and input amounts were optimized for each amplicon, resulting in threshold values between 20 and 30 cycles. Each sample was analyzed in at least three independent assays with duplicate samples. Means of the gene of interest were normalized to *GAPDH* levels. Numbers are given as mean ± SEM relative to a control sample.

Hormone Treatments. For hormone treatments, MCF-7 cells were cultured in phenol red-free MEM (GIBCO/BRL) depleted of serum for 36 h before treatment. For hormone stimulation, cells were treated with 10 nM 17-β estradiol or 10 nM progesterone (ICN Biomedicals) for 24 h and subjected to RNA analysis. Cells grown in complete media containing 10% FCS were used as controls.

Results

Generation of a Ribozyme-Library-Based Functional Selection System. The hairpin ribozyme requires a GUC triplet within the substrate RNA and cleaves 5′ of the G residue as indicated (Fig. 1*A*). By randomizing target binding domains that flank the GUC triplet (Fig. 1*B*, eight nucleotides in helix 1, four nucleotides in helix 2 of the hairpin ribozyme), we generated a library of 1.7×10^7 ($= 4^{12}$) different hairpin ribozyme molecules. The library is expressed from a retroviral vector, allowing for stable introduction into reporter cells via transduction. A reporter plasmid with the *BRCA1* promoter driving the expression of enhanced green fluorescence protein (EGFP) was constructed (see Figs. 6–8, which are published as supplemental data on the PNAS web site, www.pnas.org). Various reporter cell lines were generated by transfection of breast (SK-BR-3, T47-D) and ovarian (PA-1) cancer cell lines. Clonal cell populations derived from the transfected cells were analyzed for EGFP expression. The levels of EGFP expression correlated with the levels of endogenous *BRCA1* RNA (Fig. 1*C*), supporting the hypothesis that *BRCA1* expression is controlled by its transcriptional promoter. To functionally select for cells with altered *BRCA1* expression, an individual reporter clone based on PA-1 cells (P8EGBR3) with an intermediate expression level of EGFP and endogenous *BRCA1* was selected. P8EGBR3 reporter cells were stably transduced with retroviral vectors expressing a puromycin-
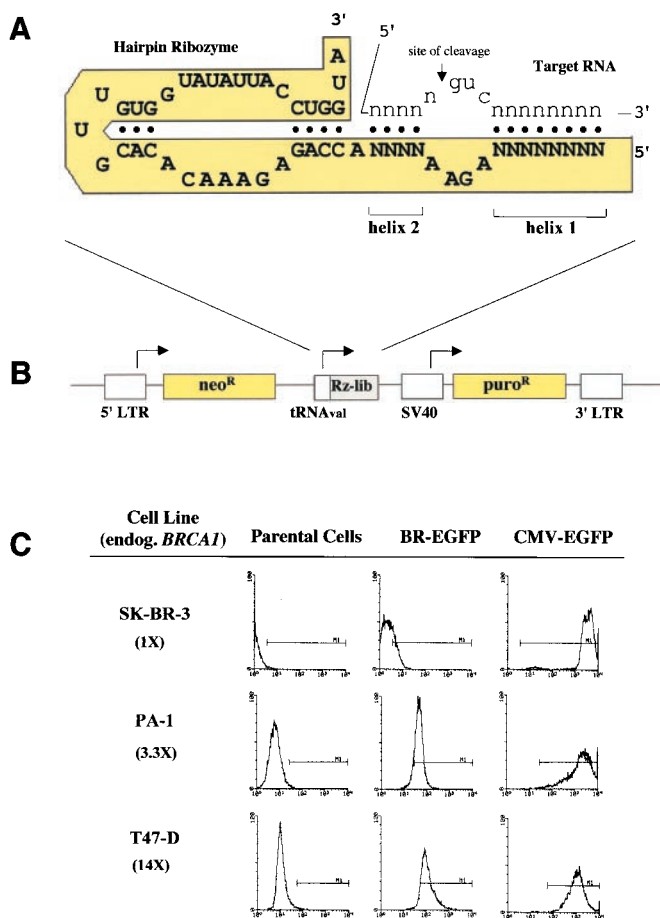
Fig. 1. The hairpin ribozyme library and gene vector. (*A*) Sequence and secondary structure of the hairpin ribozyme (capital letters), bound to its target RNA (lowercase letters). Both target-binding arms (helices 1 & 2) are randomized, generating a library with as many as 4^{12} different catalytic molecules (ribozymes). (*B*) Schematic representation of the retroviral vector following insertion of the ribozyme library DNA template. Ribozyme expression is driven by the tRNA^{val} promoter. (*C*) Flow cytometry for EGFP of SK-BR-3, PA-1, and T47-D cells. Numbers in parentheses indicate the relative expression levels of endogenous *BRCA1*. Analysis of untransfected control cells (parental cells) and dilution clones from cells, transfected with pBR-EGFP (BR-EGFP) or with pCMV-EGFP (CMV-EGFP).

resistance marker and a random ribozyme library and used for selection.

Selection of Ribozyme-Expressing Cells with Increased *BRCA1* Expression. Following puromycin selection, cells transduced with the ribozyme vector library or a control vector (as well as untransduced cells) were subjected to several rounds of FACS. Generally, to enrich for populations with increased EGFP expression, the highest three to ten percent of cells were sorted. Starting with the third sort, increasing numbers of cells with higher EGFP expression were detected in ribozyme library-transduced cells (Fig. 2*A*), but not in sorted populations of untransduced or control vector transduced cells. After five sorts, the majority of the sorted populations derived from library-transduced cells shifted toward a higher EGFP expression level compared with the controls and a greater than nine-fold increase in mean fluorescence intensity was observed (Fig. 2*A*). RNA analysis of the ribozyme-library-transduced cells confirmed the increase of *EGFP* RNA as well as endogenous *BRCA1* mRNA compared with the controls (Fig. 2*B*). These results demonstrate that the
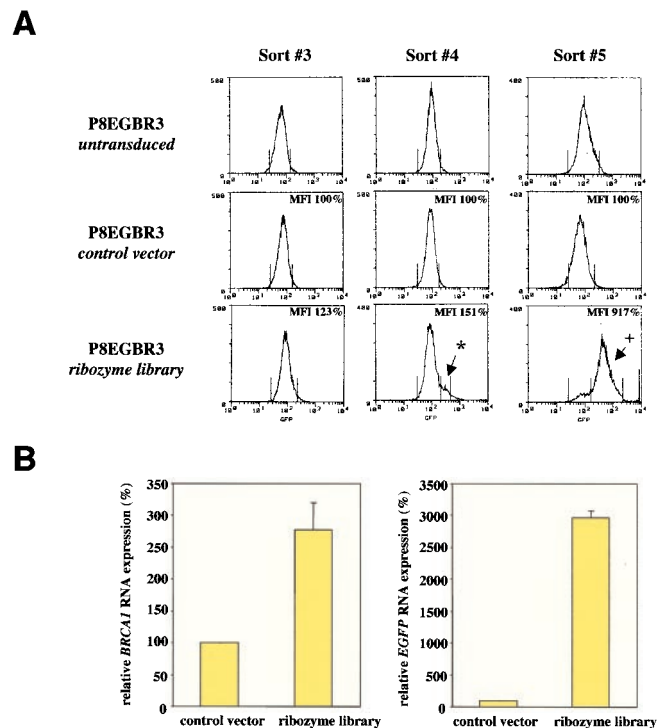

Fig. 2. Selection of ribozymes that up-regulate the BRCA1 promoter. (*A*) FACS-selection of P8EGBR3 cells (untransduced, control vector, or ribozyme-library-transduced) for cells with an increase in EGFP expression. Rz library-transduced cells show a peak on sort #4 (*) and a shift of the majority of the population on sort #5 (+), whereas control vector or untransduced cells remain unchanged throughout the experiment. MFI, mean fluorescence intensity of cell population, given in percent of control vector transduced cells. (*B*) Quantitative real-time RT-PCR analysis of *EGFP* and endogenous *BRCA1* RNA normalized to *GAPDH* RNA after sort #5 in cells transduced with control vector or ribozyme library. Numbers are given as relative RNA levels in percent compared with control vector transduced cells (mean ± SEM).

selected ribozymes conferred an increase of *BRCA1* promoter-mediated gene expression, resulting in elevated levels of both *EGFP* and endogenous *BRCA1*. PCR amplification and sequence analysis of the ribozyme genes from the shifted populations revealed the predominance of several candidate ribozymes (data not shown). The pool of enriched candidate ribozyme vectors was reintroduced into new P8EGBR3 reporter cells for further rounds of FACS-based selection and ribozyme rescue.

Identification of a Single Ribozyme to Increase *BRCA1* Expression. After several rounds of selection, a single predominant ribozyme (named Rst-RH5) was found in the population of shifted cells. Again, RNA analysis demonstrated an increase in *EGFP* and *BRCA1* RNA, confirming the involvement of this particular ribozyme in the regulation of the *BRCA1* promoter (Fig. 3*A*). Interestingly, the sorted cells also exhibited morphological changes toward a flatter, larger phenotype with a larger cytoplasm resembling normal epithelium. This was not observed with sorted cells derived from control vector transduction (Fig. 3*B*).

Additionally, anchorage-independent growth was markedly reduced in cells expressing Rst-RH5 in comparison with control vector transduced cells (Table 1), demonstrating suppression of an *in vitro* indicator of tumorigenicity.

Identification and Confirmation of *Id4* as a *BRCA1*-Regulating Gene. By using the 16 nucleotides of sequence information derived from the Rst-RH5 binding sequences and the cleavage site (8 nt
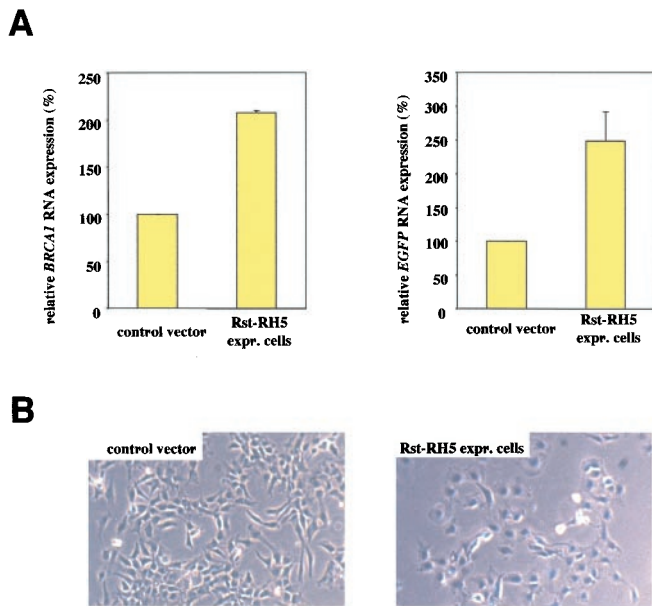
Fig. 3. BRCA1 expression and morphology of selected cells expressing Rst-RH5. Cells transduced with an enriched ribozyme library were subjected to several rounds of selection for high EGFP expression and ribozyme rescue resulting in a population expressing Rst-RH5 as the sole predominant ribozyme. These cells were examined for *BRCA1* expression and cell morphology. (*A*) Quantitative real-time RT-PCR analysis of *EGFP* and endogenous *BRCA1* RNA normalized to *GAPDH* in control vector-transduced or Rst-RH5 expressing cells. Numbers are given as relative RNA levels in percent compared with control vector transduced cells (mean ± SEM). (*B*) Morphology in monolayer culture of Rst-RH5 expressing cells compared with control vector transduced cells.

in helix 1, 4 nt in helix 2 and NGUC) to search various databases (e.g., National Center for Biotechnology Information nonredundant and EST databases), we failed to identify perfectly matching candidate genes. However, under less stringent search conditions, we identified *Id4* as a potential target. The two mismatches in helix 1 between Rst-RH5 and *Id4* mRNA (Fig. 4*A*) are expected to have a minimal effect on ribozyme binding or cleavage activity (21). In fact, *in vitro* assays confirmed cleavage activity of Rst-RH5 on synthetic *Id4* substrate RNA (data not shown). Id4 belongs to a group of four related helix–loop–helix (HLH) proteins, which are dominant negative regulators of transcription (22, 23). The mRNA sequences to *Id1*, *-2*, or *-3* have no similarities with Rst-RH5. Northern blotting analysis of parental P8EGBR3 and ribozyme-library- or control vector transduced cells after five rounds of FACS-sorting showed three *Id4* RNA transcripts (Fig. 4*B*) apparently generated by alternative usage of polyadenylation sites (24, 25). Quantification by phosphoimager revealed a 40% reduction in the major *Id4* transcript (1.7 kb, indicated by an arrow in Fig. 4*B*) normalized to *GAPDH* RNA in library-transduced cells compared with

Table 1. Anchorage-independent growth of Rst-RH5-expressing cells

Population	Number of colonies* at plating density	
	6×10^4 cells per well	9×10^4 cells per well
Control Rz	23.2, 18.7	28, 28.7
Rst-RH5	0, 0	0, 3.7

* Numbers are means of independent assays (performed in triplicate). Values are given for two experiments.
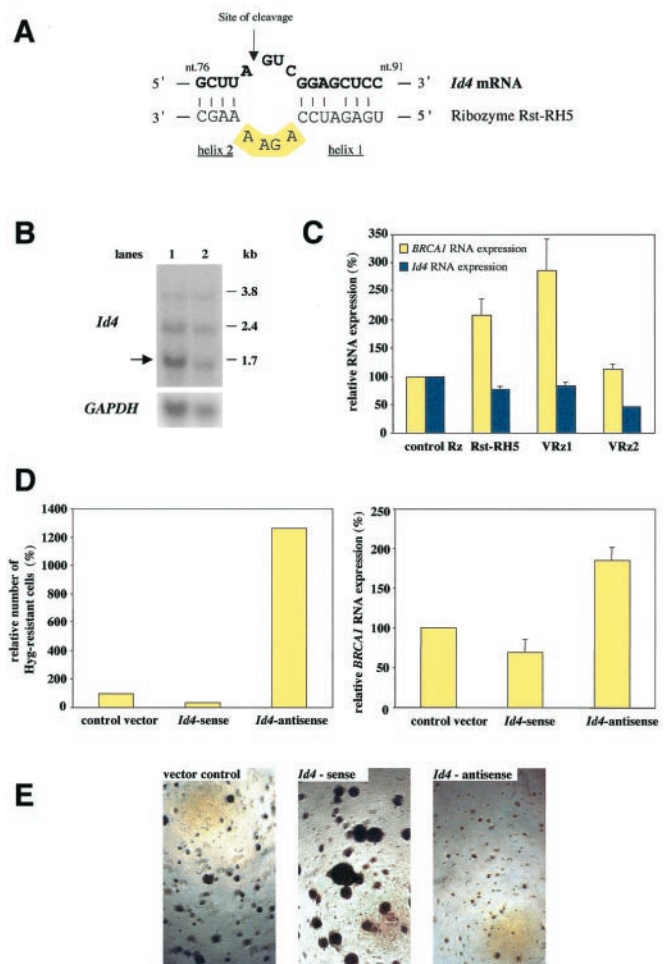
Fig. 4. Identification and validation of *Id4* as a negative regulator of the *BRCA1* promoter. (*A*) Schematic illustration of the predicted binding of Rst-RH5 to *Id4* mRNA (numbering of *Id4* nucleotides according to GenBank accession no. NM 001546). (*B*) Northern analysis for *Id4* RNA in ribozyme-library- (lane 2) or control vector (lane 1)-transduced P8EGBR3 cells after sort #5 (compare with Fig. 2). Different messages result from alternative polyadenylation. The major message is indicated (arrow). (*C*) Effects of Rst-RH5 and two validation ribozymes (VRz1 or VRz2) on endogenous *BRCA1* and *Id4* expression measured by quantitative real-time RT-PCR. Expression of *BRCA1* or *Id4* mRNA was normalized to *GAPDH* mRNA. Numbers are given as relative RNA levels in percent compared with the control ribozyme (mean ± SEM). (*D*) The Hygromycin B resistance assay. Quantitative analysis of cell survival and of endogenous *BRCA1* expression (measured by quantitative real-time RT-PCR and normalized to GAPDH message) in control vector-, *Id4*-sense-, or *Id4*-antisense-transduced cells after phenotypic selection of Hygromycin B reporter cells with Hygromycin B. Numbers are given in percent compared with control vector (mean ± SEM). (*E*) Anchorage-independent growth of P8EGBR3 cells transduced with control vector, *Id4*-sense, or *Id4*-antisense expression vectors.

the controls. Comparable reduction in endogenous *Id4* mRNA was also found in single cell clones of library-transduced cells expressing Rz Rst-RH5 (data not shown).

To confirm *Id4* as a target gene of Rst-RH5, we further examined the effects of VRz directed against additional sites within the *Id4* mRNA, located either within the coding region (VRz2) or the 3′UTR (VRz1) of *Id4*. Again, these potential ribozyme cleavage sites are not conserved within the RNA transcripts of the other *Id* genes. P8EGBR3 reporter cells were stably transduced with viral vectors containing VRz1, VRz2, Rst-RH5 or a control ribozyme and cellular RNAs were ana-

lyzed for endogenous *BRCA1* expression. A significant increase in *BRCA1* expression relative to control ribozyme-transduced cells was observed in cells expressing either Rst-RH5 or *Id4*-specific ribozymes (Fig. 4*C*). Furthermore, expression of *Id4* RNA in cells transduced with these ribozyme vectors was inversely correlated to levels of *BRCA1* expression (Fig. 4*C*).

BRCA1 regulation by *Id4* was also confirmed in a second independent reporter system, with SK-BR-3 based reporter cells expressing the hygromycin B phosphotransferase gene under control of the *BRCA1* promoter. Following stable transduction with *Id4*-sense and -antisense expression vectors or a control vector, cells were subjected to hygromycin B selection. SK-BR-3 cells constitutively express very low levels of *BRCA1*, suggesting poor activity of the *BRCA1* promoter in these cells. Consistent with this, these reporter cells are sensitive to increasing dosages of hygromycin B. Cells transfected with an *Id4*-antisense vector preferentially survived under hygromycin B selection, compared with *Id4*-sense vector or control vector transduced cells (Fig. 4*D*), suggesting that in this reporter system, suppression of *Id4* significantly increased *BRCA1*-driven hygromycin B phosphotransferase gene expression. Additionally, hygromycin B-selected *Id4*-antisense reporter cells showed a more than two-fold increase in endogenous *BRCA1* expression compared with *Id4*-sense expressing cells (Fig. 4*D*).

To further investigate the biological relevance of this regulation, we examined the effects of *Id4*-sense and -antisense overexpression on anchorage-independent growth in P8EGBR3 cells. Previous findings suggested that decreased *BRCA1* expression is associated with increased ability of cells to grow in soft agar and vice versa (9, 26–28). Therefore, *Id4*-mediated modulation of *BRCA1* expression should result in a corresponding phenotypic change. As expected, cells expressing sense-*Id4* demonstrated a significant increase in anchorage-independent growth in comparison with antisense-*Id4* expressing cells (Fig. 4*E*). Transduction with viral vectors expressing anti-*Id4* ribozymes also significantly decreased colony formation in soft agar (data not shown).

Estrogen-Dependent Regulation of *BRCA1* and *Id4* Expression. Sex hormones are known to stimulate cells through specific receptors. Furthermore, they have been shown to regulate *BRCA1* expression in cell culture (29). MCF-7 breast cancer cells contain detectable levels of the estrogen receptor, but not the progesterone receptor. Consequently, expression of *BRCA1* in these cells is responsive to estrogen, but not to progesterone. As expected, we observed an increase in *BRCA1* expression in cells treated with 17-β estradiol, but not in cells treated with progesterone (Fig. 5). In contrast, *Id4* RNA expression was reduced more than two-fold following estrogen stimulation, but did not change in progesterone-stimulated cells. The ratio of *BRCA1* RNA to *Id4* RNA increased seven-fold by stimulation with estrogen. Treatment of cells with FCS had an intermediate effect.

Discussion

BRCA1 expression has been shown to be regulated by several regions within the putative *BRCA1* promoter (30–32), and by different cellular factors, including the Rb-E2F pathway (33), the Brn-3b POU family transcription factor (34), and the GA-binding protein α/β (35). In this study, we have used a ribozyme-based selection system to target and identify a *BRCA1*-regulating gene via a *BRCA1* promoter-driven reporter system. This approach allows us to functionally select ribozymes that target key cellular factors involved in a pathway that affects a particular phenotype. Therefore, in this system, we expect to identify not only genes directly regulating the *BRCA1* promoter, but also any biologically relevant upstream regulators.

By using this approach, we were able to identify and analyze individual candidate ribozymes with the help of a FACS-based reporter system. We were particularly interested in a ribozyme,
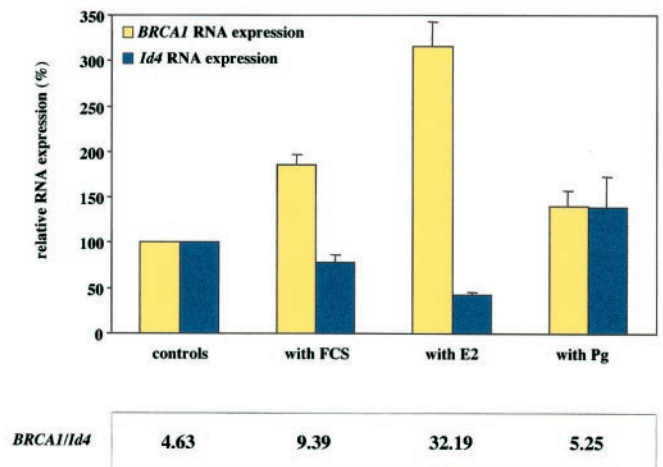
Fig. 5. Hormone stimulation of MCF-7 cells. Cells were grown in phenol-red free media without serum for 36 h before selective stimulation with 10 nM β-estradiol (E2) or 10 nM progesterone (Pg), or no stimulation (controls). Cells grown in media containing 10% FCS were used as controls (with FCS). Following stimulation, cellular RNAs were harvested and analyzed for endogenous *BRCA1* and *Id4* expression levels by using quantitative real-time RT-PCR. Numbers are given as percent (mean ± SEM) in comparison to cells grown without serum.

Rst-RH5, which not only conferred an increase in expression of both *BRCA1* promoter driven reporter gene and endogenous *BRCA1*, but also substantially changed the biological properties of the transduced cells. By using sequence information from the target-binding arms of Rst-RH5, we identified the corresponding target gene to be the HLH transcriptional regulator *Id4*.

Id proteins are dominant negative inhibitors of DNA binding. They contain functional HLH dimerization motifs, but lack the DNA-binding basic region found in the basic HLH (bHLH) proteins, thereby inhibiting DNA binding of bHLH transcription factors (22, 23, 36). Furthermore, for some Id proteins, interactions with non-HLH proteins have been described, such as retinoblastoma protein (37), MIDA1 (38), and Ets-domain transcription factors (39). Interestingly, the *BRCA1* promoter region contains several E-box elements, which are DNA recognition sequences of bHLH proteins (40, 41). In addition, the minimal *BRCA1* promoter region contains a potential binding site for ETS protein transcription factors (30) and recent data suggest that the ETS protein GABPα/β contributes to transcriptional activation of *BRCA1* (35). We have identified *Id4* as a gene functionally important for *BRCA1* regulation. However, *Id4* might be involved at any point along the upstream regulatory pathway of *BRCA1* regulation. Although bHLH proteins binding to the E-box elements of the *BRCA1* promoter or GABPα/β proteins could resemble potential dimerization partners of Id4, the precise function of *Id4* within the *BRCA1* regulatory pathway remains to be defined.

Our experiments with *Id4*-sense and -antisense expression vectors as well as ribozymes directed against additional cleavage sites within the *Id4* mRNA confirmed the significance of *Id4*-mediated regulation of *BRCA1* expression via its promoter: *Id4* modulated *BRCA1* promoter-driven reporter gene expression as well as endogenous *BRCA1* expression. In addition, our findings demonstrate that overexpression of *Id4* in target cells causes an increase in their *in vitro* tumorigenic potential as shown by anchorage-independent growth, whereas a "knock-down" of the gene by full-length antisense expression or by *Id4*-specific ribozymes results in the reverse phenotype. Although the reduction of *Id4* mRNA by *Id4*-targeting ribozymes was modest (20–40%), it is possible that cells expressing drastically reduced *Id4* mRNA (and greatly increased levels of *BRCA1*) may be

selected against, because *BRCA1* is known to be associated with decreased cell proliferation and apoptosis (26, 42). Because the ribozyme library approach is based on selection of cells with a particular altered phenotype rather than the degree of target gene knock-down, it allows us to identify cellular factors that exhibit critical thresholds for different biological functions. Previous studies using this approach showed that similar levels of target gene knock-down led to significant biological effects, namely, inhibition of HCV protein translation and increased anchorage independent cell growth respectively (18, 43).

Our data support previous observations that Id proteins function as positive regulators of cell growth and negative regulators of cell differentiation in various tissues (44–48), including mammary epithelial cells (49–51). In the latter studies, Id1 protein suppressed mammary epithelial cell differentiation and its expression correlated with an aggressive phenotype (51). Moreover, *Id* expression can be modulated by hormones (51) and Id proteins themselves can influence hormone-induced promoter activity (52). Interestingly, previous studies have also demonstrated *BRCA1* expression to be responsive to hormone stimulation (29, 53). Our observation that *Id4* and *BRCA1*

mRNA levels were inversely correlated in hormone treated MCF-7 cells suggests that Id4 may be involved in hormone-dependent regulation of *BRCA1* expression.

In summary, we have developed a ribozyme-mediated functional approach to identify genes regulating *BRCA1* expression. By using this approach we were able to identify ribozymes that conferred a phenotype characterized by an increase in *BRCA1* expression. The corresponding target gene of one particular ribozyme was identified to be the dominant negative transcriptional regulator *Id4*. Further characterization of *Id4* function demonstrated its role in regulating *BRCA1* expression in the context of breast and ovarian cancer. Work is in progress to identify the gene targets of other selected ribozymes, which may reveal additional genes in the *BRCA1* regulatory pathway. The identification of cellular genes involved in *BRCA1* regulation might facilitate the development of new therapeutic options for sporadic breast and ovarian cancer.

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