

Oncogenic BARD1 Isoforms Expressed in Gynecological Cancers

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Abstract

BARD1 is required for protein stability and tumor suppressor functions of BRCA1, which depend on the ubiquitin ligase activity of the BRCA1-BARD1 heterodimer. The NH₂-terminal RING domains of both proteins act as interaction modules and form a ubiquitin ligase, which has functions in DNA repair, cell cycle checkpoint regulation, and mitosis. Interestingly, up-regulated expression of truncated BARD1 isoforms was found to be associated with poor prognosis in breast and ovarian cancers and, in a hormonally regulated fashion, in the human cytotrophoblast, a cell type with properties reminiscent of cancer cells. We therefore performed reverse transcription-PCR to determine the structure of BARD1 isoforms in cell lines derived from hormone-dependent and hormone-independent cancers. We found a specific combination of isoforms, generated by differential splicing and alternative transcription initiation, mostly lacking the BRCA1 interaction domain, in gynecologic but not hematologic cancer cell lines. To investigate the prevalence of BARD1 isoforms in tumors, we applied immunohistochemistry to ovarian cancers, using antibodies distinguishing full-length BARD1 and isoforms. Expression of NH₂ terminally truncated BARD1 was correlated with advanced stage of cancer, and expression of spliced isoforms was typical for clear cell carcinoma, the ovarian cancer with worst prognosis, suggesting a role of BARD1 isoforms in cancer progression. To challenge this hypothesis, we silenced BARD1 isoforms in ovarian cancer cells that lacked wild-type BARD1 by siRNA interference, which led to a complete proliferation arrest. Thus, BARD1 isoform expression is required for cancer cell proliferation, which is compatible with the notion that BARD1 isoforms act as cancer maintenance genes. [Cancer Res 2007;67(24):11876–85]

Introduction

BARD1 was discovered as a protein interacting with BRCA1 (1). BARD1 and BRCA1 form a stable heterodimer, which has functions in DNA repair, transcription regulation, RNA processing, and cell cycle regulation through the ubiquitin ligase function of the heterodimer (2). Depletion of BARD1 *in vitro* leads to genomic instability, loss of polarity, development of a premalignant phenotype

(3), and embryonic lethality in knockout mice (4). As a tumor suppressor, BARD1 has also BRCA1-independent functions in mediating p53-dependent apoptosis, which is inhibited by BRCA1 (5). It binds to p53, facilitating its phosphorylation and stabilization (6).

Recently, a novel function of BARD1 in mitotic spindle assembly was described (7). The expression of BARD1 in most proliferative tissues, particularly in spleen and testis (3, 8), is consistent with its role in mitosis. Up-regulated expression of BARD1 was also observed in response to hypoxia, to genotoxic stress (5, 9), and to hormone signaling (10), suggesting that up-regulation of BARD1 might be associated with tumor suppressor pathways.

In spite of this, BARD1 mutations in tumors are infrequent. Three missense mutations, Q564H, V695L, and S761N, and five other alterations were discovered in breast, ovarian, and endometrial cancers (11). Loss-of-heterozygosity was found for Q564H and S671N mutations, substantiating the role of BARD1 as a tumor suppressor (11, 12). The V695L and S761N mutations were found in somatic breast tissue, whereas the Q564H mutation arose in the germline of a patient with clear cell ovarian carcinoma. At least six other alternations were subsequently identified (13–15), including the missense mutation C557S, which was linked to loss of p53 stability and apoptotic activity (16).

BARD1 δ , an isoform presenting a deletion of exons 2 through 6, was found in a rat ovarian cancer cell line, which is resistant to apoptosis (6), and in HeLa cells (17). BARD1 δ lacks the RING finger domain, required for BRCA1-interaction, and the ankyrin repeats, which are involved in apoptosis and p53 binding (6, 9). Aberrantly elevated expression and cytoplasmic localization of truncated forms of BARD1 was found in breast and ovarian cancer and correlated with factors of poor prognosis (18). Interestingly, spliced isoforms of BARD1 are expressed, temporally and spatially controlled, in human cytotrophoblasts, which have cancer-like properties in the first trimester of pregnancy (19).

Alternative splicing is a crucial mechanism for generating protein diversity and even proteins with novel antagonistic biological functions. Several cancer-associated alterations of splicing patterns have been reported for genes such as *RON*, *RAC1*, *CD44*, and *MDM2* (20). Because differential splicing of tumor suppressor genes, such as BARD1, could have a profound effect on tumor development and progression, we characterized the expression pattern of BARD1 isoforms in ovarian, breast, endometrial, and cervical cancer cell lines, and we investigated their potential oncogenic properties in ovarian cancer.

Materials and Methods

Cancer cell lines. Cancer cell lines were a collection of the Universitätsspital Wien.

Ovarian cancer cell lines, composed of established ovarian cancer cell lines and newly generated ones from individual tumors (O1–O32), were as follows: A2780, Caov-3, ES-2, NIH:OVCAr-3, SK-OV-3, TOV-21G, TOV-112D,

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

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OV-90, OV-MZ-1a, OV-MZ-1c, OV-MZ-2, OV-MZ-2a, OV-MZ-5, OV-MZ-6, OV-MZ-8, OV-MZ-9, OV-MZ-10, OV-MZ-12, OV-MZ-12b, OV-MZ-17b, OV-MZ-18, OV-MZ-20, OV-MZ-21, OV-MZ-22, OV-MZ-26, OV-MZ-27, OV-MZ-30, OV-MZ-32, OV-MZ-33, OV-MZ-35, OV-MZ-37, and OV-MZ-38.

Breast cancer cell lines, composed of established cell lines and newly generated ones from individual tumors (B1–B26), were as follows: MCF-7, MM231, T47D, Hs578T, SKBR3, MM435s, ZR-75-1, BT549, MM453, BT474, PA1, A2780ADR, BT20, HBL100, HMEC, MCF12A, MCF10A, MCF7/6, MCF12F, MM134VI, MM157, MM175VII, MM330, MM468, UCAA812, and MM361.

Endometrial cancer cell lines (E1–E9) were as follows: KLE, RL95-2, AN3 CA, HEC-1-B, Ishikawa, Colo 684, HEC-50, EN, and EJ.

Cervical cancer cell lines (C1–C9) were as follows: HeLa, SW756, GH354, Ca Ski, C-4 I, C-33 A, HT-3, ME-180, and SiHa.

RNA isolation and reverse transcription-PCR. Total RNA from cell lines and tissue specimens were extracted after standard protocols. For reverse transcription, 0.5 µg of RNA were used in standard assays using M-MLV-Powerscript reverse transcriptase. PCR reactions were performed with 2 µL cDNA as template for 30 cycles with primers as indicated (Supplementary Data Table S1) and *Taq DNA polymerase* in a final volume of 50 µL. Denaturation (94°C; 3 min) and final extension (72°C; 10 min) were the same for all PCR reactions, but annealing temperature and extension times varied according to different primers. PCR products (15 µL) were analyzed on 1% agarose/Tris-acetate-EDTA gels.

Determination of BARD1 cDNA 5' ends in ovarian cancer and HeLa cells. GeneRacerTM kit (Invitrogen) was used to amplify cDNA 5' ends of RNA from ovarian tumor and HeLa cells. Total RNA of 4.5 µg was used per sample, and experiments were performed according to the instructions of the supplier. 5' nested PCR was performed with 5' nested primer 5'-GGA-CACTGACATGGACTGAAGGAGTA-3' and reverse primer in exon 6 (5'-TT-TTGATACCCGGTGGTGT-3'). The PCR bands of 5' nested PCR were loaded on 1% low-melting gel, cut, and purified with the QIAEX II kit (Qiagen) followed by sequencing using 5' nested primer and reverse primer.

Western blots. BARD1 antibodies H300, directed against NH₂-terminal 300 aa (sc-7372; Santa Cruz Biotechnology), were used to detect NH₂-terminal epitopes. BARD1 JH3 (10) was used to detect epitopes encoded in exon 7. Anti-BARD1 BL518, directed against epitopes within exon 4 (Bethyl Laboratories), was used for detection of FL BARD1 and β, as described previously (21). Protein extracts from different ovarian cancer cells lines were prepared, and 40 µg of protein per lane were analyzed on 10% SDS-PAGE and blotted onto polyvinylidene difluoride filters. Membranes were blocked with 5% milk powder in TBS. Antibody incubation with purified anti-BARD1 H300 and JH3 was in a 1:500 dilution and in 1:1,000 dilution for BL518. Secondary anti-rabbit peroxidase-coupled antibodies were applied in a 1:10,000 dilution. Signal detection was performed with enhanced chemiluminescence (ECL) and/or high sensitivity ECL kit (Amersham).

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue micro arrays were deparaffinized with xylene for 48 h and rehydrated through descending ethanol (100%, 95%, 70%, and H₂O). The sections were boiled for 5 min in microwave for antigen retrieval. Slides were incubated for 24 h at 4°C in a humidifying chamber with first antibody after bovine serum albumin blocking of nonspecific epitopes. The primary antibodies for BARD1 detection were N19 (sc-7373; Santa Cruz Biotechnology), WFS, described previously (3), and C-20 (sc-7372; Santa Cruz Biotechnology), which recognize epitopes at NH₂ terminus, in exon 4, and at COOH terminus of BARD1, respectively. Secondary antibodies (goat anti-rabbit or rabbit anti-goat) conjugated with horse radish peroxidase were applied in 1:100 dilutions at room temperature for 1 h. Then 3,3'-diaminobenzidine staining was permitted for 15 min at room temperature. Slides were counterstained with hematoxylin before dehydration and mounting. Sections from human lung cancers, described previously (18), were used for positive and negative control.

To quantify BARD1 expression, staining was scored for intensity and percentage of stained cells. The value of staining intensity and percentage of positive cells was used to obtain final staining score. Statistical significance of comparisons was determined by applying Student's *t* test.

Clinical data. The pathologic diagnoses were made by experienced pathologists and staged according to the WHO and American Joint

Committee on Cancer classifications. A total of 106 cases of ovarian cancer from women of ages 32 to 87 years were analyzed, comprising 60 cases of serous carcinoma, 24 cases of endometrioid carcinoma, 16 cases of mucinous carcinoma, and 6 cases of clear cell carcinoma. According to the tumor-node-metastasis staging system, there were 38 cases in T₁, 15 cases in T₂, 53 cases in T₃, 36 cases in N₀, and 63 cases in N₁ stage. There were 25, 26, and 55 cases of pathological grade 1, 2, and 3, respectively.

Cloning of and transduction with lentiviral vectors. Lentiviral vector pLVTHM (22) was used to constitutively express the BARD1 coding region under the control of cytomegalovirus promoter.

The BARD1 siRNA construct was generated by annealing of complementary oligonucleotides and inserting into the pSuperScript vector as a BglII/HindIII fragment. Primers for si78 of human and mouse BARD1 were siRNA-F GATCCCGGTGATGCTTGGGATTCTCTTCAAGAGAGAGAATCC-CAAGCATACACTTTTTGGAAA and siRNA-R AGCTTTTCCAAAAGTG-TATGCTTGGGATTCTCTCTCTGAAGAGAATCCCAAGCATACACGGG. The pSuper fragment ClaI/EcoRI containing the BARD1 siRNA-78 were then subcloned in the lentiviral vector pLVTHM as described (22).⁴ Coexpressed tTR-KRAB locks onto the tet-on promoter. Upon doxycyclin exposure, KRAB is derepressed and allows transcription of BARD1 siRNA. The human BARD1 siRNA-34 was identical to the previously described small hairpin RNA (23).

These lentiviral vectors were then transfected into HEK293T cells with the corresponding packaging pCMV-dR8.92, pCMV-dR8.93, and envelope pMD2G plasmids. The recombinant lentiviruses were produced and purified according to standard protocols (24). HeLa-FL cells were generated by transduction with constitutive FL BARD1 for BARD1 expression control on Western blots.

SK-OV-3 and NuTu-19 cells were plated on 6-well plates (10⁴ cells per well) for 24 h and then incubated with medium containing recombinant lentiviral vectors for 48 h. To control the expression of the siRNA, cells were cotransduced with lentivirus derived from the vectors pLVTHM and pLV-tTRKRAB (22).

Results

Structure of BARD1 isoforms in cancer cells. It was previously reported that HeLa cells express BARD1 δ, an isoform derived from differential splicing (17). We compared the expression of BARD1 and its isoforms in normal human fibroblasts and in HeLa cells by applying reverse transcription-PCR (RT-PCR) with primers intended to amplify the entire *BARD1* coding region. Although full-length (FL) BARD1 was highly expressed in normal fibroblasts, in HeLa cells, smaller isoforms of BARD1 were expressed in addition to FL BARD1. We cloned and sequenced these isoforms and determined their structure and exon composition, and calculated molecular weight (*MW*; Fig. 1A and B). FL BARD1 translates into a protein of 777 amino acids (aa) with a calculated molecular weight of 87 kDa. Isoform α lacks exon 2 and could produce a 85 kDa protein of 758 aa. Isoform β, missing exons 2 and 3, translates into a putative protein of 680 aa and 75 kDa but would use a translation start in an alternative open reading frame (ORF) of exon 1 (Fig. 1C). Deletion of exon 4 in isoform γ disrupts the ORF. Isoform φ and δ, bearing deletions of exons 2 to 6 and 3 to 6, respectively, could produce 37 and 35 kDa proteins of 326 and 307 aa, respectively. The latter has been reported previously in HeLa (17) and ovarian cancer cells (6). Isoform ε lacks exons 4 to 9 and codes for a protein of 30 kDa, composed of 264 aa. Isoform η is composed of exons 1, 10, and 11. Splicing of exon 1 to exon 10 disrupts the ORF, but initiation of translation could occur in an

⁴ <http://tronolab.com/>

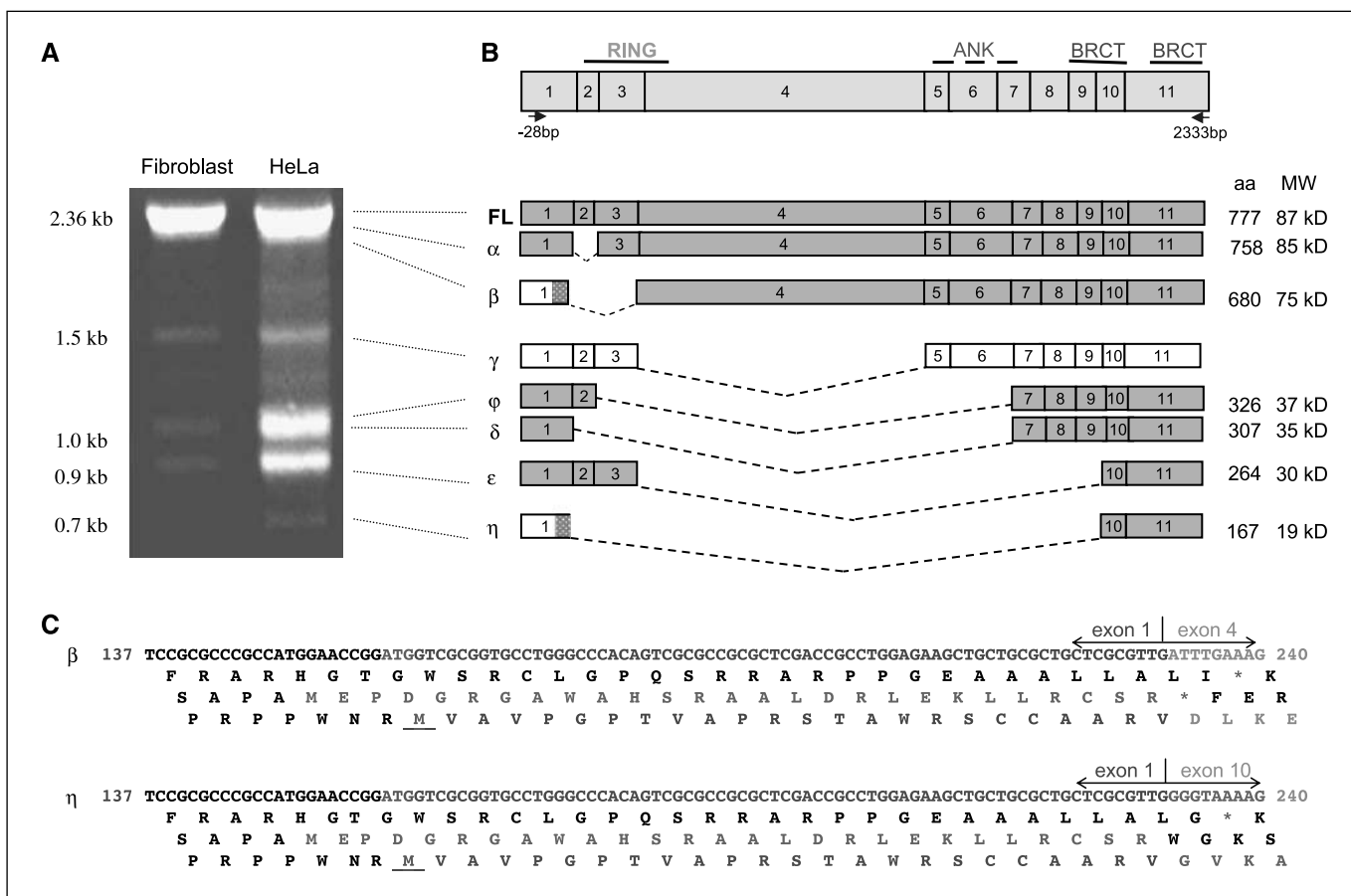


Figure 1. Structure of BARD1 isoforms. **A**, RT-PCR amplification of FL BARD1 in normal skin fibroblasts and HeLa cells. **B**, diagram of BARD1 exons and structural domains compared with exon structure of isoforms FL, α , β , γ , ϕ , δ , ϵ , and η . Approximate locations of RING, ankyrin (ANK), and BRCT are indicated. *Small arrows*, positions of forward and reverse primers used for RT-PCR. ORF, corresponding to known BARD1 sequence, is presented in green (*filled*) boxes, alternative ORF is indicated in blue (*spotted*) boxes, and out of frame, noncoding regions are unmarked. Numbers of encoded aa and calculated molecular weight are indicated on the right border. **C**, sequences of splice junctions of isoforms β and η are presented. Known BARD1 ORF is marked green, alternative ORF is marked blue. Possible translation initiation methionins are labeled red (*underlined*) within alternative ORF of isoforms β and η .

alternative ORF of exon 1 and translate into a 19 kDa protein of 167 aa (Fig. 1C). Interestingly, all these isoforms lack either the RING finger or ankyrin repeats, or both, and both regions are required for the tumor suppressor functions of BARD1 (2).

BARD expression pattern in different cancer cell lines. To investigate the pattern of BARD1 isoform expression in cells from cancers of different origin, we performed RT-PCR, on RNA from breast, ovarian, endometrial, cervical, and hematologic cancer cell lines, for amplification of the entire BARD1 coding region. RNA from HeLa cells was used as a positive control.

In ovarian cancer cells, FL BARD1 was expressed as well as isoforms β , ϕ , δ , ϵ , and η , which were often more abundant than FL BARD1. Complete absence of FL BARD1 but expression of splice isoforms was found in 5 of 32 ovarian cancer cell lines. A subgroup of ovarian cancer cell lines showed no BARD1 expression at all when forward primers in exon 1 were used for amplification of FL BARD1, but in all cases, BARD1 transcripts could be amplified by using forward primers in exon 4 (Fig. 2A). Similar results were obtained for BARD1 expression in breast and endometrial cancer cell lines (Supplementary Fig. S1).

In cervical cancer cell lines, we found neither FL BARD1 nor splice isoforms expressed when forward primers within exon 1 were used for amplification of BARD1 sequences. To amplify

potentially 5' truncated forms of BARD1, we used forward primers within exon 3, at different sites of exon 4, and within exon 5. BARD1 sequences could be amplified in some samples with primers in exon 4 and in all samples with a forward primer within exon 5 (Fig. 2B). This indicates that in all cervical cancer cell lines, BARD1 isoforms were initiated in exon 4 or 5. The control experiment, amplification from exon 1 to exon 6, was completely negative.

In summary (Table 1), FL BARD1 expression was absent in all cervical cancer cell lines tested, but BARD1 transcripts originating in exons 4 or 5 were present. In breast, ovarian, and endometrial cancer cells, FL BARD1 and splice isoforms were expressed, and splice isoforms alone were found in 15% of ovarian and in 11% of endometrial cancers. Loss of FL BARD1 and expression of transcripts initiating within exon 4 was found in 80% of breast cancer cell lines and was less frequent in ovarian (62.5%) and endometrial cancer cells (33.3%).

Thus, in all cancer cell lines derived from potentially hormone-controlled gynecologic cancers, FL BARD1 was either missing or was less abundant than isoforms, and in none of these cancer cells, BARD1 expression was absent.

To test whether BARD1 isoform expression was a particularity of gynecologic cancers, we performed RT-PCR in hematologic tumor cell lines that are unlikely to be hormonally controlled (Fig. 2C).

Table 1. BARD1 isoform expression pattern in different cancer cell lines

Type of cancer	Full-length only	Full-length and splice isoforms	Splice isoforms only	Alternative start in exon 4 only
Cervical cancer	0	0	0	100% (9/9)
Breast cancer	0	19.2% (5/26)	0	80.8% (21/26)
Ovarian cancer	0	21.9% (7/32)	15.6% (5/32)	62.5% (20/32)
Endometrial cancer	0	55.6% (5/9)	11.1% (1/9)	33.3% (3/9)
Lymphoma	100% (13/13)	0	0	Not determined

Most of these samples showed FL BARD1 expression, and only weak amplification of isoform γ , an out-of-frame splice variant, was detected.

Alternative initiation of transcription in exon 4. The results described above suggested that mRNAs transcribed from exon 4 to exon 11 use an alternative site of transcription initiation, which was consistent with the loss of NH₂-terminal epitopes, which was frequently observed in ovarian cancer samples (18). Therefore, we used forward primers located at the beginning of exon 4 (nucleotide position 428 of human BARD1 cDNA) and the end of exon 4 (nucleotide position 1280), respectively. Although in HeLa cells, both primers could amplify a BARD1 cDNA fragment of the expected length, in the ovarian cancer sample, BARD1 expression was only detected with the forward primer at the end of exon 4 (Fig. 3A).

To determine the exact start of transcription, we performed 5'race experiments to amplify the cDNA 5'ends, using RNA from ovarian cancer and from HeLa cells with reverse primers in exon 6. In HeLa cells, the amplicons corresponded to known 5'end of the human BARD1 cDNA sequence, and corresponded to FL BARD1 and isoform γ and to a cDNA derived from alternative transcription initiation within exon 4 (Fig. 3B; start 3). However, in ovarian cancer, we found two new transcription initiation sites within exon 4, mapping to nucleotide positions 458 (Fig. 3B; start 1), located at the beginning of exon 4, and 983 (start 2), in the middle of exon 4. Transcripts initiating at starts 1 and 2, designated Ω 1 and Ω 2, could be translated from ATGs at positions 1147, 1165, and 1183 within exon 4 and produce a protein of ~44 kDa; and transcripts originating at start 3, designated Ψ , could produce a protein of ~27 kDa (Fig. 3C and Supplementary Fig. S2).

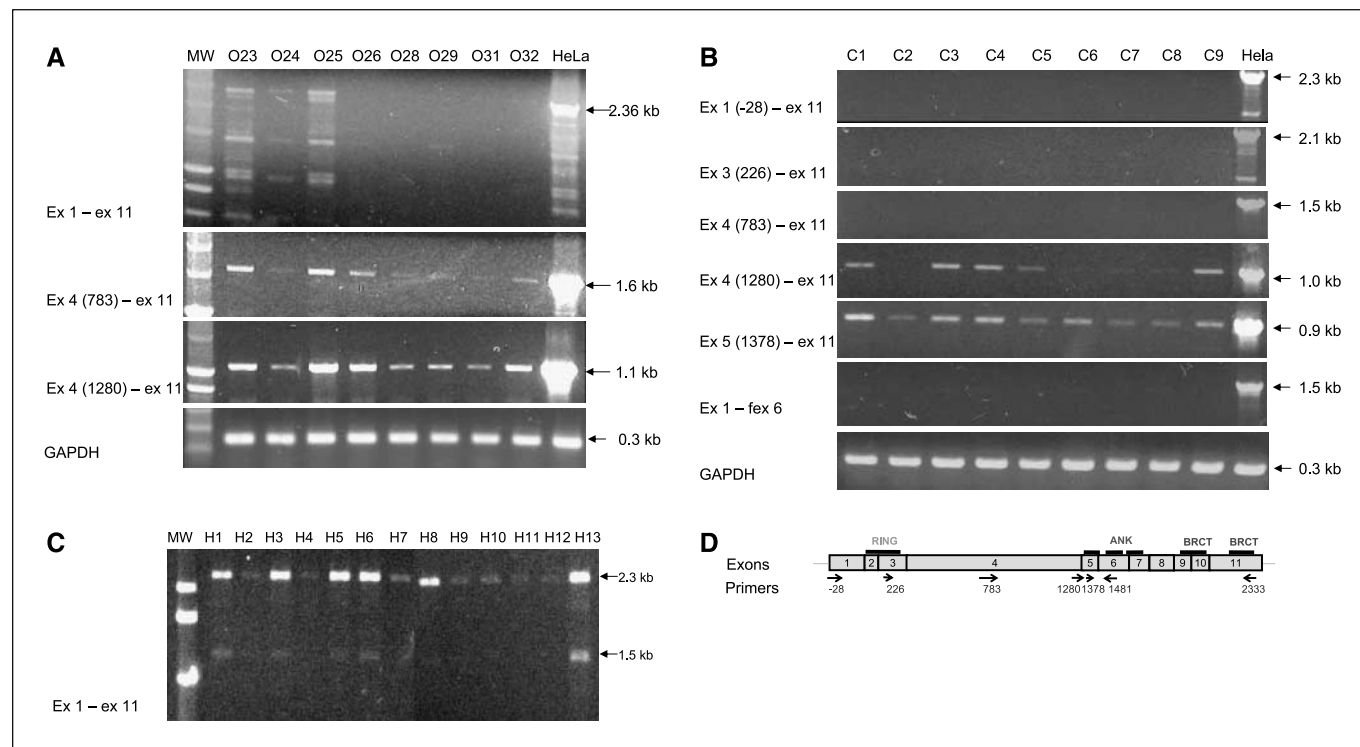


Figure 2. RT-PCR of ovarian (O23–O32), cervical (C1–C9), and hematologic cancer cell lines for amplification of *BARD1* transcripts. **A**, amplification of FL BARD1 and/or truncated isoforms in ovarian cancer cells using forward primers in exon (ex) 1 and 11, or exon 4 (positions in parentheses) and reverse primers in exon 11. HeLa cells were used as a control. Expected size of amplified band is indicated on the right side. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was probed in the same samples. **B**, amplification of BARD1 transcripts in cervical cancer. Primer positions are indicated, referring to exons and nucleotide positions within exons 4 and 5. Amplicons derived from HeLa cells were generated in parallel. GAPDH expressions are shown in the same samples. **C**, RT-PCR of BARD1 expression in hematologic tumor cell lines (H1–H13). Primers in exons 1 and 11 amplified FL BARD1 and weakly isoform γ . **D**, schematic presentation of exon structure and position of primers used in RT-PCR experiments.

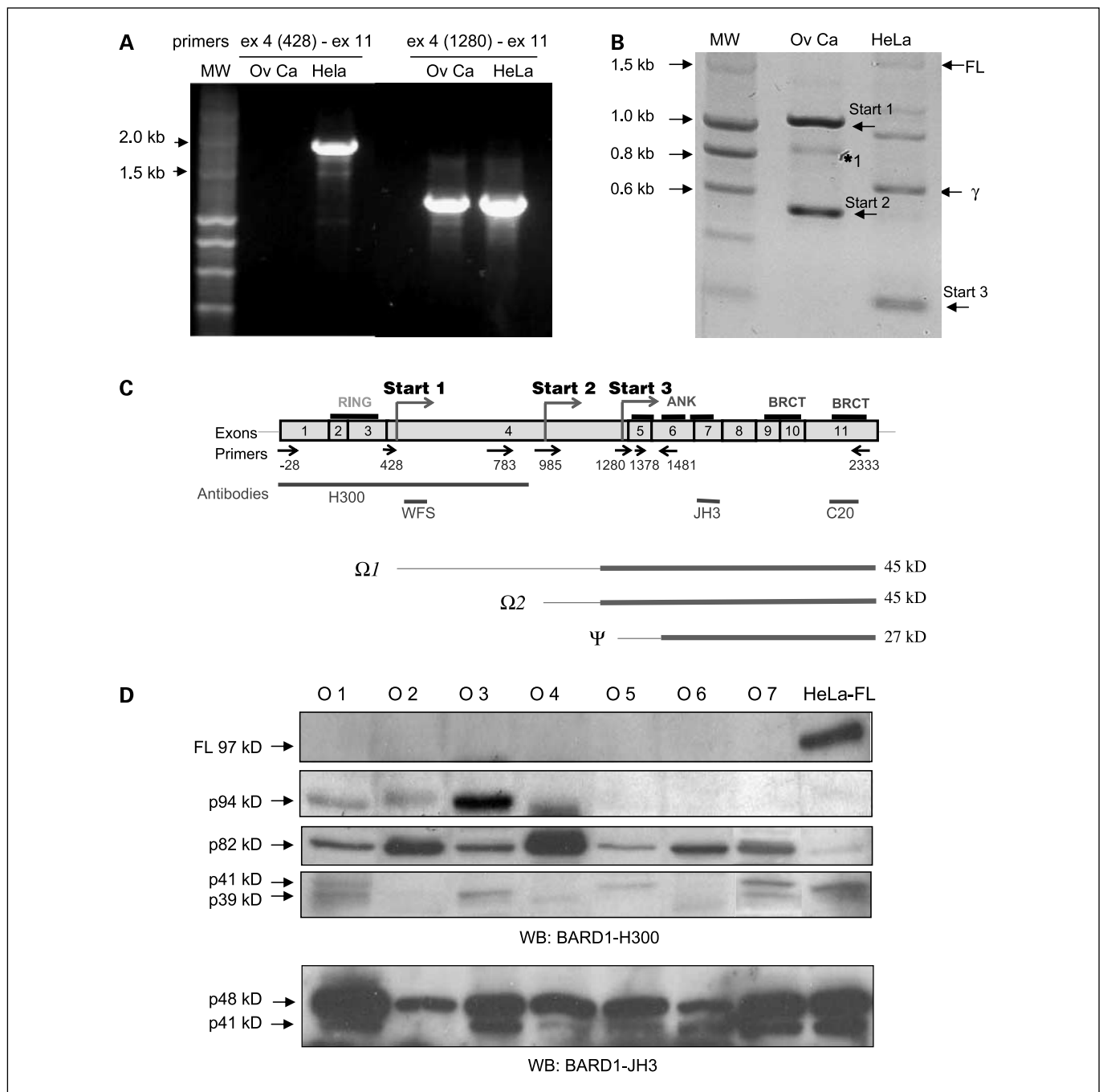


Figure 3. Alternative transcription initiation in exon 4. *A*, RT-PCR was performed with BARD1 forward primers within exon 4 (428 or 1280) and reverse primers in exon 11 with RNA from ovarian cancer sample (*Ov Ca*) and HeLa cells are shown. *B*, 5' RACE performed with RNA from ovarian cancer sample and HeLa cells. Forward primer was 5' nested primer and reverse primer was within exon 6. Arrows, bands corresponding to alternative 5' ends, labeled start 1, 2, and 3. Band *1 was identical sequence as start 1, but showed different gel migration, presumably due to secondary structures. Bands FL and γ correspond to sequences of indicated isoforms with known 5' end of BARD1. *C*, diagram of BARD1 exon structure and schematic presentation of isoforms derived from alternative transcription initiation are shown. Thick lines, translated regions; thin lines, nontranslated regions. Primers and antibodies used in experiments in Figs. 2 and 3 are indicated. *D*, Western blot analysis of ovarian cancer cell lines probed with anti-BARD1 H300 and JH3. Molecular weight of different BARD1 isoforms and presumed isoform identities are indicated. HeLa cells expressing exogenous BARD1 (*HeLa-FL*) were used as a control.

These alternative 5' ends of transcripts are consistent with the results obtained by RT-PCR (Fig. 2A): Forward primer at nucleotide position 783 could amplify isoform $\Omega 1$, and forward primer at nucleotide position 1280 could amplify isoforms $\Omega 1$ and $\Omega 2$. In cervical cancer cells, $\Omega 2$ was present in five of nine samples, and Ψ was present in the remaining four samples

(Fig. 2B). Interestingly, in ovarian, breast, and endometrial cancer cell lines, $\Omega 1$ or $\Omega 2$ were detected in all samples that lacked expression of FL BARD1. It is possible, but cannot be determined by RT-PCR, that Ω isoforms are also expressed in cells that express FL BARD1. These data show that gynecologic cancers express deletion-bearing BARD1 isoforms, derived from

differential splicing and from alternative transcription initiation within exon 4.

Identification of BARD1 protein isoforms in ovarian cancer cell lines. Because we found different *BARD1* isoforms transcribed in cancer cells, we were interested in determining whether these isoforms were translated. We analyzed protein extracts from ovarian cancer cell lines and HeLa cells overexpressing exogenous FL BARD1 (HeLa-FL) as a control on Western blots (Fig. 3D). We used anti-BARD1 H300, directed against the NH₂-terminal 300 aa of BARD1 (Fig. 3C), and antibody JH3 directed against a peptide antigen within exon 7 (10, 19, 25), to detect BARD1 and BARD1 isoforms. H300 detected FL BARD1, which migrates as a 97 kDa protein on SDS-PAGE, only in extracts from HeLa-FL cells but in none of the ovarian cancer cell lines (Fig. 3D). A 94-kDa protein was detected in a few of the ovarian cancer samples and an 82-kDa protein was present in all samples. Based on the cDNA structure of BARD1 isoforms (Fig. 1B), the 94 kDa and 82 kDa bands might be translation products of isoform α (deletion exon 2) and isoform β (deletion of exons 2 and 3), respectively, assuming that, like FL BARD1, they migrate slightly higher than predicted from their calculated molecular weight. In some of the samples, we observed two smaller bands of about 40 kDa, which might be isoforms φ (deleted of exons 3 to 6) and δ (deleted of exons 2 to 6). Western blots probed with JH3 resulted in very strong staining of a 48 kDa protein, which was not detected by H300. This protein might correspond to the protein translation product of isoforms Ω 1 and Ω 2 (44 kDa). Importantly, in nonmalignant tissue, JH3 recognizes FL BARD1 and BARD1 β , as was shown previously for BARD1 expression in rat testis (10). JH3 did not detect FL BARD1 in any of the cell lines and only showed weak staining for BARD1 β with exposure times applied for detection of Ω , indicating that Ω was more abundant than BARD1 β . Isoform Ω cannot be detected by anti-BARD1 H300 because it cannot recognize *in vitro* generated BARD1 deletion mutants that use the same translation initiation codons as Ω isoforms (Supplementary Fig. S3). The smaller proteins of about 41 kDa detected by JH3 could be isoforms φ and δ , which were also detected by H300. Thus, RT-PCR and Western blot analyses show, at the mRNA and at the protein level, that little or no expression of FL BARD1 but high expression of isoforms is found in ovarian cancer cells.

BARD1 expression in ovarian cancer. To investigate BARD1 expression in tumors and its relevance for tumor growth, we performed immunohistochemistry on a large sample collection of ovarian tumors prepared as tissue arrays. We used antibodies detecting epitopes at the NH₂ terminus (N19), within exon 4 (WFS), and at the COOH terminus (C-20) of BARD1, which were all described before (N19 and C-20, refs. 5, 18, 19; WFS, ref. 3). A total of 106 cases of ovarian cancer were analyzed. With antibodies N19 and C-20, we observed high expression of BARD1 in some of the patient samples and weak in others, but in a third group, staining of N19 was lost or decreased (Fig. 4A). Antibody WFS was weakly expressed in most of the samples. Interestingly, loss of N19 expression mostly happened in ovarian cancer of T₃ stage or cancers with lymph node metastasis (N₁; $P < 0.05$; Fig. 4B). Thus, loss of NH₂-terminal epitopes is correlated with advanced stage of ovarian cancer. However, we did not find a correlation of BARD1 expression and pathologic tumor grade (data not shown). Expression of COOH terminus and loss of NH₂ terminus are compatible with the expression of Ω isoforms. Interestingly, we found strong staining for N19 and C-20 but no staining for WFS in clear cell carcinoma (Fig. 4B and C), which is the type of ovarian

cancer with the worst prognosis. This staining pattern is consistent with the expression of isoforms φ , δ , and ϵ .

RT-PCR, performed in ovarian cancer cell lines derived from clear cell carcinoma, SK-OV-3 and TOV-21G, confirmed this hypothesis. Elevated expression of isoforms φ , δ , and ϵ , but not FL BARD1, was found in SK-OV-3 and TOV-21G cells (Fig. 4D). Western blots, using BARD1 H300 and JH3 antibodies on extracts from ovarian cancer cell lines, including SK-OV-3 and TOV-21G (Fig. 3D; O5 and O6), showed absence of FL BARD1 and presence of isoforms. To further support this finding, we used BARD1 antibody BL518, directed against epitopes encoded on exon 4 (26), which could only detect FL BARD1 and BARD1 β , to analyze BARD1 expression in SK-OV-3 and TOV-21G cells. Although we found abundant FL BARD1 expression in HEK 293 cells, no FL BARD1 was found in SK-OV-3 and TOV-21G cells, but BARD1 β was present in all (Fig. 4D). Thus, SK-OV-3 and TOV-21G cells express BARD1 isoforms but not FL BARD1.

BARD1 isoforms are essential for tumor cell growth. Because SK-OV-3 and TOV-21G cells expressed BARD1 isoforms, we were interested in elucidating the functions of these isoforms. We performed repression assays in SK-OV-3 with drug-inducible siRNAs (22). Cells were transduced with control lentiviral vector pLV or lentiviral vector si78, expressing siRNA-targeting sequences in exon 9 of human BARD1, present on isoforms β , φ , and δ (Fig. 5). Both vectors are coexpressing green fluorescent protein (GFP) as reporter gene. Cell growth was monitored and cell numbers were determined at 4 and 9 days after induction of siRNA expression. At 9 days of siRNA expression, we observed pronounced repression of cell growth and only few cells transduced with si78 were attached, whereas cells transduced with control vector proliferated normally even after 9 days (Fig. 5A). Interestingly, these differences in cell proliferation were not observed after 4 days of repression (Fig. 5B). This delay in response of siRNA repression might be explained by the slow decrease of BARD1 β after siRNA induction (Fig. 5B). This observation is consistent with increased stability of *in vitro* generated NH₂ terminal deletion-bearing BARD1 isoforms (9). With the increased stability of BARD1 β , we also observed in time curves of BARD1 repression in HeLa cells, although repression of FL BARD1 is observed after 4 days, repression of isoform β is only seen after 9 days (data not shown). Therefore, these experiments show that BARD1 β , and presumably φ and δ (not detectable with anti-BARD1 BL518), are important for cancer cell growth, and that repression of these isoforms leads to proliferation block and cell death.

To further investigate this function of BARD1 isoforms in proliferation, in other than human cancer cells, we performed a similar repression experiment in a rat ovarian cancer cell line, NuTu-19, which was previously shown to abundantly express alternatively spliced isoforms BARD1 β and δ but not FL BARD1 protein (ref. 6; Supplementary Fig. S4A). We transduced NuTu-19 cells with lentiviral vectors, containing BARD1 control siRNAs si34, targeting sequences in human but not rat exon 2, and si78, targeting a sequence in exon 9, which is identical in human and rat. After transduction and induction of siRNA expression, RT-PCR was performed and showed that si78 completely repressed all BARD1 isoforms (Supplementary Fig. S4B). Fluorescence microscopy, showing 4',6-diamidino-2-phenylindole (DAPI) staining and GFP expression for transduction control, illustrates that NuTu-19 cells transduced with si78 do not proliferate and the few surviving cells became big and flat. Cells transduced with the control si34 had a normal morphology and high proliferation rate (Fig. 5C and D).

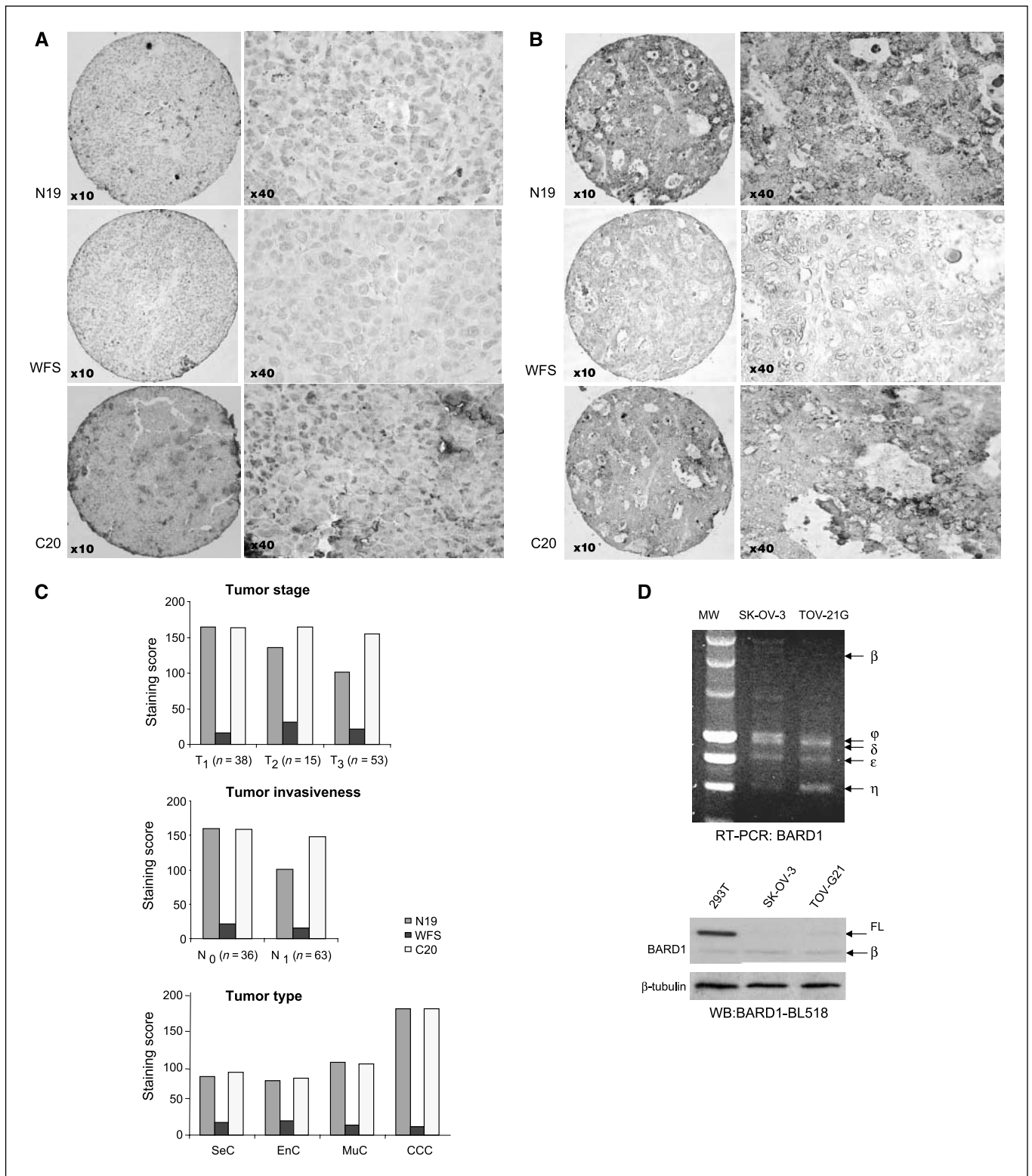


Figure 4. Analysis of BARD1 expression in ovarian cancer tissue arrays. Total of 106 ovarian cancers were analyzed with BARD1 antibodies N19, WFS, and C-20. **A**, example of immunohistochemistry of stage T₃ tumor is shown with negative staining for N19 and WFS but positive staining for C-20. **B**, immunohistochemistry of clear cell carcinoma shows strong staining with both N19 and C-20 but negativity for WFS. **C**, correlation of BARD1 expression and primary tumor stage (T₁, tumor is limited to one or both ovaries; T₂, tumor involves one or both ovaries with spread into the pelvis; and T₃, tumor involves one or both ovaries, with peritoneal metastasis outside the pelvis and/or to nearby lymph nodes), tumor invasiveness (N₀, regional lymph nodes contain no metastases; N₁, evidence of lymph node metastasis), and with different pathologic types of ovarian cancer (SeC, serous carcinoma; EnC, endometrioid carcinoma; MuC, mucinous carcinoma; and CCC, clear cell carcinoma). Note that clear cell carcinoma has the highest score. **D**, RT-PCR and Western blot analysis for detection of BARD1 isoforms in clear cell carcinoma cell lines SK-OV-3 and TOV-21G. RT-PCR shows that splice variants are more abundant than FL BARD1. Western blot shows FL BARD1 and β expression in HEK 293T (293T) but only β in SK-OV-3 and TOV-21G cells, when anti-BARD1 BL518 (directed against epitope within exon 4) was used and visualized with normal ECL and short exposure (5 min).

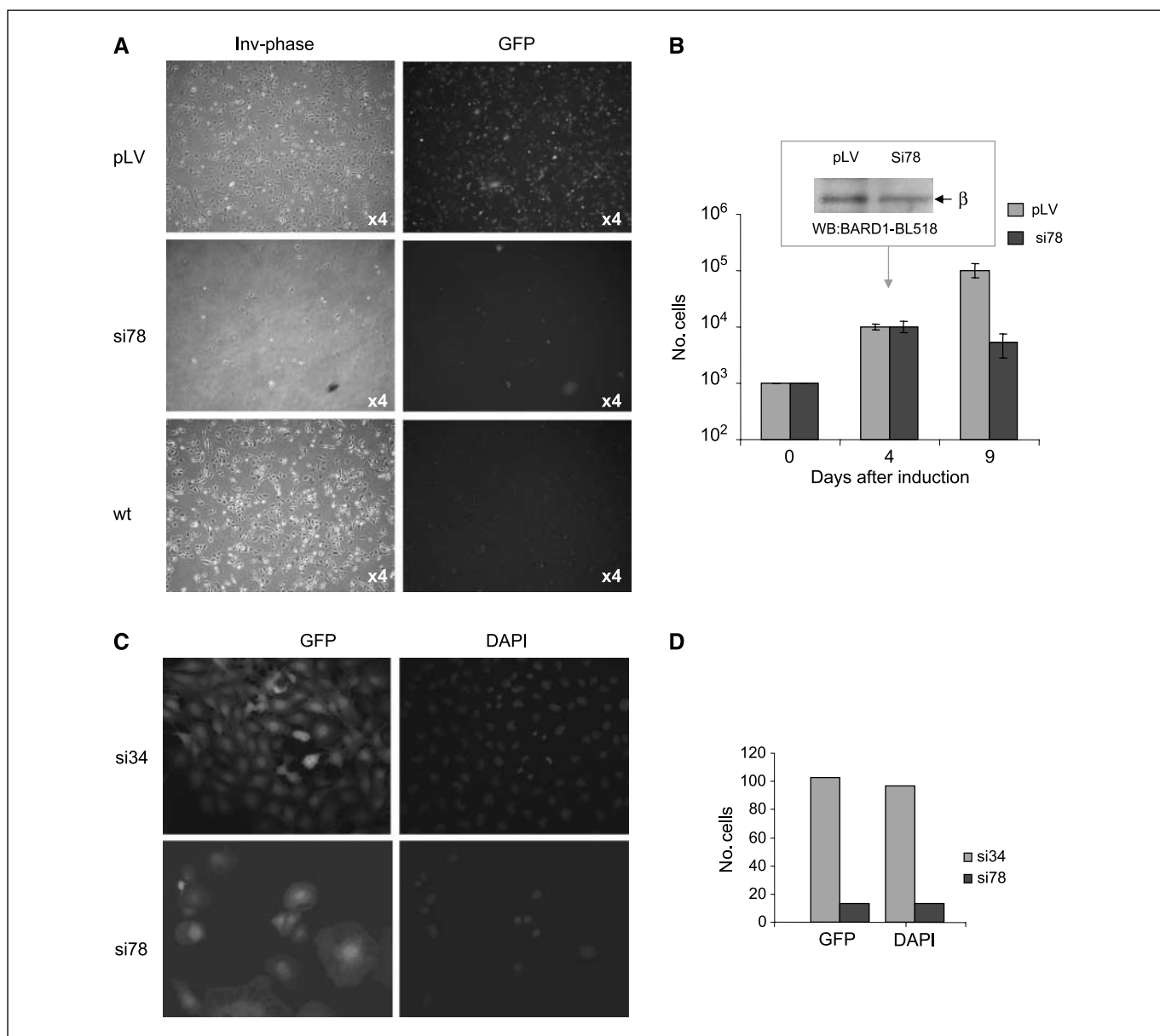


Figure 5. BARD1 isoforms required for proliferation of ovarian cancer cells. *A*, images of inverse phase microscopy and GFP expression of SK-OV-3 cells transduced with control virus (*pLV*), BARD1 siRNA-78 (*si78*), or untransduced (*wt*) are shown. *B*, histogram of cell survival after 4 and 9 d of induction of siRNA expression by doxycyclin. *Inset*, Western blot of SK-OV-3 cells transduced with *pLV* or *si78* after 4 d of siRNA induction by doxycyclin. Anti-BARD1 BL518 detected isoform β in *si78* cells although in reduced amounts compared with *pLV*-transduced cells (control). Repression after 9 d could not be monitored because no *si78*-transduced cells survived after 9 d of siRNA induction. *C*, images of GFP expression and DAPI staining of NuTu-19 cells, transduced with BARD1 siRNA *si34* (control) and *si78* show growth arrest and loss of adherent cells in *si78* but not *si34*-transduced cells. *D*, histogram shows quantification of surviving cells at 4 d after induction of siRNA.

Discussion

Research on BARD1 has intensified in the last few years, and evidence is accumulating that it is a tumor suppressor both as a binding partner of the breast cancer protein BRCA1 and independently of BRCA1 (2). Inherited predisposition to breast and ovarian cancer is associated in up to 50% of cases with mutations in BRCA1 and BRCA2. BARD1 mutations associated with inherited and spontaneous cases of breast and ovarian cancer, however, are very infrequent (13, 27). This could be interpreted as BARD1 being involved in pathways affecting cell viability.

Indeed, rather than absence of BARD1 due to mutations, overexpressed aberrant forms of BARD1 were found in breast

and ovarian cancer (18). Here we report that cancer cells express a complex pattern of BARD1 isoforms, namely α , β , γ , δ , ϵ , and η . All of them are likely to code for proteins, except for isoform γ , which does not have a continuous ORF. All these isoforms are also expressed in cytotrophoblasts of early pregnancy (19), which share invasive properties with cancer cells. In cytotrophoblasts, the expression of BARD1 isoforms is temporally and spatially regulated, suggesting that they play a role in proliferation and invasion (19).

In cancer cell lines derived from various types of gynecologic cancers, we found a specific pattern of differentially spliced isoforms and isoforms derived from transcription initiation in

exon 4, which was in strong contrast to hematologic cancer cell lines, where FL BARD1 was expressed in all cell lines tested (Table 1). BARD1 isoforms are either deprived of the BRCA1-interacting RING finger domain, i.e., isoforms β , δ , φ , η , and Ω , or, like isoforms ϵ and η , have lost the ankyrin motif or part of the BRCT region, which suggests loss of other tumor suppressor functions involving binding to these regions (6, 28, 29). NH₂-terminally truncated forms of BARD1, derived from transcription initiation within exons 4 or 5, were found in all gynecologic cancer cell lines. Such transcripts could encode a 44 kDa protein, which is indeed highly expressed in all ovarian cancer cell lines, although FL BARD1 protein is absent. This finding suggests that FL BARD1, even when identified by RT-PCR, is proportionally less efficiently translated or less stable than isoforms.

BARD1 isoforms might be specifically expressed in gynecological cancers, suggesting that their expression might be linked to hormone-signaling pathways. It is well-established that exposure to female reproductive hormones is a central factor for the development of breast (30–32) and ovarian cancer (33, 34). Interestingly, a region of the *BARD1* gene within intron 8 confers estrogen responsiveness, and *BARD1* mRNA and protein levels can be increased by estrogen (35). Recently, the estrogen receptor α was identified as a substrate for the BRCA1-BARD1 ubiquitin ligase (36), which is in line with the observations that BRCA1 is an activator of estrogen receptor transcriptional activity (37, 38). The estrogen-regulated expression of *BARD1*, however, links the ubiquitin ligase activity of the BRCA1-BARD1 heterodimer to estrogen levels. The up-regulated expression of BARD1 isoforms lacking the BRCA1 interaction domain disrupts this regulatory circle and provides an explanation for BARD1 isoform expression in gynecological cancers and their correlation with poor prognosis. It is possible that BARD1 isoforms act like dominant negative mutations in an estrogen-signaling pathway and promote cancer development and progression. Further experiments will be needed to establish the estrogen-dependent expression of each particular isoform and to identify the regulatory pathways with which they interfere.

It was interesting that in all cancer cell lines tested, we always could detect BARD1 isoforms or FL BARD1 but never found cells that completely lacked BARD1 expression. In contrast to hypermethylation of BRCA1, which was observed in sporadic breast cancers (39, 40), no promoter methylation of *BARD1* was found in 50 primary cell lines generated from ovarian cancer samples and 34 ovarian cancer cell lines (data not shown). This suggests that BARD1 or isoforms might be essential for cancer cell growth. Importantly, siRNA repression experiments, targeting isoforms in cell lines that lack FL BARD1, prove that isoforms are required for cell growth (Fig. 5). Hence, cancer-

specific BARD1 isoforms might have lost tumor suppressor functions but retained or acquired functions that play a role in cell viability and tumor growth.

Immunohistochemistry of 106 ovarian cancers confirmed the importance of aberrant expression of BARD1 isoforms. Specifically, expression of isoforms missing NH₂-terminal epitopes, such as β and Ω , was associated with tumors of T₃ (tumor invades tissue outside of the pelvis) and N₁ (positive for lymph node metastasis) stages, suggesting that expression of β and Ω is associated with advanced stage of ovarian carcinoma and might be a negative prognostic factor in ovarian cancer.

A type of ovarian cancer with poor prognosis and poor response to platinum-based chemotherapy is clear cell carcinoma (41, 42). We found a specifically high expression of BARD1 isoforms β , φ , δ , and ϵ in clear cell carcinoma. *In vitro* repression assays, performed in clear cell carcinoma-derived cells and in cells from rat ovarian cancer, clearly show that BARD1 isoforms are essential for cell growth. Because the siRNAs were targeting sequences within exon 9, expression of β , φ , and δ was affected but not ϵ and η . In both cell types, the repression of these isoforms led to complete growth arrest and cell death. Thus, isoforms β , φ , and δ might have essential functions in proliferation of clear cell carcinoma, the ovarian cancer with the worst prognosis.

Loss of FL BARD1 and expression of isoforms that lack the RING finger and are deficient for BRCA1 interaction (43) entails the loss of BRCA1-BARD1 ubiquitylation functions, which are important for tumor suppressor functions but will also influence BRCA1 stability and/or nuclear localization (44–46). Therefore, lack of FL BARD1 and expression of isoforms lacking the RING domain could have a similar cancer predisposition effect as mutations in BRCA1 when occurring in healthy tissue.

To summarize, BARD1 isoforms are more expressed than FL BARD1 in gynecological cancers. All isoforms lack regions that are required for BRCA1 interaction and tumor suppressor functions, but might have acquired novel functions in favor of tumor growth. Because repression of isoforms in ovarian cancer cells leads to growth arrest, this suggests that BARD1 isoforms act as tumor maintenance genes in gynecological cancers.

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References

1. Wu LC, Wang ZW, Tsan JT, et al. Identification of a RING protein that can interact *in vivo* with the BRCA1 gene product. *Nat Genet* 1996;14:430–40.
2. Irmingier-Finger I, Jefford CE. Is there more to BARD1 than BRCA1? *Nat Rev Cancer* 2006;6:382–91.
3. Irmingier-Finger I, Soriano JV, Vaudan G, Montesano R, Sappino AP. *In vitro* repression of Brca1-associated RING domain gene, Bard1, induces phenotypic changes in mammary epithelial cells. *J Cell Biol* 1998;143:1329–39.
4. McCarthy EE, Celebi JT, Baer R, Ludwig T. Loss of Bard1, the heterodimeric partner of the Brca1 tumor suppressor, results in early embryonic lethality and chromosomal instability. *Mol Cell Biol* 2003;23:5056–63.
5. Irmingier-Finger I, Leung WC, Li J, et al. Identification of BARD1 as mediator between proapoptotic stress and p53-dependent apoptosis. *Mol Cell* 2001;8:1255–66.
6. Feki A, Jefford CE, Berardi P, et al. BARD1 induces apoptosis by catalysing phosphorylation of p53 by DNA-damage response kinase. *Oncogene* 2005;24:3726–36.
7. Joukov V, Groen AC, Prokhorova T, et al. The BRCA1/BARD1 heterodimer modulates ran-dependent mitotic spindle assembly. *Cell* 2006;127:539–52.
8. Ayi TC, Tsan JT, Hwang LY, Bowcock AM, Baer R. Conservation of function and primary structure in the BRCA1-associated RING domain (BARD1) protein. *Oncogene* 1998;17:2143–8.
9. Jefford CE, Feki A, Harb J, Krause KH, Irmingier-Finger I. Nuclear-cytoplasmic translocation of BARD1 is linked to its apoptotic activity. *Oncogene* 2004;23:3509–20.
10. Feki A, Jefford CE, Durand P, et al. BARD1 expression during spermatogenesis is associated with apoptosis and hormonally regulated. *Biol Reprod* 2004;71:1614–24.

11. Ghimenti C, Sensi E, Prescittini S, et al. Germline mutations of the BRCA1-associated ring domain (BARD1) gene in breast and breast/ovarian families negative for BRCA1 and BRCA2 alterations. *Genes Chromosomes Cancer* 2002;33:235–42.
12. Thai TH, Du F, Tsan JT, et al. Mutations in the BRCA1-associated RING domain (BARD1) gene in primary breast, ovarian and uterine cancers. *Hum Mol Genet* 1998;7:195–202.
13. Karppinen SM, Barkardottir RB, Backenhorn K, et al. Nordic collaborative study of the BARD1 Cys557Ser allele in 3956 patients with cancer: enrichment in familial BRCA1/BRCA2 mutation-negative breast cancer but not in other malignancies. *J Med Genet* 2006;43:856–62.
14. Ishitobi M, Miyoshi Y, Hasegawa S, et al. Mutational analysis of BARD1 in familial breast cancer patients in Japan. *Cancer Lett* 2003;200:1–7.
15. Karppinen SM, Heikkinen K, Rapakko K, Winqvist R. Mutation screening of the BARD1 gene: evidence for involvement of the Cys557Ser allele in hereditary susceptibility to breast cancer. *J Med Genet* 2004;41:e114.
16. Sauer MK, Andrulis IL. Identification and characterization of missense alterations in the BRCA1 associated RING domain (BARD1) gene in breast and ovarian cancer. *J Med Genet* 2005;42:633–8.
17. Tsuzuki M, Wu W, Nishikawa H, et al. A truncated splice variant of human BARD1 that lacks the RING finger and ankyrin repeats. *Cancer Lett* 2006;233:108–16.
18. Wu JY, Vlastos AT, Pelte MF, et al. Aberrant expression of BARD1 in breast and ovarian cancers with poor prognosis. *Int J Cancer* 2006;118:1215–26.
19. Li L, Cohen M, Wu J, et al. Identification of BARD1 splice-isoforms involved in human trophoblast invasion. *Int J Biochem Cell Biol* 2007;39:1659–72.
20. Srebrow A, Kornblihtt AR. The connection between splicing and cancer. *J Cell Sci* 2006;119:2635–41.
21. Schuchner S, Tembe V, Rodriguez JA, Henderson BR. Nuclear targeting and cell cycle regulatory function of human BARD1. *J Biol Chem* 2005;280:8855–61.
22. Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* 2003;77:8957–61.
23. Fabbro M, Savage K, Hobson K, et al. BRCA1-1 complexes are required for p53Ser-15 phosphorylation and a G₁-S arrest following ionizing radiation-induced DNA damage. *J Biol Chem* 2004;279:31251–8.
24. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nat Biotechnol* 1997;15:871–5.
25. Gautier F, Irminger-Finger I, Gregoire M, Meflah K, Harb J. Identification of an apoptotic cleavage product of BARD1 as an autoantigen: a potential factor in the antitumoral response mediated by apoptotic bodies. *Cancer Res* 2000;60:6895–900.
26. Tembe V, Henderson BR. BARD1 translocation to mitochondria correlates with Bax oligomerization, loss of mitochondrial membrane potential and apoptosis. *J Biol Chem* 2007;282:2513–22.
27. Stacey SN, Sulem P, Johannsson OT, et al. The BARD1 Cys557Ser variant and breast cancer risk in Iceland. *PLoS Med* 2006;3:e217.
28. Kleiman FE, Manley JL. Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50. *Science* 1999;285:1576–9.
29. Kleiman FE, Manley JL. The BARD1-CstF-50 interaction links mRNA 3' end formation to DNA damage and tumor suppression. *Cell* 2001;104:743–53.
30. Gallicchio L, Berndt SI, McSorley MA, et al. Polymorphisms in estrogen-metabolizing and estrogen receptor genes and the risk of developing breast cancer among a cohort of women with benign breast disease. *BMC Cancer* 2006;6:173.
31. Wang J, Higuchi R, Modugno F, et al. Estrogen receptor α haplotypes and breast cancer risk in older Caucasian women. *Breast Cancer Res Treat* 2007;106:273–80.
32. Conway K, Parrish E, Edmiston SN, et al. Risk factors for breast cancer characterized by the estrogen receptor α A908G (K303R) mutation. *Breast Cancer Res* 2007;9:R36.
33. Gupta PB, Proia D, Cingoz O, et al. Systemic stromal effects of estrogen promote the growth of estrogen receptor-negative cancers. *Cancer Res* 2007;67:2062–71.
34. Wirtenberger M, Tchatchou S, Hemminki K, et al. Associations of genetic variants in the estrogen receptor coactivators PPARGC1A, PPARGC1B and EP300 with familial breast cancer. *Carcinogenesis* 2006;27:2201–8.
35. Creekmore AL, Ziegler YS, Boney JL, Nardulli AM. Estrogen receptor α regulates expression of the breast cancer 1 associated ring domain 1 (BARD1) gene through intronic DNA sequence. *Mol Cell Endocrinol* 2007;267:106–15.
36. Eakin CM, Maccoss MJ, Finney GL, Klevit RE. Estrogen receptor α is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci U S A* 2007;104:5794–9.
37. Fan S, Ma YX, Wang C, et al. Role of direct interaction in BRCA1 inhibition of estrogen receptor activity. *Oncogene* 2001;20:77–87.
38. Zheng L, Annab LA, Afshari CA, Lee WH, Boyer TG. BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. *Proc Natl Acad Sci U S A* 2001;98:9587–92.
39. Jing F, Zhang J, Tao J, et al. Hypermethylation of tumor suppressor genes BRCA1, p16 and 14-3-3sigma in serum of sporadic breast cancer patients. *Onkologie* 2007;30:14–9.
40. Scardocci A, Guidi F, D'Alo F, et al. Reduced BRCA1 expression due to promoter hypermethylation in therapy-related acute myeloid leukaemia. *Br J Cancer* 2006;95:1108–13.
41. Tammela J, Geisler JP, Eskew PN, Jr., Geisler HE. Clear cell carcinoma of the ovary: poor prognosis compared to serous carcinoma. *Eur J Gynaecol Oncol* 1998;19:438–40.
42. Goff BA, Sainz de la Cuesta R, Muntz HG, et al. Clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. *Gynecol Oncol* 1996;60:412–7.
43. Brzovic PS, Rajagopal P, Hoyt DW, King MC, Klevit RE. Structure of a BRCA1-1 heterodimeric RING-RING complex. *Nat Struct Biol* 2001;8:833–7.
44. Joukov V, Chen J, Fox EA, Green JB, Livingston DM. Functional communication between endogenous BRCA1 and its partner, BARD1, during *Xenopus laevis* development. *Proc Natl Acad Sci U S A* 2001;98:12078–83.
45. Fabbro M, Rodriguez JA, Baer R, Henderson BR. BARD1 induces BRCA1 intranuclear foci formation by increasing RING-dependent BRCA1 nuclear import and inhibiting BRCA1 nuclear export. *J Biol Chem* 2002;277:21315–24.
46. Rodriguez JA, Schuchner S, Au WW, Fabbro M, Henderson BR. Nuclear-cytoplasmic shuttling of BARD1 contributes to its proapoptotic activity and is regulated by dimerization with BRCA1. *Oncogene* 2004;23:1809–20.