

PRL-3 Phosphatase Is Implicated in Ovarian Cancer Growth

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Abstract Purpose: The PRL-3 phosphatase has been found expressed at higher levels in metastasis than in primary tumors of patients with colorectal cancer. In the present study, we evaluated the expression of PRL-3 in ovarian cancer tissue and its role in ovarian cancer cell growth.

Experimental Design: PRL-3 phosphatase expression was evaluated in 84 ovarian tumor samples. PRL-3 expression has been knocked down using specific small interfering RNAs to determine its role in ovarian cancer cell growth *in vitro*.

Results: In ovarian cancers, PRL-3 expression correlates with disease progression, being higher in advanced (stage III) than in early (stage I) tumors. *In situ* measurements of PRL-3 expression showed that it was confined to the epithelial neoplastic cells. The molecular mechanism underlying PRL-3 overexpression in ovarian cancers is independent from amplification of the corresponding genomic locus. Ovarian cancer cells growing in culture have high levels of expression of this phosphatase. PRL-3-specific knockdown using small interfering RNA severely impaired the growth of cells without affecting the expression of the closely related homologue PRL-1. Intriguingly, the growth of human colon carcinoma cells expressing lower levels of the PRL-3 was not affected by the PRL-3 knockdown.

Conclusions: Altogether, these results show that PRL-3 expression is associated with ovarian cancer progression and point to a key role for this phosphatase in the control of ovarian cancer cells growth. This strongly suggests that PRL-3 should be considered as a target for the discovery of new anticancer agents to be tested against this malignancy.

PRL-3 phosphatase, also known as PTP4A3, belongs to a small class of tyrosine phosphatases, which includes PRL-1 and PRL-2 (1). These proteins are prenylated *in vitro* and *in vivo* and this posttranslational modification is important for their intracellular distribution (1, 2). Increasing importance in the cancer field has been gained by PRL-3 phosphatase, which has been reported to be overexpressed in colorectal metastasis in respect to nonmetastatic tumor or normal colorectal epithelium (3, 4). Liver metastasis from other cancers such as pancreas, esophagus, or stomach did not express high levels of PRL-3, suggesting

a degree of specificity for colorectal cancer (4). The overexpression of PRL-3 in colorectal cancer metastasis was associated with gene amplification in a small subset of cases (3). Normal Chinese hamster ovary cells transfected with PRL-3 showed increased mobility and invasiveness and these effects were markedly reduced when a catalytically inactive mutant was used (5). Furthermore, cells ectopically expressing PRL-3 are able to form experimental metastasis in mice and this effect is dependent on the catalytic activity of the phosphatase (6, 7). These results indicate that PRL-3 could be an important factor contributing to the invasive-metastatic properties of cancer cells. In the present study, we have investigated the involvement of the PRL-3 phosphatase in primary ovarian cancer and its metastasis.

Materials and Methods

Human tumor samples. Tumor tissues were obtained at first laparotomy before any chemotherapy; freed of necrotic, hemorrhagic, and connective tissue; and immediately put in liquid nitrogen and stored at -80°C until processed. A total of 84 tumor samples (61 stage I and 23 stage III) were analyzed. Twenty-two percent of the tumors were grade 1, 29.9% grade 2, 37.7% grade 3, and 10.4% borderline. As for the histologic type, 41 were serous, 16 mucinous, 9 clear cells, 10 endometrioid, 2 undifferentiated, and 6 unknown.

Total RNA was extracted from ovarian cancer tissue homogenate using SV total isolation system (Promega, Milan, Italy). Single-stranded cDNA was generated using MultiScribe Reverse Transcriptase (Applied Biosystem, Milan, Italy) following the directions of the manufacturer. Quantitative PCR was done with an ABI Prism 5700 cyler (Applied Biosystem) using SYBR Green PCR Master Mix (Applied Biosystem). Primer sets and amplification conditions were those described (3, 4). Gene expression was normalized to that of β -actin in each sample. For

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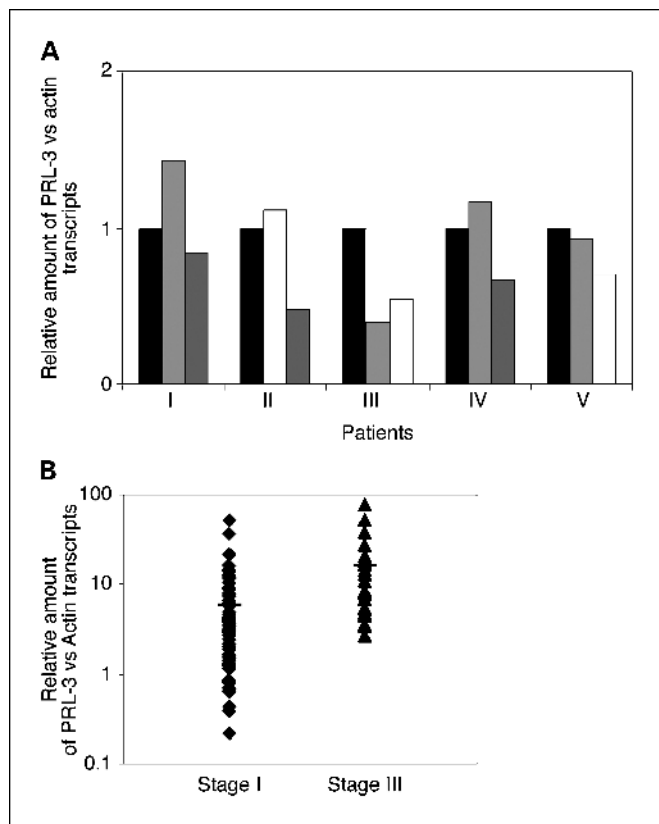


Fig. 1. Expression of PRL-3 phosphatase in human ovarian tumors and metastasis. **A**, quantitative analysis of PRL-3 mRNA in five primary tumors and corresponding peritoneal metastases. The expression of PRL-3 in primary tumor was arbitrarily set to 1 for each patient. *Black columns*, primary tumor; *light gray columns*, omentum metastases; *dark gray columns*, pelvic metastases; *white columns*, Douglas metastases. **B**, expression of PRL-3 mRNA by real-time PCR in primary ovarian cancer. Samples were obtained from patients with stage I and III ovarian cancer. A total of 61 stage I and 23 stage III tumors were analyzed.

in situ hybridization analysis, paraffin-embedded tumor samples were used and staining was done as previously reported (3). Digoxigenin-labeled antisense RNA probes were generated by PCR amplification of multiple PCR products (500-800 bp in length) corresponding to the entire PRL-3 transcript. *In vitro* transcription was performed using digoxigenin RNA labeling reagents and T7 RNA polymerase according to the instructions of the manufacturer (Roche, Indianapolis, IN). Six-micrometer-thick sections obtained from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, treated with pepsin, blocked with *in situ* hybridization solution (DAKO, Carpinteria, CA), and incubated with RNA probes (100 ng/mL) overnight at 55°C. After washing, sections were incubated at 37°C with RNase mixture (Ambion, Austin, TX) and stringently washed twice in a mixture of one part deionized formamide and one part 2× SSC (pH 7.5), then once with 0.1× SSC at 55°C. Before immunodetection, tissues were treated with peroxidase blocking reagent (DAKO) and blocked with 1% blocking reagent (DIG Nucleic Acid Detection kit; Roche) containing purified nonspecific rabbit immunoglobulins (DAKO). For signal amplification, a horseradish peroxidase rabbit antidigoxigenin antibody (DAKO) was used to catalyze the deposition of Biotin-Tyramide (GenPoint kit; DAKO). Additional amplification was achieved by adding horseradish peroxidase-rabbit anti-biotin (DAKO), biotin tyramide, and then alkaline phosphatase rabbit anti-biotin (DAKO). Signal was detected with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma, St. Louis, MO). All sections were exposed for 10 minutes. Cells were counterstained with Nuclear Fast Red (Biogenex, San Ramon, CA) and mounted with Crystal/Mount.

Human cell lines and cell growth. A2780, SKOV-3, OVCAR 3, OVCAR 5, OVCAR 8, OVCA 432, and Igrov-1 ovarian cancer cells were cultured in RPMI medium supplemented with 10% FCS. Human colocal carcinoma cell line HCT-116 was maintained in Iscove's medium supplemented with 10% FCS.

Cells were seeded at 6×10^4 cells/well in a 24-well plate. Twenty-four hours later, the cells were transfected with 8.3 nmol/L small interfering RNA (siRNA) duplexes using LipofectAMINE 2000 (Invitrogen, Milan, Italy) according to the protocol of the manufacturer. To analyze growth rate of the siRNA-treated cells compared with untreated

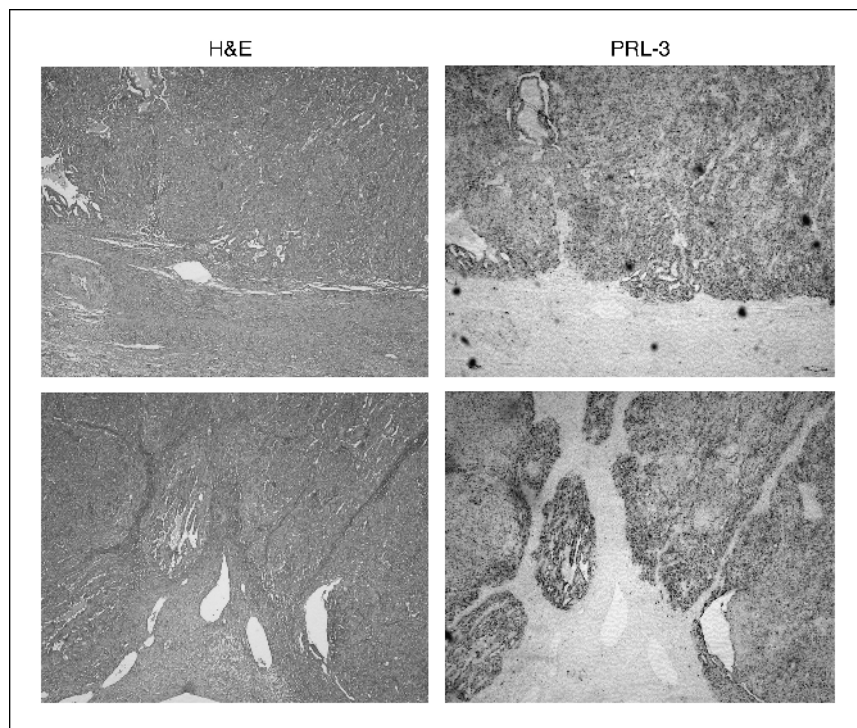
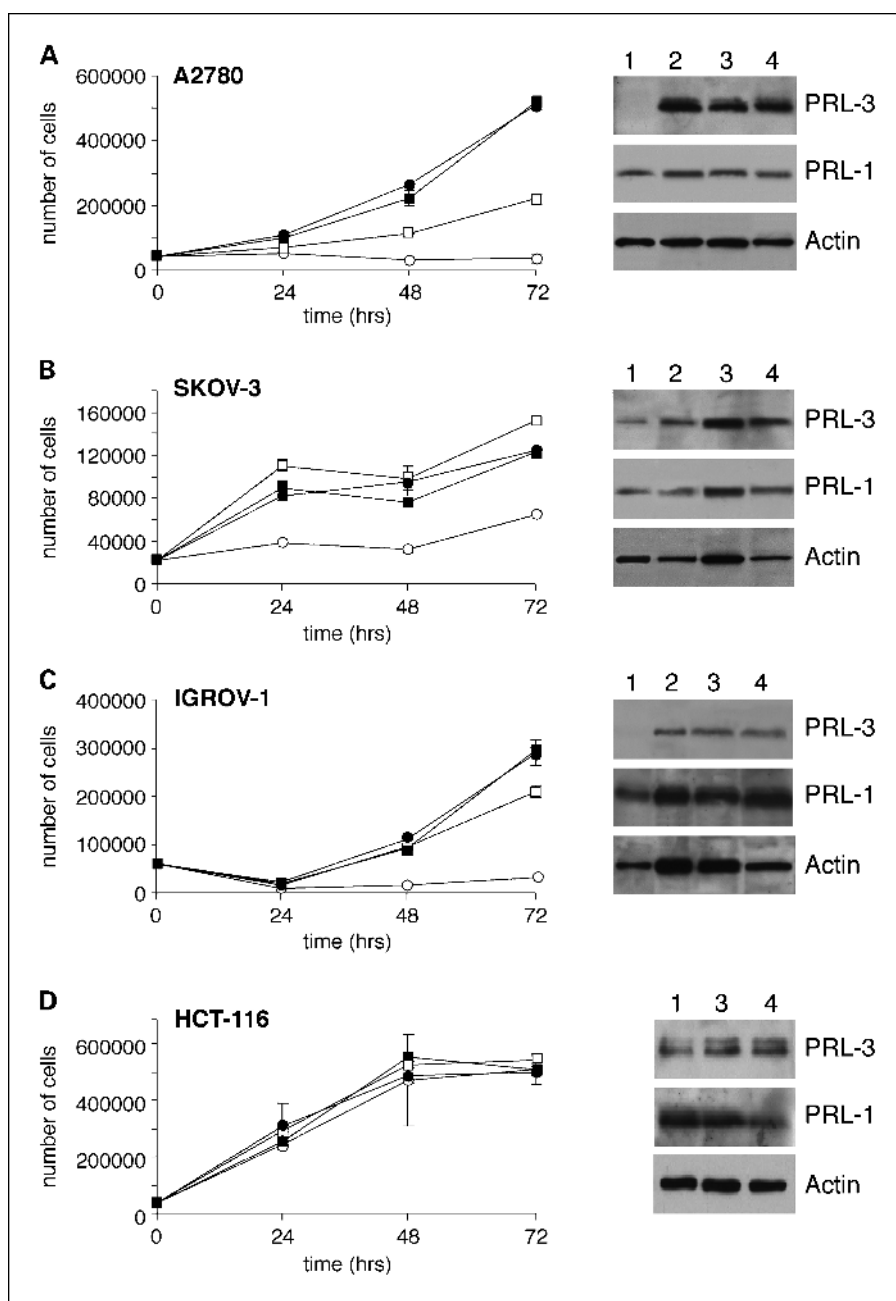


Fig. 2. Representative *in situ* hybridization on tissue slides from two stage III ovarian tumors. Starting from the left, the panel reports H&E staining and PRL-3 expression in consecutive sections for two patients.

Fig. 3. PRL-3 knocking down impairs the growth of human ovarian cancer cells *in vitro*. **A**, A2780 ovarian cancer cells were cultured in RPMI medium supplemented with 10% FCS. Cells were seeded at 6×10^4 cells/well in a 24-well plate. Twenty-four hours later, the cells were transfected with 8.3 nmol/L siRNA duplexes (against PRL-3 or scrambled) using LipofectAMINE 2000 (Invitrogen), according to the protocol of the manufacturer. To analyze growth rate of the siRNA-treated cells (○) compared with scrambled siRNA-treated cells (□), untreated (●), or mock-transfected (■) cells, the total cell number was counted. Three independent samples per point were analyzed. **B to D**, effect of PRL-3 siRNA on the growth of human ovarian cancer cells SKOV-3 and Igrrov-1 and human colon cancer cells HCT-116. The experimental conditions were the same as for A2780 cells. *Left*, the levels of PRL-3, PRL-1, and actin measured by Western blotting in each cell lines are reported. *Lane 1*, PRL-3 siRNA; *lane 2*, scrambled siRNA; *lane 3*, mock-transfected; *lane 4*, untreated.



or mock-transfected cells, the total cell number was counted using a Coulter Counter (Coulter Channelyser²⁵⁶; Beckman Coulter, Fullerton, CA). For time point 0 (24 hours after seeding), and for all subsequent time points, cells were washed twice in PBS, trypsinized, collected, and counted. Three independent samples per point were analyzed.

To determine siRNA-induced growth inhibition at longer times, cells were seeded in six-well plates and treated 24 hours later with 8.3 nmol/L PRL-3 siRNA or with LipofectAMINE 2000 alone. After 1 week, the cells were washed in PBS and stained with crystal violet. After extensive washings, the plates were dried and photographed.

Small interfering RNA preparation. The human PRL-3 cDNA was scanned to identify sequences of the AA(N₁₉)UU type that fulfill the criteria for siRNA design (8, 9). BLAST analysis showed no homology with other known human genes for two of such sequences, which were investigated for their ability to down-regulate PRL-3 expression using the Silencer siRNA Construction Kit (Ambion). The two PRL-3 mRNA

target sequences were 5'-AAATCTCGTTTCTCTTGACA-3' (siRNA6) and 5'-AAATTATTAGACCCCGGGCA-3' (siRNA31). The experiments reported were conducted with the siRNA6 oligo. Similar results were obtained with the siRNA31 oligo (data not shown). Scrambled siRNA was generated using the sequence 5'-AAAATCGACTCGTTTTTGCTC-3' as target.

Western blotting analysis. Protein expression was assessed in total cell extracts 24, 48, and 72 hours after transfection with PRL-3 siRNA or liposomes alone. Cells were lysed in 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 0.1% Nonidet NP40, 5 mmol/L EDTA, 50 mmol/L NaF in the presence of aprotinin, leupeptin, and phenylmethylsulfonyl fluoride as protease inhibitors for 30 minutes on ice. Insoluble material was pelleted at $13,000 \times g$ for 10 minutes at 4°C and the protein concentration was determined in the supernatant (containing both cytoplasmic and nuclear proteins) using a Bio-Rad assay kit (Bio-Rad, Milan, Italy). Twenty micrograms of total cellular proteins were separated on SDS-PAGE and electrotransferred to nitrocellulose. Immunoblotting

was carried out with the following antibodies: (a) monoclonal anti PRL-3 antibody (kindly provided by Dr. B. Vogelstein, Johns Hopkins University, Baltimore, MD), (b) anti hPRL-1 antiserum (kindly given by Dr. R. Herbst, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA), and (c) antiactin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was revealed by peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence (ECL; Amersham, Milan, Italy).

Results and Discussion

To gain insight into the molecular basis of ovarian cancer progression, we assessed the mRNA expression levels of PRL-3 in peritoneal metastasis and primary (stage III) tumors from each of five different patients. Figure 1A shows that in all patients, no significant differences in the expression between primary tumor and peritoneal metastasis were found. Interestingly, we found that in stage III ovarian tumors the levels of PRL-3 were already measurable. This suggested to us that, differently from what has been found in the colorectal cancer model, PRL-3 could be implicated in the early phases of ovarian cancer progression.

We therefore decided to look for differences in PRL-3 expression between early (I) and late (III-IV) stages. The analysis was done in 61 stage I and 23 stage III primary ovarian cancers and a clear difference in the expression was found, with advanced ovarian cancers expressing higher levels of the phosphatase than stage I ovarian cancers (Fig. 1B). The median expression level (relative to actin mRNA) was 5.9 for stage I tumors and 16.3 for stage III tumors ($P = 0.007$ ANOVA). When the expression of PRL-3 was related to the histotype or to the differentiation state, no differences were found (data not shown). By *in situ* hybridization, we found that the expression of PRL-3 phosphatase in stage III human ovarian cancer was confined to cancer cells, meaning that those cells were responsible for the increased PRL-3 expression found by quantitative PCR (Fig. 2). The differences were confirmed at the protein level by Western blotting (data not shown). DNA analysis by PCR in five tumor samples overexpressing the phosphatase showed no differences from stage I-derived DNA samples (data not shown), suggesting that the increased expression of PRL-3 in stage III ovarian cancer was not related to gene amplification.

Having shown that PRL-3 overexpression was associated with more advanced ovarian cancers, we examined the level of expression of this phosphatase in ovarian cancer cell lines. This is a prerequisite to assess the biological role of PRL-3 in the progression of these tumors. All the cell lines tested (A2780, SKOV-3, OVCAR 3, OVCAR 5, OVCAR 8, OVCA 432, Igrov-1) expressed detectable levels of PRL-3, assessed by either real-time PCR or Western blotting. Notably, expression was higher than in a panel of human colorectal cancer cells (data not shown). The contribution of PRL-3 expression in ovarian cancer cells growth was assessed by knockdown experiments with siRNA, targeting a region of the PRL-3 sequence with very low homology to the two closely related phosphatases PRL-2 and PRL-1. Figure 3A shows that treatment with PRL-3-specific siRNA dramatically reduced the growth of the ovarian cancer cells A2780 cells *in vitro*. Experiments conducted with different concentrations of the siRNA showed that a >50% inhibition was observed at a concentration as low as 1.7 nmol/L. This powerful effect was associated with a strong decrease in the expression of PRL-3 in cells treated with the siRNA in conditions in which the levels of actin protein and the related phosphatase PRL-1 did not change significantly. The growth and expression levels were compared with cells transfected with LipofectAMINE alone or with a scrambled siRNA. As it can be seen from Fig. 3A, a certain degree of growth inhibition was achieved when a scrambled siRNA was used. This does not seem to be related to the sequence per se because an unrelated luciferase siRNA gave similar results. Anyway, the effect observed with the scrambled siRNA was always well below the one obtained with the specific siRNA. Moreover, this effect was much less evident in two other ovarian cancer cell lines, SKOV-3 (Fig. 3B) and Igrov-1 (Fig. 3B), whereas the specific PRL-3 siRNA inhibited significantly the growth of these cells.

PRL-3 siRNA treatment reduced the expression of the PRL-3 protein levels both in SKOV-3 and Igrov-1 with little or no effect on PRL-1 and actin levels. Again, the scrambled siRNA did not modify PRL-3 expression. The same treatment conditions (either with the specific or with scrambled siRNA) did not affect the growth of the human colocal carcinoma cell line HCT-116, which expresses very low basal levels of PRL-3 (Fig. 3D).

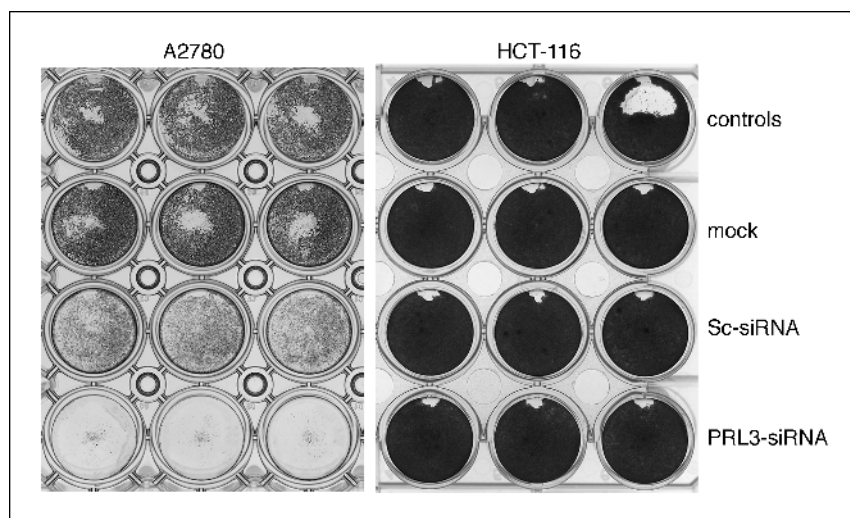


Fig. 4. Long-term effects of PRL-3 siRNA on the growth of the human ovarian cancer cell line A2780 and of human colon carcinoma cells HCT-116. Cells were seeded in 24-well plates and 24 hours later treated with 8.3 nmol/L of PRL-3 siRNA or scrambled (Sc) siRNA. Cells were incubated for 7 days and stained with crystal violet.

To assess whether the effect of the PRL-3 siRNA was also observed at longer times, A2780 and HCT-116 cells were plated in 24-well plates and treated 24 hours later with the PRL-3 siRNA or with the scrambled siRNA. After 1 week, the cells were washed and stained with crystal violet. As shown in Fig. 4, in A2780 cells, but not in HCT-116 cells, the PRL-3 siRNA completely abolished cell proliferation whereas control or mock-transfected cells grew normally. In HCT-116 cells, there was no difference between control and siRNA-treated cells. The scrambled siRNA did not alter the growth of HCT-116 cells, but had a weak but detectable effect against A2780 cells.

Altogether, these results show that PRL-3 plays an important role in the growth of ovarian cancer cells. Although the use of siRNA could activate unspecific effects leading to a slow down of cell proliferation, a clear specific, antiproliferative effect due to the inhibition of PRL-3 expression can be evidenced. Differently from colorectal cancer, in ovarian cancer PRL-3 is not overexpressed in metastasis compared with primary tumor, and its expression

seems to be associated with the transition from stage I to stage III ovarian cancer. It might, therefore, be associated with the invasive properties of ovarian cancer cells. Given that standard chemotherapy of ovarian cancer has limited effect on survival (10–12), our results suggest that PRL-3 might be a valuable target for the development of new anticancer strategies for this malignancy. This would be an additional potential use of anti PRL-3 drugs, together with their potential activity in halting the progression of cardiac hypertrophy and heart failure and in metastatic spread of the tumors (7, 13). In this respect, data already available on the structure of PRL-3 (14–16) will certainly be of great help in designing compounds with selective ability to inhibit PRL-3 activity and that might serve as a basis for anticancer agents for advanced ovarian cancer.

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