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14. ABSTRACT Mutations in mitochondrial DNA (mtDNA) are frequent in prostate cancer and they seem to occur early during prostate malignant transformation. Depletion of mtDNA in prostate cancer cells has been linked to acquisition of androgen-independence, progression to an invasive phenotype that is resistant to conventional chemotherapies, as well as induction of epithelial-mesenchymal transition leading to cancer metastasis. Using long-range genomic polymerase chain reaction, large deletion of mtDNA can be detected in prostate cancer tissues but not benign or normal prostate tissues. Noticeably, our study excludes the germ-line origin of the mutant mtDNA pattern in prostate cancer patient through analysis of the blood of the corresponding patient. Our data conclude that mtDNA deletion is due to carcinogenesis process in somatic prostate cells. In addition, our data have unveiled the molecular alteration in prostate cancer cells resulted from mtDNA deletion. For example, Skp2 protein elevation is often associated in prostate cells with loss of mtDNA. Also, the presence of Skp2 expression can decrease the expression of BRCA2 protein as an early biomarker of prostate neoplastic transformation, which is due to BRCA2 proteolysis.					
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INTRODUCTION

Depletion of the mitochondrial DNA (mtDNA) has been shown to promote malignant progression in prostate cancer cells. However, the molecular mechanisms underlying the association between mutant mtDNA and prostate cancer progression remain obscure. Mutant mtDNA has been associated with increased genomic DNA double-strand breaks. The resulting genomic instability could account for the multiple phenotypic effects observed in prostate cells harboring mutations/depletion of mtDNA, i.e. increased migration, acquisition of androgen-independence and progression to an invasive phenotype that is resistant to conventional chemotherapies. *BRCA2* is among the few genes known to be involved in repair of DNA double-strand breaks, and its loss is known to confer a significantly elevated risk to develop aggressive, rapidly progressing, high-grade prostate carcinoma. Our preliminary data indicated that *i)* a high proportion of sporadic human prostate carcinomas display heteroplasmic large deletion mutant mtDNA, which is associated with reduced *BRCA2* protein levels, and *ii)* experimental induction of mtDNA depletion in normal and cancer human prostate cell lines results in decreased *BRCA2* protein levels, overall suggesting that *BRCA2* loss might be an important molecular determinant of prostate cancer progression induced by mutant mtDNA. The goal of this proposal is to dissect the molecular mechanisms involved in suppression of *BRCA2* by mutant mtDNA and assess its role in mtDNA-related prostate cancer progression.

BODY

During the first year we have accomplished most of the planned experimental tasks. A portion of the experiments planned for the second year have been already performed during the first year, as detailed below.

Aim 1. Investigate the association between mutations in mtDNA and loss of *BRCA2* protein in prostate cancer specimens *in vivo* (Months 1-19)

Task 1. Analyze mtDNA mutations in human prostate cancer specimens (Months 1-11)

1.a Extract total DNA from 12 BPH and 12 PCA frozen biopsies and buffy coat samples, analyze mtDNA large deletions by long-PCR, mtDNA depletion by real-time PCR and mtDNA point mutations by sequencing (*Months 1-10*)

1.b Analyze the correlation between type/number of mtDNA mutations and clinical stage (*Months 10-11*)

We extracted total DNA from 6 BPH and 40 PCA frozen biopsies and buffy coat samples. Buffy coat samples were available for PCA but not for BPH patients. We analyzed mtDNA large deletions by long-PCR as previously described (1) and we found the presence of mtDNA large deletions in PCA but not in BPH biopsies (Table 1). Analysis in buffy coat samples from the same PCA patients showed absence of large deletions or a pattern different than that found in PCA, thus indicating the somatic origin of the PCA mtDNA profile and excluding a germline derivation. A typical mtDNA long-PCR pattern is reported in Figure 1. On a set of samples (6 BPH and 12 PCA) we also performed sequencing of the mtDNA coding regions in collaboration with the University of Bologna (Italy). As shown in Table 2, mtDNA point mutations were found both in PCA and BPH samples. No statistically significant correlation was found between mtDNA point mutations and clinical stage. Instead, a significant correlation was found between number of mtDNA large deletions and

Gleason grade ($p < 0.01$). Real-time PCR performed as described in (2) on 20 PCA and 6 BPH showed a decrease in mtDNA content in PCA compared with BPH (Figure 2).

Task 2. Measure the activity of mitochondrial respiratory complexes in human prostate cancer specimens (Months 9-15)

2.a Prepare total homogenate from 20 BPH and 20 PCA frozen biopsies and measure the activity of mitochondrial respiratory complexes spectrophotometrically (*Months 9-12*)

The activity of the mitochondrial respiratory complexes I/III, II/III, and IV was measured in isolated mitochondria prepared from 6 BPH and 20 PCA frozen biopsies as described in (3). Briefly, the electron transfer activities of complex I/III (NADH dehydrogenase/cytochrome bc_1 complex: catalyzes the electron transfer from NADH to ferricytochrome c) and complex II/III (succinate dehydrogenase/cytochrome bc_1 complex: catalyzes the electron transfer from succinate to ferricytochrome c) in isolated mitochondria were assayed by measuring ferricytochrome c reduction (26), using NADH and succinate as substrates, respectively. Specific activity (nmol ferricytochrome c reduced/min/mg protein) was calculated using the cytochrome c molar extinction coefficient of $21 \text{ mM}^{-1}\text{cm}^{-1}$ (3). The electron transfer activity of complex IV (cytochrome c oxidase: catalyzes the final step of the respiratory chain by transferring electrons from ferrocytochrome c to oxygen) was assayed by measuring ferrocytochrome c oxidation (3). Specific activity (nmol ferrocytochrome c oxidized/min/mg protein) was calculated with a molar extinction coefficient of $21 \text{ mM}^{-1}\text{cm}^{-1}$. As shown in Table 3, the activity of the complex I/III was significantly reduced in PCA compared with BPH ($0.01 < p < 0.001$; one-way ANOVA test).

Task 3. Measure BRCA2 protein expression in human prostate cancer specimens (130 samples) (Months 12-19)

3.a Prepare total protein lysates from low-grade vs. high-grade PCA frozen biopsies and analyze BRCA2 protein levels by immunoblotting (*Months 12-18*)

3.b Analyze BRCA2 protein expression in paraffin-embedded prostate specimens by immunohistochemistry (*Months 12-18*)

3.c Investigate the correlation between loss of BRCA2, mtDNA mutations, tumor stage (*Months 18-19*)

Tasks 3.a and 3.b have been completed during this first year, even though they were planned in the second year. Part of the results has been published (4). BRCA2 protein was analyzed by immunohistochemical analysis on a prostate tissue microarray containing cores from 80 prostate cancer specimens. We assessed the specificity of BRCA2 immunohistochemistry by testing a section of keratinizing squamous epithelium (we determined it expresses BRCA2 consistently and strongly) and a known BRCA2-positive breast carcinoma. As shown in Figure 3A, while the skin section showed almost exclusively nuclear staining, the breast carcinoma section showed both cytoplasmic and nuclear staining, as well as positive staining within the lumina of breast ductules, in agreement with a prior report.(5) To further test for the possibility of nonspecific antibody binding, we generated a peptide bearing the specific epitope sequence recognized by the antibody and used it to pre-adsorb the anti-BRCA2 antibody before application onto the breast cancer section and PCa tissue microarray. As expected, nuclear and cytoplasmic reactivity were successfully abolished in both tissues, thereby

confirming the specificity of the BRCA2 immunostaining (Figure 3, A-B). We performed BRCA2 immunohistochemistry in normal prostate, BPH, PIN, low-grade, and high-grade PCa specimens obtained from 80 patients with prostate cancer in a tissue microarray platform (Figure 3). After elimination of cores with significant tissue loss during processing, 48 cores of normal prostate, 79 cores showing BPH, 30 cores showing high-grade PIN, 78 cores with low-grade (Gleason 1-3) and 62 cores with high-grade (Gleason 4-5) PCa were examined. As shown in Figure 3B, BRCA2 immunostaining highlights the nuclei of both luminal and basal epithelial layers in normal and BPH, where it appears to accumulate immediately under the nuclear membrane. Weak cytoplasmic staining of the prostate epithelium, as well as scattered positive stromal cells and small lymphocytes were also observed. High-grade PIN and PCa showed almost complete loss of nuclear BRCA2 in basal and luminal cells in comparison with normal and hyperplastic prostate tissue ($P < 0.0001$, Figure 3B). There was an inverse correlation between frequency of BRCA2 staining and Gleason grade, albeit did not reach statistical significance ($P = 0.05$ for the inverse correlation; threshold of significance: $P < 0.05$). Quantitative immunostaining data including the percentage of incidence of BRCA2-positive specimens among each specimen group and cell labeling frequency (%) are summarized in Tables 4 and 5.

To confirm that decreased BRCA2 levels in sporadic PCa were not due to the presence of BRCA2-truncating mutations, we performed Western blot analysis in a cohort of normal and PCa specimens using antibodies recognizing three different regions of the BRCA2 protein, i.e. the N-terminal, an internal and the C-terminal region (Figure 4A). Western blot using the antibody against the N-terminal region, which is expected to recognize any BRCA2-truncation variants, showed full-length BRCA2 protein and a degradation fragment of approximately 268 kDa both in normal and PCa tissues, but no truncation variants (Figure 4B). Of note, the relative amount of the 268-kDa degradation product was higher in PCas than in normal prostates. All PCa specimens analyzed by Western blotting exhibited a statistically significant decrease of full-length BRCA2 protein compared to the normal prostate tissue ($0.001 < P < 0.007$; Figure 4C) which is consistent with the immunohistochemistry results. Western blot using antibodies against an internal and the C-terminal region of BRCA2 showed similar results (Figure 4B). In addition to the 390 kDa full-length BRCA2 protein, the C-terminal antibody used in immunohistochemical analysis detected also smaller BRCA2 degradation products (approx. 280-300 kDa) in PCa but not in normal prostate (Figure 4B), suggesting activation of BRCA2 proteolytic processes in cancer tissues.

We previously described that Skp2 mediates BRCA2 ubiquitination and degradation in prostate cells.(6) Given the biochemical link between BRCA2 and Skp2, we asked whether a correlation between Skp2 and nuclear BRCA2 levels could be demonstrated *in vivo* in PCa biopsies. Our experiments demonstrated that while Skp2 expression was almost undetectable in normal and BPH prostate tissue, 48% of high-grade PIN, 70% of LGPCa, and 89% of HGPCa showed strong Skp2 staining [Figure 3C; (4)]. In agreement with prior reports,(7-9) the observed Skp2 pattern of expression included nuclear and cytoplasmic staining. The nuclear staining was assessed in terms of number of positive nuclei (labeling frequency %) and staining intensity (index) on each specimen. They were then compared with the nuclear BRCA2 labeling frequency and index on the same set of specimens (Figure 5). A statistically significant inverse correlation was found between

BRCA2 and Skp2 labeling frequency (Spearman's $\rho = -0.42$, $p < 0.0001$) and intensity index (Spearman's $\rho = -0.38$, $p < 0.0001$).

We prepared additional protein lysates from 6 BPH and 20 PCA (10 high-grade and 10 low-grade PCA) and we are now analyzing the correlation between loss of BRCA2 protein and presence/number of mtDNA large deletions in PCA (Aim 1, task 3.c).

Aim 2. Identify the molecular mechanisms of down-regulation of BRCA2 expression by mutant mtDNA (Months 10-24)

Task 1. Investigate the regulation of BRCA2 expression by mutant mtDNA at the transcription, translational, post-translational level (Months 10-19)

1.a Generate PNT1A/C4-2 trans-mitochondrial cybrids and analyze BRCA2 protein levels in cybrids versus PNT1A wild-type (Months 10-12)

We are in the process of generating the PNT1A/C4-2 trans-mitochondrial cybrids.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of presence of large deletion mtDNA mutants in PCA
- Demonstration of reduced activity of mitochondrial respiratory complexes I/III in PCA versus BPH
- Demonstration of reduction of BRCA2 protein levels early during prostate neoplastic transformation (high-grade PIN)
- Inverse correlation between loss of BRCA2 protein and increase in Skp2 levels in PCA tissues

REPORTABLE OUTCOMES

Publication

Arbini AA, Greco M, Yao JL, Bourne P, Marra E, Hsieh JT, di Sant'agnese PA, Moro L. Skp2 overexpression is associated with loss of BRCA2 protein in human prostate cancer. *Am J Pathol.* 2011 May;178(5):2367-76.

CONCLUSION

Our results show the presence of mtDNA large deletions in PCA but not BPH or normal prostate tissues. Point mutations were instead found both in benign prostates and in PCA, indicating that they are not specific for prostate neoplastic transformation. To our knowledge, this is the first time that a complete study of the pattern of mtDNA mutations in PCA has been performed. More importantly, this study excludes the germline origin of the mutant mtDNA pattern in PCA through analysis of the blood of the corresponding patient.

Finally, our data have shown for the first time that loss of BRCA2 protein is an early biomarker of prostate neoplastic transformation and that it is inversely correlates with Skp2 protein levels, an oncoprotein overexpressed in prostate carcinoma which is involved in BRCA2 proteolysis.

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APPENDICES

Manuscript: Arbini AA, Greco M, Yao JL, Bourne P, Marra E, Hsieh JT, di Sant'agnese PA, Moro L. Skp2 overexpression is associated with loss of BRCA2 protein in human prostate cancer. *Am J Pathol*. 2011 May;178(5):2367-76.

SUPPORTING DATA

Figure legends

Figure 1. Occurrence of mtDNA large deletions in PCa. Total DNA was extracted from BPH and PCa frozen biopsies and long-PCR of the whole mtDNA was performed using specific back-to-back primers. For PCa samples, mtDNA long PCR analysis was also performed in the blood of the corresponding patient. PCR products were analyzed by 0.8% agarose gel electrophoresis. λ xH: DNA marker

Figure 2. mtDNA content in PCa is reduced compared with BPH. Total DNA was extracted from 6 BPH and 20 PCa tissues and mtDNA content was analyzed by real-time PCR using specific primers. mtDNA content in PCa tissues is reported in relative units compared with the mean of 6 BPH, set as 1.

Figure 3. Loss of BRCA2 protein expression in PCa. **A:** Almost exclusive nuclear localization of BRCA2 observed in squamous surface epithelium (x400). Nuclear and cytoplasmic localization of BRCA2 in breast carcinoma was suppressed by preincubation of the antibody with a competing peptide. The lumina of breast ductules were also positive for BRCA2 staining (x200). **B-C:** Tissue microarray sections were stained using either anti-BRCA2 antibody (**B**) or anti-Skp2 antibody (**C**). **B: Top panel:** Nuclear and cytoplasmic BRCA2 localization in normal prostate and BPH (x400). *Lower panel:* Significant loss of nuclear BRCA2 in high-grade PIN and PCa. Two different PCa samples are shown (x400). Cytoplasmic and nuclear localization of BRCA2 in prostate carcinoma was suppressed by preincubation of the antibody with a competing peptide. PIN: x200, with enlarged panel at x400. **C:** Skp2-positive cells were rarely seen in benign glandular epithelium. In contrast, increased levels of Skp2 were seen in high-grade PIN and PCa. Weak or moderate Skp2 cytoplasmic immunoreactivity could be seen in addition to predominant nuclear reactivity in PIN and PCa cells. BPH, PIN and PCa in the middle: x200; PCa on the right: x400.

Figure 4. Sporadic PCas show decreased BRCA2 protein expression and absence of BRCA2 truncation products. **A:** Epitopes recognized by the anti-BRCA2 antibodies used for Western blotting analysis of prostate tissues and cells. **B-C:** Total protein extracts (50 μ g) from 6 normal prostates (N) and 11 PCa specimens were analyzed for BRCA2 expression and presence of BRCA2 protein truncation products by Western blot analysis using different antibodies recognizing either the N-terminal, an internal or the C-terminal region of BRCA2. The antibody generated against the N-terminal region of BRCA2 is expected to recognize potential BRCA2 truncation variants resulting from *BRCA2* gene mutations. In **B**, representative blots for 2 normal prostates and 7 PCa samples are shown. The antibody against the N-terminal recognized the full-length BRCA2 protein and also a degradation fragment of approximately 268 kDa both in normal and in carcinoma tissues. The antibody against the C-terminal region recognized full-length BRCA2 in normal and PCa samples and a degradation fragment of approximately 280-300 kDa only in PCa

specimens. Tubulin was used as loading control. In **C**, a quantification of full-length BRCA2 protein (390 kDa) in PCa specimens is reported as percentage of the normal prostate. The results are the mean $\pm$ SE of three different blots using the N-terminal antibody. Similar results were obtained using the internal and the C-terminal antibodies. Different sets of filters were prepared to be blotted with each antibody. **D**: Total protein extracts (50 μ g) from normal prostate primary epithelial cells (PrEc), and from PNT1A, PC-3, LNCaP and DU145 prostate cell lines were analyzed for BRCA2 protein expression as described in **B**.

Figure 5. Correlation of BRCA2 with Skp2 protein levels. **A**: BRCA2 labeling frequency was negatively correlated with Skp2 labeling frequency ($p < 0.00001$; Spearman's correlation coefficient testing). **B**: BRCA2 labeling intensity was negatively correlated with Skp2 labeling intensity ($p < 0.00001$; Spearman's correlation coefficient testing). Each box and the associated bars represent the values of middle 50% and the range of data, respectively. The dark line within a box indicates the median. N = number of cores.

Figure 1

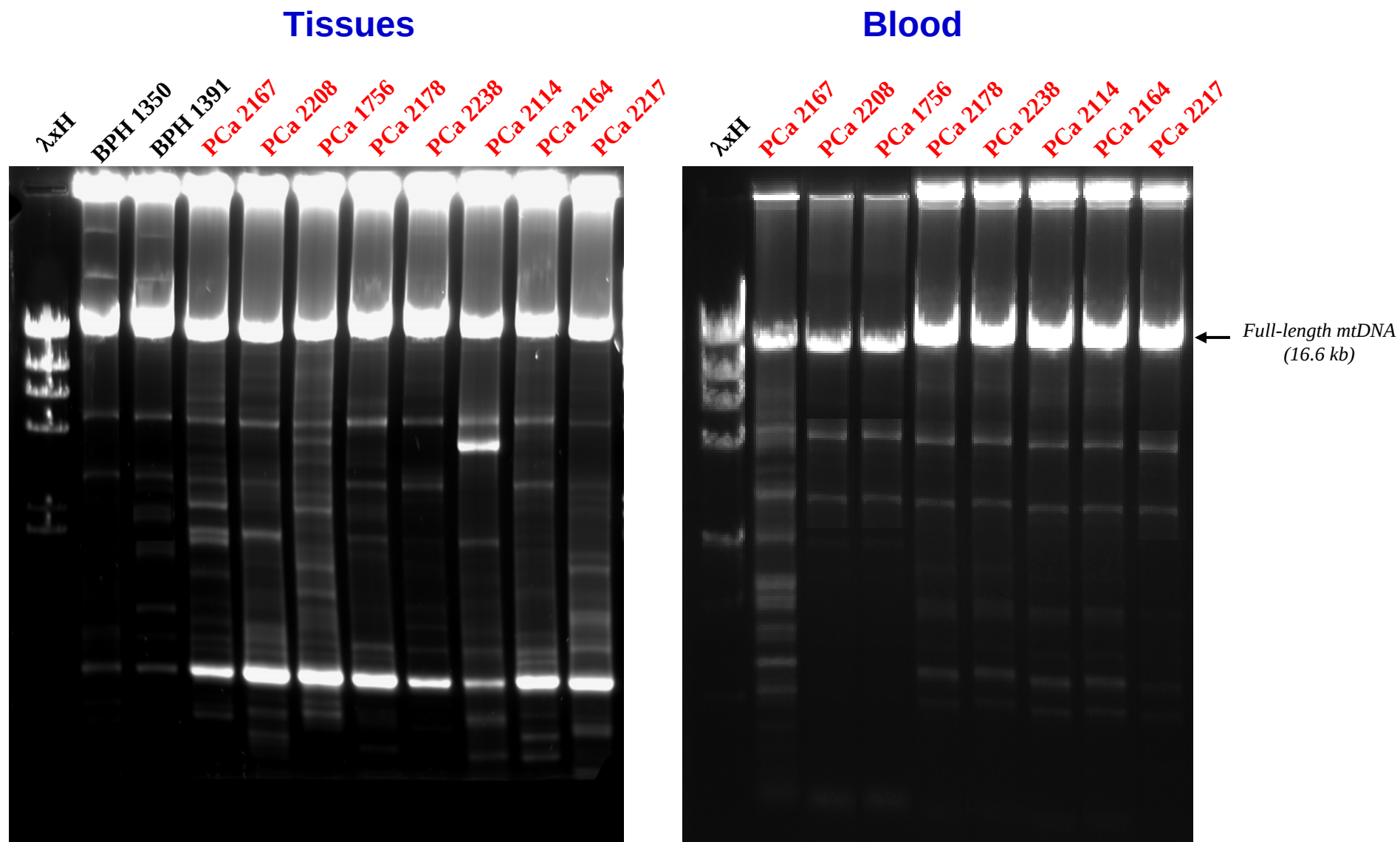
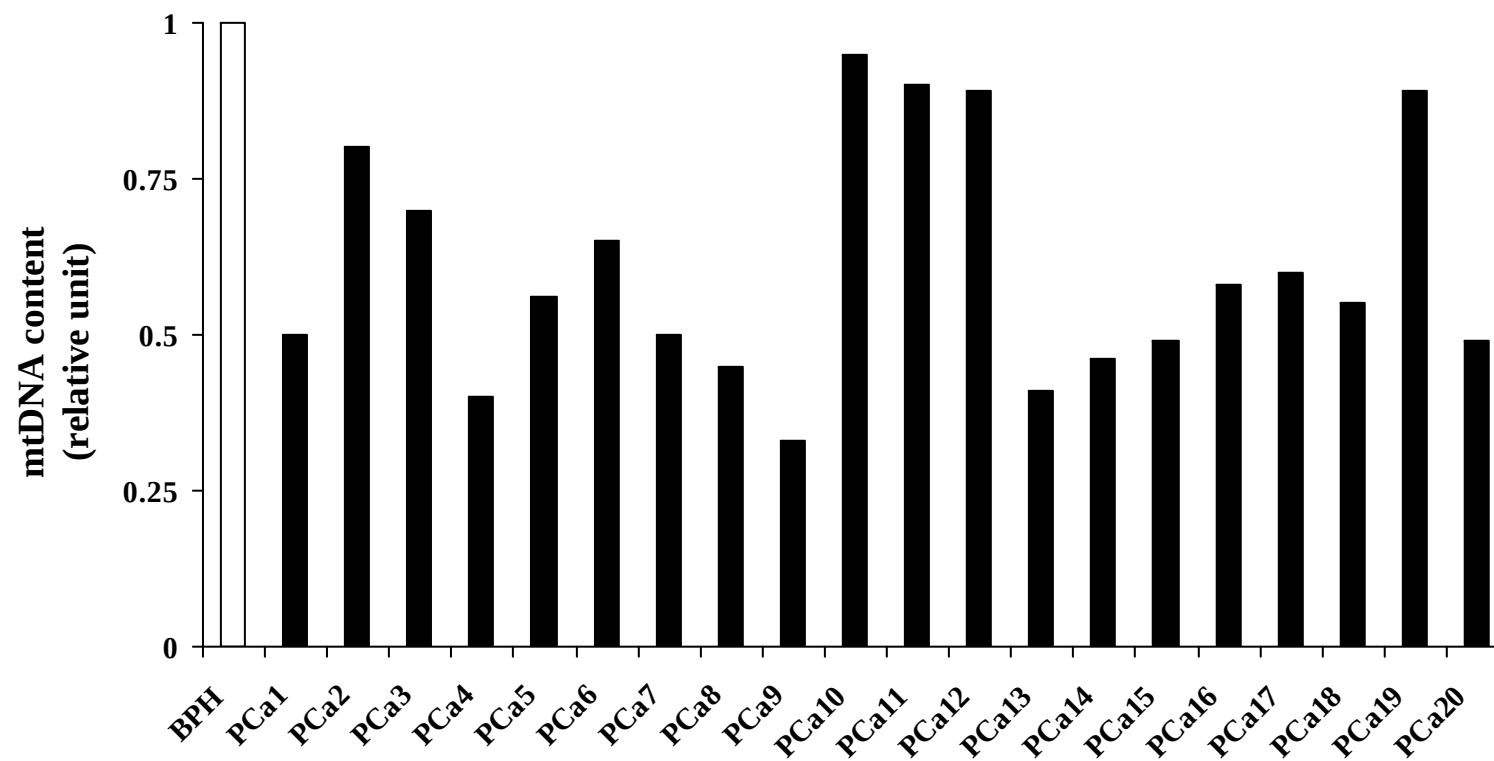


Figure 2



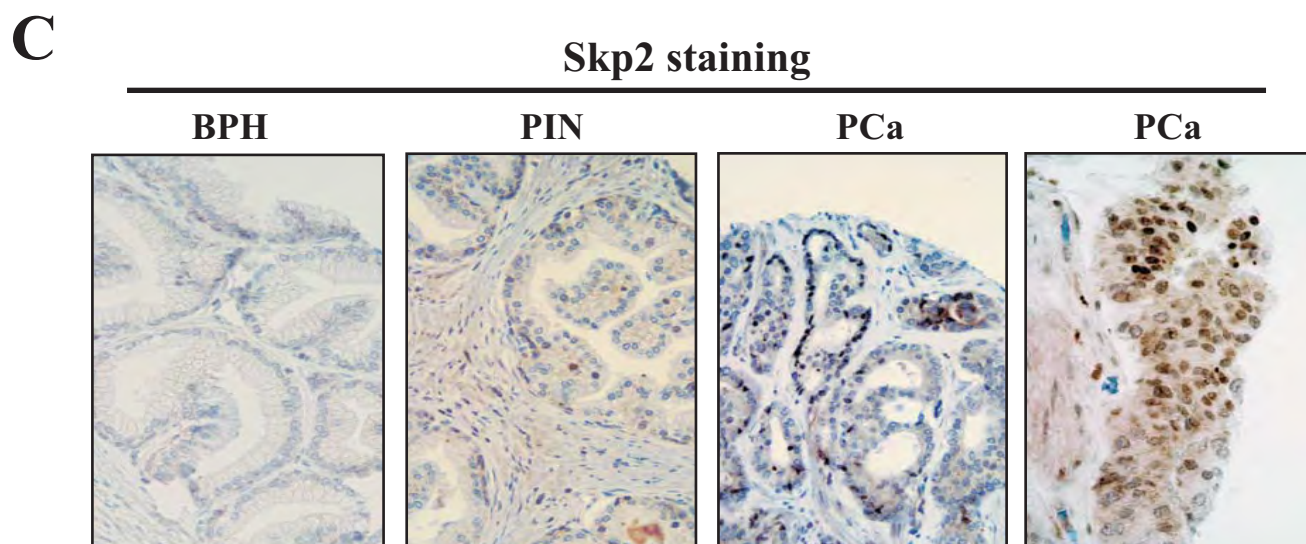
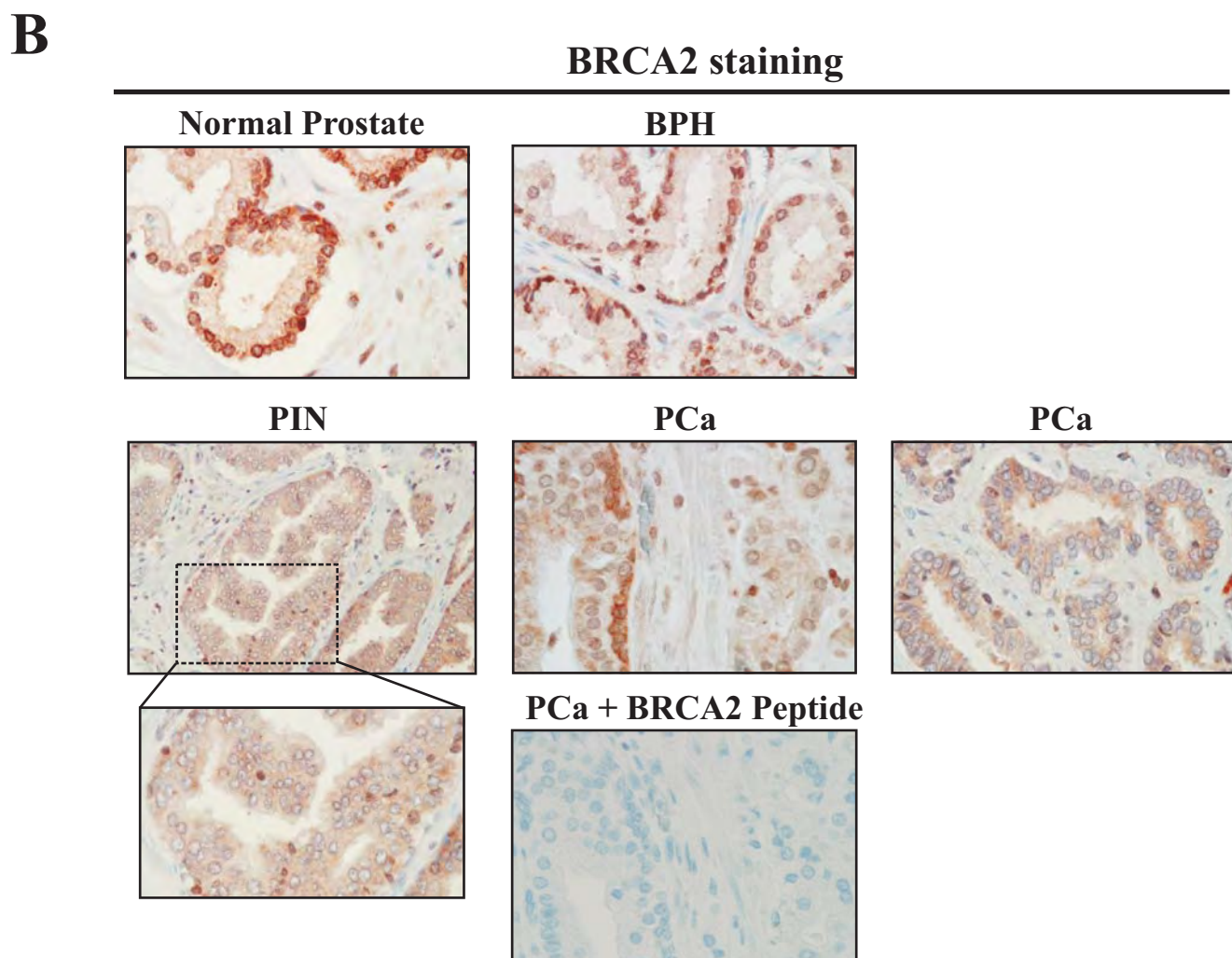
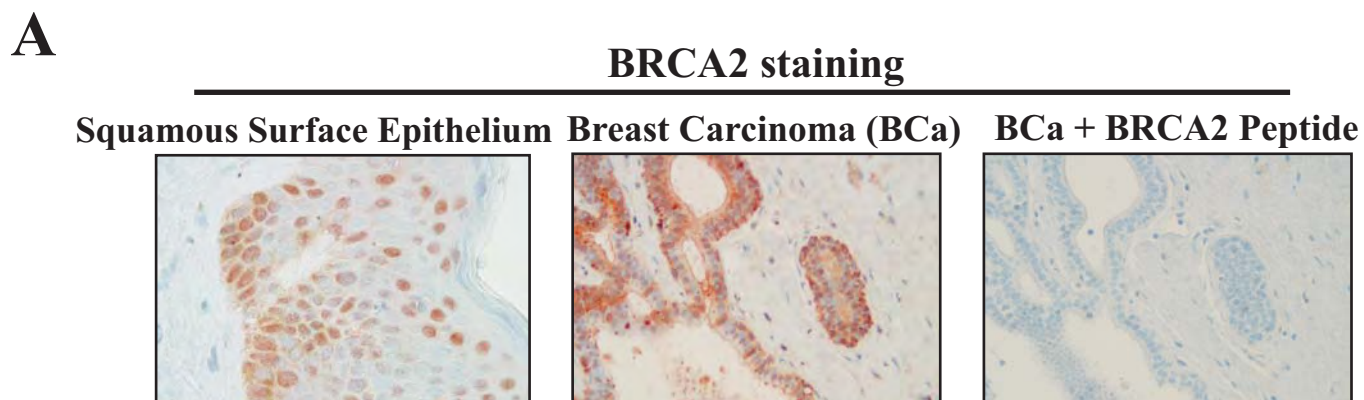


Figure 4

A**BRCA2 antibodies**

Name	Epitope (aminoacids)
a-BRCA2 I-17	N-terminal (Within the first 50 aminoacids)
a-BRCA2 H300	Internal (2520-2819)
a-BRCA2 Ab-2	C-terminal (3245-3418)

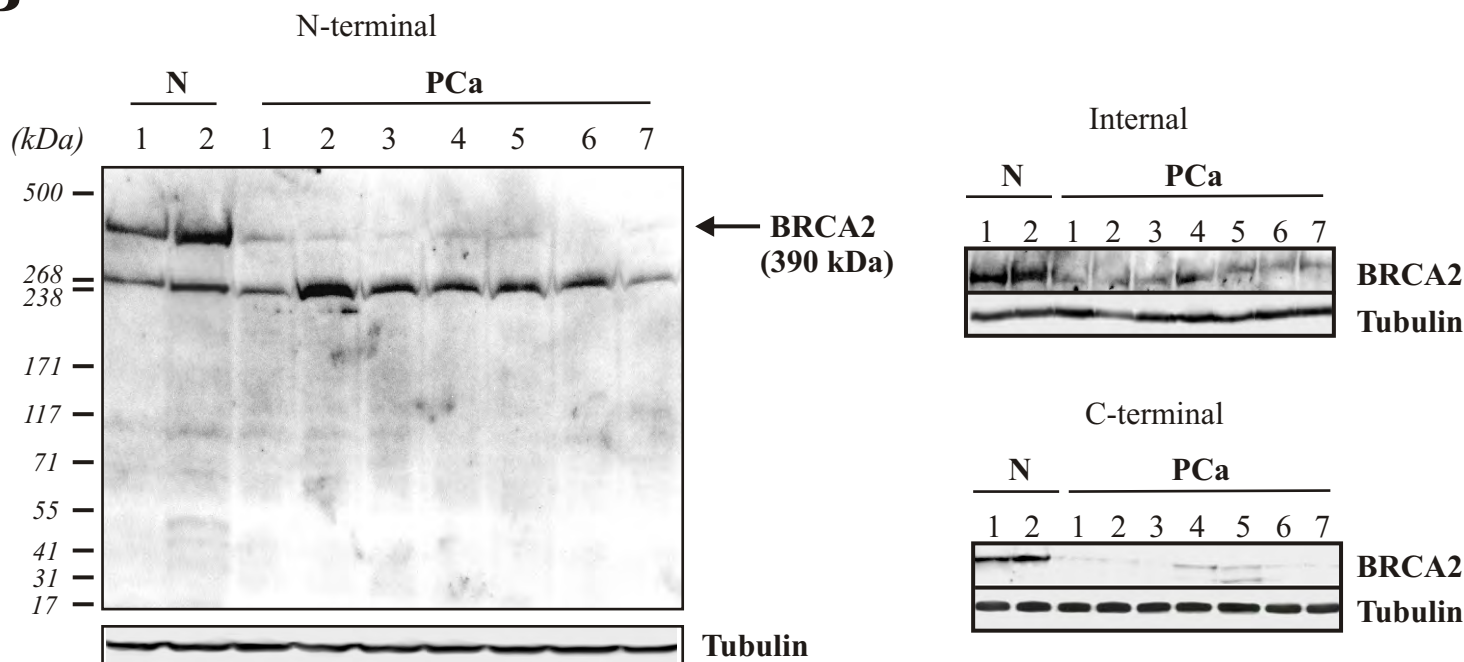
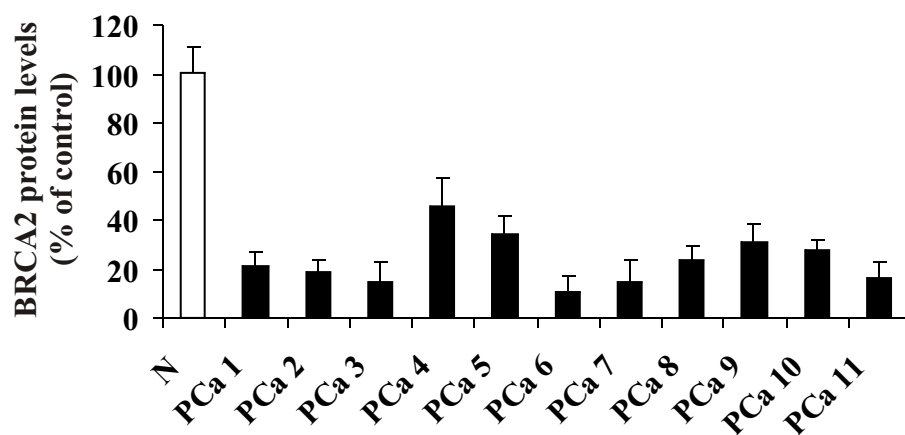
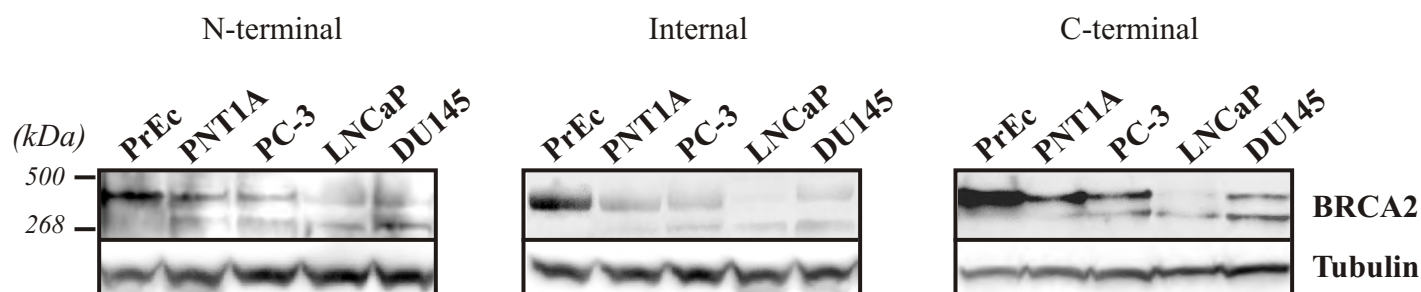
B**C****D**

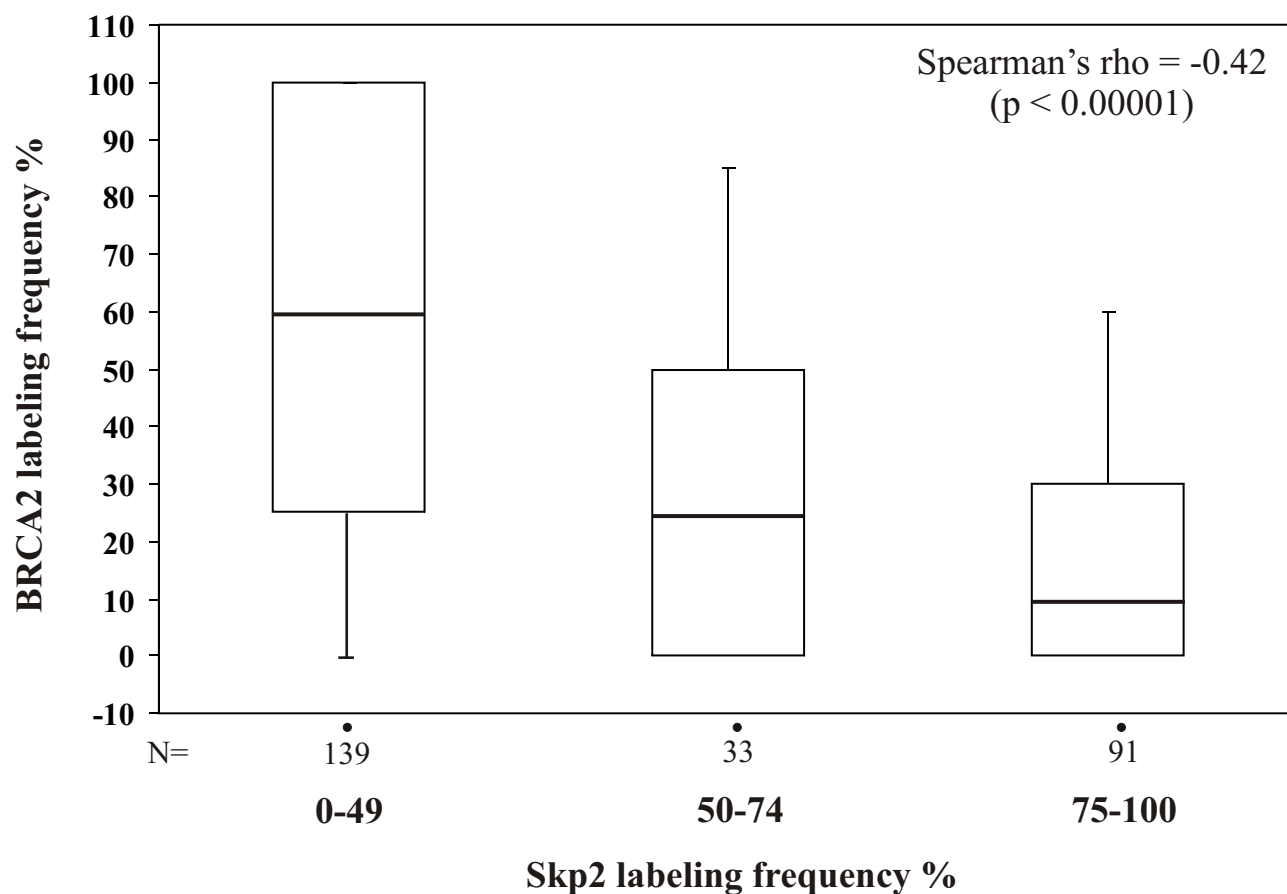
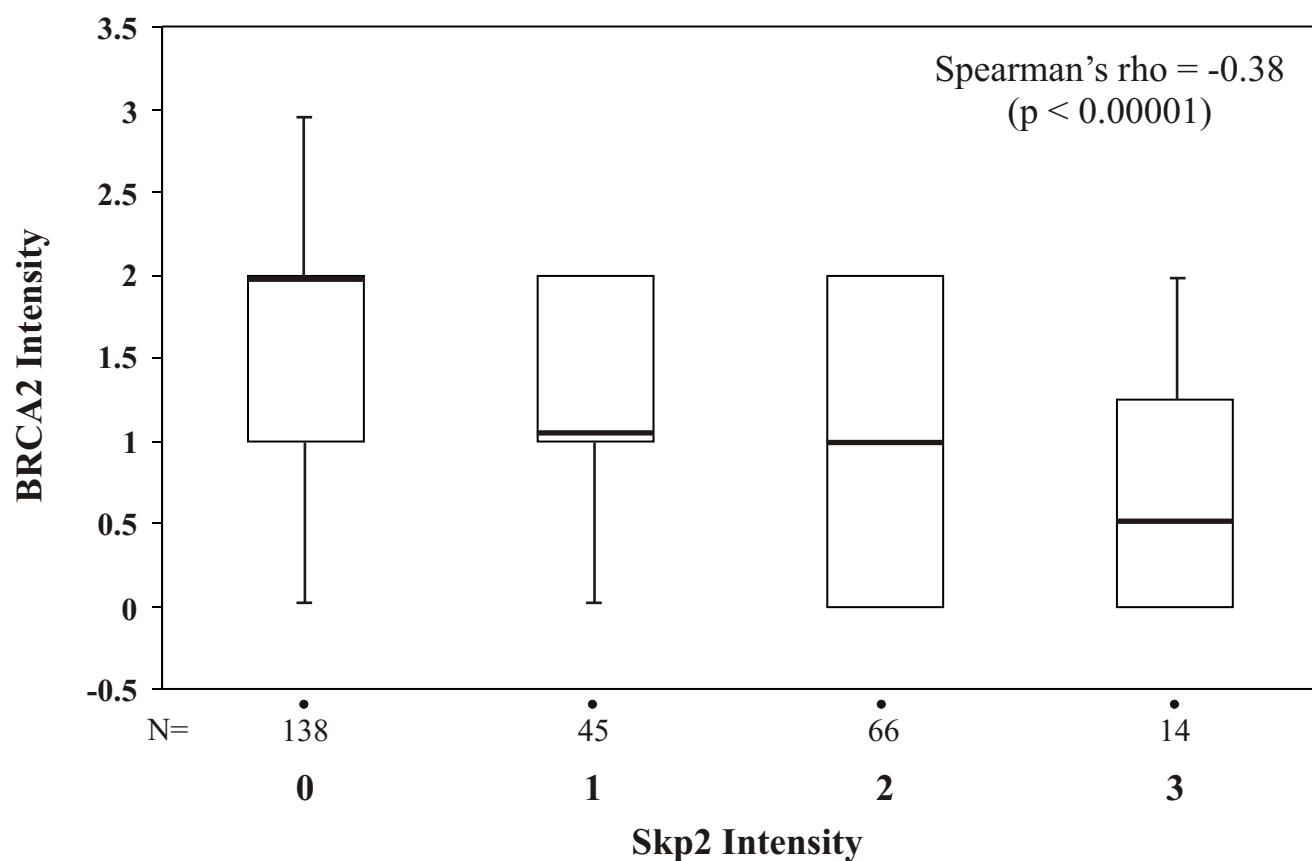
Figure 5**A****B**

Table 1. Patients' Clinicopathological Data

Patient No.	Age (y)	PSA (ng/ml)	Histopathological Diagnosis (Gleason Grade)	mtDNA Deletions in Tissue (No.)	mtDNA Deletions in Blood (No.)
0859	27	NR	Normal	0	n.a.
51883	35	NR	Normal	0	n.a.
50744	20	NR	Normal	0	n.a.
12387	18	NR	Normal	0	n.a.
49593	19	NR	Normal	0	n.a.
49160	29	NR	Normal	0	n.a.
1350	55	4.9	BPH	0	n.a.
1391	61	3.3	BPH	0	n.a.
1465	67	3.89	BPH	0	n.a.
1519	58	2.7	BPH	1	n.a.
1526	61	3.5	BPH	2	n.a.
1116	59	5.4	PCa (4)	4	n.a.
2105	52	5.7	PIN (Gleason 6)	1 Hom.	0
2217	53	5.1		8	0
1734	59	33.9	PCa (6)	0	0
1766	65	0.05	PCa (6)	9	0
1782	55	7.8	PCa (6)	3	0
1800	63	4.36	PCa (6)	6	0
1804	56	4.2	PCa (6)	7	3
2050	49	2.2	PIN (Gleason 6)	10	0
2081	58	2.9		5	0
1756	66	17	PCa (7)	11	0
1844	58	5.2	PCa (7)	9	0
2011	59	4.7	PCa (7)	10	0
2023	58	2.67	PCa (7)	9	0
2024	64	6.15	PCa (7)	7	3
2030	61	NR	PCa (7)	6	0
2130	69	6.46	PCa (7)	8	2
2131	65	7	PCa (7)	9	0
2148	60	3.6	PCa (7)	3	0
1150	66	7.7	PCa (7)	3	n.a.
1153	56	2.28	PCa (7)	0	n.a.
1208	69	5.3	PCa (7)	5	n.a.
1248	65	8.38	PCa (7)	4	n.a.
1285	70	18	PCa (7)	5	n.a.
1752	48	28.8	PCa (7)	5	0
2114	58	9.7	PCa (7)	6	0
2160	62	6.15	PCa (7)	5	1
2178	60	15	PCa (7)	5	0
2208	62	5.1	PCa (7)	6	0
2238	59	5.1	PCa (7)	0	0
1147	66	6	PCa (8)	16	n.a.
1838	74	5.3	PCa (8)	14	4
1211	66	55	PCa (9)	7	n.a.
1216	63	5.42	PCa (9)	5	n.a.
1314	64	13	PCa (9)	8	n.a.
1727	61	18	PCa (9)	10	7
1898B	60	45.3	PCa (9)	14	9
2096	63	8.5	PCa (9)	16	0
2164	53	8.8	PCa (9)	13	4

2167	76	7.8	PCa (9)	15	12
2371	76	7.8	PCa (9)	13	3
2472	53	11	PCa (9)	16	13

NR: Not Recorded; n.a.: not available; Hom.: Homoplasmic

Table 2. mtDNA point mutations in PCA and BPH specimens.

Sample		Base change	AA change	Gene
BPH1	1350T	A15929G	-	<i>MT-TT</i>
BPH2	1365T	T12713C	I126T	<i>MT-ND5</i>
BPH3	1391T	G9300A	A32T	<i>MT-COIII</i>
BPH4	1465T	T14501C	I58T	<i>MT-ND6</i>
BPH5	1519T	A12634G	I100V	<i>MT-ND5</i>
		A13630G	T432A	<i>MT-ND5</i>
		T8668C	W48R	<i>MT-ATP6</i>
BPH6	1526T	T5160C	S231P	<i>MT-ND2</i>
PT1	2167T			
PT2	2208T	T11204C	F149L	<i>MTND4</i>
PT3	2160T			
PT4	2178T			
PT5	2238T	C9739T	A178V	<i>MTCOIII</i>
PT6	2114T	T11253C	I195T	<i>MTND4</i>
PT7	2164T	G15428A	D228N	<i>MTCYTB</i>
		T12631G	S99A	<i>MTND5</i>
PT8	1838T			
PT9	1727T			
PT10	1898BT			
PT11	2371T			
PT12	2472T	G9738A	A178T	<i>MTCOIII</i>
PT13	2024T			
PT14	2131T	A14220C	L152P	<i>MTND6</i>
		G5849A	-	<i>MT-TY</i>
PT15	2096T			

Table 3. Activity of mitochondrial respiratory complexes

Prostate Tissues	Complex I/III <i>(nmol/min/mg protein)</i>	Complex II/III <i>(nmol/min/mg protein)</i>	Complex IV <i>(nmol/min/mg protein)</i>
BPH (1-6)	52 ± 4	21 ± 4	11 ± 3
PCa 1	26 ± 4	16 ± 3	6 ± 2
PCa 2	39 ± 3	21 ± 2	9 ± 2
PCa 3	41 ± 2	18 ± 4	9 ± 1
PCa 4	21 ± 4	12 ± 3	7 ± 3
PCa 5	19 ± 3	16 ± 3	6 ± 3
PCa 6	21 ± 2	18 ± 1	8 ± 1
PCa 7	23 ± 4	9 ± 3	9 ± 3
PCa 8	29 ± 3	11 ± 2	6 ± 2
PCa 9	31 ± 2	17 ± 2	7 ± 1
PCa 10	41 ± 5	18 ± 3	10 ± 2
PCa 11	39 ± 1	21 ± 3	8 ± 2
PCa 12	42 ± 4	18 ± 4	5 ± 1
PCa 13	21 ± 4	11 ± 3	7 ± 3
PCa 14	19 ± 1	20 ± 3	6 ± 3
PCa 15	14 ± 2	18 ± 1	8 ± 1
PCa 16	17 ± 4	16 ± 3	5 ± 1
PCa 17	19 ± 3	11 ± 2	6 ± 2
PCa 18	35 ± 5	17 ± 4	7 ± 1
PCa 19	50 ± 4	22 ± 1	10 ± 2
PCa 20	36 ± 3	17 ± 2	7 ± 1

Table 4. Nuclear BRCA2 protein expression in human prostatic tissues ^a

Prostate Tissue	<i>n</i> ^b	BRCA2 labeling frequency (%)			%
		Median	Mean ± SE	Incidence	
Normal	48	75	68.23 ± 4.75	39/48	81
BPH	79	50	63.16 ± 3.50	63/79	80
PIN	30	25 ^c	37.59 ± 5.87	17/30	57
Carcinoma	140	10 ^d	21.71 ± 2.35	32/140	23

^a Nuclear BRCA2 immunostaining was demonstrated in normal prostatic epithelial cells, BPH, PIN lesions, and in carcinomas

^b Number of cores

^c Frequency was significantly lower than in normal prostate tissue (P = 0.0007)

^d Significantly lower than normal prostate tissue and PIN lesions (P = $5.32e^{-13}$ and P = $6.79e^{-08}$, respectively)

Table 5. BRCA2 protein expression in prostatic carcinoma tissues^a

	<i>n</i> ^b	BRCA2 labeling frequency (%)			
		Median	Mean ± SE	Incidence	%
PCa	140	10	21.71 ± 2.35	32/140	23
LGPCa	78	10	18.97 ± 2.61	20/78	26
HGPCa	62	0 ^c	25.16 ± 4.16	12/62	19

^a BRCA2 levels expressed as nuclear labeling frequency of cancer cells in tissue microarray composed of cores from radical prostatectomy specimens stratified according to their pathological features.

^b Number of cores

^c Frequency was not significantly different than low-grade carcinomas (P = 0.05)

Tumorigenesis and Neoplastic Progression

Skp2 Overexpression Is Associated with Loss of BRCA2 Protein in Human Prostate Cancer

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BRCA2 (breast cancer 2, early onset) is a tumor suppressor gene that confers increased susceptibility for prostate cancer (PCa). Previous *in vitro* experiments demonstrated that Skp2, an E3 ubiquitin ligase aberrantly overexpressed in PCa, is involved in the proteolytic degradation of BRCA2 in PCa cells, suggesting that the BRCA2-Skp2 interaction may play a role in prostate tumorigenesis. Herein, we investigated BRCA2 and Skp2 expression during PCa development using a prostate TMA. Although luminal and basal benign prostate epithelium exhibited moderate to strong nuclear BRCA2 immunostaining, the intensity and number of positive nuclei decreased significantly in high-grade prostatic intraepithelial neoplasia and PCa. Decreased frequency and intensity of nuclear BRCA2 labeling were inversely correlated with Skp2 expression in high-grade prostatic intraepithelial neoplasia and PCa. To functionally assess the effects of BRCA2 and Skp2 expression on prostate malignant transformation, we overexpressed Skp2 in normal immortalized prostate cells. Skp2 overexpression reduced BRCA2 protein and promoted cell growth and migration. A similar phenotype was observed after reduction of BRCA2 protein levels using specific BRCA2 small-interfering RNA. Forced BRCA2 expression in Skp2-overexpressing stable transfectants inhibited the migratory and growth properties by >60%. These results show that loss of BRCA2 expression during prostate tumor development is strongly correlated with both migratory behavior and cancer growth and include Skp2

as a BRCA2 proteolytic partner *in vivo*. (Am J Pathol 2011, 178:2367–2376; DOI: 10.1016/j.ajpath.2011.01.050)

Prostate cancer (PCa) is a leading cause of morbidity and mortality among men in the Western world. Despite increased awareness and improved methods for early detection, many patients succumb to disseminated cancer that is resistant to conventional therapies.¹ The identification of early molecular events in PCa represents the first step in devising early diagnostic tools and new anti-cancer therapies aimed at preventing disease progression and dissemination.

BRCA2 (breast cancer 2, early onset) is a tumor suppressor gene that, when mutated, confers an increased susceptibility to developing PCa.^{2,3} The inheritance of one defective allele confers PCa predisposition; and neoplastic cells from predisposed individuals frequently exhibit loss of heterozygosity in the remaining wild-type allele,⁴ consistent with a critical role of BRCA2 in prostate tumor suppression. The major identified function of the BRCA2 protein is to form complexes with Rad51 in the nuclei, which orchestrate homologous recombinational repair of double-stranded DNA breaks.⁵ In addition to its role in mediating DNA repair, BRCA2 plays a role in the stabilization of stalled DNA replication forks, centrosome duplication, mammalian gametogenesis, cytokinesis, telomere replication, and transcriptional regulation.^{6–8} In addition to cancer predisposition, recent clinical evidence suggests that patients with PCa who carry germline BRCA2 mutations display a more aggressive phenotype with worse survival rates than noncarrier patients^{9–14}; we have provided mechanistic evidence demonstrating that loss of BRCA2 pro-

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motes PCa cell proliferation and invasion in experimental cell-line models.^{15–18} These latter findings are important despite the fact that mutations in *BRCA2* are rare in sporadic PCas^{2,9,19,20}; in sporadic prostate tumors, non-mutational functional inactivation of *BRCA2* may occur through different mechanisms, including down-regulation of its expression.^{15–18,21} Indeed, there is preliminary evidence indicating that *BRCA2* protein is significantly reduced in most sporadic PCas.¹⁸

Researchers have previously reported that *BRCA2* protein levels are regulated by ubiquitin-mediated proteolysis^{16,22} in a process that requires its association with the E3 ubiquitin ligase Skp2,¹⁶ an oncogenic protein that is up-regulated in prostatic intraepithelial neoplasia (PIN) and PCa.^{23,24} In this study, we investigated the relationship between *BRCA2* and Skp2 expression in human PCa specimens. Our results demonstrate that nuclear *BRCA2* levels are significantly decreased in sporadic PCa, and this correlates with increased expression of Skp2. Furthermore, decreased expression of *BRCA2* is consistently seen in premalignant lesions referred to as high-grade PIN, suggesting that *BRCA2* loss may constitute an early event in prostate neoplastic transformation. Finally, functional studies demonstrate that elevated Skp2 protein and loss of *BRCA2* expression in preneoplastic prostate cells are strongly associated with increased cell motility and proliferation.

Materials and Methods

Prostate Specimens and IHC

The construction of prostate TMAs was approved by the University of Rochester's Research Subjects Review Board and has been previously described.^{25–27} Briefly, archival prostatectomy cases (from 2002 to 2003) in the Department of Pathology at the University of Rochester Medical Center, Rochester, NY, were reviewed; 80 cases were selected for microarray construction. One or more cores were taken from areas of normal prostatic tissue ($n = 50$ cores) and areas with benign prostatic hyperplasia (BPH; $n = 82$ cores), high-grade PIN ($n = 35$ cores), low-grade prostate carcinoma (Gleason grades 2 and 3; $n = 104$ cores), and high-grade prostate carcinoma (Gleason grades 4 and 5; $n = 82$ cores), averaging four to six cores per case. The FFPE tissues were sectioned at 5- μ m thickness, deparaffinized, and quenched with 3% hydrogen peroxide for 6 minutes. Antigen heat retrieval was accomplished in a steamer containing citrate buffer (pH 6.1; Dako, Glostrup, Denmark) at 95°C to 99°C for 30 minutes. After rinsing with Tris-buffered solution, 10 μ g/mL rabbit polyclonal antibody against amino acids 3245 to 3418 of human *BRCA2* (Ab-2; Calbiochem, San Diego, CA) or monoclonal antibody to Skp2 (Zymed/Invitrogen Immunodetection, San Francisco, CA) at a dilution of 1:50 was applied overnight at 4°C, followed by 30 minutes remove incubation of biotinylated secondary linking antibody and streptavidin-bound peroxidase enzyme complex. Slides were developed with 3-Amino-9-ethylcarbazole (AEC⁺; Dako) for 10 minutes, rinsed in

running distilled water, counterstained in modified Mayer's hematoxylin, blued in 0.3% ammonia water, and examined under a light microscope. For TMA examination, each tissue core was separately scored; those with <50% of tissue present were discarded. Scoring included percentage of staining and staining intensity (0, +1, +2, or +3). Results were recorded as positive ($\geq 5\%$ +1 to +3) or negative (<5% staining or staining intensity 0). Paraffin-embedded sections of squamous surface epithelium and breast carcinoma provided the positive control for *BRCA2* staining. Competition studies were performed by preincubating the *BRCA2* antibody overnight at 4°C with the immunizing peptide (Abgent, San Diego, CA) at fivefold molar excess.

For Western blotting analysis, primary cancer specimens (Gleason grade 6 to 9) were obtained from prostatectomy performed at the Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX. The Institutional Review Board approved the tissue procurement protocol in this study, and appropriate informed consent was obtained from all patients. Six normal prostate specimens from organ donors who died from May 9, 2003, to November 26, 2004, were obtained from the National Disease Research Interchange (Philadelphia, PA).

Cell Cultures

Normal immortalized prostate epithelial cells PNT1A (from the European Collection of Cell Cultures; used at passages 8 to 9) and the PCa cell lines LNCaP clone FGC (from the European Collection of Cell Cultures), DU145, and PC-3 (from American Type Culture Collection) were kept in culture as previously described.^{28,29} A primary prostatic epithelial cell (PrEC) line derived from a 17-year-old juvenile prostate, was maintained in a chemically defined medium purchased from Lonza (Basel, Switzerland) and used at passage 2.

Western Blotting

Total protein extracts from cultured cells were prepared as previously described¹⁵ in PBS (pH 7.4) containing 1% Nonidet P-40, 2 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 5 mmol/L sodium pyrophosphate (lysis buffer). Total protein extracts from tissues were prepared as follows: either normal or neoplastic frozen tissue specimens were pulverized and homogenized in lysis buffer containing 0.1% SDS, 1% Nonidet P-40, 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 200 mmol/L LiCl, 5 mmol/L ethylenediaminetetraacetic acid, 10% glycerol, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 5 mmol/L sodium pyrophosphate. The homogenate was sonicated twice for 10 seconds and then centrifuged for 30 minutes at 14,000 $\times g$ at 4°C. β -Mercaptoethanol (1%) was added for 20 minutes at 4°C to further solubilize potentially cross-linked molecules. The protein content of each lysate was quantified using a DC protein assay reagent (Bio-Rad, Hercules, CA). *BRCA2* antibodies I-17

(against the N terminus) and H-300 (against an internal region) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Ab-2 (against the C terminus) was from Calbiochem. Antibodies to Skp2, poly(ADP-ribose) polymerase (PARP), and β -tubulin were previously described.^{15,17,29} Antibody to p85 was from Sigma (St Louis, MO).

Preparation of Nuclear and Cytoplasmic Fractions

Cells at 70% confluence were collected and resuspended in ice-cold hypotonic buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 300 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 0.1% NP40] supplemented with protease and phosphatase inhibitors (2 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 5 mmol/L sodium pyrophosphate). After swelling on ice for 20 minutes, plasma membranes were disrupted by repeated pipetting. Plasma membrane breakage was assessed by microscopic observation (Trypan blue exclusion). Samples were centrifuged at 3000 rpm for 10 minutes at 4°C to collect the cytoplasmic fractions (supernatant). The pellets were resuspended in hypertonic buffer [20 mmol/L HEPES (pH 7.4), 250 mmol/L NaCl, 1 mmol/L EDTA, 0.5% deoxycholic acid, 1 mmol/L dithiothreitol, 1% Triton X-100, and 0.1% SDS] containing the previously listed protease and phosphatase inhibitors, incubated on ice for 30 minutes, and resuspended by repeated pipetting. The nuclear fraction (supernatant) was recovered by centrifugation at top speed for 10 minutes at 4°C.

Transient Transfections

Stable and transient transfections with Skp2 cDNA (a gift from Professor Sylvain Meloche, Institut de Recherche en Immunologie et Cancérologie, Université de Montréal, Montreal, QC, Canada) or empty vector (pcDNA3; Invitrogen) were performed using a reagent (Lipofectamine; Invitrogen) according to the manufacturer's instructions. Transient transfection with BRCA2 cDNA was performed as previously described.¹⁸ BRCA2 small-interfering RNA (siRNA) samples were purchased from Santa Cruz Biotechnologies, Inc., and used for transient transfections according to the manufacturer's instructions. Negative control (nonspecific) siRNA (Santa Cruz Biotechnologies, Inc.) was also transfected. siRNA-mediated suppression of BRCA2 expression was confirmed by Western blotting analysis.

Cell Growth Assay

Cells (5×10^3) were seeded in triplicate in 96-well plates. Cell growth was assessed after 24, 48, and 72 hours using a crystal violet staining assay, as previously described.²⁹

Cell Migration Assay

Cells (1×10^5) were seeded on an 8- μ m pore-sized transwell Boyden chamber (Corning Costar, Lowell, MA) coated overnight at 4°C with 10 μ g/mL laminin-1. After 6 hours at 37°C, migrated cells were fixed with 3% paraformaldehyde and stained with crystal violet; the number of cells per square millimeter on the bottom was counted (average and SE of 10 random fields). Each experiment was performed in duplicate.

Statistical Analysis

Differences in BRCA2 and Skp2 labeling were compared among normal prostate, BPH, PIN, and cancer specimens by using the Mann-Whitney *U*-test and Fisher's exact test, with similar results. The relationship of BRCA2 with Gleason grade was evaluated using the Spearman correlation coefficient. Spearman correlation coefficient testing was also used to determine the relationship of BRCA2 with Skp2. $P < 0.05$ was considered significant in all analyses. The Student's *t*-test was used for experiments with cell lines.

Results

BRCA2 IHC Assay Is Specific for BRCA2

In preliminary experiments, we determined ideal experimental conditions and investigated BRCA2 expression in a variety of human tissues (data not shown). Afterward, we assessed the specificity of BRCA2 IHC by testing a section of keratinizing squamous epithelium (we determined it expresses BRCA2 consistently and strongly) and a known BRCA2-positive breast carcinoma. As shown in Figure 1A, although the skin section showed almost exclusive nuclear staining, the breast carcinoma section showed both cytoplasmic and nuclear staining and positive staining within the lumina of breast ductules, in agreement with a prior study.³⁰ To further test for the possibility of nonspecific antibody binding, we generated a peptide bearing the specific epitope sequence recognized by the antibody and used it to preadsorb the anti-BRCA2 antibody before application onto the breast cancer section and PCa TMA. As expected, nuclear and cytoplasmic reactivities were successfully abolished in both tissues, thereby confirming the specificity of the BRCA2 immunostaining (Figure 1, A and B).

BRCA2 Expression Is Significantly Reduced in Cancer Cells and in High-Grade PIN

A previous study¹⁸ demonstrated that BRCA2 protein levels are reduced in PCa. Thus, to better understand the relationship between BRCA2 expression and neoplastic transformation *in vivo*, we performed BRCA2 IHC in normal prostate and BPH, PIN, low-grade, and high-grade PCa specimens obtained from 80 patients with PCa in a TMA platform (Figure 1). After elimination of cores with significant tissue loss during processing, 48 cores of

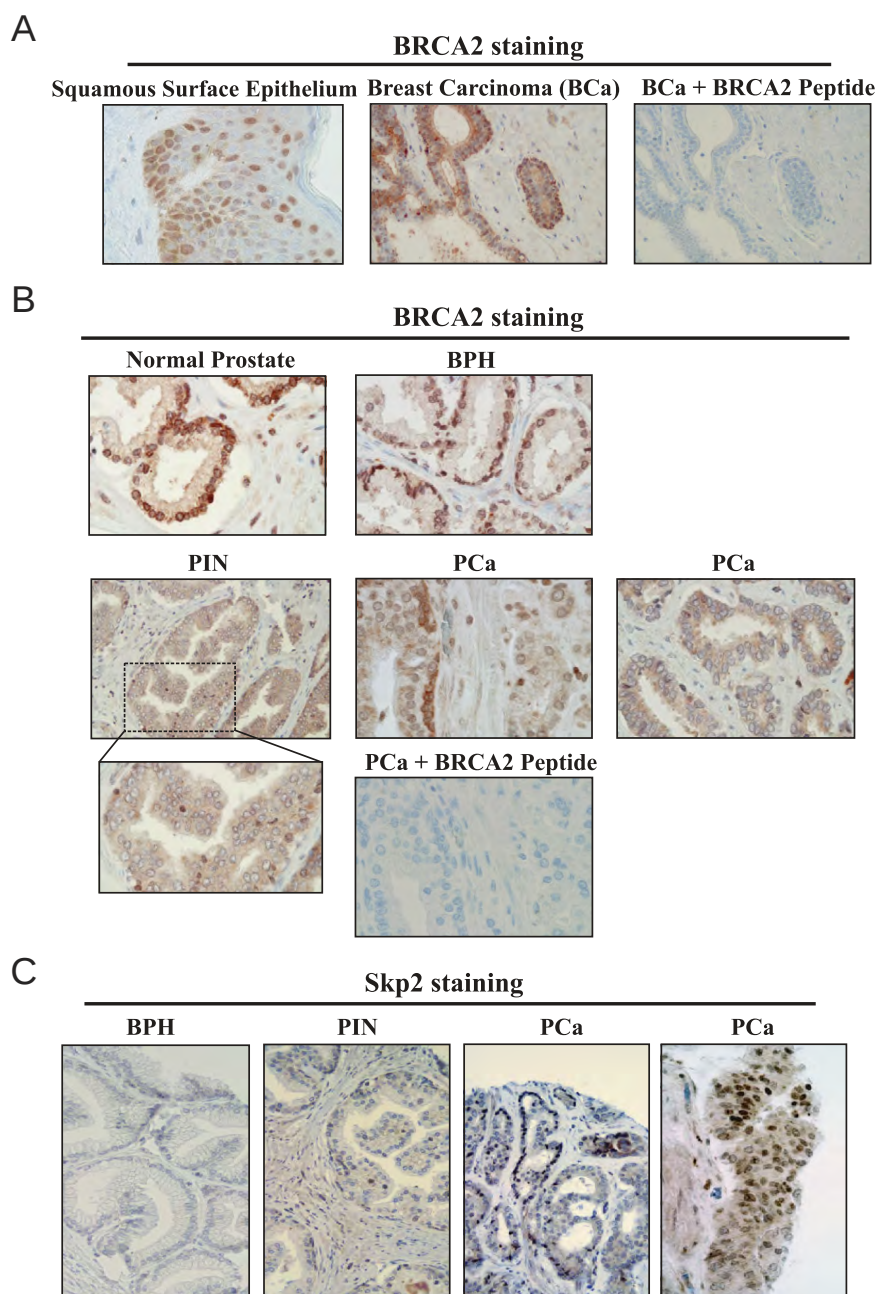


Figure 1. Loss of BRCA2 protein expression in PCa. **A:** Almost exclusive nuclear localization of BRCA2 observed in squamous surface epithelium (original magnification, $\times 400$). Nuclear and cytoplasmic localization of BRCA2 in breast carcinoma (BCa) was suppressed by preincubation of the antibody with a competing peptide. The lumina of breast ductules were also positive for BRCA2 staining (original magnification, $\times 200$). **B and C:** TMA sections were stained using either anti-BRCA2 antibody (**B**) or anti-Skp2 antibody (**C**). **B: Top:** Nuclear and cytoplasmic BRCA2 localization in normal prostate and BPH (original magnification, $\times 400$). **Lower:** Significant loss of nuclear BRCA2 in high-grade PIN and PCa. Two different PCa samples are shown (original magnification, $\times 400$). Cytoplasmic and nuclear localization of BRCA2 in prostate carcinoma was suppressed by preincubation of the antibody with a competing peptide (PIN: original magnification, $\times 200$; enlarged panel at original magnification, $\times 400$). **C:** Skp2-positive cells were rarely seen in benign glandular epithelium. In contrast, increased levels of Skp2 were seen in high-grade PIN and PCa. Weak or moderate Skp2 cytoplasmic immunoreactivity could be seen in addition to predominant nuclear reactivity in PIN and PCa cells. Original magnification: $\times 200$ (BPH, PIN, and PCa in the **middle**); $\times 400$ (PCa on the **right**).

normal prostate, 79 cores showing BPH, 30 cores showing high-grade PIN, 78 cores with low-grade PCa (Gleason grade 1 to 3), and 62 cores with high-grade PCa (Gleason grade 4 to 5) were examined. As shown in Figure 1B, BRCA2 immunostaining highlights the nuclei of both luminal and basal epithelial layers in normal tissue and BPH, where it appears to accumulate immediately under the nuclear membrane. Weak cytoplasmic staining of the prostate epithelium and scattered positive stromal cells and small lymphocytes were also observed. High-grade PIN and PCa showed almost complete loss of nuclear BRCA2 in basal and luminal cells compared with normal and hyperplastic prostate tissue ($P < 0.0001$, Figure 1B). There was an inverse correlation between frequency of BRCA2 staining and Gleason grade that did

not reach statistical significance ($P = 0.05$ for the inverse correlation; threshold of significance, $P < 0.05$). Nevertheless, cancer specimens retained weak cytoplasmic BRCA2 immunoreactivity. Quantitative immunostaining data, including the percentage of incidence of BRCA2-positive specimens among each specimen group and cell labeling frequency (percentage), are summarized in Tables 1 and 2.

The patients included in our study were not selected for PCa family history or early-onset PCa; thus, the decreased BRCA2 levels found in 77% of PCas are unlikely to be because of the presence of BRCA2-truncating mutations, which have been reported to be present in 5% of PCas in familial clusters,³¹ 2.3% of early-onset PCas in the UK population,² and 0.73% of early-onset PCas in the

Table 1. Nuclear BRCA2 Protein Expression in Human Prostate Tissues

Prostate tissues	No. of cores	BRCA2 labeling frequency			
		Median (%)	Mean $\pm$ SE (%)	Incidence	
				No./total	%
Normal	48	75	68.23 $\pm$ 4.75	39/48	81
BPH	79	50	63.16 $\pm$ 3.50	63/79	80
PIN	30	25*	37.59 $\pm$ 5.87	17/30	57
Carcinoma	140	10 [†]	21.71 $\pm$ 2.35	32/140	23

Nuclear BRCA2 immunostaining was demonstrated in normal prostate epithelial cells, BPH, PIN lesions, and carcinomas.

*Significantly lower than in normal prostate tissue ($P = 0.0007$).

[†]Significantly lower than in normal prostate tissue and PIN lesions ($P = 5.32e^{-13}$ and $P = 6.79e^{-08}$, respectively).

US population.⁹ To confirm that decreased BRCA2 levels in sporadic PCas were not because of the presence of BRCA2-truncating mutations, we performed Western blot analysis in a cohort of normal and PCa specimens using antibodies recognizing three different regions of the BRCA2 protein (ie, the N-terminal, an internal, and the C-terminal regions; see Supplemental Figure S1A at <http://ajp.amjpathol.org>). A Western blot using the antibody against the N-terminal region, which is expected to recognize any BRCA2 truncation variants, showed a full-length BRCA2 protein and a degradation fragment of approximately 268 kDa in both normal and PCa tissues but no truncation variants (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). The relative amount of the 268-kDa degradation product was higher in PCas than in normal prostates. All PCa specimens analyzed using Western blots exhibited a statistically significant decrease of full-length BRCA2 protein compared with normal prostate tissue ($0.001 < P < 0.007$; see Supplemental Figure S1C at <http://ajp.amjpathol.org>), which is consistent with the IHC results. Western blot analysis using antibodies against an internal and the C-terminal region of BRCA2 showed similar results (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). In addition to the 390-kDa full-length BRCA2 protein, the C-terminal antibody used in immunohistochemical analysis detected smaller BRCA2 degradation products (approximately 280 kDa to 300 kDa) in PCa but not in normal prostate (see Supplemental Figure S1B at <http://ajp.amjpathol.org>), suggesting

Table 2. BRCA2 Protein Expression in Prostate Carcinoma Tissues

Tissues	No. of cores	BRCA2 labeling frequency			
		Median (%)	Mean $\pm$ SE (%)	Incidence	
				No./total	%
PCa	140	10	21.71 $\pm$ 2.35	32/140	23
LGPCa	78	10	18.97 $\pm$ 2.61	20/78	26
HGPCa	62	0*	25.16 $\pm$ 4.16	12/62	19

BRCA2 levels expressed as nuclear labeling frequency of cancer cells in TMA composed of cores from radical prostatectomy specimens stratified according to their pathological features.

LGPCa, low-grade prostate carcinoma; HGPCa, high-grade prostate carcinoma.

*Not significantly different from LGPCa ($P = 0.05$).

Table 3. Skp2 Protein Expression in Human Prostate Tissues

Prostate tissue	No. of cores	Skp2 labeling frequency			
		Median (%)	Mean $\pm$ SE (%)	Incidence	
				No./total	%
Normal	44	0	13.33 $\pm$ 5.18	6/44	14
BPH	81	0	4.88 $\pm$ 2.07	6/81	7
PIN	31	25*	38.71 $\pm$ 7.68	15/31	48
LGPCa	90	50 [†]	45.78 $\pm$ 4.22	61/90	70
HGPCa	64	60 [‡]	56.02 $\pm$ 4.10	57/64	89

LGPCa, low-grade prostate carcinoma; HGPCa, high-grade prostate carcinoma.

*Significantly higher than in normal prostate tissue ($P = 0.006$).

[†]Significantly higher than in normal prostate tissue and PIN lesions ($P = 2.26e^{-06}$ and $P = 0.04$, respectively).

[‡]Significantly higher than in normal prostate tissue and PIN lesions ($P = 1.83e^{-09}$ and $P = 0.02$, respectively).

activation of BRCA2 proteolytic processes in cancer tissues.

BRCA2 Nuclear Expression Inversely Correlates with Skp2 in PIN and Cancer

The overexpression of the ubiquitin ligase Skp2 has been implicated in prostate oncogenesis.^{23,24} Previously, Skp2 has been shown to mediate BRCA2 ubiquitination and degradation in prostate cells.¹⁶ Given the biochemical link between BRCA2 and Skp2, we asked whether a correlation between Skp2 and nuclear BRCA2 levels could be demonstrated *in vivo* in PCa biopsy specimens. Our experiments demonstrated that, although Skp2 expression was almost undetectable in normal and BPH prostate tissue, 48% of high-grade PIN, 70% of low-grade prostate carcinoma, and 89% of high-grade prostate carcinoma showed strong Skp2 staining (Figure 1C and Table 3). In agreement with prior reports,^{23,24,32} the observed Skp2 pattern of expression included nuclear and cytoplasmic staining. The nuclear staining was assessed for number of positive nuclei (labeling frequency and percentage) and staining intensity (index) of each specimen. The results were then compared with the nuclear BRCA2 labeling frequency and index for the same set of specimens (Figure 2). A statistically significant inverse correlation was found between BRCA2 and Skp2 labeling frequency (Spearman's $\rho = -0.42$, $P < 0.0001$) and intensity index (Spearman's $\rho = -0.38$, $P < 0.00001$).

To further investigate the correlation between BRCA2 and Skp2 levels in prostate cells and to analyze whether cytosolic BRCA2 immunoreactivity was because of full-length BRCA2 and/or BRCA2 degradation fragments, we performed Western blot analysis in PrECs and neoplastic (DU145, LNCaP, and PC-3) and preneoplastic (PNT1A) prostate epithelial cell lines. As shown in Figure 3 and Supplemental Figure S1D (available at <http://ajp.amjpathol.org>), BRCA2 protein was decreased in LNCaP, DU145, and PC-3 cells compared with PrECs. PNT1A cells, an immortalized normal epithelial prostate cell line, also exhibited reduced BRCA2 levels when compared with PrECs. In addition to the 390-kDa wild-type BRCA2 intact protein, the antibody (C terminal) also detected smaller BRCA2

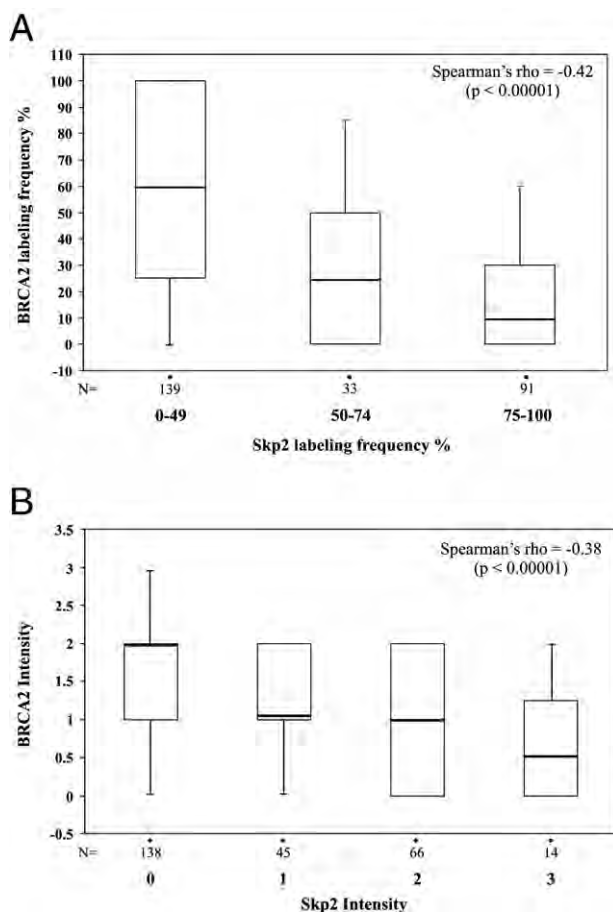


Figure 2. Correlation of BRCA2 with Skp2 protein levels. **A:** BRCA2 labeling frequency was negatively correlated with Skp2 labeling frequency ($P < 0.00001$, Spearman's correlation coefficient testing). **B:** BRCA2 labeling intensity was negatively correlated with Skp2 labeling intensity ($P < 0.00001$, Spearman's correlation coefficient testing). Each box and the associated bars represent the values of the middle 50% and the range of data, respectively. The dark line within a box indicates the median. N indicates number of cores.

degradation products (approximately 280 kDa to 300 kDa) in transformed prostate cell lines but not in normal PrECs, which is consistent with the results obtained in PCa specimens (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). Compared with BRCA2, Skp2 protein showed an inverse pattern of expression, displaying levels lower than detection in PrECs but highly expressed in LNCaP and DU145 neoplastic prostate cells. Skp2 levels in PC-3 cells were comparable to those in DU145 cells (data not shown). Cellular subfractionation experiments confirmed the presence of full-length BRCA2 and Skp2 proteins in the cytoplasm of normal and transformed prostate cells (Figure 3).

Down-regulation of BRCA2 expression in preneoplastic prostate cells promotes cell growth and migration. To functionally assess the effects of BRCA2 and Skp2 expression on prostate malignant transformation, we overexpressed Skp2 in the preneoplastic PNT1A cells and measured BRCA2 levels, cell migration, and cell growth. PNT1A cells are nontumorigenic³³ and exhibit molecular and biochemical properties close to normal prostate epithelium^{15,27,28,34}; they may be considered preneoplastic because they have experienced an initial genetic hit (ex-

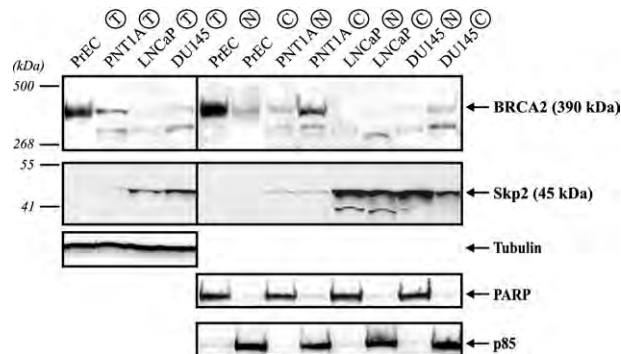


Figure 3. Subcellular localization of BRCA2 and Skp2 protein in normal and transformed prostate cells. Total (T), nuclear (N), and cytoplasmic (C) extracts were prepared from PrECs from normal donor, a normal immortalized epithelial prostate cell line (PNT1A), and two PCa cell lines (LNCaP and DU145); and subjected to Western blot analysis using anti-BRCA2 and anti-Skp2 antibodies. Tubulin was used as the loading control for total protein extracts, the nuclear protein PARP was used to assess potential contamination of the cytosolic fraction by nuclei, and the cytosolic protein p85 was used to assess potential contamination of the nuclear fraction by cytosol. BRCA2 and Skp2 proteins in full-length form were seen in both the nuclei (ie, N) and the cytosol (ie, C) of normal and transformed prostate cells. BRCA2 was significantly lost, whereas Skp2 was up-regulated, in neoplastic prostate cells compared with PrECs ($P < 0.001$).

pression of SV40T for immortalization) that renders them susceptible to progression toward malignant transformation. As shown in Figure 4A, Skp2 overexpression resulted in reduced levels of BRCA2 protein, consistent with previous results obtained with PCa cells,¹⁶ and promoted cell growth (41% increase after 72 hours: $P < 0.005$) and cell motility on laminin-1, a basement membrane protein (75% increase: $P < 0.01$). Similarly, down-regulation of BRCA2 expression by siRNA increased both cell growth (42% increase after 72 hours: $P < 0.005$) and migration (93% increase: $P < 0.008$) of PNT1A cells (Figure 4B). To confirm the causal link between loss of BRCA2 and up-regulated proliferation and motility in Skp2-overexpressing cells, we transiently transfected PNT1A cells stably overexpressing Skp2 with two different concentrations of BRCA2 cDNA and measured cell growth and migration. As shown in Figure 4, C and D, restoration of BRCA2 protein in Skp2-overexpressing cells resulted in a dose-dependent inhibition of cell growth and migration. Complete recovery of BRCA2 to the levels of wild-type PNT1A cells (in BRCA2 #2 transfectants) was able to reduce cell proliferation by 70% after 72 hours ($P < 0.001$) and cell motility by 63% ($P < 0.002$). Overall, these results suggest that loss of BRCA2 may confer a proliferative and migratory advantage to preneoplastic prostate cells and imply that Skp2 may be a key regulator of BRCA2 levels in prostate cells.

Discussion

One of the milestones in molecular oncology has been the development of therapeutic strategies aiming at selectively disabling molecular pathways that are operative in tumor cells, with little effect on normal tissues, thus limiting adverse effects. This approach has yielded great clinical success in the treatment of myeloid leukemias.³⁵

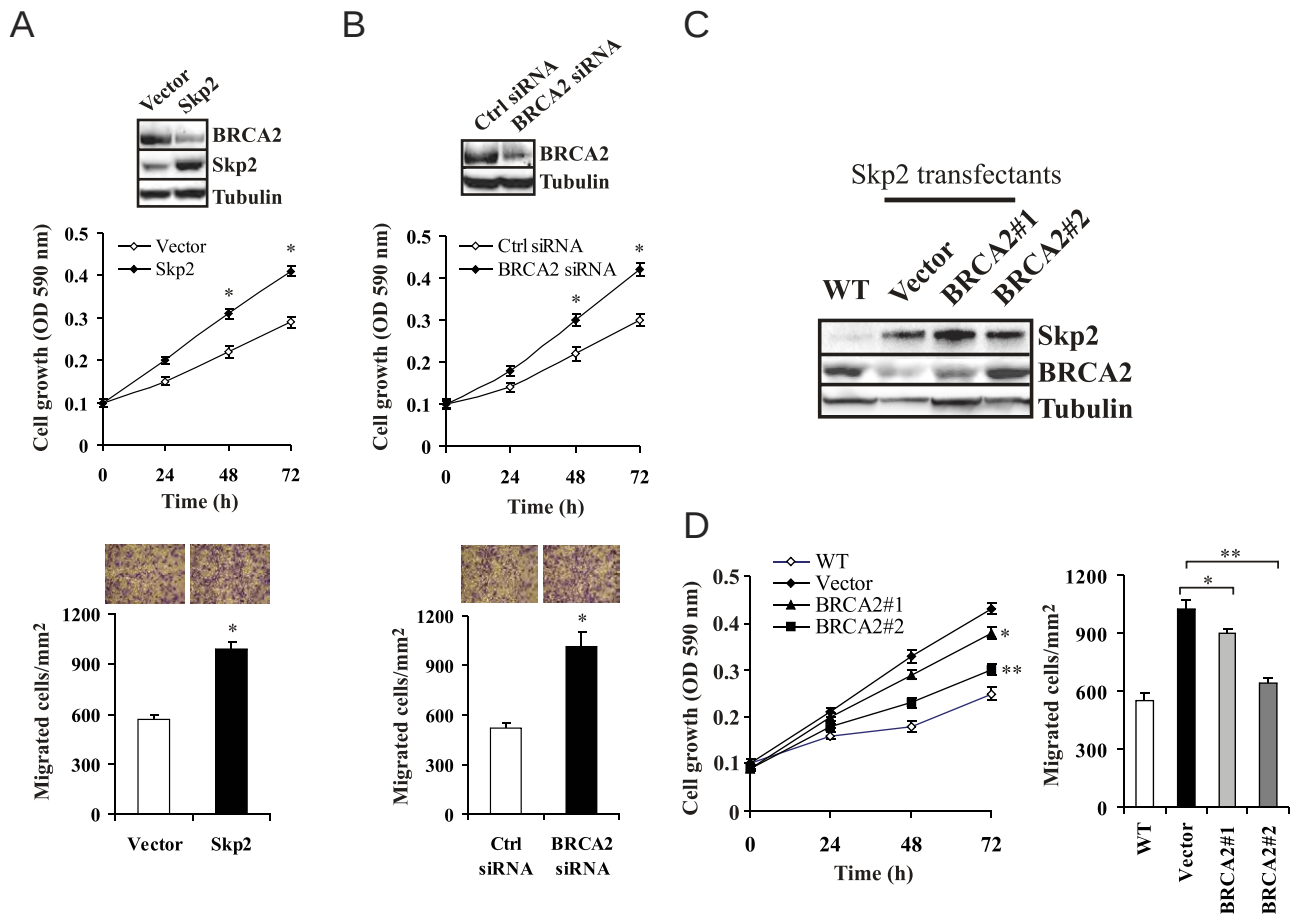


Figure 4. Down-regulation of BRCA2 expression in preneoplastic prostate cells promotes cell growth and migration. **A:** PNT1A cells were transiently transfected with Skp2 cDNA (*Skp2*) or empty vector (*Vector*) for 48 hours; then, Western blot, cell growth, and migration assays were performed. **Top:** Total protein extracts were subjected to Western blot analysis using anti-BRCA2 and anti-Skp2 antibodies. Tubulin was used as the loading control. For cell growth assays, cells (5×10^3 per well) were plated in 96-well plates and, at the indicated time, fixed, stained with crystal violet, and read in an enzyme-linked immunosorbent assay reader at an absorbance of 590 nm. Cell migration assays were performed by plating 1.0×10^5 cells onto transwell insert filters coated with $10 \mu\text{g/mL}$ laminin-1. After 6 hours, cells at the lower side of the filter were fixed, stained with crystal violet, photographed, and counted. A representative experiment of three is shown. Data are given as the mean $\pm$ SE of 10 random fields. * $P < 0.005$ for cell growth and $P < 0.01$ for cell migration. **B:** PNT1A cells were transiently transfected with BRCA2 siRNA or nonspecific siRNA (*Ctrl siRNA*) for 48 hours; then, Western blot, cell growth, and migration assays were performed, as described in **A**. * $P < 0.005$ for cell growth and $P < 0.008$ for cell migration. **C and D:** PNT1A cells stably overexpressing Skp2 were transiently transfected with $1 \mu\text{g}$ (*BRCA2#1*) or $2 \mu\text{g}$ (*BRCA2#2*) BRCA2 cDNA or empty vector; 48 hours after transfection, the cells were analyzed for Skp2, BRCA2, and tubulin expression by Western blotting analysis (**C**) and cell growth and migration (**D**). Wild-type PNT1A cells (*WT*) were included as control. * $P < 0.01$ and ** $P < 0.001$ for cell growth; * $P < 0.01$ and ** $P < 0.002$ for cell migration versus cells transfected with empty vector.

However, similar efforts targeting epithelial cancers remain underdeveloped, in part because the biological mechanisms involved in this type of cancer are less well understood. By using PCa cell lines, a recent study¹⁶ demonstrated that the Skp1–cullin–F-box ubiquitin ligase Skp2 interacts with and promotes ubiquitination of BRCA2, targeting it for degradation in the proteasome; in addition, the loss of BRCA2 promotes PCa cell proliferation and invasion.^{15–18} However, although experimental models based on cultured cell lines are amenable for the assessment of signaling cascades and molecules that are selectively deregulated in cancer, *in vivo* validation remains a stepping stone in translating relevant scientific findings to the patients' bedside. In the current study, we investigated whether the expression of Skp2 correlates with BRCA2 protein levels in prostate epithelial neoplasms *in vivo*; we also assessed the functional role of Skp2 overexpression and loss of BRCA2 protein in the early stages of prostate neoplastic transformation.

By using a TMA platform, we show that loss of BRCA2 expression is a relevant event in many sporadic PCa cases. We found that, although 80% of normal and BPH specimens showed strong nuclear BRCA2 expression and moderate cytoplasmic BRCA2 levels, 43% of high-grade PIN and 77% of PCa exhibited decreased or absent nuclear BRCA2 protein. Nevertheless, cytoplasmic BRCA2 was retained in PCa glands and PIN; Western blotting analysis of PCa cell lines confirmed the presence of full-length BRCA2 in the cytosol. Although localization of BRCA2 in the nucleus has been associated with its role in DNA repair and genome stability,³⁶ the functional role of cytoplasmic BRCA2 remains to be investigated. However, its presence in normal and neoplastic breast³⁰ (Figure 1) and prostate undermines the possibility of BRCA2 playing a significant role in neoplastic transformation. In contrast to our investigations, a previous immunohistochemical study³⁷ reported that BRCA2 protein was virtually absent in normal prostate. In all likelihood, the neg-

ative finding is related to the different antibody that was used in that particular study. The commercial antibody used in our study was generated against the BRCA2 C-terminal domain and detected a 390-kDa band corresponding to a full-length BRCA2 protein in Western blotting analysis of protein extracts from normal and carcinoma prostate tissues and cell lines. Western blotting experiments using two other antibodies against BRCA2 confirmed the presence of full-length BRCA2 in normal prostate cells and a significant reduction of BRCA2 levels in PCa tissues and cell lines. In the immunohistochemical analysis, we further confirmed the specificity of the immune reaction by preadsorbing the antibody with a peptide containing the C-terminal epitope used in the generation of the antibody before incubation onto the microarrays. As expected, the peptide completely suppressed any positive immunostaining in breast and prostate sections. In addition, we observed positive BRCA2 staining of neoplastic glandular epithelium and ductules in human breast cancer sections, in agreement with the results of a prior study³⁰ that used different antibodies.

In addition to conferring an increased risk for PCa,^{9,11,13,14} there is recent evidence suggesting that germline mutations in *BRCA2* predispose to developing highly aggressive PCa with a higher histological grade than noncarrier patients.^{9–14} However, we were not able to discern a statistically significant relationship between cancer histological grade and severity of *BRCA2* loss in sporadic PCas. Our results show that nuclear BRCA2 protein is lost early and significantly during prostate neoplastic transformation; premalignant PIN already exhibits significant reduction of BRCA2 protein, and no further reduction was observed when comparing Gleason low- with high-grade PCa, even if a nonsignificant trend toward an inverse correlation between frequency of BRCA2 staining and Gleason grade was evident. Prospective studies aimed at establishing whether loss of BRCA2 detected by immunohistochemical analysis of early lesions and carcinoma has a deleterious effect on clinical evolution and prognosis are necessary to better understand the significance of these findings. Nevertheless, to our knowledge, this is the first time that loss of nuclear BRCA2 expression is shown to be an early biomarker of sporadic prostate carcinogenesis.

Loss of BRCA2 protein in sporadic PCas may theoretically involve several molecular mechanisms, including allelic loss at the *BRCA2* locus, the presence of protein-truncating mutations in the *BRCA2* gene, and activation of protein degradation pathways.^{15,16} *BRCA2* is located on chromosome 13q, a frequently deleted region in PCa. However, several studies^{38–40} have shown that *BRCA2* is not the target of these deletions in sporadic PCas. In addition, no somatic mutations were detected in the *BRCA2* coding region of sporadic PCa tissues exhibiting loss of heterozygosity at the *BRCA2* locus,³⁹ thus excluding inactivation or alteration of the *BRCA2* gene in clinically localized sporadic PCa. We confirmed the absence of protein-truncating *BRCA2* mutations in sporadic PCa by analyzing protein extracts from normal and carcinoma prostate tissues and cell lines for the presence of protein fragments using an antibody to the BRCA2 N-terminal

region. Our analysis failed to show any BRCA2 fragment present selectively in PCa but not in normal prostates. Furthermore, immunoblotting analysis using antibodies that recognized either an internal or the C-terminal region of BRCA2 confirmed the presence of the full-length BRCA2 protein in PCas. Overall, our results support previous studies³⁹ showing lack of deleterious mutations in the *BRCA2* gene in sporadic PCas, thus pointing to a different mechanism of down-regulation of BRCA2 levels in these cancers.

Overexpression of the E3 ubiquitin ligase Skp2 has previously been observed in PCas²³ and other types of human tumors, including colorectal carcinoma,⁴¹ breast cancer,⁴² lymphoma,⁴³ and various soft tissue sarcomas⁴⁴; in addition, it has been associated with carcinogenesis and metastasis.^{43,45–47} We found a significant increase in Skp2 protein levels already in preneoplastic prostate lesions (high-grade PIN), in agreement with a previous study,⁴⁸ and a pattern of expression that was inversely related to BRCA2 protein during prostate neoplastic transformation. Skp2 exhibited both a nuclear and a cytoplasmic pattern of expression in PCa glands and human prostate cell lines, in agreement with previous findings.^{24,32} However, nuclear, but not cytoplasmic, BRCA2 protein was lost during prostate neoplastic transformation, suggesting that other factors may stabilize BRCA2 in the cytosol or prevent its interaction with Skp2. Two recent studies^{32,49} showed that aberrant localization of Skp2 in the cytoplasm during cancer progression is because of its Akt-dependent phosphorylation. Thus, the possibility exists that posttranslational modifications of Skp2 occurring in the cytosol could prevent or weaken the interaction with its substrates, including BRCA2.

The reverse pattern of expression of BRCA2 and Skp2 during prostate neoplastic transformation is of particular relevance because several lines of evidence suggest that Skp2 overexpression may contribute to tumorigenesis. Indeed, Skp2 cooperates with H-Ras to transform primary rodent fibroblasts⁵⁰; Skp2 overexpression cooperates with N-Ras to induce T-cell lymphomas,⁴³ and prostate-specific expression of Skp2 causes PIN in mice.⁵¹ In addition, Skp2 promotes degradation of the cell cycle inhibitor p27, thus supporting cell cycle entry in PCa cells.^{23,52} Our *in vivo* and *in vitro* results indicate that, in addition to p27, Skp2 is a key regulator determining the abundance of BRCA2 in prostate cells. Moreover, we show, for the first time to our knowledge, that Skp2 overexpression confers a migratory and proliferative advantage to preneoplastic prostate cells through, at least in part, reduction of BRCA2 protein levels, suggesting an important role of the BRCA2-Skp2 axis in promoting or facilitating prostate malignant transformation. These findings are further corroborated by the *in vivo* results that demonstrate significant overexpression of Skp2 and loss of BRCA2 protein already in preneoplastic prostate lesions (high-grade PIN). Our findings are also consistent with recent data⁵³ obtained on murine models in which pharmacological inactivation of Skp2 was able to suppress the growth of preformed PCa tumors derived from PC3 cells. There are ongoing clinical trials of therapeutic regimens that combine small-molecule drugs that target

the proteasome, a general component of the ubiquitin-mediated proteolytic system, with conventional chemotherapeutic agents (eCancerTrials; at <http://ecancertrials.com>, last accessed March 13, 2011). Because Skp2 is amenable to pharmacological inhibition and its complete inactivation is not lethal in mice,⁵³ our findings suggest that novel small inhibitors targeting Skp2 enzymatic activity or specifically counteracting Skp2/BRCA2 interaction may open new promising therapeutic venues for patients with PCA.

In summary, we have presented strong clinical evidence demonstrating that loss of BRCA2 is an early event in prostate carcinogenesis and is associated with overexpression of Skp2. These results, along with *in vitro* studies^{15,16,18,54,55} demonstrating that loss of BRCA2 stimulates cancer cell proliferation and invasion (Figure 4), make the BRCA2-Skp2 oncogenic pathway an attractive target for therapeutic intervention.

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