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Down-regulation of BRCA2 Expression by Collagen Type I Promotes Prostate Cancer Cell Proliferation*

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WITHDRAWN
May 20, 2014

***BRCA2* is a tumor suppressor gene that when mutated confers an increased susceptibility to developing breast and prostate carcinoma. Besides its role in mediating DNA repair, new evidence suggests that BRCA2 may also play a role in suppressing cancer cell growth. Because altered interactions between neoplastic cells and the surrounding extracellular matrix (ECM) play a pivotal role in unchecked cancer cell proliferation and metastatic progression, we hypothesized that the ECM may have an effect in BRCA2 expression. By using normal and prostate carcinoma cell lines, we demonstrated that although normal cells transiently increase BRCA2 protein levels when adhering to the ECM protein collagen type I (COL1), carcinoma cells exhibit a significant r[illegible] duction in BRCA2 protein. This aberrant effect is [illegible] pendent from *de novo* protein synthesis and re[illegible] COL1-β_1 integrin signaling through phos[illegible] tol (PI) 3-kinase leading to BRCA2 ub[illegible] degradation in the proteasome. B[illegible] tion after cancer cell adhesion [illegible] interference assays trig[illegible] trophic effect that is al[illegible] expression. Blocki[illegible] nase, or proteasom[illegible] COL1-mediated DNA[illegible] cells, the transient in[illegible] dependent from β_1 in[illegible] effect in cell proliferat[illegible] unravel a novel mechan[illegible] noma cell proliferation is [illegible] lation of BRCA2 expressi[illegible] COL1, a major component of [illegible] static sites.**

Germ line mutations in the *BRCA2* g[illegible]ult in breast and ovarian cancer susceptibility in women a[illegible] prostate carcinoma in men (1–4). Inheritance of one defective allele confers cancer predisposition, and neoplastic cells from predisposed individuals frequently exhibit loss of heterozygosity in the wild-type allele (5, 6). The major identified function of the BRCA2 protein is to form complexes with Rad51, which orchestrate homologous recombination repair of DNA double-strand breaks (7). Inactivation of the murine homologue *Brca2* leads to embryonic lethality (8–11), and *Brca2*-null murine embryonic fibroblasts exhibit spontaneous accumulation of chromosomal abnormalities (12, 13) and defective cytokinesis (14). In addition to its role in mediating DNA repair and genome stability, new evidence suggests that BRCA2 could s[illegible]ppress tumor development by inhibiting cancer cell gro[illegible]

The majority of br[illegible]nd prostate cancer cases is sporadic (16, 1[illegible]utational inactivation of *BRCA2* is [illegible] both gene copies to be affecte[illegible] functional suppression co[illegible] such as binding of [illegible]poradic breast and [illegible]er of environmen- [illegible]n during cancer [illegible] regulation of [illegible]l as in other [illegible]harted.

[illegible]ral impor- [illegible]ality and inva- [illegible]ation through mod- [illegible]ed, altered interaction [illegible]ng ECM is recognized as a [illegible] cells (19). The adhesion of cancer [illegible]get organs has been implicated in the [illegible] specific metastasis (20, 21). For instance, [illegible] metastasis of prostate cancer to the bone has [illegible]ggested to involve collagen type I (COL1) (20), the [illegible] structural component of the bone (22). Collagens and [illegible]her ECM proteins interact with cells through integrins (23), a family of heterodimeric transmembrane receptors composed of an α and a β subunit (24). These interactions activate cytoplasmic kinases, such as members of the mitogen-activated protein kinase/extracellular signal-regulated kinase-1 and -2 (25, 26) and the phosphatidylinositol (PI) 3-kinase/AKT (27, 28), which modulate the expression of genes exerting stringent control on cell proliferation (23).

In this study, we have elucidated a novel mechanism whereby adhesion of prostate carcinoma cells to the ECM protein COL1 results in BRCA2 protein degradation, which stimulates cancer cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture—PNT1A cells (a human prostate normal cell line established by immortalization of normal adult prostate epithelial cells),

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[1] The abbreviations used are: ECM, extracellular matrix; COL1, collagen type I; PI 3-kinase, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline solution; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PL, plastic; PLL, polylysine; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; siRNA, small interference RNA; RT, reverse transcription.

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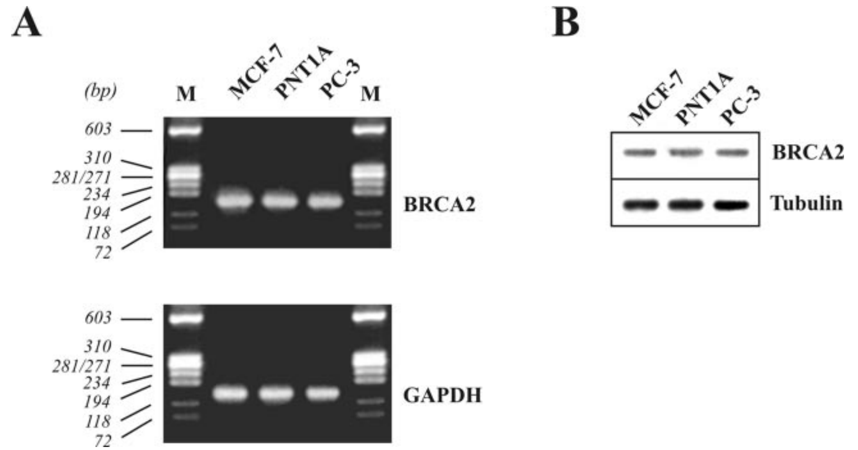

FIG. 1. **BRCA2 mRNA and protein expression is comparable in normal and cancer prostate cells.** *A*, semi-quantitative RT-PCR of BRCA2 mRNA from PNT1A and PC-3 cells. RT-PCR of BRCA2 mRNA from MCF-7 cells was included as positive control. RT-PCR of GAPDH mRNA was used as internal control. *M*, molecular size marker. Results are representative of three independent experiments. *B*, immunoblotting analysis of BRCA2 protein. MCF-7, PNT1A, and PC-3 cell protein extracts (100 μg) were electrophoresed on 6% SDS-polyacrylamide gel under reducing conditions and immunostained using an antibody to BRCA2. β-Tubulin signals were used as loading controls. Autoradiographs are representative of five independent experiments.

PC-3 cells (a human prostate carcinoma cell line derived from a bone metastasis), and MCF-7 (a human breast adenocarcinoma cell line) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PNT1A and PC-3 cells were kept in culture as described previously (29). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% inactivated fetal bovine serum (BioSpa, Milan, Italy), 2 mM glutamine (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen), at 37 °C in the presence of 5% CO_2.

RNA Extraction and RT-PCR—Cells were grown to 60–70% confluence and washed twice with cold phosphate-buffered saline (PBS), and total cellular RNA was isolated using the TRIzol reagent (Invitroge following the manufacturer's instructions. The amount of R mRNA present in the cells was measured by semi-quantitati transcription. Total RNA was reverse-transcribed, an cDNA was amplified by PCR using the Titan One T (Roche Applied Science), as described previousl (1 μg) was reverse-transcribed for 30 mi (Roche Applied Science), 5 units of R Italy), 1× RT-PCR buffer (Roch primer (Invitrogen), 0.6 μM enzyme mix (avian my proofreading polymeras sis, a cycle of denaturati denaturation at 94 °C for tion at 72 °C for 60 s were r the BRCA2 cDNA were used, dehyde-3-phosphate dehydroge control, as described by Wu *et a* using a PCR system (PerkinElme were analyzed on a 2% agarose gel,

Immunoblotting Analysis and Imm and MCF-7 cells were grown either on 10 μg/ml; Sigma, Milan, Italy), COL1 (10 μ vating β_1 integrin antibody P4G11 (1 μg/ml; ional, Temecula, CA) and then lysed in PBS (pH g 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 aprotinin, 10 μg/ml leupeptin, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate. In some experiments, cells were added with either the protein synthesis inhibitor cycloheximide (12.5 μM; Sigma) or the lysosomal inhibitors leupeptin (100 μM; Sigma) or pepstatin (100 μM; Sigma) or NH_4Cl (50 mM), or the calpain inhibitor ALLN (10 μM; Calbiochem), or the proteasome inhibitor MG132 (10 μM; Calbiochem), or the vehicle alone (Me_2SO). Where indicated, cells were pretreated either for 1 h on ice with the blocking β_1 integrin antibody P4C10 (ascites 1:200; Chemicon) or the monoclonal antibody 1C10 to a vascular endothelial surface protein (ascites 1:200; Invitrogen) as a negative control, or for 1 h at 37 °C with wortmannin (0.1 μM; Sigma) or LY294002 (10 μM; Calbiochem), two different PI 3-kinase inhibitors, or the solvent alone (Me_2SO). The protein content of each lysate was quantified using the Bio-Rad Dc protein assay reagent (Bio-Rad) according to the manufacturer's protocol. Protein extracts (100 μg) were electrophoresed through 6 or 10% SDS-polyacrylamide gel under reducing conditions according to Laemmli (31) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) in 25 mM Tris and 192 mM glycine, pH 8.3, containing 20% methanol. Membranes were blotted with either 1 μg/ml polyclonal rabbit anti-BRCA2 antibody (H-300; Santa Cruz Biotechnology, Santa Cruz, CA), 10 μg/ml monoclonal antibody to β-tubulin (Sigma), 1 μg/ml monoclonal antibody to β_1 integrin (BD Transduction Laboratory), 1 μg/ml polyclonal rabbit anti-phospho-AKT-Ser-473 antibody (Santa Cruz Biotechnology), 1 μg/ml polyclonal rabbit anti-AKT 1/2 antibody (H-136; Santa Cruz Biotechnology), 1:400 dilution of anti-p110 antibody (H-239; Santa Cruz Biotechnology), or 1:1000 dilution of anti-p85α rabbit antiserum (Sigma),

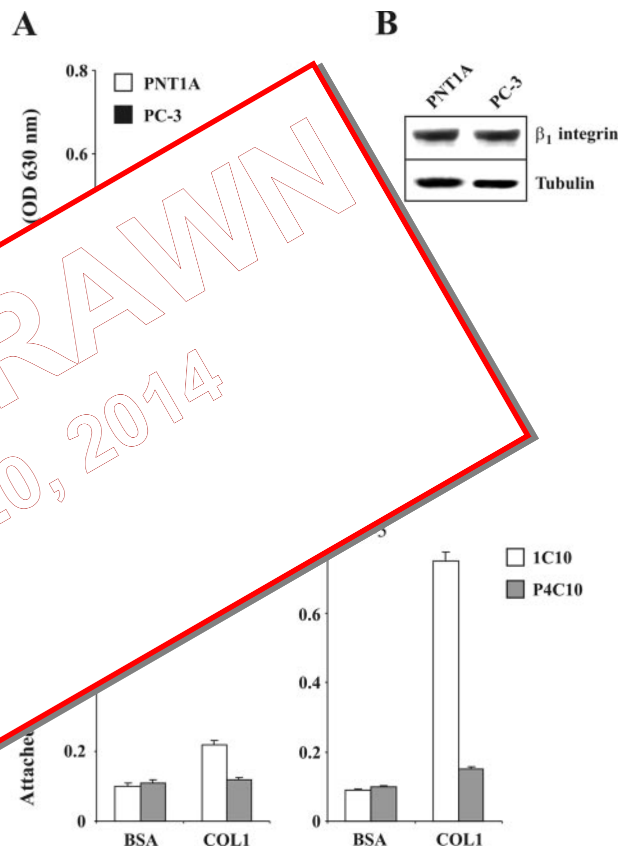

FIG. 2. **Cancer cells adhere more efficiently to COL1 than normal prostate cells.** *A*, to analyze cell adhesion, 1.5 × 10^5 PNT1A or PC-3 cells were allowed to attach to BSA (10 μg/ml), PLL (10 μg/ml), or COL1 (10 μg/ml) at 37 °C for 1 h, washed, and stained with crystal violet. Data are expressed as mean ± S.E. of triplicate wells. A representative experiment of three is shown. *B*, immunoblotting analysis of β_1 integrin protein. Total cell lysates (100 μg) from PNT1A and PC-3 cells were processed for immunoblotting. Membranes were probed with anti-β_1 integrin monoclonal antibody. β-Tubulin signals were used as loading controls. *C*, to analyze the involvement of β_1 integrin in cell adhesion, 1.5 × 10^5 PNT1A or PC-3 cells were incubated for 1 h on ice in the presence of the β_1 integrin blocking antibody P4C10 (ascites 1:200) or 1C10 monoclonal antibody (ascites 1:200) as control and allowed to adhere to BSA or COL1, in the presence of the antibodies, as described in *A*. Data are expressed as mean ± S.E. of triplicate wells. A representative experiment of three is shown.

WITHDRAWN
May 20, 2014

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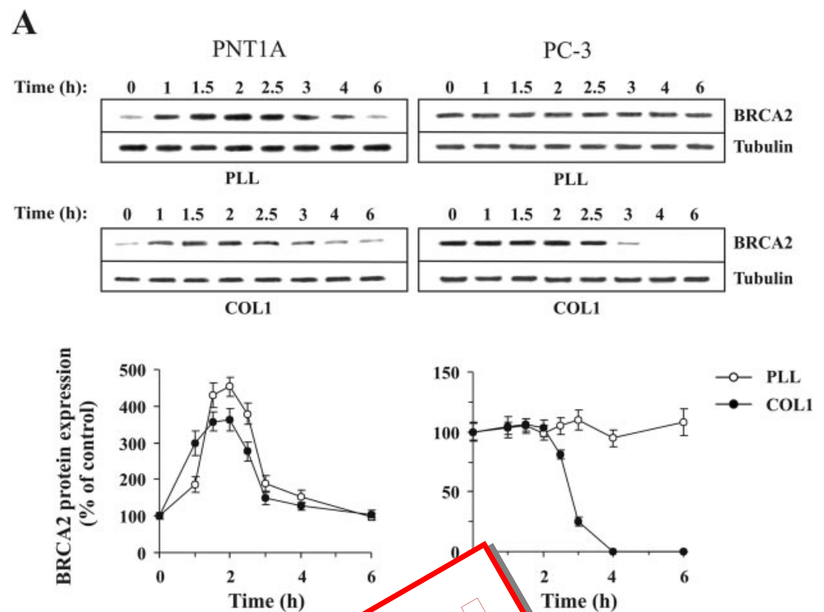

B

FIG. 3. **Adhesion of PC-3 cells to COL1 decreases BRCA2 protein levels in a β_1 integrin-dependent manner.** *A*, PNT1A and PC-3 cells were allowed to attach to PLL or COL1 at 37 °C for 0–6 h, washed, lysed, and processed for immunoblotting. Membranes were probed with anti-BRCA2 antibody. β-Tubulin signals were used as loading controls. The blots are representative of three independent experiments. At the bottom, a quantification of BRCA2 protein levels is reported as percentage of the protein levels at 0 h of cell adhesion to PLL or COL1 (control), set at 100. Data are expressed as mean ± S.E. of three independent experiments. *B*, PNT1A and PC-3 cells were incubated for 1 h on ice in the presence or absence of P4C10 (ascites 1:200) and then allowed to adhere to the β_1 integrin activating antibody P4G11 or to COL1 at 37 °C for the indicated times, washed, lysed, and processed for immunoblotting. Membranes were probed with anti-BRCA2 antibody. The blots are representative of three independent experiments.

WITHDRAWN
May 20, 2014

for 16 h at 4 °C in Tris-buffer... pH 7.5, 150 mM NaCl, 0.1% ... washed three times in TBS-T a... horseradish peroxidase-conjugate... either rabbit or mouse IgG (Amersh... room temperature. After three washe... ized using the enhanced chemilumine... Biosciences) according to the manufactu... ric values for immunoreactive bands we... a GS-700 Imaging densitometer (Bio-Rad).

To perform a coupled immunoprecipitati... immunoblotting assay, the whole cell extract (0.25–0.5 mg of protein) was first precleared and then incubated overnight at 4 °C with 1 μg of polyclonal antibody to BRCA2 (H-300). Immunocomplexes were recovered by binding to protein A-Sepharose (Sigma) and washed five times with PBS containing 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate. Immunocomplexes were analyzed by 6% SDS-PAGE under reducing conditions followed by transfer to polyvinylidene difluoride membrane. Filters were immunoblotted using a 1:500 dilution of monoclonal antibody to ubiquitin (Sigma) or 2 μg/ml monoclonal antibody to BRCA2 (clone 5.23; Chemicon) following the manufacturer's instructions.

Cell Adhesion—Cell adhesion assays were carried out using 96-well tissue culture plates. Plates were pre-coated with different concentrations of COL1 (0.1–10 μg/ml) or P4G11 (0.5–1 μg/ml) for 16 h at 4 °C. Coating with 10 μg/ml bovine serum albumin (BSA; Sigma) was performed as a negative control. Coating with 10 μg/ml PLL was performed as a positive control. Adventitious binding sites in the wells were blocked with 1 mg/ml BSA in PBS for 1 h at 37 °C. Cells (150,000 cells/well) were plated on coated wells for 1 h at 37 °C, washed twice with PBS to remove unattached cells, fixed with 3% paraformaldehyde in PBS for 30 min at 4 °C, and stained with 0.5% crystal violet in PBS. ...antified by measuring the absorbance at 630 nm in ...a immunosorbent assay reader. Inhibition assays were ...y incubating cells for 1 h on ice in the presence of either ...the monoclonal antibody to human β_1 integrin (ascites 1:200), or ...0 (ascites 1:200) as a negative control. Triplicate observations were performed in each experiment.

Plasmid Expression Vectors—Expression vectors for wild-type PI 3-kinase p85 regulatory subunit (wt-p85), Δp85, a dominant negative form of PI 3-kinase, and p110*, a constitutively active form of PI 3-kinase, were generously provided by Dr. Robert Freeman (University of Rochester, Rochester, NY). The expression vector containing the wild-type BRCA2 cDNA was a kind gift of Dr. M. C. Hung (University of Texas M. D. Anderson Cancer Center, Houston). pCMV-β-gal vector expressing β-galactosidase was from Stratagene.

Transient Transfection—PNT1A and PC-3 cells were seeded into 6-well dishes at 4–5 × 10^5 cells/well. After 24 h, transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The DNA sequence against which small interfering RNA (siRNA) for BRCA2 was created is AACAACAAUUACGAACCAAACUU (32). Duplexed RNA oligomer for BRCA2 was synthesized by Qiagen (Milan, Italy). Double-stranded RNA was transfected into the cells as 0.3 μg of siRNA/well. pCMV-β-gal (0.5 μg/well) was transfected along with 4.5 μg/well of either one of the PI 3-kinase variant cDNAs constructs or the empty vector (pcDNA3). Cells were harvested 36–96 h after transfection and used in immunoblotting, immunoprecipitation, and [^{3}H]thymidine incorporation assays. Transfected cells were also plated in 48-well plates and stained for β-galactosidase expression to determine transfection efficiency. The β-galactosidase staining was performed as described by Manes *et al.* (33).

[^{3}H]Thymidine Incorporation—Thymidine incorporation assay was used to measure the cell proliferative index. Cells were starved for 24 h, detached, plated in a 96-well plate (10,000 cells/well), and grown for

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15 h either on PL-, PLL (10 μg/ml)-, COL1 (10 μg/ml)-, or P4G11 (1 μg/ml)-coated wells. Where indicated, cells were preincubated for 1 h on ice with P4C10 (ascites 1:200) or 1C10 (ascites 1:200) as a negative control, or added with MG132 (10 μM), or preincubated for 1 h at 37 °C with wortmannin (0.1 μM) or the solvent alone (Me_2SO) before plating. During the last 3 h of the 15-h culture, cells were pulsed with 1 μCi/ml methyl-[^{3}H]thymidine (Amersham Biosciences), washed three times with PBS at 0 °C, and dissolved with 10% SDS. Radioactivity was measured by using a scintillation counter (Beckman Instruments).

Statistical Analysis—Data are reported as the mean ± S.E. Statistical analysis was performed by the Student's *t* test. All experiments were repeated at least twice.

RESULTS

In this study a normal human cell line (PNT1A), established by immortalization of normal adult prostate epithelial cells, non-tumorigenic in mice (34), and the prostate cancer epithelial cell line PC-3, derived from a bone metastasis (35, 36), were used as the experimental model. Both PNT1A and PC-3 cells expressed comparable levels of BRCA2 mRNA and protein as measured by RT-PCR (Fig. 1*A*) and Western blot assays (Fig. 1*B*), respectively. MCF-7, a breast cancer cell line that expresses wild-type BRCA2 (37), was included as a positive control (Fig. 1, *A* and *B*).

Cancer Cells Adhere More Efficiently to COL1 than Normal Prostate Cells—We proceeded to investigate the adhesive properties of PNT1A and PC-3 cells to COL1. Adhesion to BSA and PLL, an integrin-independent adhesive substrate, served as negative and positive controls, respectively. Serial dilutions were performed with the coating substrates, and the con... tration that supported maximal adhesion, reported i... was used for all subsequent experiments. Both c... played comparable adhesion to PLL (Fig. 2A)... differed significantly when interacting... PNT1A cells adhered to COL1 at a... adhesion to PLL, PC-3 cells ad... 141% of PLL levels after 1... periods did not result i... Because cell adhesio... integrin receptors, we... cells expressed similar l... bation with P4C10, a bloc... PNT1A and PC-3 cell adhe... and 92 ± 6% ($p < 0.001$), re... adhesion after P4C10 treatme...

Adhesion of PC-3 Cells to C... Levels in a β_1 Integrin-dependent... cell adhesion to COL1 had any effe...els. PNT1A cells transiently increased ...n on integrin-dependent (COL1) and -indep...LL) adhesive substrates (Fig. 3*A*). Levels surged 4.5 ...-fold on PLL ($p < 0.001$) and 3.6 ± 0.2-fold on COL1 ($p < 0.001$) after 2 h and returned to basal levels after 6 h. In PC-3 cells, adhesion to PLL did not affect BRCA2 levels. However, adhesion to COL1 reduced BRCA2 levels by 75 ± 4% ($p < 0.0001$) after 3 h and completely depleted them after 4 h. BRCA2 protein levels did not recover after 12–15 h of cell adhesion (data not shown). To investigate whether this effect was directly linked to the interaction between COL1 and β_1 integrin, we set up similar experiments in which PNT1A and PC-3 cells were either preincubated with the β_1 integrin blocking antibody P4C10 or allowed to adhere to P4G11, a β_1 integrin-activating antibody (Fig. 3*B*). Cell adhesion to P4G11 (1 μg/ml) was similar to cell adhesion to COL1 (10 μg/ml) for both cell types (data not shown). After PC-3 cell adhesion to P4G11, BRCA2 levels decreased by 42 ± 5% at 2.5 h ($p < 0.001$) and became undetectable at 4 and 6 h. However, BRCA2 protein depletion was partly prevented by pretreating the cells with P4C10 before adhesion to COL1. Indeed, after 4 h, BRCA2 levels were 39 ±

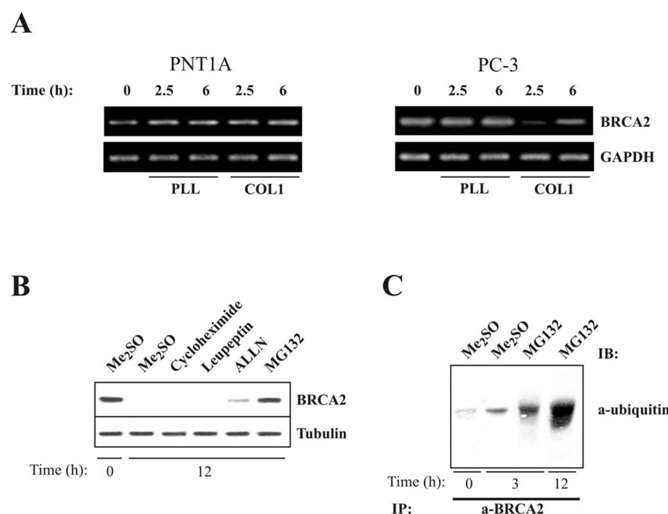

FIG. 4. **BRCA2 protein undergoes ubiquitination and degradation in the proteasome in PC-3 ...lls.** *A*, PNT1A and PC-3 cells were allowed to adhere to PLL o... 37 °C for 0, 2.5, or 6 h, washed, and processed for BRCA... quantitative RT-PCR. GAPDH mRNA RT-PCR wa...ntrol. Results are representative of two inde... PC-3 cells were allowed to adhere to C...imes in the presence of the translat...ml), the lysosomal inhibitor ...LLN (10 μM), the protea... alone (Me_2SO). Cell ...otein by immunoblot... ...endent experiments. ...presence of MG132 ...imes before being ...ith an antibody ...y to ubiquitin. ...riments.

WITHDRAWN
May 20, 2014

...). In PNT1A cells, ... P4C10 did not change ...ression observed on COL1.

...*Ubiquitination and Degradation* ...*-3 Cells*—Because variations at the ... could provide an explanation for the ...A2 protein levels, we evaluated the eventual ...mRNA levels upon PNT1A and PC-3 cell adhesion ...1 or PLL (Fig. 4*A*). In PNT1A cells, BRCA2 mRNA ...creased 1.4- and 1.5-fold 2.5 h after adhesion to PLL and COL1, respectively, and remained elevated at 6 h. In PC-3 cells, although adhesion to PLL did not affect BRCA2 mRNA levels, adhesion to COL1 transiently abated BRCA2 mRNA to ~10% after 2.5 h and then to later increase to ~65% of basal levels after 6 h. However, the increase in mRNA 6 h after adhesion (Fig. 4*A*) did not result in an increase in BRCA2 protein (Fig. 3*A*). These results suggested that post-transcriptional mechanisms might be mainly responsible for BRCA2 protein depletion by COL1 in PC-3 cells.

We next proceeded to investigate whether *de novo* protein synthesis and/or protein degradation contributed to the reduction of BRCA2 protein levels in PC-3 cells after adhesion to COL1. Cycloheximide treatment of PC-3 cells did not prevent BRCA2 loss within 12 h of adhesion to COL1, indicating that *de novo* protein synthesis was not necessary for BRCA2 depletion (Fig. 4*B*). The involvement of lysosomal-, calpain-, and ubiquitin-proteasome-mediated proteolysis was investigated by measuring BRCA2 protein levels after allowing PC-3 cells to adhere to COL1 in the presence of the lysosomal inhibitor leupeptin, the calpain inhibitor ALLN, or the proteasome inhibitor MG132 (Fig. 4*B*). MG132 prevented BRCA2 depletion almost completely, with levels that were 89 ± 7% after 12 h of adhesion. Treatment of PC-3 cells with leupeptin failed to

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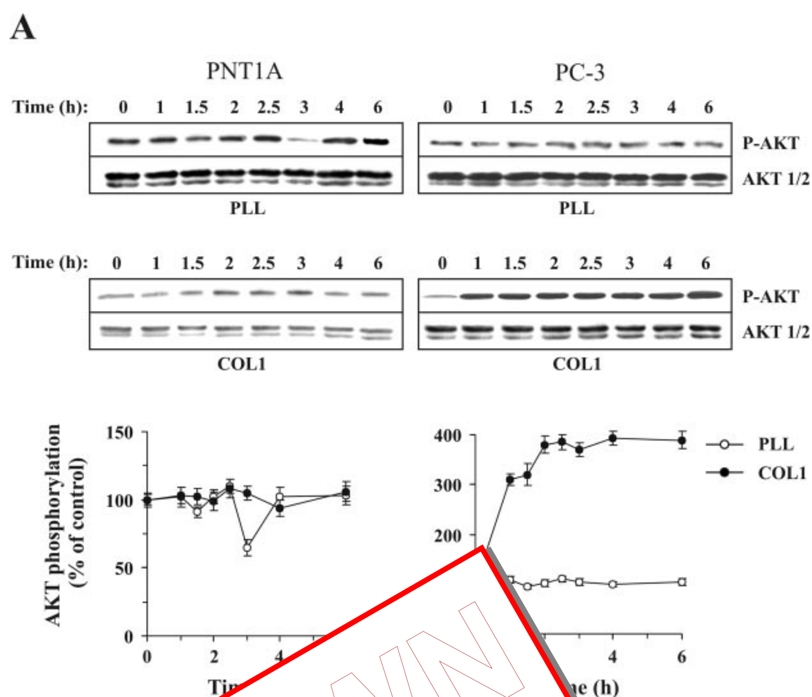

FIG. 5. **COL1 enhances the PI 3-kinase activity in cancer cells.** *A* and *B*, PNT1A and PC-3 cells were allowed to attach to PLL or COL1 at 37 °C for 0–6 h, washed, and lysed. *B*, PC-3 cells were preincubated with the β_1 integrin blocking antibody P4C10 (ascites 1:200) or 1C10 (ascites 1:200) as a negative control, before plating on COL1. Cell protein extracts (100 μg) were electrophoresed on 10% SDS-polyacrylamide gel under reducing conditions, and membranes were probed with anti-phospho-Ser-473 AKT antibody (*P-AKT*) or anti-AKT 1/2 antibody (*AKT 1/2*) as control, after stripping of the membrane. The blots are representative of three independent experiments. At the bottom of *A*, a quantification of AKT phosphorylation is reported as percentage of phosphorylation at 0 h of cell adhesion to PLL or COL1 (control), set at 100. Data are expressed as mean ± S.E.

WITHDRAWN
May 20, 2014

prevent BRCA2 depletio[illegible] lysosomal inhibitor pepst[illegible] NH_4Cl yielded similar resu[illegible] minor effect in preventing BR[illegible] 4% after 12 h. Cell viability, as [illegible] was >98% in all the experiments [illegible] solvent Me_2SO did not affect BRC[illegible]

To investigate whether BRCA2 [illegible]teasome degradation through ubiquitination, [illegible]C-3 cells to adhere to COL1 in the presence of MG1[illegible] solvent alone, and cell extracts were immunoprecipitated with an antibody to BRCA2, followed by Western blotting with an antibody against ubiquitin (Fig. 4*C*). Before adhesion (0 h), PC-3 cells showed minimal BRCA2 ubiquitination. After 3 h of adhesion to COL1, BRCA2 ubiquitination increased steadily at a pace that mirrored the reduction in BRCA2 protein levels seen in Fig. 3*A*. Treatment with MG132 further increased the levels of ubiquitinated BRCA2.

PI 3-Kinase Activation after PC-3 Cell Adhesion to COL1 Promotes BRCA2 Ubiquitination—Integrin engagement has been shown to trigger the activation of the PI 3-kinase signaling pathway (27, 28), which can modulate gene expression (23). Thus, we asked whether PI 3-kinase/AKT mediated the down-regulation of BRCA2 after PC-3 cell adhesion to COL1. The PI 3-kinase activity was measured by Western blotting with anti-phospho-Ser-473 AKT antibody following adhesion of PNT1A and PC-3 cells to COL1 or PLL (Fig. 5*A*). Both PNT1A and PC-3 cells showed basal AKT phosphorylation. Whereas PNT1A cells [illegible]crease in PI 3-kinase activity 3 h after [illegible] and no significant change after adhesion to [illegible] cells exhibited a 3.8-fold increase in AKT phospho[illegible] at 2 h of adhesion to COL1. These high levels remained [illegible]changed throughout the 6 h of cell adhesion (Fig. 5*A*) and after 12 h of adhesion (data not shown). On the contrary, there was no change in AKT phosphorylation after PC-3 cell adhesion to PLL. The increase in AKT phosphorylation upon PC-3 cell adhesion to COL1 was dependent on β_1 integrin, as P4C10 pretreatment resulted in ~80% inhibition as compared with 1C10-treated cells (Fig. 5*B*). To investigate whether activation of the PI 3-kinase/AKT was involved in the down-regulation of BRCA2 expression, we determined the effect of PI 3-kinase inhibitors on BRCA2 protein levels. In PC-3 cells, inhibition of the PI 3-kinase activity through wortmannin (Fig. 6*A*) or LY294002 (data not shown) caused a 3.8-fold increase of BRCA2 expression after 2.5 h of adhesion to COL1 but had no significant effect in PNT1A cells. After 6 h, BRCA2 levels were slightly higher (~23%) than those observed before cell attachment (0 h). Treatment with wortmannin did not affect BRCA2 protein levels in PC-3 cells adherent to PLL. As expected, wortmannin or LY294002 significantly inhibited AKT phosphorylation both in PNT1A and PC-3 cells (data not shown). These results were further validated by transiently transfecting PC-3 cells with either a dominant negative (Δp85) or a constitutively active (p110*) form of the PI 3-kinase before adhesion to COL1. Transfection with Δp85 completely prevented BRCA2 protein degradation, with 97 ± 5% of starting levels at 12 h (Fig. 6*B*).

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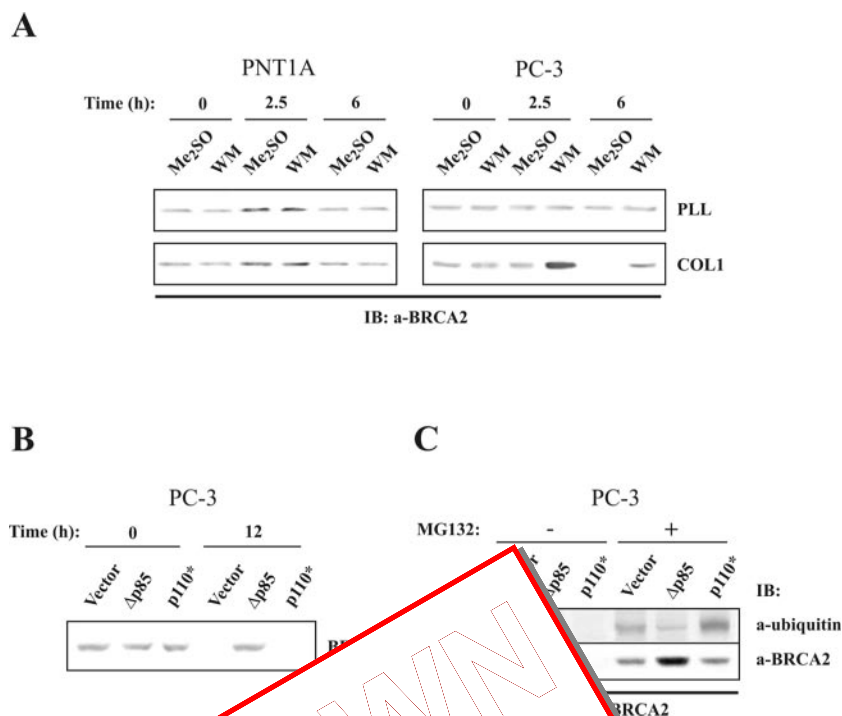

FIG. 6. **PI 3-kinase activation after PC-3 cell adhesion to COL1 promotes BRCA2 ubiquitination.** *A*, PNT1A and PC-3 cells were pre-treated for 1 h at 37 °C with 0.1 μM wortmannin (*WM*), a PI 3-kinase inhibitor, or the solvent alone (*Me$_2$SO*), and then allowed to adhere to PLL or COL1 at 37 °C for 0, 2.5, or 6 h, washed, lysed, and processed for immunoblotting (*IB*). Membranes were probed with anti-BRCA2 antibody. *B* and *C*, PNT1A and PC-3 cells were transiently transfected with empty vector (pcDNA3), dominant negative PI 3-kinase p85 subunit (*Δp85*), or constitutively active PI 3-kinase p110 subunit (*p110**). Thirty-six hours after transfection, the cells were allowed to adhere to COL1 at 37 °C for the indicated times in the presence or absence of the proteasome inhibitor MG132 (10 μM) or the solvent alone (*Me$_2$SO*) and then lysed. Cell lysates were processed for immunoblotting with an antibody to BRCA2 (*B*) or first immunoprecipitated (*IP*) with an antibody to BRCA2 and then immunoblotted with a monoclonal antibody to ubiquitin or to BRCA2 (*C*). The blots are representative of two independent experiments.

WITHDRAWN May 20, 2014

Transfections with p110* (Fig. 6*B*) or a wild-type form of t[illegible] p85α subunit of the PI 3-kinase (data not shown) did n[illegible] BRCA2 depletion by COL1. Overall, these resul[illegible] compelling evidence that β_1 integrin signalin[illegible] nase/AKT mediated the depletion of BR[illegible] cell adhesion to COL1. Transfecti[illegible] ments was about 80–85% as[illegible] staining, and overexpressi[illegible] was confirmed by im[illegible] antibody, respective[illegible]

To investigate wheth[illegible] pathway after PC-3 cell[illegible] to the ubiquitin-proteaso[illegible] PC-3 cells were transient[illegible] Δp85, or p110* and allowed[illegible] the proteasome inhibitor MG1[illegible] immunoprecipitated with an [illegible] lowed by immunoblotting analy[illegible] ubiquitin (Fig. 6*C*). Upon treatme[illegible]ans-fected cells showed BRCA2 ubiquiti[illegible] with the results reported in Fig. 4*C*. Transfecti[illegible]5 resulted in 86% decrease in ubiquitinated BRCA2[illegible]reas the constitutively active p110* increased BRCA2 ubiquitination by 32% when compared with mock-transfected cells. Δp85 transfectants treated with MG132 showed lower BRCA2 ubiquitination than untreated cells.

Down-regulation of BRCA2 Expression by COL1 Increases DNA Synthesis in Prostate Cancer Cells—To investigate whether the ECM had any effect on prostate cell proliferation, we measured [^{3}H]thymidine incorporation into DNA in PNT1A and PC-3 cells growing onto different substrates (Fig. 7*A*). PNT1A cells exhibited similar DNA synthesis when growing onto COL1 or PL, *i.e.* their own matrix, whereas adhesion to PLL decreased DNA synthesis by 39 ± 5% of the levels seen on PL ($p < 0.03$). On the contrary, COL1 stimulated PC-3 cell proliferation when compared with PL or PLL. We immediately posed the question whether the differences in DNA synthesis on COL1 were related to BRCA2 depletion in PC-3 cells. This was first addressed by small RNA interference assays. We transfected PC-3 cells with BRCA2 double-stranded RNA, [illegible] days. Forty-eight [illegible]n of BRCA2 was [illegible]his point, PC-3 [illegible]thymidine in-[illegible]the levels of [illegible]se results [illegible]versely corre-[illegible]is hypothesis was [illegible]ype BRCA2 cDNA into [illegible]er transfection the [^{3}H]thy-[illegible] of cell adhesion to PL, PLL, or [illegible] absence of MG132 (Fig. 7*C*). Mock-[illegible]s served as a control. Thirty six hours after [illegible]CA2 protein levels increased ~2.2-fold and [illegible]onstant upon adhesion to PL (data not shown) or [illegible]t decreased by 39 ± 8% after adhesion to COL1 ($p <$ [illegible]02) (Fig. 7*C*, *top panel*). Treatment of BRCA2 overexpressing cells with MG132 did not affect BRCA2 protein levels upon adhesion to PLL (data not shown) but resulted in a 210 ± 16% increase upon adhesion to COL1 ($p < 0.001$) (Fig. 7*C*, *top panel*). The expression of recombinant BRCA2 protein did not affect [^{3}H]thymidine incorporation in PC-3 cells adherent to PL or PLL but completely abrogated the increase in DNA synthesis in response to adhesion to COL1 (Fig. 7*C*, *bottom panel*). Consistently, treatment with MG132 did not affect DNA synthesis in BRCA2-transfected cells adherent to PLL but decreased [^{3}H]thymidine incorporation in control and BRCA2 transfectants adherent to COL1 by 46 and 57%, respectively ($p < 0.003$).

Inhibition of PI 3-Kinase/AKT Abrogates COL1-β_1 Integrin Stimulatory Effect on DNA Synthesis in PC-3 Cells—In order to establish whether a link exists between β_1 integrin, PI 3-kinase/AKT activation, and the increase in DNA synthesis after PC-3 cell adhesion to COL1, we measured [^{3}H]thymidine incorporation in PNT1A and PC-3 cells either preincubated with P4C10 before adhesion to COL1 or allowed to adhere to P4G11 (Fig. 8*A*) and in cells allowed to adhere to COL1 for 12 h in the presence or absence of wortmannin (Fig. 8*B*). Blocking of β_1 integrin by P4C10 decreased by 27% the incorporation of [^{3}H]thymidine in PC-3 cells ($p < 0.01$). Conversely, PC-3 cell

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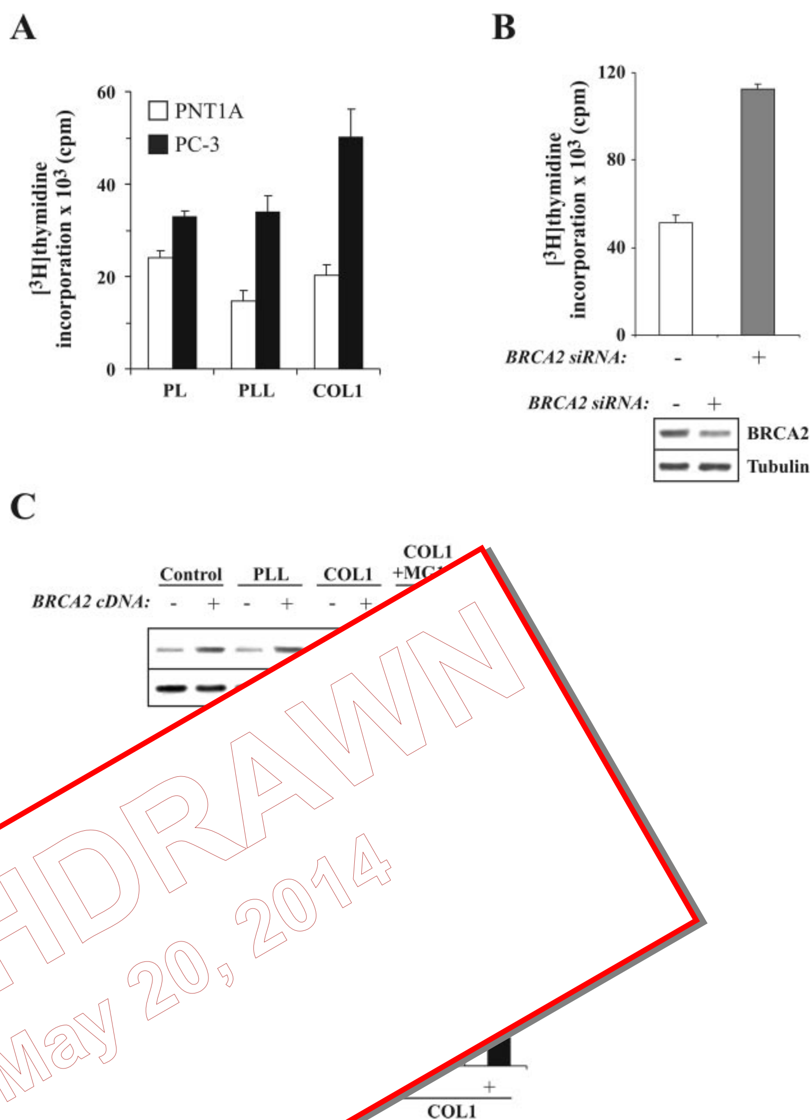

FIG. 7. **Down-regulation of BRCA2 expression by COL1 increases DNA synthesis in prostate cancer cells.** *A*, PNT1A and PC-3 cells were allowed to adhere to PL, PLL, or COL1 for 15 h and pulsed with 1 μCi/well methyl-[^{3}H]thymidine during the last 3 h of the 15-h culture. Thereafter, the "hot" medium was washed out, the cells were lysed with SDS, and cell remnants were collected into scintillation tubes to measure incorporated radioactivity. *B*, PC-3 cells either transfected to express an interfering RNA for BRCA2 or treated with the transfection reagent alone, 48 h after transfection, were allowed to adhere to COL1 for 15 h, and pulsed with methyl-[^{3}H]thymidine as described in *A*. At the bottom, BRCA2 protein knock-down was determined by immunoblotting 48 h after transfection. β-Tubulin signals were used as loading controls. *C*, PC-3 cells were transfected to express the wild-type BRCA2 cDNA or the empty vector (pcDNA3), and 36 h after transfection, BRCA2 protein expression was determined by immunoblotting before (*Control*) and after 12 h of adhesion to PLL or to COL1 in the presence or absence of the proteasome inhibitor MG132 (10 μM). β-Tubulin signals were used as loading controls. At the bottom, PC-3 cells transfected to express the wild-type BRCA2 cDNA (*BRCA2 cDNA*) or the empty vector (pcDNA3) 36 h after transfection were allowed to adhere to COL1 for 15 h in presence of MG132 (10 μM) or the solvent alone (Me_2SO), and pulsed with me... [^{3}H]thymidine as described in *A*. ... expressed as mean ± S.E... wells. A representati... three is shown.

WITHDRAWN May 20, 2014

adhesion to a β_1 integrin a... ~40% [^{3}H]thymidine incorpor... bition nor activation of β_1 inte... response of PNT1A cells as con... Inhibition of PI 3-kinase with wor... [^{3}H]thymidine incorporation in PC-3 cells b... but had no effect in PNT1A cells. This was fu...med by transfections with Δp85 dominant negative ...inase form, which decreased DNA synthesis in PC-3 cells adherent to COL1 by 36% ($p < 0.001$; Fig. 8*C*). Transfection with wt-p85 or p110* did not affect DNA synthesis.

DISCUSSION

We have uncovered evidence suggesting that ECM proteins enriched at metastatic bone sites have a stimulatory effect on prostate cancer cell proliferation. This proliferative effect is linked to BRCA2 protein depletion via β_1 integrin-dependent activation of the PI 3-kinase pathway, which promotes BRCA2 ubiquitination and degradation in the proteasome.

Prostate carcinoma has an exquisite tendency to metastasize to the bone with as many as 90% of patients with advanced disease suffering from osseous metastasis (38). The selective nature of this process is suggestive of a unique microenvironment that provides prostate cancer cells with the factors necessary to sustain metastatic capabilities. The non-mineral osseous ECM consists mostly of COL1, a protein that serves as a ...ve substrate through interaction with $\alpha_1\beta_1$, $\alpha_2\beta_1$, ...$_3\beta_1$ integrins. Prior work has suggested that PC-3 cells, which are derived from a human bone metastasis (35), adhere to the collagenous component of the osseous matrix to a great extent through the $\alpha_2\beta_1$ integrin (20). We observed that PC-3 cells exhibited an increased adhesion to COL1 compared with the normal prostate cell line PNT1A. This property hinged on the presence of β_1 integrin as it was entirely prevented by a β_1 integrin blocking antibody. It has been postulated that elevated cell adhesion to COL1 may partly explain the osteotropism displayed by metastatic prostate tumor cells (20, 39). Interestingly, both normal and cancer cells displayed comparable amounts of β_1 integrin protein. Thus, it is conceivable that the different adhesive properties of PNT1A and PC-3 cells to COL1 could be because of the differential expression of specific α integrin subunits between the two cell types (40).

In addition to providing osteotropic properties to prostate cancer cells, we show for the first time that β_1 integrin-mediated adhesion to an ECM protein has a negative effect on BRCA2 protein levels. Although in normal cells the adhesion to PLL and COL1 elicits a transient increase in BRCA2 protein, PC-3 cells exhibit a complete loss of BRCA2 after adhesion to COL1 but not to PLL. A few hours after adhesion, there is a sizable decrease in BRCA2 mRNA levels, which should contribute at least initially to lowering BRCA2 protein levels. How-

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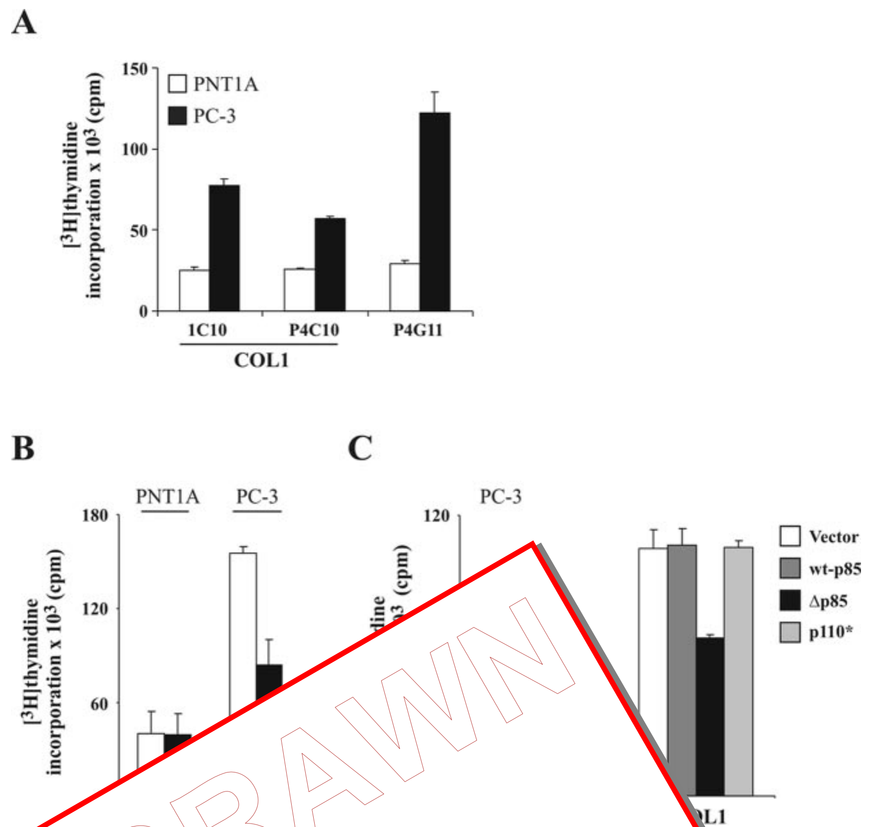

FIG. 8. **Inhibition of PI 3-kinase/AKT abrogates COL1-β_1 integrin stimulatory effect on DNA synthesis in PC-3 cells.** *A*, PNT1A and PC-3 cells preincubated for 1 h on ice in the presence or absence of the β_1 integrin blocking antibody P4C10 (ascites 1:200) or 1C10 monoclonal antibody (ascites 1:200), as a negative control, were allowed to adhere to COL1 or P4G11 for 15 h, and pulsed with 1 μCi/well methyl-[^{3}H]thymidine during the last 3 h of the 15-h culture. Thereafter, the hot medium was washed out; the cells were lysed with SDS, and cell remnants were collected into scintillation tubes to measure incorporated radioactivity. *B*, PNT1A and PC-3 cells, pretreated for 1 h at 37 °C with 0.1 μM wortmannin (*WM*), a PI 3-kinase inhibitor, were allowed to adhere to COL1 for 15 h and pulsed with methyl-[^{3}H]thymidine as described in *A*. *C*, PC-3 cells were transiently transfected with empty vector (pcDNA3), wild-type PI 3-kinase p85 subunit (*wt-p85*), dominant negative PI 3-kinase p85 subunit (*Δp85*), or constitutively active PI 3-kinase p110 subunit (*p110**). Thirty six hours after transfection, the cells were allowed to adhere to PLL or COL1 at 37 °C for 15 h and pulsed with methyl-[^{3}H]thymidine as described in *A*. Data are expressed as mean ± S.E. of triplicate wells. A representative experiment of three is shown.

WITHDRAWN
May 20, 2014

ever, although mRNA recovers after 4 h ... levels, BRCA2 protein levels remain ... fair to assume that transcription ... minor role in the sustained ... adhesion to COL1. Th ... transcriptional dow ... been shown that p53 ... moter causing reductio ... response to DNA damage ... is mutated in PC-3 cell ... mRNA expression is likely t ... mechanism. Furthermore, the ... els after cancer cell adhesion ... exclusively by a COL1-β_1 integ ... proteins may have an analogous e ... as been reported for *BRCA1*, a tumor ... nctionally related to *BRCA2* (42), after adh ... carcinoma cells to laminin-1 and collagen type IV ... us, it is conceivable that BRCA1 and BRCA2 proteins are at the cross-roads of abnormal cancer cell response to signaling from the extracellular environment.

We proceeded to dissect the biological mechanisms involved in BRCA2 disappearance in response to PC-3 cell adhesion to COL1. It had been reported previously that the BRCA2 protein depletion that occurs after ultraviolet irradiation requires new protein synthesis (44). However, adhesion experiments in the presence of the translational inhibitor cycloheximide failed to prevent BRCA2 degradation, implicating a more conventional proteolytic process. Two major proteolytic systems exist in mammalian cells, the lysosomal and the non-lysosomal systems (45); our experiments excluded any involvement of the former in the COL1-mediated depletion of BRCA2 protein. Among the latter, calpain- and ubiquitin-mediated proteolysis ... involved in ... –48). As in ... asal levels of ... levels increased ... COL1 and increased ... bition, thus providing circ ... A2 depletion under these con ... ubiquitin-proteasome degradation. ... has been shown to play a role in down ... CA2 expression in response to mitomycin C- ... damage (49) but not in ultraviolet irradiation- ... BRCA2 depletion (44), suggesting that the ... chanisms of regulation of BRCA2 expression are complex and can vary in different physiopathological conditions. Calpain-mediated proteolysis of BRCA2 protein was observed in our conditions but to a much lesser extent than proteolysis mediated by the ubiquitin-proteasome system.

Ligation of β_1 integrins can activate both the mitogen-activated protein kinase/extracellular signal-regulated kinase and the PI 3-kinase/AKT signaling pathway (50, 51). In normal cells, the transient increase in BRCA2 expression after attachment to an adhesive substrate is accompanied by a transient increase in extracellular signal-regulated kinase phosphorylation.[3] In contrast, PC-3 cell adhesion to COL1 triggers a conspicuous increase in AKT phosphorylation along with the decrease in BRCA2 levels. These two events were proven to be linked to each other in experiments whereby chemical inhibition of AKT phosphorylation with wortmannin or LY294002 or by transfection of a dominant negative form of PI 3-kinase prevented any ensuing BRCA2 protein degradation from happening. Transfection of a constitutively active form of PI 3-kinase had the opposite effect and promoted BRCA2 ubiquitination and degradation upon PC-3 cell adhesion to COL1. These experiments provide compelling evidence that the PI 3-kinase

[2] L. Moro, A. A. Arbini, E. Marra, and M. Greco, manuscript in preparation.

[3] L. Moro, A. A. Arbini, E. Marra, and M. Greco, unpublished data.

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pathway promotes ubiquitination of BRCA2 protein in cancer cells, thus targeting it to the proteasome for proteolysis. Recent studies have shown that the PI 3-kinase/AKT pathway can control the expression of proteins such as tuberin, FOXO, p53, the androgen receptor, and insulin receptor substrate-1 through ubiquitination (52–55). AKT has been shown to activate the ubiquitin ligase activity of murine double minute-2 that has been implicated in AKT-stimulated increase in p53 and androgen receptor degradation (53, 54, 56). However, the mechanism whereby PI 3-kinase/AKT targets substrates such as BRCA2 for ubiquitination remains to be investigated.

In normal prostate cells we did not find any consistent correlation between cell proliferation and the transient increase in BRCA2 expression resulting from attachment to an adhesive substrate. Adhesion to PLL diminishes the proliferative index, which could be rather attributed to the absence of specific mitogenic signals derived from the ECM (57). On the contrary, in cancer cells we demonstrate that BRCA2 protein degradation after adhesion to the ECM results in a conspicuous increase in DNA synthesis, an index of increased cell proliferation. This was corroborated by demonstrating that BRCA2 protein depletion after transfection with small interfering RNA did enhance DNA synthesis on COL1, and this effect was reversed by the expression of recombinant wild-type BRCA2 or by incubating the cells with a proteasome inhibitor. An increase in cell proliferation after PC-3 cell adhesion to COL1 had been noted in a previous report that showed an increase in the number of PC-3 cells after attachment to COL1 (39). M[...] over, our findings are in line with previous reports [...] down-regulation of BRCA2 mRNA expression du[...] liferative expansion of a nodal marginal zone [...] (58), and inhibition of the neoplastic ph[...] cancer cell line once expressing wil[...]

A number of studies have i[...] receptors, in having a reg[...] (50, 59, 60). Others ha[...] 3-kinase signaling [...] (61). Finally, it has bee[...] invasiveness is associate[...] (62–64). The results pres[...] play a central role in bri[...] BRCA2 also becomes a mem[...] molecules that are regulated [...] signaling cascade (61, 65) thro[...] proteolytic system (29, 66), whic[...] cological manipulation by PI 3-kina[...]tors in anticancer therapy (65–67).

Overall, our findings supports the c[...]hat COL1 not only serves as a major adhesive substr[...]r prostate cancer cells but also provides a permissive substrate for enhanced cancer cell proliferation at metastatic osseous sites, a paramount event in the natural history of prostate carcinoma. Further elucidation of the regulatory mechanisms involved in BRCA2 expression by environmental signals will provide a better understanding of the molecular pathways that contribute to prostate cancer progression and metastasis.

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Down-regulation of BRCA2 Expression by Collagen Type I Promotes Prostate Cancer Cell Proliferation

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