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Up-regulation of Skp2 after Prostate Cancer Cell Adhesion to Basement Membranes Results in BRCA2 Degradation and Cell Proliferation*

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Aberrant interaction of carcinoma cells with basement membranes (BM) is a fundamental pathophysiological process that initiates a series of events resulting in cancer cell invasion and metastasis. In this report, we describe the results of our investigations pertaining to the events triggered by the adhesion of normal (PNT1A) and highly metastatic (PC-3) prostate cells onto BM proteins. Unlike PNT1A, PC-3 cells adhered avidly to Matrigel BM matrix as well as to isolated collagen type IV, laminin, and heparan sulfate proteoglycan perlecan, main BM components. This aberrantly increased cancer cell adhesion resulted in sustained BRCA2 protein depletion and vigorous cell proliferation, a cascade triggered by β_1 integrin-mediated phosphatidylinositol 3-kinase activation leading to BRCA2 degradation in the proteasom latter effect was orchestrated by phosphatidylinositol pendent up-regulation of Skp2, a subunit of the protein ubiquitin complex that directly as demonstrated by coimmunoprecipi ubiquitination, and ultimately dation. Inhibition of Skp2 prevented BRCA2 d upon cell proliferation dence on the role of BRC and elucidate the molecul regulation in cancer cells w step in the biology of metasta of this molecular pathway may p therapeutic strategies aimed at m prostate carcinoma.

Basement membranes (BM)[2] are thi ers of specialized extracellular matrix (ECM) that surround and closely associate with epithelial and endothelial cells, muscle fibers, and nerves. They consist mostly of collagen type IV (COL4) admixed with laminins (LN), nidogens, and the heparan sulfate proteoglycan perlecan (PLN) and may contain small amounts of fibronectin (FN) (1, 2). Although the BM tructural role in defining tissue architecture and compa lization has long been recognized, its dynamic ulation of cell behavior has only recently

Aberra h BM proteins play a crucial ancer cells must be able ons and increase cell come motile, which ing a BM directly cell behavior is sage of various s are trans- that not only o play a pivotal role sponse to environmental molecules such as growth fac- , 7), MAPK/ERK 1/2 (8, 9), and PI hese various interactions help in modu- of genes exerting stringent control upon cell ty, and cell proliferation (12).

ellular protein degradation via the ubiquitin-proteasome pathway is a prime pathway through which cells normally regulate processes involved in cell growth and proliferation (13, 14). There is evidence that a number of growth inhibitory molecules and tumor suppressor proteins, such as p53, p21, p27, p130, the β_{1C} integrin, and FOXO1, are preferentially degraded by the ubiquitin-proteasome system in carcinoma cells (14–17). Furthermore, E3 ubiquitin ligase family members Skp2 and Mdm2 have been shown to play a role in prostate cancer development and progression (18–20). In a previous report, we provided evidence for a novel pathological mechanism whereby prostate carcinoma cell adhesion to collagen type I (COL1), a major ECM protein at osseous metastatic sites, promotes cancer cell proliferation through depletion of BRCA2 protein, the product of a tumor suppressor gene whose inactivation accounts for an increased risk in cancer development (21–23). This newly described effect resulted from β_1 integrin-dependent activation of the PI 3-kinase pathway, which promoted BRCA2 ubiquitination and degradation in the proteasome (24).

In this study, we extended our investigations to elucidate the mechanisms by which β_1 integrin signaling in prostate cancer

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[2] The abbreviations used are: BM, basement membranes; ECM, extracellular matrix; COL4, collagen type IV; LN, laminin; FN, fibronectin; PLN, perlecan; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI 3-kinase, phosphatidylinositol 3-kinase; Me_2SO, dimethyl sulfoxide; E3, ubiquitin-protein isopeptide ligase; siRNA, small interference RNA.

cells resulted in BRCA2 protein degradation in the proteasome. We also provide evidence demonstrating that the BRCA2-associated trophic effect is not restricted to the osseous environment but is quite active in mediating cancer cell proliferation after interaction with BM proteins.

EXPERIMENTAL PROCEDURES

Cell Culture—PNT1A cells (a human prostate normal cell line established by immortalization of normal adult prostate epithelial cells) and PC-3 cells (a human prostate carcinoma cell line derived from a bone metastasis) were kept in culture as described previously (16, 24).

Cell Adhesion—Cell adhesion assays to ECM proteins were carried out using 96-well tissue culture plates as described previously (24). Plates were precoated with different concentrations of FN (3 μg/ml; Sigma), LN (10 μg/ml; Invitrogen), PLN (10 μg/ml; Sigma), or COL4 (10 μg/ml; Sigma) for 16 h at 4 °C. Coating with 10 μg/ml bovine serum albumin (Sigma) served as negative control.

Adhesion to the BM matrix Matrigel (Sigma) was tested in 96-well plates coated with 50 μl/well of a 1:3 dilution in RPMI medium (Invitrogen) before cell plating. Cells were starved in serum-free methionine/cysteine-deficient RPMI 1640 (Sigma) for 45 min at 37 °C before labeling with 100 μCi/ml ^{35}S protein labeling mix (Amersham Biosciences) in 1 ml of methio cysteine-free RPMI medium containing 5% fetal bov After 24 h, 100 μl of a 0.2 × 10^6 cell suspension adhere for 1 h onto Matrigel or bovine μg/ml) at 37 °C and were washed th were lysed in 100 μl of 150 mM N and 2 mM EDTA containi phate-buffered saline lation counter (Beckm

Inhibition assays we 1 h on ice in the prese antibody to human β_1 i Temecula, CA), or the mon vascular endothelial surface gen) used as a negative control recorded for each experiment.

Immunoblotting Analysis and Im Cells were grown either onto FN (3 μg/ml), , PLN (10 μg/ml), or COL4 (10 μg/ml) and lyse protein extracts were analyzed by immunoblotting as described previously (24). Where indicated, cells were pretreated for 1 h with either P4C10 or 1C10, or the PI 3-kinase inhibitors wortmannin (0.1 μM; Sigma), LY294002 (10 μM; Calbiochem), or solvent alone (Me_2SO), or added with the proteasome inhibitor MG132 (10 μM). The following antibodies were used: 1 μg/ml anti-BRCA2 polyclonal antibody (H-300; Santa Cruz Biotechnology, Santa Cruz, CA), 10 μg/ml monoclonal antibody to β-tubulin (Sigma), 1 μg/ml polyclonal antibody to Skp2 (H-435; Santa Cruz Biotechnology), 2 μg/ml monoclonal antibody to Mdm2 (D-12; Santa Cruz Biotechnology), 1 μg/ml anti-phospho-AKT-Ser-473 polyclonal antibody (Santa Cruz Biotechnology), 1 μg/ml anti-AKT 1/2 polyclonal antibody (H-136; Santa Cruz Biotechnology), 0.2 μg/ml anti-phospho-ERK monoclonal antibody (E-4; Santa Cruz Biotechnology), 0.2 μg/ml anti-ERK2 polyclonal antibody (C-14; Santa Cruz Biotechnology), or 1:1000 dilution of anti-p85α rabbit antiserum (Sigma).

Analysis of BRCA2 ubiquitination was performed as described previously (24). To analyze BRCA2 association with Skp2, cell extracts were precleared and incubated overnight with 2 μg of polyclonal antibody to BRCA2. Immunocomplexes were recovered with protein A-Sepharose (Sigma), washed five times with phosphate-buffered saline containing 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate and were subjected to 10% SDS-PAGE under reducing conditions followed by transfer to polyvinylidene difluoride membranes. Filters were immunoblotted using 1 μg/ml monoclonal antibody to Skp2 (Zymed Laboratories Inc., San Francisco, CA) or 2 μg/ml monoclonal antibody to BRCA2 (clone 5.23; Chemicon) following the manufacturers' instructions. Alternatively, whole cell extracts were immunoprecipitated with 2 μg of monoclonal antibody to Skp2 (Zymed atories Inc.) and separated by 6% SDS-PAGE, and f munoblotted using 1 μg/ml anti-BRCA2 po 1 μg/ml polyclonal antibody to Sk

Tr midine Incorporation— RCA2 cDNA (a kind xas M. D. Anderson negative form of man, University tor (pcDNA3; usly (24). A pecific siR- nology and used the manufacturer's ion assays were performed previously (24).

ata are reported as the mean ± S.E. as performed by the Student's *t* test. All re repeated at least twice.

LTS

Prostate Normal and Carcinoma Cells Adhere Differently to BM—We investigated the adhesive properties of PNT1A and PC-3 cells to LN, PLN, and COL4, major components of BM, and to FN, a widely expressed extracellular matrix protein (25) enriched at the prostatic stroma but a minor component in BM. As shown in Fig. 1*A*, PNT1A cells adhered efficiently to FN, to a lesser extent to LN and PLN (~60% of FN levels), and showed no adhesion to COL4. Highly metastatic PC-3 cells exhibited a reversed affinity pattern, with partial loss of adhesion to FN and newly gained adhesion capabilities to BM proteins. Because cell adhesion to FN, LN, COL4, and PLN (26) is mediated by integrin receptors that share a common β_1 subunit ($\alpha_5\beta_1$ to FN, $\alpha_6\beta_1$ to LN, $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ to COL4, $\alpha\beta_1$ to PLN), we tested the effect of the β_1 integrin-blocking antibody P4C10 on cell adhesion. As shown in Fig. 1*B*, adhesion to FN was reduced by 42 ± 6% ($p < 0.002$) and 39 ± 7% ($p < 0.001$) in PNT1A and PC-3 cells, respectively. Adhesion to LN was inhibited by ~25% in PNT1A and ~75% in PC-3 cells, adhesion to PLN was almost completely inhibited in both cell types, and onto COL4 PC-3

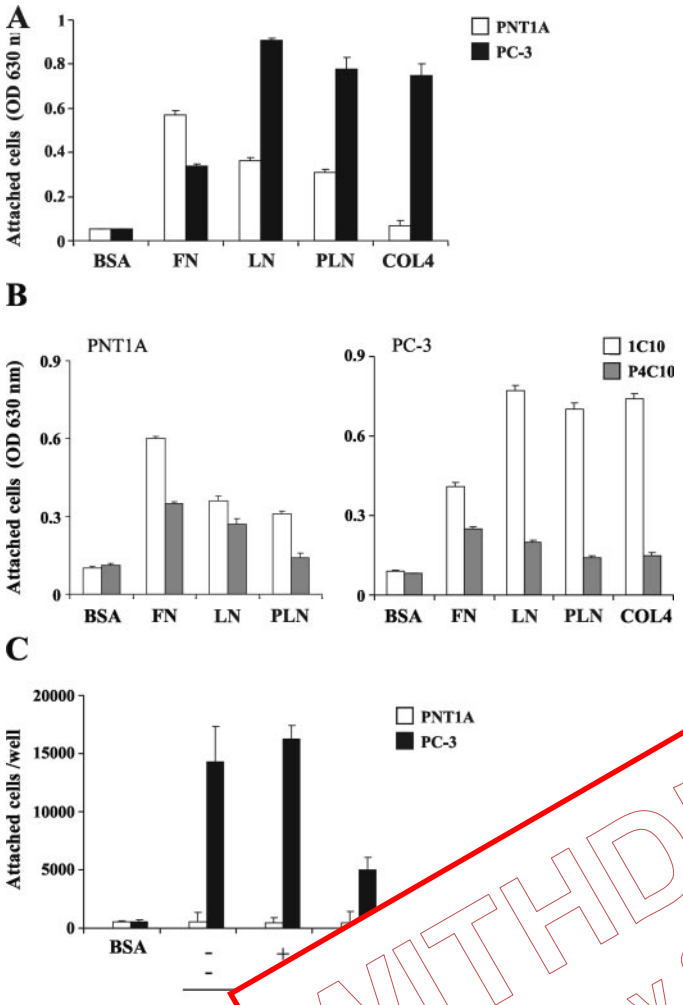


FIGURE 1. **Prostate normal a**
A, 1.5 × 10^5 PNT1A or PC-3 c
albumin (*BSA*) (10 μg/ml), FN (
COL4 (10 μg/ml) at 37 °C for 1 h
violet. Cell adhesion was quantifie
in an enzyme-linked immunosorben
cells were incubated for 1 h on ice in t
antibody P4C10 or 1C10 as control an
albumin (*BSA*), FN, LN, PLN, or COL4 as c
labeled PNT1A or PC-3 cells were incubate
absence of P4C10 or 1C10 before being allo
mg of protein/well) for 1 h at 37 °C. Follow
saline washes, cells were lysed and the radioac
expressed as mean ± S.E. of triplicate wells. A r
three is shown.

cell adhesion was almost completely inhibited (90 ± 7%, $p <$ 0.0001).

To investigate whether these findings could be reproduced onto naturally occurring BM layers, we tested the adhesive properties of normal and cancer prostate cells to Matrigel, a reconstituted BM preparation (27). As shown in Fig. 1*C*, whereas PC-3 cells adhered efficiently to Matrigel in a manner that was significantly inhibited by the anti-β_1 integrin-blocking antibody (~70% inhibition, $p < 0.004$), normal prostate cell adhesion was nil in the time period considered.

Adhesion of PC-3 Cells to BM Proteins Decreases BRCA2 Protein Levels in a β_1 Integrin-dependent Manner—We asked whether PC-3 cell adhesion to BM proteins exercised a modulatory effect upon BRCA2 expression. To this effect, PNT1A and PC-3 cells were grown onto plates coated with FN, LN, PLN, COL4, or Matrigel and BRCA2 levels were assessed. As depicted in Fig. 2*A*, whereas PNT1A cells transiently increased BRCA2 protein after adhesion to BM proteins (2.2 ± 0.3-fold onto FN after 2.5 h, $p < 0.002$), PC-3 cells exhibited almost complete (PLN) or complete (LN, COL4, Matrigel) loss of detectable BRCA2 after adhesion for 6 h. The strongest down-regulatory effect was observed onto COL4, which caused BRCA2 protein levels to decrease after only 2.5 h by 68 ± 4% ($p < 0.01$). BRCA2 protein levels did not recover after 12 h of cell adhesion (data not shown). The β_1 integrin-blocking antibody P4C10 partly rescued BRCA2 protein to 45 ± 6% ($p <$ 0.001) after 6 h onto COL4 (Fig. 2*B*) as well as onto other BM proteins (data not shown). As COL4 is the most abundant component of the BM, most of the subsequent experiments were performed onto this BM protein.

BRCA2 Protein Depletio r Cancer Cell Adhesion to BM Increases DNA Synthe stigate whether the signaling cascade initiated d any effect on cell proliferation, we ation of [^{3}H]thymidine in PNT1A s substrates (Fig. 3*A*). W ed no variations irre- to COL4 enhanced tic by 291 ± 24% ive response was PLN (~175%). a not shown). he response 35% (data not ated and confirmed g. 3*B*, adhesion to Matri- 509 ± 27% and P4C10 inhib- 5% ($p < 0.0002$). The increase in hesion to BM proteins could be reversed C-3 cells with wild-type BRCA2 cDNA (Fig. e experiments, PC-3 cells were transiently trans- with BRCA2 cDNA or empty vector for 36 h, after which measured BRCA2 protein levels (*upper panel*) and [^{3}H]thymidine incorporation upon 12 h of cell adhesion to COL4 in the presence or absence of the proteasome inhibitor MG132 (*lower panel*). After transfection, BRCA2 protein levels increased 2.3-fold compared with mock-transfected cells. Upon COL4 adhesion, BRCA2 decreased by 44 ± 8% ($p < 0.03$) in transfected cells, but this reduction had no effect in [^{3}H]thymidine incorporation, which remained at basal levels throughout the experiment. On the contrary, mock-transfected cells exhibited complete disappearance of BRCA2 protein upon adhesion to COL4, which resulted in a burst in [^{3}H]thymidine incorporation. This trophic effect could be reversed with the proteasome inhibitor MG132, which decreased [^{3}H]thymidine incorporation by 94% ($p < 0.003$). Treatment of BRCA2-transfected cells with MG132 resulted in BRCA2 accumulation to 245 ± 29% ($p <$ 0.001).

Skp2 Promotes BRCA2 Protein Depletion in PC-3 Cells Adherent to BM—Because it is known that F-box proteins Skp2 and Mdm2 mediate the ubiquitin-dependent degradation of several negative regulators of cell proliferation in cancer (17, 28,

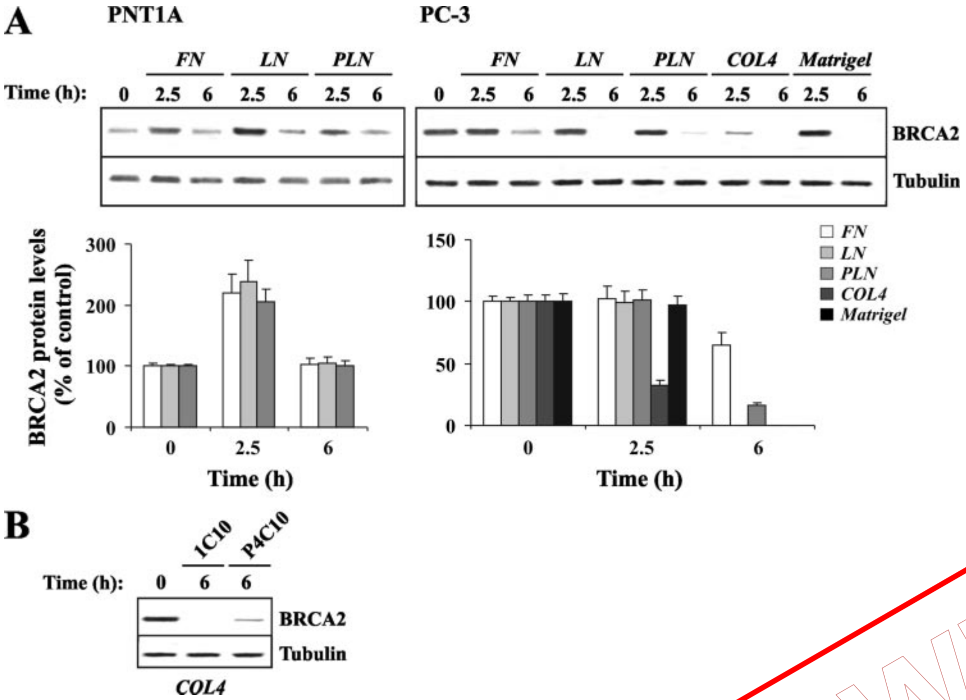


FIGURE 2. **PC-3 cell adhesion to BM proteins decreases BRCA2 protein levels ... manner.** *A*, PNT1A and PC-3 cells were allowed to adhere to FN (3 μg/ml), LN ... (10 μg/ml), or Matrigel at 37 °C for 0–6 h and were washed and processe... antibody. β-Tubulin signals were used as loading controls. The blot... experiments. *Bottom*, BRCA2 protein levels were quantitated... protein at 0 h, set to represent 100%. Data are expressed a... PC-3 cells were incubated for 1 h on ice in the presen... control, allowed to adhere to COL4 (10 μg/ml) a... branes were probed with anti-BRCA2 antibo... representative of three independent ex...

29), we hypothesized ... involved in BRCA2 u... tion in prostate cancer c... cell adhesion to COL4 ha... tein levels. As depicted in F... triggered Skp2 and Mdm2 pr... 184% at 2.5 h and 393 and 20... regulatory effect was significan... blocking antibody for Skp2 but no... to investigate whether manipulatio... levels had an effect upon BRCA2 protein le... synthesis. As shown in Fig. 4*B*, Skp2 knock dow...NAs resulted in Skp2 protein cell depletion (*upper panel*) and concomitant rescue of BRCA2 protein levels after adhesion to COL4 (*lower panel*). Furthermore, these newly induced changes blunted much of the new DNA synthesis upon PC-3 cell adhesion to COL4 (Fig. 4*C*). BRCA2 protein depletion was independent from any increase in Mdm2 protein levels upon adhesion to COL4, and Mdm2 knock down by siRNAs did not rescue BRCA2 protein levels (data not shown).

Skp2 Directly Interacts with BRCA2, Promoting Its Ubiquitination—Next we examined whether changes in Skp2 expression had any effect upon BRCA2 ubiquitination in PC-3 cells after COL4 adhesion. To this effect, cell extracts from PC-3 cells transfected with Skp2 siRNAs or mock transfectants were prepared at various time points and immunoprecipitated with anti-BRCA2 antibody, followed by Western blotting with an antibody against ubiquitin (Fig. 5*A*). Before adhesion (0 h), PC-3 cells showed minimal BRCA2 ubiquitination, as previously described (24). After 2.5 h of adhesion to COL4, cells transfected with nonspecific siRNAs exhibited steady increase in BRCA2 ubiquitination at a pace that mirrored the reduction in BRCA2 protein levels shown in Fig. 2*A*. In contrast, knock down of Skp2 expression resulted in a dramatic reduction in BRCA2 ubiquitination at all time points. This effect results from a direct interaction between the two proteins as demonstrated by coimmunoprecipitation assays. In the ...iment shown in Fig. 5*B*, after ... adhesion amounts of Skp2- ... complex immunoprecipi- ...h anti-BRCA2 sharply ...om detectable levels at ...al amounts at 2.5 h to ...decrease after 6 h, ...l BRCA2 protein ...havior mirrored ...depicted in Fig. ...etected when ...was used for ... (data not

...PI 3-Kinase Activation in ...er—We also investigated the ...ERK and the PI 3-kinase signaling ...g BRCA2 protein depletion upon cancer ...BM. In Western blotting with anti-phospho-...K/ERK activity in PC-3 cells was nil at rest and did ...ease after adhesion to COL4 (Fig. 6*A*). On the contrary, ...3-kinase activity increased by 2-fold, remaining highly phosphorylated for as long as 6 h as measured by Western blotting with anti-phospho-Ser-473 AKT. This response was dependent on β_1 integrin as pretreatment with the blocking antibody P4C10 resulted in ~80% inhibition in AKT phosphorylation onto COL4 (Fig. 6*B*). AKT phosphorylation affected BRCA2 levels as demonstrated by inhibition of PI 3-kinase activity with wortmannin (Fig. 6*C*) or LY294002 (data not shown), which increased BRCA2 protein by 4.3-fold after 2.5 h of adhesion to COL4.

Degradation of BRCA2 by Skp2 Requires PI 3-Kinase—To investigate whether the increase in Skp2 protein levels leading to BRCA2 ubiquitination after PC-3 cell adhesion to COL4 results from aberrant PI 3-kinase activation, we transiently transfected PC-3 cells with a dominant negative (Δp85) form of the PI 3-kinase before adhesion to COL4 and analyzed protein levels of BRCA2, Skp2, and Skp2-BRCA2 complex and [^{3}H]thymidine incorporation. As depicted in Fig. 7, transfection with Δp85 completely prevented BRCA2 protein degradation and Skp2 protein up-regulation (*panel A*) as well as the increase in

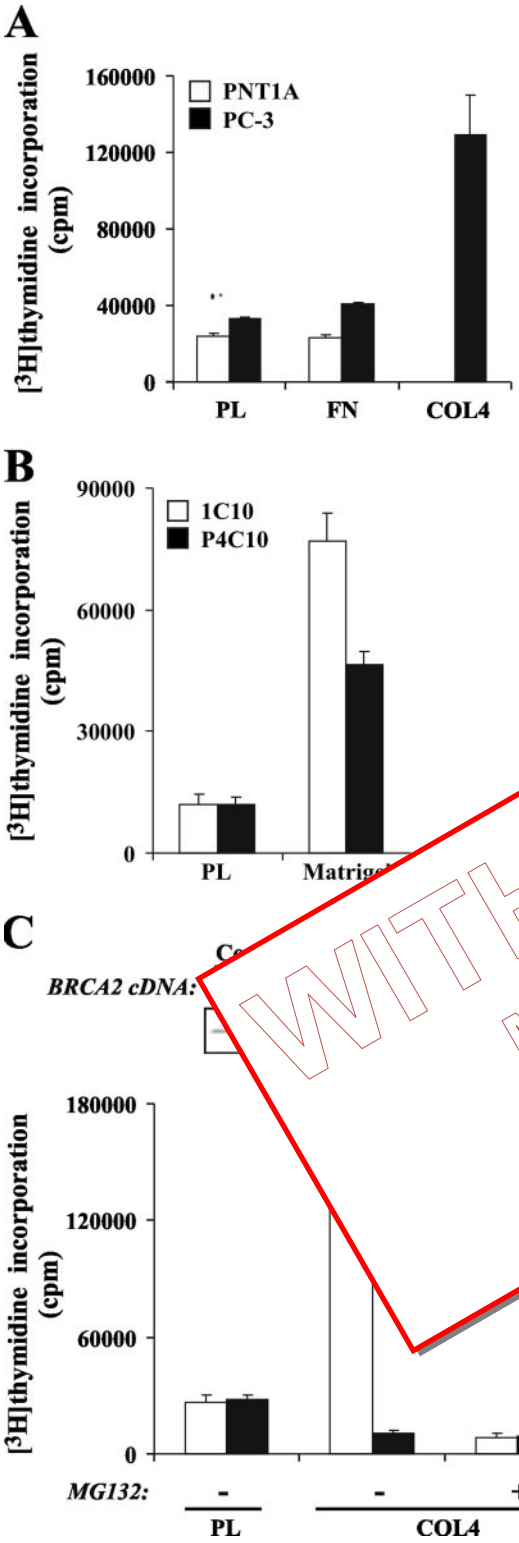


FIGURE 3. **BRCA2 protein depletion after cancer cell adhesion to BM proteins increases DNA synthesis.** *A* and *B*, PNT1A or PC-3 cells were preincubated for 1 h on ice in the presence or absence of β_1 integrin-blocking antibody P4C10 or 1C10 as control and cultured onto PL, FN (3 μg/ml), COL4 (10 μg/ml), or Matrigel (~0.2 mg of protein/well) for 15 h. A pulse with 1 μCi/well of methyl-[^{3}H]thymidine was applied for the last 3 h. After washing with cold medium, cells were solubilized and collected into scintillation vials for quantification of incorporated radioactivity. Data are expressed as mean ± S.E. of triplicate wells. A representative experiment of three is shown. *C*, PC-3 cells were transiently transfected with wild-type BRCA2 cDNA or empty vector (pcDNA3) for 36 h, after which BRCA2 protein expression was determined by immunoblotting before (*Control*) and after 12 h of adhesion to COL4 in the presence or absence of the proteasome inhibitor MG132 (10 μM). *Bottom*, PC-3 cells transiently transfected for 36 h with BRCA2 cDNA or vector alone were allowed to adhere onto plastic or COL4 in presence of MG132 or the solvent alone (−) and pulsed with methyl-[^{3}H]thymidine as described in *panels A* and *B*. Data are expressed as mean ± S.E. of triplicate wells. A representative experiment of three is shown.

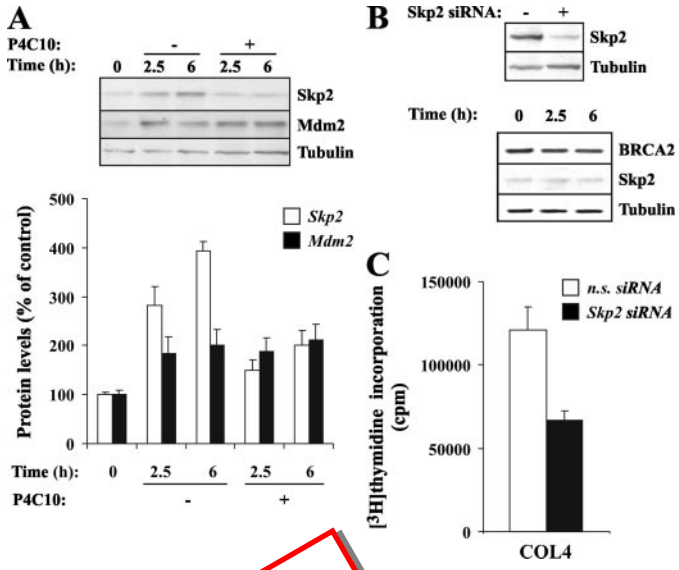


FIGURE 4. **Skp2 promotes B… depletion in PC-3 cells adherent to BM.** *A*, PC-3 cells … for 1 h on ice in the presence or absence of β_1 int… 4C10, allowed to attach to COL4 for the indic… rocessed for immunoblotting. Membra… Mdm2, and β-tubulin as loading … independent experiments. … ntitated and reported as … epresent 100%. Data are … eriments. *B*, PC-3 cells … r siRNAs for Skp2, and … for Skp2 36 h after … fection with Skp2 … es and processed … control. *C*, PC-3 … or nonspecific … and pulsed with … sed as mean ± S.E. of … is shown.

(*panel B*) and DNA synthesis … on to COL4.

… y of carcinoma *in situ* to overcome the surrounding … al BM and spread into the underlying stroma has long … fined the progression to invasive carcinoma (30). In turn, for local and distant metastasis to occur, locally invasive carcinoma cells must be able to colocalize and eventually invade muscle, nerves, and blood vessels, all of which are separated from the adjacent stroma by continuous sheets of BM. Hence, a fundamentally abnormal interaction of carcinoma cells with BM proteins must be at the core of their metastatic capabilities. Our experimental paradigm confirmed that unlike normal PNT1A, highly invasive PC-3 carcinoma cells exhibited inappropriate adhesion onto the main components of the BM as well as on a reconstituted BM matrix system. On the other hand, the ECM glycoprotein FN enriched at the prostatic stroma and a minor component of BM was a poor adhesive substrate for neoplastic

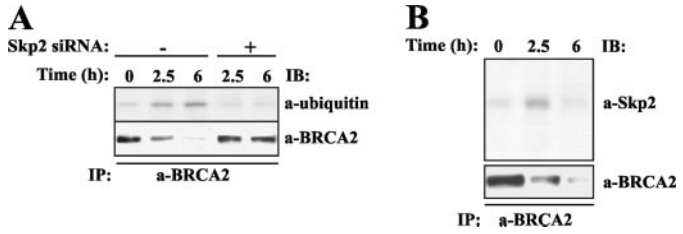
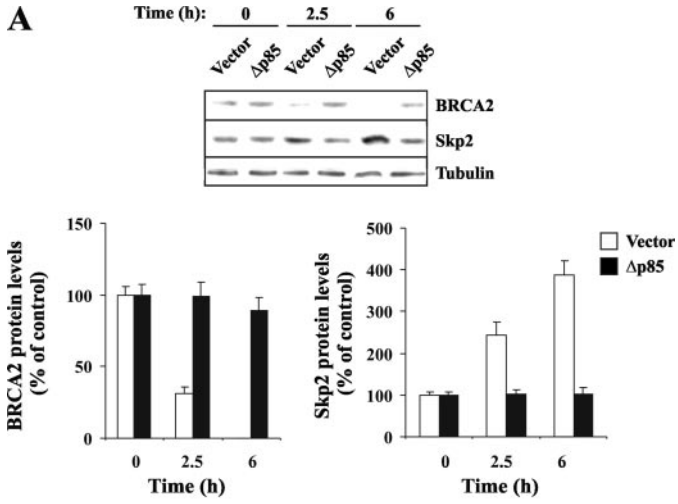
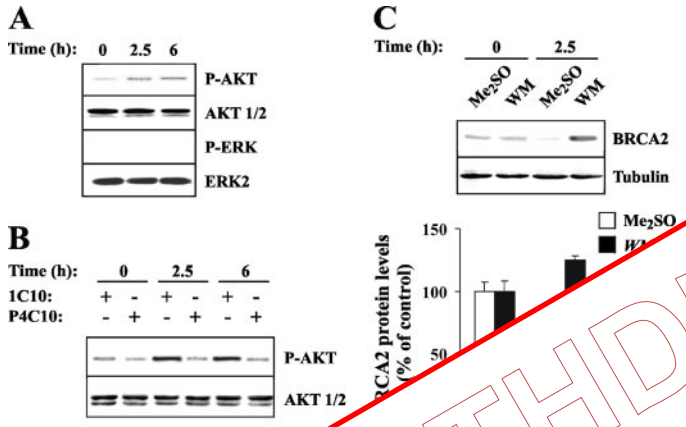


FIGURE 5. **Skp2 directly interacts with BRCA2, promoting its ubiquitination.** *A*, PC-3 cells were transfected with a pool of siRNAs for Skp2 or nonspecific siRNAs (−); 36 h after transfection cells were allowed to adhere to COL4 for the indicated times and lysed, and protein extracts were immunoprecipitated (*IP*) with a BRCA2 antibody. Immunoprecipitates were analyzed by immunoblotting (*IB*) with anti-ubiquitin and anti-BRCA2 antibody. Blots are representative of two independent experiments. *B*, PC-3 cells were allowed to adhere to COL4 for the indicated times and lysed, and protein extracts were immunoprecipitated with anti-BRCA2 antibody and immunoblotted with antibodies for Skp2 and BRCA2. Blots are representative of three independent experiments.





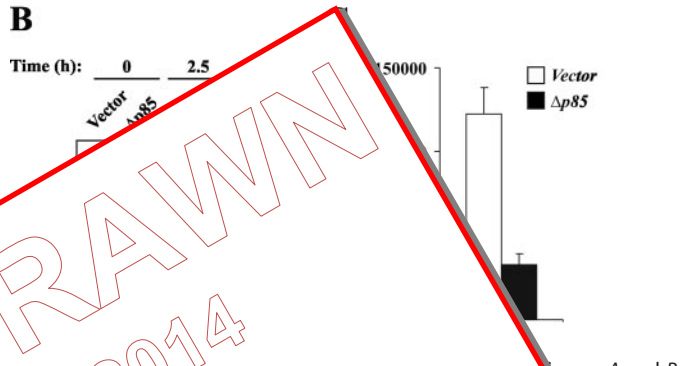


FIGURE 6. **PC-3 cell adhes… β_1 integrin-dependent m…** COL4 at 37 °C for 0–6 h, wash… were electrophoresed onto … conditions. Membranes were pr… or anti-phospho-ERK (*P-ERK*). Sub… blotted with anti-AKT 1/2 (*AKT 1/2*)… are representative of three independ… incubated with the β_1 integrin-blocki… before plating onto COL4 and being p… blots are representative of two independ… pretreated for 1 h at 37 °C with 0.1 μM wor… itor, or the solvent alone (*Me_2SO*). Cells were… COL4 at 37 °C for 0 or 2.5 h and were washed, lysed, … immunoblotting. Membranes were probed with anti-BRCA… and β-tubulin as loading control. The blots are representative … independent experiments. *Bottom*, BRCA2 protein levels were quantified and reported as percentage of the amount of protein at 0 h, set to represent 100%. Data are expressed as mean ± S.E. of two independent experiments.

…nase. *A* and *B*, …mant negative PI… to adhere to COL4 …ssed for immunoblotting … as loading control (*A*) or first …and then immunoblotted (*IB*) with …tin (*B*). The blots are representative of … the bottom of *panel A*, BRCA2 and Skp2 …ed and reported as percentage of the amount of …esent 100%. Data are expressed as mean ± S.E. of two …ments. *C*, PC-3 cells transiently transfected with domi… 3-kinase or vector alone were allowed to adhere to COL4 for … pulsed with methyl-[^{3}H]thymidine for the last 3 h. Data are …ssed as mean ± S.E. of triplicate wells. A representative experiment of …ree is shown.

PC-3 cells when compared with normal PNT1A cells. This latter observation could be partly correlated with the lower expression of the FN receptor β_3 integrin in PC-3[3] when compared with PNT1A cells.

Another crucial finding is that after adhesion to isolated BM proteins, particularly to COL4, highly invasive carcinoma cells exhibit a burst in [^{3}H]thymidine incorporation, a sensitive measurement of new DNA synthesis and cell proliferation. This trophic response seems to be elicited by β_1 integrin adhesion, as a similar phenomenon was previously demonstrated onto COL1, a ligand for β_1 integrin receptors (24). The proliferative response after adhesion to LN and PLN was slightly weaker than with COL4, and no effect was noticeable onto FN. When allowed to adhere onto the BM matrix Matrigel, prostate cancer cells exhibited a proliferation surge that was stronger than with isolated components, suggesting cooperation rather than competition among various proteins in activating proliferative signals.

The abnormal proliferative response following PC-3 cell adhesion onto BM was linked to a sustained BRCA2 protein depletion and could be completely averted by wild-type BRCA2 cDNA transfection. This pathway seems to switch cell proliferation on and off depending on a critical amount of BRCA2 protein, as suggested by the unresponsiveness to mild reductions in BRCA2 protein after PC-3 cell adhesion to FN. Conversely, normal cell adhesion to FN resulted in an increase in BRCA2 expression with no discernible effect upon DNA syn-

[3] L. Moro, unpublished data.

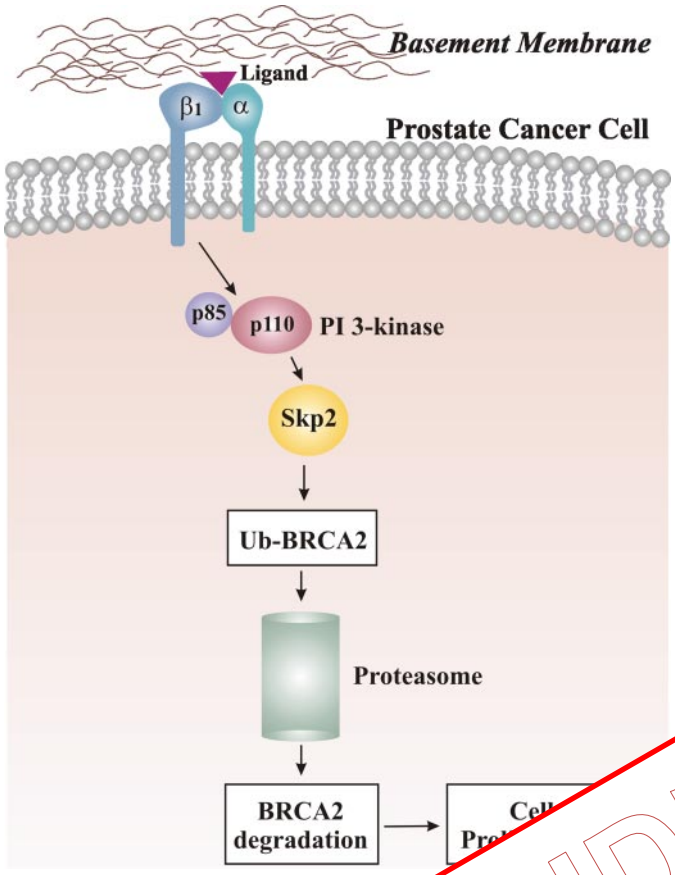


FIGURE 8. **Hypothesized role of BRCA2 in P… cancer cell proliferation.** BM-dependent… 3-kinase, increasing Skp2 protein leve… ing its ubiquitination (*Ub-BRCA2*) … dation. Ultimately, BRCA2 d… the BM interfaces.

thesis. Our findings add t… gesting that BRCA2 also fu… eration and tumor growth. … recombinant BRCA2 overexp… pancreatic adenocarcinoma cell… stantial decrease in tumorigenicity… mice (31). More recently, Miyamot… that Capan-1 cells exhibited an increased … ter adhesion to COL4. The mechanisms involve… yet well understood. Normally, part of the cellular resp…se to DNA damage involves the activation of an ATM/p53/Mdm2 feedback loop that regulates cell cycle progression and/or apoptosis in response to relative amounts of a DNA repair complex containing BRCA2 (33–35). However, involvement of this mechanism seems unlikely because our experiments demonstrate no relationship between β_1 integrin signaling and Mdm2 levels and suggest an alternative pathway involving Skp2 (see below). Furthermore, PC-3 (and Capan-1) cells exhibit inactivating mutations in the *p53* gene (36, 37).

Depletion of BRCA2 protein upon cancer cell adhesion to BM proteins and osseous COL1 (24) occurs through protein degradation in the proteasome. However, the precise mechanisms involved had not been elucidated before. In the present study, we have provided evidence that this event is mediated by Skp2, an F-box protein that associates with Skp1, Cul1, and Roc1/Rbx1 to form the SCF(Skp2) ubiquitin ligase complex (28). Indeed, down-regulation of Skp2 by siRNA was sufficient to rescue BRCA2 levels inhibiting the burst in cell proliferation upon cancer cell adhesion to BM. Evidence that this mechanism may be relevant in prostate cancer has also been provided in immunohistochemical studies performed on 622 radical prostatectomy specimens that demonstrate that Skp2 levels and cell-labeling frequency increase dramatically in both premalignant prostatic intraepithelial lesions and prostate carcinoma (19). Changes in Skp2 levels are dependent upon β_1 integrin-mediated signaling for Skp2 up-regulation, and subsequent BRCA2 degradation could be prevented by a β_1 integrin-blocking antibody. Evidence demonstrating that cell adhesion to the ECM results in Skp2 mRNA and protein up-regulation had been provided previously (38). We confirm those results and demonstrate for the first time that β_1 integrin-mediated signaling is necessary for ECM-dependent changes in Skp2 expressi…

The up-regulation of … follows β_1 integrin adhesion is signaled through … and could be abrogated by transfecting … of PI 3-kinase. These observations … horts implicating PI 3-ki… 27^{kip1} and FOXO1 (17, … /AKT in BRCA2 pro… of this pathway has … cell proliferation … matory evidence … prostate intra… e carcinoma … croarrays com…

…trated that Skp2 directly … degradation of BRCA2. As … cess requires PI 3-kinase/AKT acti… integrin-mediated signaling following … ction with BM proteins, a crucial physiopatho… omenon at the beginning of the metastatic cascade … and distant organs. Furthering the understanding of … molecular pathway may prove valuable in designing new therapeutic strategies aimed at modifying the natural history of prostate carcinoma.

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