Loss of β_4 Integrin Subunit Reduces the Tumorigenicity of MCF7 Mammary Cells and Causes Apoptosis upon Hormone Deprivation

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Abstract

Purpose: The $\alpha_6\beta_4$ integrin, a laminin receptor, has been implicated from many studies in tumor progression and invasion. We showed that the β_4 integrin subunit associates with the ErbB-2 tyrosine kinase in human mammary carcinoma cell lines and that its overexpression in NIH3T3/ErbB-2–transformed cells causes a constitutive activation of phosphatidylinositol 3-kinase (PI3K), inducing a strong increase of their invasive capacity. In this study, we investigated the biological consequences of interference with the endogenous β_4 integrin subunit expression.

Experimental Design: *In vitro* and *in vivo* tumor growth and the biochemical consequences of β_4 integrin inactivation were studied in mammary tumor cells by using short hairpin RNA approach.

Results: Our data show that tumor growth of mammary tumor cells strictly depends on β_4 expression, confirming the relevance of β_4 protein in these cells. Moreover, interference with β_4 expression significantly reduces endogenous PI3K activity and AKT and mammalian target of rapamycin phosphorylation. Accordingly, with these results and considering that PI3K activity in mammary tumor plays a relevant role in hormone resistance, we asked whether β_4 expression might be relevant for hormone responsiveness in these cells. Data reported indicate that the interference with endogenous β_4 expression, upon hormone deprivation, induces caspase-9 and cytochrome *c*–mediated apoptosis, which is enhanced upon tamoxifen treatment. On the other hand, the expression of myr-AKT in MCF7 β_4–short hairpin RNA cells rescues the cells from apoptosis in the absence of hormones and upon tamoxifen treatment.

Conclusions: Overall, these results confirm the relevance of β_4 expression in mammary tumors and indicate this integrin as a relevant target for tumor therapy.

Integrin $\alpha_6\beta_4$ is a laminin receptor essentially expressed in epithelial tissues where it participates to the formation and stabilization of hemidesmosomes (1). The expression level of the β_4 subunit of the receptor increases in several epithelial tumors compared with normal tissues (2–4). There are many studies demonstrating the contribution of $\alpha_6\beta_4$ integrin to breast carcinoma progression (5, 6). In particular, it has been found that high expression level of the α_6 subunit correlates with reduced survival and metastatic phenotype of human breast cancer cells (7) and that the coexpression of laminin and $\alpha_6\beta_4$ integrin correlates with poor prognosis of women affected by breast cancers (6). Further strong evidence implicating $\alpha_6\beta_4$ in invasion was provided by the finding that the expression of exogenous β_4 subunit in the β_4-deficient carcinoma cell lines increased their ability to invade *in vitro* (8). Several reports show that $\alpha_6\beta_4$ integrin contributes to tumor progression by its participation to signaling events that control functions such as migration, proliferation, survival, invasion, and angiogenesis (9–16).

In mammary tumor cells, it has been shown that $\alpha_6\beta_4$ activates phosphatidylinositol 3-kinase (PI3K) promoting their invasive capability (14). It is also well known that mammary tumor cells express high level of ErbB-2 oncogene. Others and us have reported that β_4 integrin and ErbB-2 receptor colocalize and associate in mammary and ovarian carcinoma cells (17, 18). Furthermore, we have also shown that $\alpha_6\beta_4$ integrin and ErbB-2 receptor cooperate to promote a PI3K-dependent invasion (15). In the present study, by using short hairpin RNA (shRNA) interference, we showed that β_4 integrin expression is required for mammary tumor cell growth and that the abrogation of endogenous β_4 integrin expression is responsible for a significant reduction in the endogenous PI3K activity and AKT and mammalian target of rapamycin (mTOR) phosphorylation. Interestingly, this event occurs in spite of mutation present in PIK3CA, the catalytic subunit of PI3K,

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thus suggesting further level of PI3K activity control in mammary tumor cells. According to these results and in view of the fact that the relevance of PI3K activity signaling for mammary tumor cells in response to hormone treatment is well known (19, 20), we asked whether β_4 plays a role in this event. Our data indicate that interference with β_4 expression induces apoptotic effect generated by hormone deprivation that increases upon tamoxifen treatment. However, the expression of myr-AKT in MCF7 β_4-shRNA cells rescues the apoptotic effect generated upon hormone deprivation and tamoxifen treatment.

Materials and Methods

Cell lines, transfection, and treatment. The human mammary carcinoma (MCF7 and BT549) cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI or DMEM supplemented with 10% FCS (Invitrogen, Milan, Italy). NIH3T3 cells were maintained in DMEM supplemented with 10% FCS. The inactivation of β_4 was obtained by LipofectAMINE PLUS method (Invitrogen) using pSUPER.retro vector containing β_4-shRNA or a scramble RNA (scr-shRNA) sequences as previously described (21). Selection of puromycin-positive cells was carried out using 500 μg/mL puromycin (Sigma, Milan, Italy). After selection, the negative clones were analyzed by Western blot. MCF7 β_4-shRNA cells were transfected with pECE expression vector alone or carrying HA-myr-Akt protein (22). Selection of G418-positive cells was carried out using 1 μg/mL G418 (Life Technologies, Milan, Italy). The expression of myr-AKT was measured by Western blot analysis using α-HA antibody (Invitrogen). Rat bladder epithelial cell line 804G was cultured in MEM supplemented with 10% FCS and used for laminin 5 (LM5)–rich matrix preparation (23). To perform estrogen treatment, the cells, 5 days before adding tamoxifen, were switched to medium supplemented with hormone-deprived serum (24). Then, 10^{-7} mol/L tamoxifen was added to the cells for the indicated times.

Antibodies and matrix proteins. The anti-β_4 antibodies (439-9B and 450-11A) and the anti-ErbB-2 (W6/100) were prepared as described (15, 25). The anti-ErbB-2 antibody (clone 3B5) used in Western blot experiment was from Transduction Laboratories (Lexington, KY). The anti–estrogen receptor α (ERα; HC-20) and anti-ERβ (PPG5/10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and UCS Diagnostic (Rome, Italy), respectively. The anti-total and phospho-AKT (Ser^{473}), the anti-total and phospho-mTOR (Ser^{2448}), and anti-cleaved caspase-9 (Asp^{315}) were purchased from Cell Signaling (Beverly, MA). The anti-HA (3F10), anti–α_3 integrin subunit (P1B5), anti–β1 integrin subunit (P4C10), anti–cytochrome *c* (6H2.B4), anti-α-tubulin (TU-01), anti-actin (JLA20), and anti-Hsp70 (N27F3-4) were purchased from Calbiochem (Milan, Italy), Invitrogen, BD Biosciences (Milan, Italy), Immunological Sciences (Milan, Italy), and Stressgen (Victoria, CA), respectively. FITC and peroxidase-conjugated anti-IgGs were purchased from Cappel (Milan, Italy) and Bio-Rad (Milan, Italy). The LM5-rich matrix from 804G cells was prepared as described previously (23). In brief, 804G cells were plated onto 100-mm dishes or 96-well plate and were allowed to reach confluence. The cells were washed in sterile PBS and were removed from their matrix by treatment for 10 minutes in 20 mmol/L NH_4OH at 4°C. The remaining cells were removed by washing thrice with sterile PBS. The poly-L-lysine (Calbiochem) was used as negative control at the concentration of 10 μg/mL.

Reverse transcription-PCR, PCR, sequencing, and mutational analysis. Total RNA was prepared from MCF7 using RNAzol B according to the procedure of the manufacturer (Invitrogen). Human ERβ mRNA for reverse transcription-PCR analysis was carried out using specific primers as previously described (26). The housekeeping aldolase mRNA was used as an internal control. Genetic analysis of the *PIK3CA* gene was done by PCR and sequencing of exons 9 and 20, which are known to contain most of the mutations thus far detected in breast carcinomas as previously described (27). For sequence analysis, PCR products were purified and subjected to bidirectional dye terminator sequencing. Sequencing fragments were detected by capillary electrophoresis using the ABI Prism 3100 DNA analyzer (Applied Biosystems, Milan, Italy). Amplified PCR products were electrophoresed on a agarose gel containing ethidium bromide (0.5 μg/mL) and visualized under UV light.

Adhesion assay. The cells were seeded at a concentration of 5×10^3 per well coated with LM5 or poly-L-lysine and allowed to adhere for 1 hour at 37°C. After washing (0.1% bovine serum albumin in DMEM), the cells were fixed with 4% paraformaldehyde and incubated at room temperature for 10 to 15 minutes. Then, the cells were washed with washing buffer and stained with crystal violet for 10 minutes. After washing, the cells were lysed with 1% SDS for 10 minutes at room temperature. The absorbance was measured at 595 nm in a plate reader. For stimulation assay, 1×10^6 cells, after serum starvation for 12 hours, were seeded onto 100-mm dishes coated with LM5-rich matrix preparation from 804G cells. After stimulation, the cells were washed with PBS and lysed in NP40 buffer [1% NP40, 10% glycerol, 137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L Na_3VO_4, aprotinin (5 μg/mL), leupeptin (10 μg/mL), and pepstatin A (4 μg/mL)]. Total cell lysate was subjected to SDS-PAGE and analyzed by Western blot for AKT and mTOR phosphorylation status.

Immunoprecipitation and Western blot analysis. Purified 439-9B and W6/100 antibodies were cross-linked to activated immune affinity supports Affi-Gel 10/15 (Bio-Rad) according to the procedures from the manufacturer. The beads were then washed with 0.1 mol/L Tris-HCl (pH 8) and suspended in PBS containing 0.03% sodium azide (NaN_3). MCF7 scr-shRNA and β_4-shRNA transfectants were lysed in 20 mmol/L Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 137 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, aprotinin (5 μg/mL), leupeptin (10 μg/mL), and pepstatin A (4 μg/mL). Lysates were clarified by centrifugation and the immunocomplexes were purified by affinity chromatography using monoclonal antibodies cross-linked to Affi-Gel (bead-conjugated antibodies). The immunocomplexes were analyzed by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). The blots were probed with the following antibodies: 2 μg/mL anti-ErbB-2 monoclonal antibody or 2 μg/mL purified mouse anti-β_4 (clone 450-11A). To analyze the phosphorylation status of AKT and mTOR, MCF7 scr-shRNA and β_4-shRNA cells, after serum starvation, were plated onto LM5-rich matrix for the indicated times, rinsed twice with ice-cold PBS, and lysed with NP40 buffer. Total proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-phospho and anti–total AKT and mTOR antibodies. Filters were washed and developed with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (Amersham). Autoradiographies were done with Hyperfilm enhanced chemiluminescence (Amersham, Milan, Italy).

PI3K activity. To assay PI3K activity, the cells, after serum starvation for 24 hours, were lysed and aliquots of cell extracts containing equivalent amounts of protein were subjected to PI3K assay as previously described (15). The phosphorylated lipids were resolved by TLC plates (Merck, Milan, Italy) and subjected to autoradiography.

Soft agar assay. Both MCF7 scr-shRNA and β_4-shRNA cells were plated into soft agar to determine the efficiency of anchorage-independent growth. Cells (1×10^4) were plated in 5 mL of 0.3% agar in DMEM with 10% FCS overlaid onto a solid layer of 1.2% agar in DMEM. The cultures were maintained for 4 weeks. Then, the colonies were stained with neutral red solution (Sigma-Aldrich, Milan, Italy) to color the viable colonies and then counted.

Experiments in vivo. Experiments *in vivo* have been done in 45-day-old female nude mice (*nu/nu* Swiss; Charles River Laboratories, Wilmington, MA). At day 0, the animals were fully anesthetized by i.m. injection of 1.0 mg/kg Zoletil (Virbac) and 0.12% Xylor (Xylazine),

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and 17-β-estradiol pellets (1.7 mg/pellet 60 days release, Innovative Research of America, Sarasota, FL) were s.c. implanted into the intrascapular region of mice. The day after, 5.0×10^6 cells per mouse of exponentially growing cells MCF7 scr-shRNA or MCF7 β_4-shRNA were inoculated s.c. in 0.1 mL Matrigel (BD Biosciences). Tumor development was followed twice a week by caliper measurements along two orthogonal axes: length (L) and width (W). The volume (V) of tumors was estimated by the formula $V = L \times (W^2)/2$. All the procedure involving animals and their care were conducted in conformity with the institutional guidelines.

Cell cycle analysis and apoptosis assay. The MCF7 scr-shRNA and β_4-shRNA cells were hormone deprived for 5 days; then, 10^{-7} mol/L tamoxifen was added for the indicated times. The cells were fixed in cold methanol/acetone (1:4) for 30 minutes at 4°C and stained in PBS containing propidium iodide and RNase A for 30 minutes at room temperature. DNA content was measured by fluorescence-activated cell sorting (FACS). For Annexin V stain, 10^6 cells were washed with PBS, centrifuged, and resuspended in 100 μL Annexin V-biotin labeling solution (Boehringer, Milan, Italy) in the presence of propidium iodide and incubated for 15 minutes. After washing, the cells were incubated in SA-FLUOS solution for 20 minutes at 4°C, centrifuged, washed, and analyzed by FACS. The MCF7 β_4-shRNA/pECE and β_4-shRNA/pECE-HA-mryAKT cells were hormone deprived for 5 days, then 10^{-7} mol/L tamoxifen was added for the indicated times. Then, the apoptosis was measured by FACS analysis as described above.

Analysis of caspase-9 and cytochrome c. To analyze the activated form of caspase-9, the cells were hormone deprived as indicated above and, after washing in PBS, were resuspended in CHAPS buffer [50 mmol/L PIPES (pH 6.5), 2 mmol/L EDTA, 0.1% CHAPS, 5 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, protease inhibitors] followed by freeze and thaw. To detect cytochrome c, cytosolic fractions were prepared as follows. The cells (5×10^6) were washed in PBS and suspended in buffer containing 250 mmol/L sucrose, 50 mmol/L PIPES-NaOH (pH 7.4), 50 mmol/L KCl, 5 mmol/L EGTA, 2 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors. The cells were homogenized and centrifuged. Total proteins and the cytosolic fractions were separated on SDS-PAGE and subjected to Western blot analysis.

Statistical analysis. All the experiments were done at least thrice. Phosphorylation levels of lipids and proteins were evaluated by NIH Image program. The results relative to cell cycle analysis and apoptosis (±SD) were obtained from three independent experiments. Tumor development was followed twice a week by caliper measurements along two orthogonal axes: length (L) and width (W). The volume (V) of tumors was estimated by the formula $V = L \times (W^2)/2$. Statistical significance of *in vivo* data was evaluated by Student's t test ($P < 0.05$).

Results

The interference with β_4 expression inhibits tumor growth. To investigate the loss of function of β_4 integrin signaling, we used RNA interference to block the expression of β_4 subunit as previously described (21). We first analyzed the expression of β_4 integrin subunit upon shRNA interference. As shown on Fig. 1A, we obtained two stable negative β_4-shRNA clones (*lanes 3* and *4*), whereas the control MCF7 scr-shRNA clone express β_4 integrin subunit at the same extent of parental cells (*lanes 1* and *2*). To evaluate the biological consequences of β_4 depletion, we verified whether the loss of β_4 integrin could affect the adhesion of MCF7 cells to LM5. As shown on Fig. 1B, MCF7 β_4-shRNA cells loose 25% of adhesion to LM5 compared with scr-shRNA cells, indicating that although the decrease of adhesion is statistically significative, the cells still substantially adhere to LM5. In fact, we found that >95% of MCF7 cells express high level of $\alpha_3\beta_1$ integrin, indicating that this integrin, as previously described, is involved in the adhesion of MCF7 β_4-shRNA cells to LM5 (28). Then, we asked whether the loss of β_4 expression could affect the capability of MCF7 β_4-shRNA cells to grow in an anchorage-independent fashion. To this end, scr-shRNA and β_4-shRNA cells were overlaid onto a solid layer of agar and maintained for 4 weeks before counting. Our results show that β_4 depletion causes a strong reduction of colony formation (75% of reduction compared with control cells) as well as colony size (Fig. 2A and B). The percentages of colony formation of three independent experiments are reported on Fig. 2B. Then, we tested the capability of MCF7 β_4-shRNA cells to grow *in vivo*. Results of xenograft experiments show that the depletion of β_4 integrin subunit weakens the aggressiveness of MCF7 cells, significantly reducing the tumor growth and tumor uptake in mice bearing MCF7 β_4 shRNA cells with respect to the control MCF7 scr-shRNA cells (Student's t test, $P < 0.05$; Fig. 2C and D). Altogether, these results indicate that β_4 integrin has an important role contributing to the tumorigenicity of MCF7 cells.

The interference with β_4 expression affects endogenous PI3K activity. Given our previous demonstration of a physical association between $\alpha_6\beta_4$ and ErbB-2 in mammary tumor cells (18), we verified the interaction in MCF7 cells. To this purpose, $\alpha_6\beta_4$ and ErbB-2 complexes were purified by affinity chromatography in MCF7 scr-shRNA and β_4-shRNA cells. The presence of ErbB-2 or β_4 proteins in the immunocomplexes were

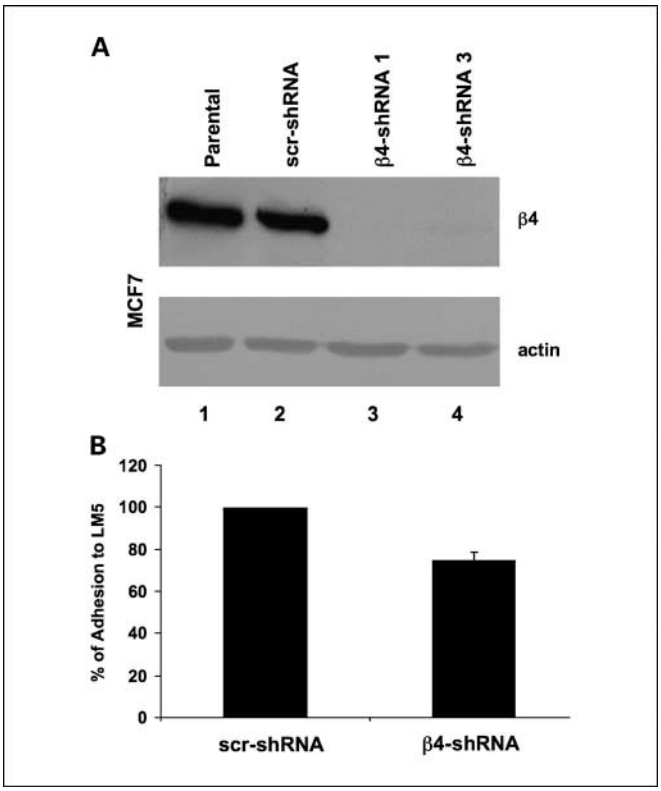

Fig. 1. Adhesion to LM5 of MCF7 cells is not substantially affected by β_4-shRNA. *A*, total cell extracts were prepared from parental MCF7 cells, scr-shRNA, and β_4-shRNA MCF7 clones, and equal amounts of protein were resolved by SDS-PAGE and subjected to Western blot analysis to evaluate the expression of β_4. α-Actin protein was used as loading control. *B*, the cells were seeded at a concentration of 5×10^3 per well coated with LM5 and allowed to adhere for 30 minutes at 37°C. Columns, percentages of adhesion obtained from three independent experiments; bars, SD.

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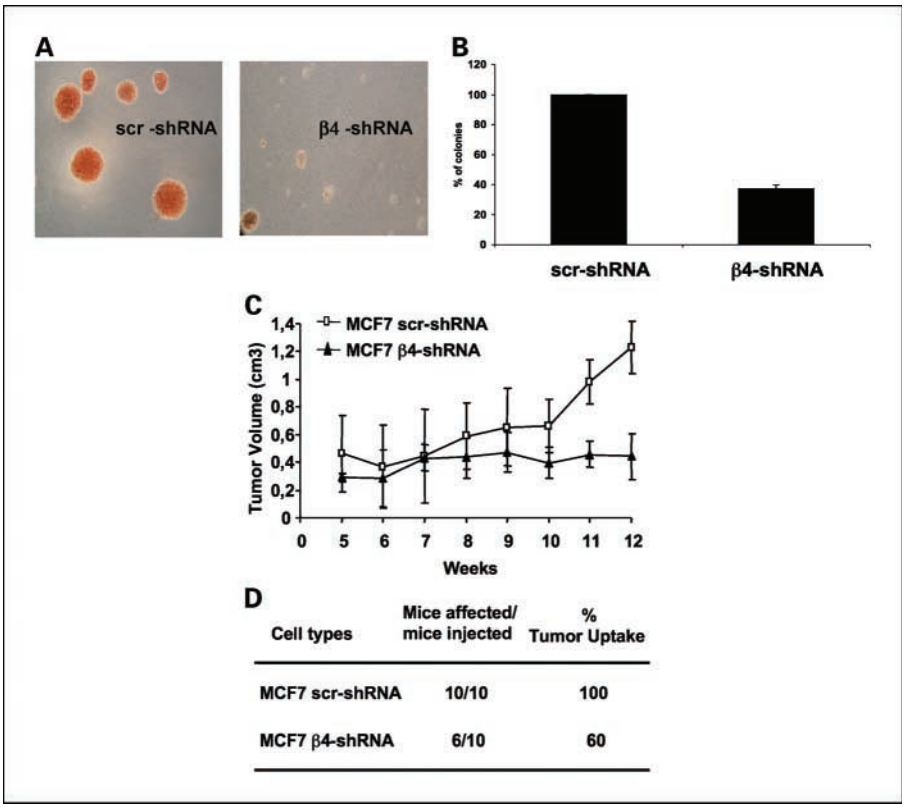

Fig. 2. *In vitro* and *in vivo* growth of MCF7 cells strictly depend on β_4 expression. *A,* to determine the efficiency of anchorage-independent growth, 1×10^4 MCF7 scr-shRNA and β_4-shRNA cells in 0.3% soft agar were plated onto a solid layer of 1.2% agar in DMEM supplemented with 10% FCS. The cultures were maintained for 4 weeks; the colonies were stained with neutral red solution and were counted. *B,* columns, percentage of colonies obtained from three independent experiments; bars, SD. *C,* to determine the *in vivo* tumor growth rate, 5.0×10^6 cells per mouse of exponentially growing MCF7 scr-shRNA or MCF7 β_4-shRNA cells were inoculated s.c. in 0.1 mL Matrigel. Tumor development was followed twice a week by caliper measurements. *D,* percentage of tumor uptake.

determined by Western blot experiments. Using this approach, we detected the ErbB-2 protein in the β_4 immunocomplexes and β_4 subunit in ErbB-2 immunocomplexes derived from MCF7 scr-shRNA cells, whereas, as expected, no interaction was found in MCF7 β_4-shRNA cells (Fig. 3A). Based on the finding that the activation of PI3K by the $\alpha_6\beta_4$ and ErbB-2 interaction has been shown to promote PI3K-dependent invasion (15) and that $\alpha_6\beta_4$ promotes PI3K-dependent survival and invasion of transformed cells (13, 14), we verified whether PI3K activity might be involved in the reduction of the tumor growth upon β_4 depletion. To this end, total cell lysates from MCF7 β_4-shRNA and scr-shRNA cells were tested for PI3K activity.

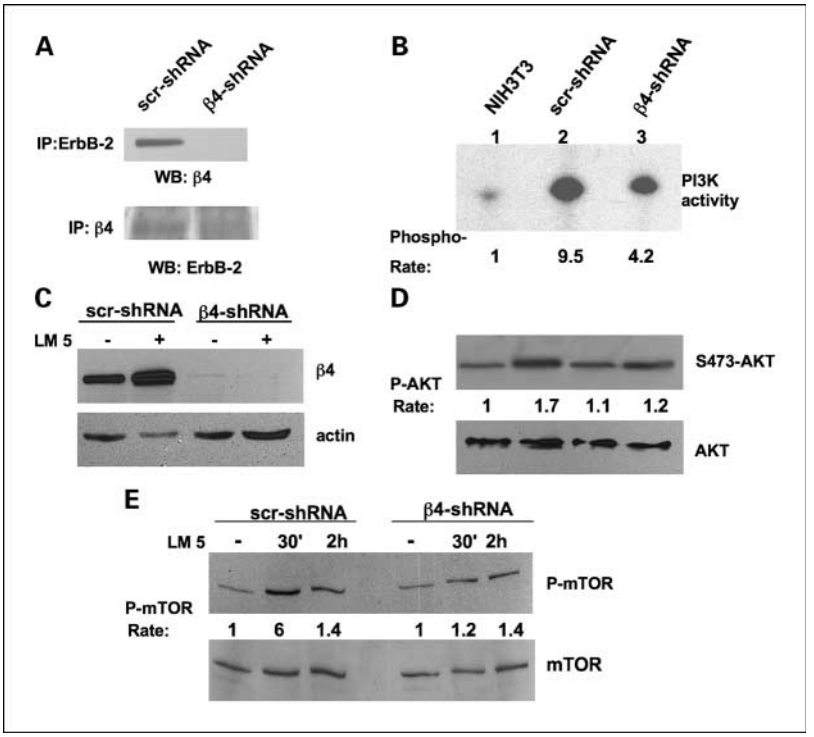

Fig. 3. The inactivation of β_4 in MCF7 cells inhibits PI3K activity and AKT and mTOR phosphorylation. *A,* total cell lysates from MCF7 scr-shRNA and β_4-shRNA cells were incubated with bead-conjugated anti-β_4 and ErbB-2 antibodies. The interaction between the two molecules was analyzed by Western blot experiments. *B,* the MCF7 scr-shRNA, β_4-shRNA cells, and NIH3T3 (negative control) cells were serum starved for 24 hours. Then, aliquots of cell extracts containing equivalent amount of protein were subjected to PI3K assay. The phosphorylated lipids were resolved by TLC. *C* to *E,* same number of MCF7 scr-shRNA and β_4-shRNA cells, after serum starvation, were spread onto LM5 or onto poly-L-lysine, used as negative control. Same amounts of total protein were resolved by SDS-PAGE and subjected to Western blot analysis to reveal the expression of β_4 integrin subunit (*C*), phospho-AKT (*D*), and phospho-mTOR (*E*), respectively (*top*). The normalization of the loaded proteins was done using α-actin (*C*), anti-total AKT (*D*), and anti-total mTOR (*E*) antibodies (*bottom*). Phosphorylation levels of lipids and proteins were evaluated by NIH Image program.

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We found a strong down-regulation of the kinase activity in β_4-shRNA cells compared with control MCF7 scr-shRNA cells (phosphorylation rate 4.2 versus 9.5; Fig. 3B, *lanes 3* and *2*). As a negative control, we used NIH3T3 cells (Fig. 3B, *lane 1*). These results indicate, for the first time, that the interference with β_4 expression destroying β_4/ErbB-2 interaction significantly reduces the endogenous PI3K activity of tumor cells. It has been previously reported that mammary tumor cells, and in particular MCF7 cells, express a mutated form of *PIK3CA*, the catalytic subunit of PI3K (27, 29, 30), and that this mutation confers to the molecules a phosphorylation status higher than the wild-type molecule (29). To verify whether β_4 depletion could exert its effect on PI3K activity independently of mutation, we analyzed the somatic mutations of exons 9 and 20 of *PI3KCA* that are most frequently described and confirmed the mutation of the exon 9 (E545K; ref. 27; data not shown). This result indicates that although *PI3KCA* is mutated in MCF7 cells, the absence of β_4 causes the inhibition of the endogenous PI3K activity, suggesting further levels of PI3K activity control in mammary cells.

The interference with β_4 expression inhibits AKT and mTOR phosphorylation. It is known that PI3K activity sustains signaling pathway responsible for survival and proliferation through the phosphorylation of target proteins such as AKT and mTOR (19). To verify whether β_4 interference by reducing PI3K activity also affects downstream signaling of PI3K, we assayed AKT and mTOR phosphorylation upon LM5 stimulation. Results reported on Fig. 3D and E show that the phosphorylation status of both PI3K effectors is reduced in β_4-shRNA cells with respect to scr-shRNA cells, indicating that the absence of β_4 signal causes the inhibition of the endogenous PI3K/AKT pathway.

β_4 inactivation causes apoptosis upon hormone deprivation. Because the interference with β_4 causes a strong down-regulation of PI3K activity pathway, we evaluated whether the loss of β_4 could affect the proliferation of MCF7 cells. The analysis of the proliferation rate of scr- and β_4-shRNA cells revealed that the loss of β_4 did not affect the proliferation of MCF7 cells (data not shown). It is well known that mammary cells proliferate and survive under hormone stimuli and that the hormones activate PI3K activity (19). Consequently, we asked whether the interference with β_4 is relevant in cells growing in the presence of hormones. To this end, we first verified the expression of ER receptors in MCF7 parental cells. As shown on Fig. 4A (*lane 2*), we confirmed the expression of ERα in MCF7 cells. By reverse transcription-PCR, we found that they express ERβ (Fig. 4B, *lane 2*), whereas by immunocytochemistry we confirmed the expression at the protein level (Fig. 4C, *top*). As negative control, BT549 cell line was used (Fig. 4A, *lane 1*; B, *lane 1*; and C, *bottom*). Then, we evaluated, by FACS analysis, the cell cycle progression of β_4-shRNA and scr cells in the presence or in the absence of hormones. Interestingly, we found that β_4-shRNA cells in the absence of hormone show accumulation of the cells in sub-G_0-G_1 cell cycle phase (17%), whereas the same cells in normal growth culture condition do not show significant cell death (Fig. 4D). Furthermore, MCF7 control cells in the absence of hormones show a very low level of cell death compared with β_4-shRNA cells (5% versus 17%), indicating that β_4 molecule is relevant for the survival of MCF7 cells (Fig. 4D). Moreover, the addition of tamoxifen to β_4-shRNA cells strongly enhances cell death

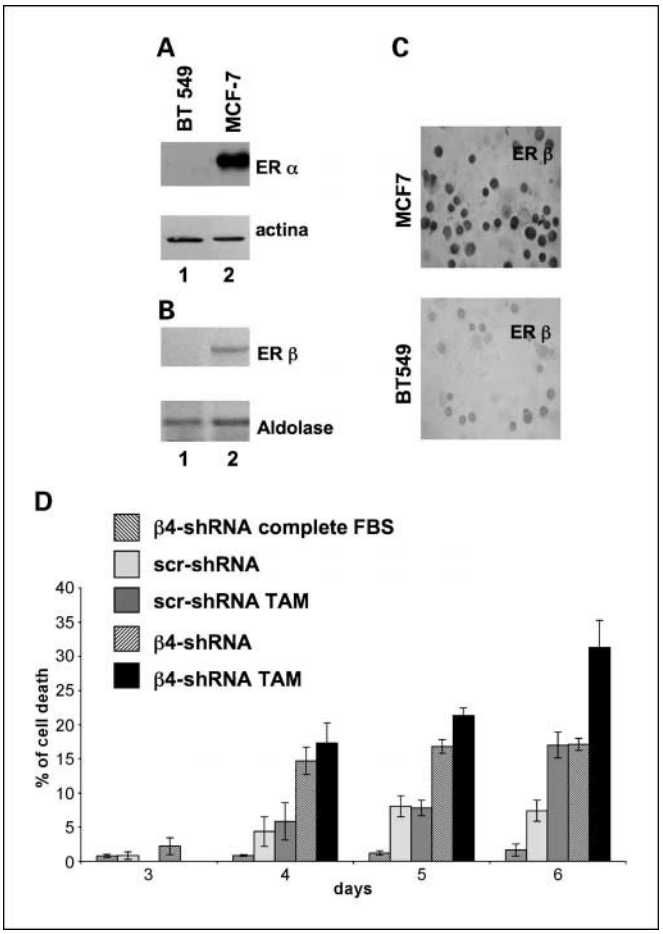

Fig. 4. The inactivation of β_4 in MCF7 cells induces apoptosis upon hormone deprivation. *A,* Western blot analysis of ERα on MCF7 cells and BT549 cells used as negative control. *B,* reverse transcription-PCR to analyze ERβ expression in MCF7 and BT549 cell lines. *C,* immunocytochemistry to evaluate the expression of ERβ protein. The α-actin protein and the *aldolase* gene were used to normalize both protein and mRNA levels (*A* and *B*). *D,* the same number of MCF7 scr and β_4-shRNA cells was grown in medium supplemented with 10% FBS or with hormone-deprived FBS for 5 days. Then, the same number of cells was seeded onto 100-mm dishes in the presence or in the absence of tamoxifen for the indicated times. DNA contents of control and β_4-shRNA cells were analyzed daily by FACS analysis. Columns, percentage of cell death obtained from three independent experiments; bars, SD.

(35%), whereas when tamoxifen is added to scr-shRNA cells, a lower level of cell death is induced (15%; Fig. 4D). The cell cycle data were confirmed by Annexin V staining, indicating that in the absence of hormones β_4-shRNA cells were apoptotic (Fig. 5A) and that the addition of tamoxifen increases this apoptotic effect (Fig. 5A). However, MCF7 scr-shRNA cells do not show apoptosis when cultured in the absence of hormones and show a very low level of apoptosis (7%) when treated with tamoxifen (Fig. 5A). As expected, MCF7 β_4-shRNA as well as scr-shRNA cells do not undergo apoptosis in the presence of hormones (Fig. 5A).

β_4 inactivation causes apoptosis mediated by caspase-9. The cell death results we obtained prompted us to investigate the apoptotic signals induced upon hormone deprivation and β_4 inactivation. To this end, we analyzed the activation of caspases in both β_4-shRNA and scr cells growing in the presence and in the absence of hormones. We found that upon β_4 depletion, there is a strong activation of caspase-9, which, in agreement

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with cell cycle and Annexin V data, increases upon tamoxifen treatment (Fig. 5B, *lanes 5* and *6*). As expected, we did not find activation of caspase-9 in scr cells, whereas a very mild activation of caspase-9 occurs in scr cells upon tamoxifen treatment (Fig. 5B, *lanes 3* and *4*). Then, to understand the pathway through which caspase-9 activates the apoptotic program, we analyzed the integrity of mitochondria measuring the release of cytochrome *c* into the cytoplasm. As shown on Fig. 5C, we found release of cytochrome *c* in β_4-shRNA cells (*lane 5*), which, in agreement with caspase-9 data, increases after tamoxifen treatment (*lane 6*). However, we did not find any release of cytochrome *c* in scr cells and a mild release in the same cells upon tamoxifen treatment (*lanes 2* and *3*). Both MCF7 scr-shRNA and β_4-shRNA cells do not show caspase-9 activation and release of cytochrome *c* when cultured in the presence of hormones (Fig. 5B and C, *lanes 1* and *2*).

Overall, these data show that the inactivation of β_4 integrin by RNA interference causes a strong down-regulation of PI3K activity and apoptosis in the absence of hormone stimuli.

Myr-AKT rescues the apoptotic effect caused by β_4 inactivation. Because β_4 depletion causes the down-regulation of PI3K activity that correlates with apoptosis during hormone withdrawal,

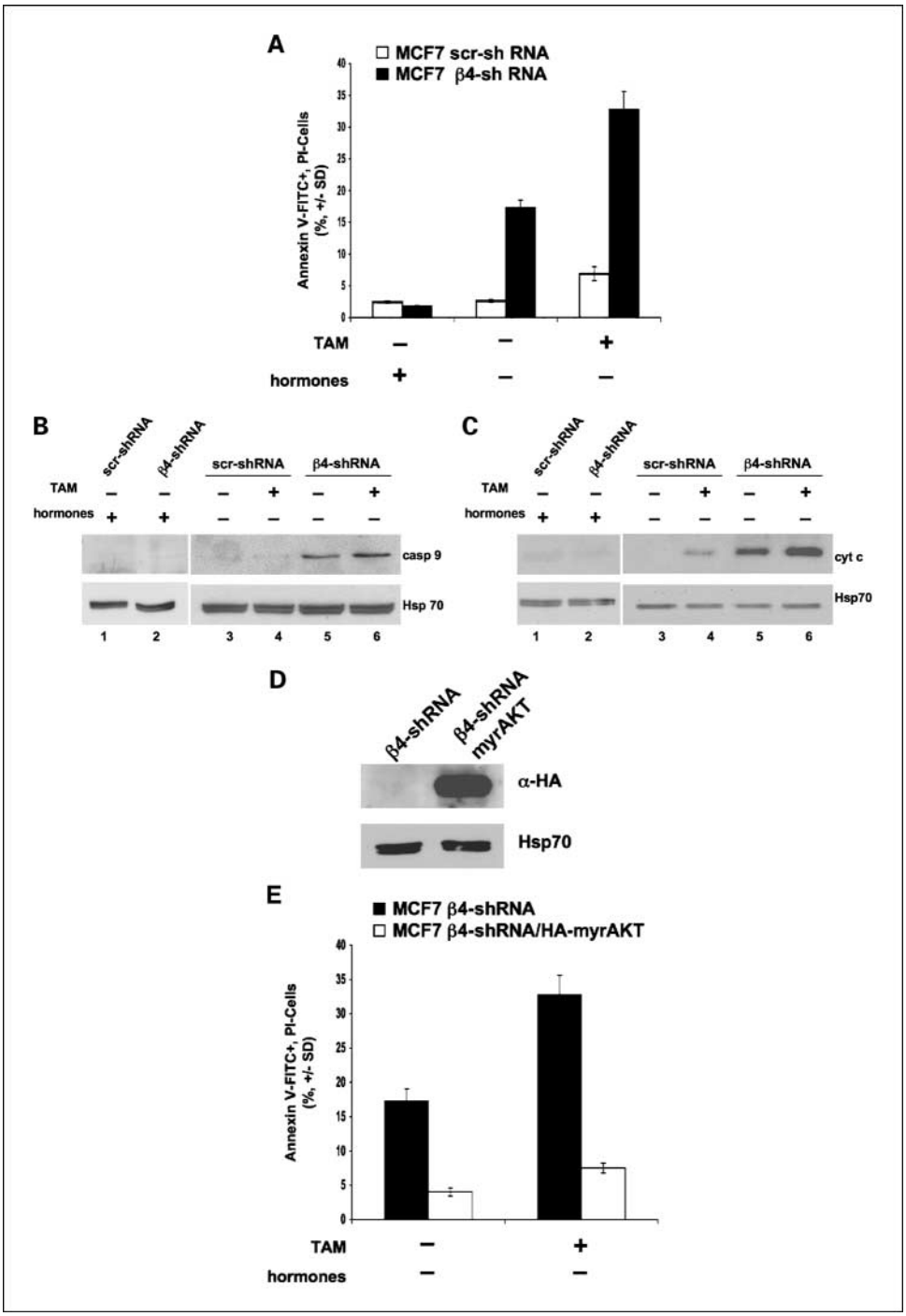

Fig. 5. Following hormone deprivation, the loss of β_4 causes apoptosis mediated by the activation of caspase-9 and release of cytochrome *c*. *A,* for Annexin V analysis, scr-shRNA and β_4-shRNA cells, after hormone deprivation, were left in culture in the absence or in the presence of tamoxifen for 6 days. Then, apoptosis was analyzed by FACS. *B,* MCF7 scr-shRNA and β_4-shRNA cells were grown in medium supplemented with 10% FBS or in medium supplemented with hormone-deprived serum. Then, the cells were left in culture in the absence or in the presence of tamoxifen for 6 days. Total cell lysates from MCF7 scr-shRNA and β_4-shRNA cells were analyzed by Western blot for the expression of cleaved caspase-9. *C,* the cytosolic fractions from the same cells were analyzed by Western blot for the expression of cytochrome *c*. The proteins were normalized by using anti-Hsp70 antibody (*B* and *C, bottom*). *D,* MCF7 β_4-shRNA cells were transfected with pECE expression vector alone or carrying the myr-AKT cDNA. *E,* apoptosis was evaluated by Annexin V.

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we asked whether an active form of AKT was able to rescue the apoptotic effect we found in MCF7 β_4-shRNA cells. To this end, MCF7 β_4-shRNA cells were stably transfected with expression vector alone or carrying myr-AKT cDNA. We first evaluated the expression of exogenous myr-AKT protein as shown on Fig. 5D (*lanes 2*). Then, the cells were hormone deprived and treated with tamoxifen to evaluate the apoptosis by Annexin V. As shown on Fig. 5E, the expression of myr-AKT rescues the apoptosis in MCF β_4-shRNA cells either when they are cultured in the absence of hormones and upon tamoxifen treatment. However, control cells (MCF7 β_4-shRNA/pECE) still undergo to apoptosis. These data clearly show that β_4 activating PI3K sustains the survival of mammary cells when they are hormone deprived.

Discussion

The results presented here highlight the role of $\alpha_6\beta_4$ contributing to the tumorigenicity of mammary tumor cells. Our finding that the interference with β_4 inhibits the growth in anchorage-independent fashion and *in vivo* tumorigenicity is in agreement with recent data (31) showing that the depletion of β_4 integrin in another mammary cell line strongly reduces the *in vivo* tumorigenicity. Our data are also in agreement with a number of increasing studies showing the involvement of $\alpha_6\beta_4$ integrin in tumor progression and survival (5). We have also shown that the interference with β_4 molecule strongly reduces the endogenous PI3K activity and down-regulates the phosphorylation of the endogenous AKT and mTOR, key molecules for cell survival and proliferation (19). Altogether, these data are in agreement with previous work showing that $\alpha_6\beta_4$ sustains the survival of cancer cells by activating PI3K/AKT pathway (11, 14, 15). Indeed, it has been shown that the overexpression of $\alpha_6\beta_4$ integrin in breast carcinoma cells inhibits p53-inducing apoptosis and contributes to tumor invasion by activating PI3K (11, 14). We have also previously shown that the cooperative signaling between $\alpha_6\beta_4$ integrin and ErbB-2 receptor is required to promote PI3K-dependent invasion (15). It is well known that the activity of PI3K/AKT pathway is involved in many physiologic processes and plays an important role in cancer (19, 20). In many breast cancers, the efficacy of hormone therapy is abrogated by the PI3K pathway that remains highly active (20) and numerous studies showed that PI3K and in particular its downstream substrate AKT molecule are critical for cell survival by phosphorylation and inactivation of downstream molecules, such as Bad and caspase-9 (32, 33). Recently, it has been found that *PIK3CA* gene, which encodes the catalytic subunit of the PI3K complex, is mutated in colon, brain, gastric, lung, and breast tumors and in tumor cell lines (27, 29, 30). The mutations of *PIK3CA* are well characterized and it has been shown that some mutations induce to the molecule a phosphorylation status higher than wild-type molecule, suggesting that some mutations could confer oncogenic function to the kinase (29). Although we recently confirmed the somatic mutation of exon 9 in MCF7 cells (27) and that this mutation has been shown to confer resistance to tumor necrosis factor–related apoptosis-inducing ligand in colon cancer (29), we found that the interference with β_4 in these cells down-regulates the activity of PI3K independently from the mutation of *PIK3CA*, thus indicating the complexity of PI3K activation pathway, and suggest further levels of PI3K activity control in mammary cells. Our finding that the loss of β_4 inhibits *in vitro* and *in vivo* tumor growth and causes the down-regulation of AKT and mTOR phosphorylation following the adhesion to LM5 indicates that $\alpha_6\beta_4$ integrin mediates the survival of mammary tumor cells through its binding to LM5. These data are in agreement with previous studies indicating that the anchorage-independent growth of mammary tumors is mediated by autocrine LM5 secretion and binding to $\alpha_6\beta_4$ integrin (12) and that the prognostic value of $\alpha_6\beta_4$ expression in breast cancers is affected by laminin production (6). Furthermore, it has been shown that β_4 integrin promotes the survival of carcinoma cells stimulating the phosphorylation of 4E-BP1, a direct target of mTOR, by activating PI3K pathway (13).

An important aspect of our study is that the interference with β_4 causes apoptosis in the absence of hormones. Estrogens are, for mammary cells, important controllers of cell proliferation and survival (24, 26), and although MCF7 cells are also able to proliferate in the absence of hormone, the finding that β_4 loss induces apoptosis upon hormone deprivation indicates that $\alpha_6\beta_4$ integrin plays an important role in sustaining the cell survival of mammary tumor cells. Furthermore, the activation of caspase-9 that we found in hormone-deprived cells and upon tamoxifen treatment is consistent with the reported role of AKT-inducing cell survival signal by inactivating pro-caspase-9 (33) and with the finding showing that mTOR activity restores tamoxifen treatment in cells expressing aberrant AKT activity (34). In agreement with these data, we also found that the expression of myr-AKT molecules in MCF7 β_4-shRNA rescues the apoptotic effect caused by β_4 depletion.

In summary, our data show that $\alpha_6\beta_4$ integrin is required for mammary tumor cell growth. Moreover, β_4 integrin participating in the activation of PI3K survival pathway plays an important role sustaining the survival of mammary tumor cells in the absence of ER stimuli; this indicates that $\alpha_6\beta_4$ integrin is a relevant target for therapy.

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Loss of β_4 Integrin Subunit Reduces the Tumorigenicity of MCF7 Mammary Cells and Causes Apoptosis upon Hormone Deprivation

Giulia Bon, Valentina Folgiero, Gianluca Bossi, et al.

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