

Integrin $\alpha 5\beta 1$ Promotes Survival of Growth-Arrested Breast Cancer Cells: An *in Vitro* Paradigm for Breast Cancer Dormancy in Bone Marrow

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ABSTRACT

The mechanisms of long-term survival of occult breast cancer cells in the bone marrow microenvironment are not known. Using selected bone marrow stromal components with demonstrated roles in promoting growth arrest and survival of breast cancer cells, we reconstituted an *in vitro* model for dormancy of breast cancer cells in bone marrow. According to this model, basic fibroblast growth factor, a mammary differentiation factor abundant in the bone marrow stroma, induces growth arrest of relatively well-differentiated breast cancer cells, induces a spread appearance, and restricts their survival to fibronectin by up-regulating integrin $\alpha 5\beta 1$. Most of the basic fibroblast growth factor-arrested cells fail to establish optimal ligation to fibronectin and undergo cell death. Cells that do attach to fibronectin, another major constituent of the bone marrow microenvironment, stay alive and growth-arrested for many weeks. Although capable of adhering to other stromal proteins collagen and laminin, dormant cells do not gain a survival advantage from these interactions. Using function-blocking peptides, we show a specific contribution of $\alpha 5\beta 1$ -fibronectin interaction in maintaining survival of growth-arrested cells, potentially by negatively modulating apoptotic response via signaling pathways. Blocking of phosphatidylinositol 3'-kinase and Akt inhibits survival of dormant clones, demonstrating this as one of those pathways. Experiments with human bone marrow stroma cocultures confirm the role of fibronectin ligation in maintaining survival of dormant clones.

INTRODUCTION

Breast cancer cells metastasize to the bone marrow early in the course of the disease. Although most metastatic cells die upon reaching the marrow microenvironment, some breast cancer cells are found in the marrow at the time of diagnosis (1). These cells can remain dormant, or growth arrested without loss of viability, for extended time periods (2). They remain protected from death and, in fact, survive multiple rounds of adjuvant chemotherapy administered for the very purpose of eradicating them (3). Some of these cells begin to proliferate years later and result in death to the patient (1). Although mechanisms of dormancy remain largely unknown, a variety of growth factors and ligands of cellular integrins in the bone marrow microenvironment may influence the fate of the metastatic cell. These factors have well-established modulatory effects on cell behavior, including protection of hematopoietic stem cells (4, 5). The response of a metastatic cell to these factors depends on its state of de-differentiation. Dormant cancer cells are generally well differentiated, with traits considered as good prognostic indicators (6). Highly de-differentiated breast cancer cells, however, appear to resist these factors and often proliferate soon after reaching the marrow.

Among the growth factors endemic to the bone marrow microenvironment, basic fibroblast growth factor (FGF-2) is one of the can-

didates likely to play a significant role in the initiation of dormancy. FGF-2, a peptide growth factor with roles in the morphogenic differentiation of mammary ducts (7), can inhibit growth (8–12) and induce a more differentiated state in breast cancer cells (13, 14). FGF-2 inhibits proliferation in breast cancer cells by inducing the expression of cyclin-dependent kinase p21^{WAF1/Cip1} that results in the inactivation of G₁ cyclin complexes and dephosphorylation of Rb (10). These events occur in the context of transient activation of extracellular signal-regulated kinase (ERK) 1 and 2 (9), in contrast to their sustained activation in cells that exit dormancy (15). The antiproliferative effects of FGF-2 are restricted to well-differentiated breast cancer cells, whereas its overall civilizing effects on malignant behavior extend to highly aggressive breast cancer cells (13).

Here, we present *in vitro* evidence for a paradigm in which FGF-2, present in high concentrations in the bone marrow microenvironment (16–20), imparts a more differentiated state to micrometastatic breast cancer cells. This encompasses cell cycle arrest of well-differentiated breast cancer cells, changes in the integrin repertoire, and death to cells with improperly ligated integrins such as $\alpha 5\beta 1$, previously shown to be up-regulated by FGF-2 in fibroblasts and endothelial cells (21, 22). Although most breast cancer cells die in the bone marrow microenvironment, appropriate binding of up-regulated integrin $\alpha 5\beta 1$ to its ligand fibronectin, a significant component in bone marrow stroma (23), may promote survival of FGF-2-responsive cells. Accordingly, the survival effects of integrin $\alpha 5\beta 1$ ligation have been described in a variety of cell types including breast cancer (24, 25). Our paradigm provides support for individual components of this model and implicates phosphatidylinositol 3'-kinase (PI3k) and Akt pathway signaling in the survival of dormant breast cancer cells.

MATERIALS AND METHODS

Cells and Culture, Dormancy, and Adhesion Assays. MCF-7, T-47D, and MDA-MB-231 cells were cultured in DMEM/10% FCS ± 10 ng/ml rhFGF-2 or rhEGF (R&D Systems, Minneapolis, MN) on plastic or extracellular matrix (ECM)-coated plates (BioCoat, Becton Dickinson, Lincoln Park, NJ), with medium changed every 3 days. Cells were seeded at a clonogenic density of 1,000 cells/well in 24-well plates. After 3, 5, 6, 8, 10, or 15 days, floating and loosely attached cells were washed away, and attached cells were stained with freshly made 0.1% crystal violet in 2% ethanol/10 mM sodium borate (pH 9.0). Growing (≥ 8 , 24, or 100 cells on days 3, 5, or 10/15, respectively) or dormant (4 ± 2 cells on day 3 and ≤ 10 cells thereafter) clones were counted. All experiments were done in quadruplicate plates and done at least twice.

In peptide-blocking experiments, cells were incubated with FGF-2 on various substrata. After 3 days, blocking peptides were added with fresh medium and FGF-2. Dormant clones were counted on day 6. Studies used blocking peptides to fibronectin [GRGDSP (P1)], collagen [CQDSETRTFY (P3)], laminin [YIGSR (P4)], and a nonbinding control [GRGES (P2); American Peptide Co., Sunnyvale, CA] at 1.5 nM, a concentration previously established in dose-finding experiments. Similarly, in signal-blocking experiments, cells were cultured for 3 days on plastic or fibronectin-coated 24-well plates with and without FGF-2. After 3 days, the medium was replaced; and fresh FGF-2 was added along with variable concentrations of PI3k inhibitor LY294002, its inactive control LY303511, or Akt inhibitor I (1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate; Calbiochem, La Jolla, CA). On day 6, cells were fixed and stained, and dormant and growing clones were counted, as before.

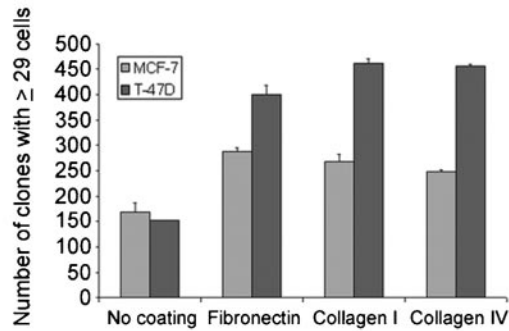
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A



B

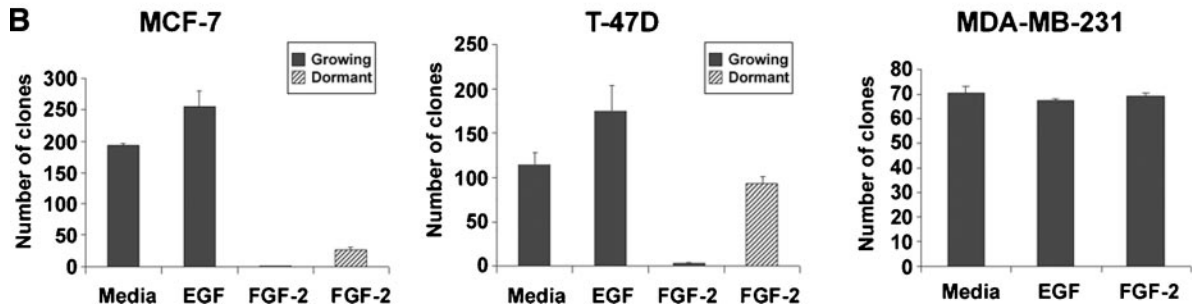
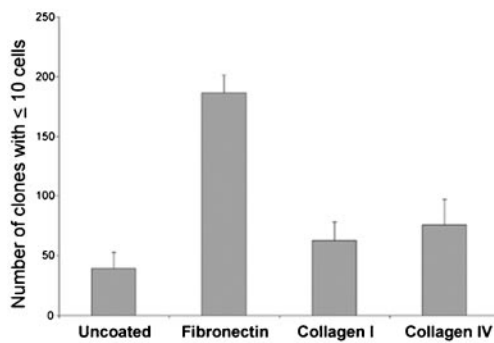


Fig. 1. *A*, effects of ECM proteins on the clonogenic efficiency of MCF-7 and T-47D cells. Cells were incubated on uncoated 24-well plates or plates coated with fibronectin, collagen I, or collagen IV at clonogenic densities of 1,000 cells/well. Crystal violet-stained colonies containing at least 29 cells were counted after 6 days in culture. *B*, effects of epidermal growth factor and FGF-2 on the clonogenic potential of well and poorly differentiated breast cancer cells in tissue culture. MCF-7 and T-47D cells (1,000 cells/well) and MDA MB-231 cells (200 cells/well) were incubated in 24-well plates \pm 10 ng/ml epidermal growth factor or FGF-2 for 6 days and stained with crystal violet, and clones with ≥ 29 cells (growing clones; ■) or with ≤ 10 -well spread, growth arrested cells (dormant clones; ▨) were counted. Error bars indicate \pm SD.

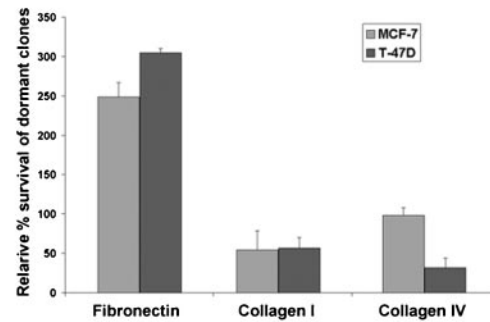
In adhesion studies, cells were cultured on plastic or fibronectin-coated plates \pm FGF-2 for 3 days, detached using Cell Dissociation solution (Sigma, St. Louis, MO), washed, and counted. Fifty thousand cells were incubated with 2 μ g/ml blocking monoclonal antibodies to integrins $\alpha 1$,

$\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αv , $\alpha 2\beta 1$, and $\alpha 5\beta 1$ (Chemicon) or mouse IgG for 30 min at 37°C before incubation in 24-well variably coated plates for 45 min at 37°C. Unattached cells were aspirated; attached cells were washed and fixed with freshly made crystal violet solution for 20 min, washed in

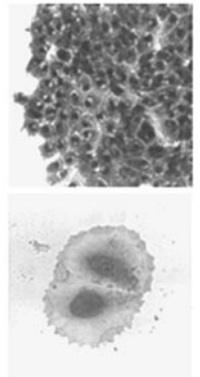
A



B



C FGF-2



D

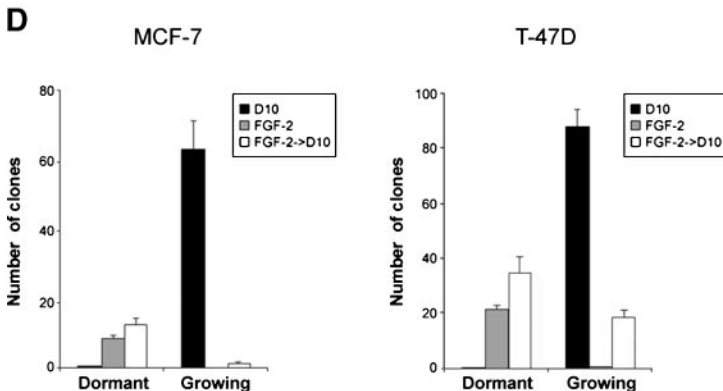


Fig. 2. Fibronectin rescues the survival of FGF-2-treated dormant MCF-7 and T-47D cells. Cells were incubated in 6-well variably coated plates (5,000 cells/well) with FGF-2 10 ng/ml for 5 days (*A*; MCF-7 cell data are shown) to 15 days and stained with crystal violet, and colonies of ≤ 10 cells were counted. *B*, ratios of dormant clones on fibronectin, collagen I, and collagen IV to those on tissue culture plates after 15 days. Clones on tissue culture are normalized to 100%. *C*, differential appearance of growing and spread out dormant MCF-7 cells on fibronectin; magnification, $\times 200$. *D*, growth potential of dormant cells after removal of FGF-2. MCF-7 and T-47D cells were incubated on fibronectin-coated plates with or without FGF-2 (*D10*). After 3 days, FGF-2 was replenished in one-half the plates (*FGF-2*) and replaced by standard medium on the other plates (*FGF-2*->*D10*). Growing and dormant colonies shown were counted at 9 days. Error bars indicate \pm SD.

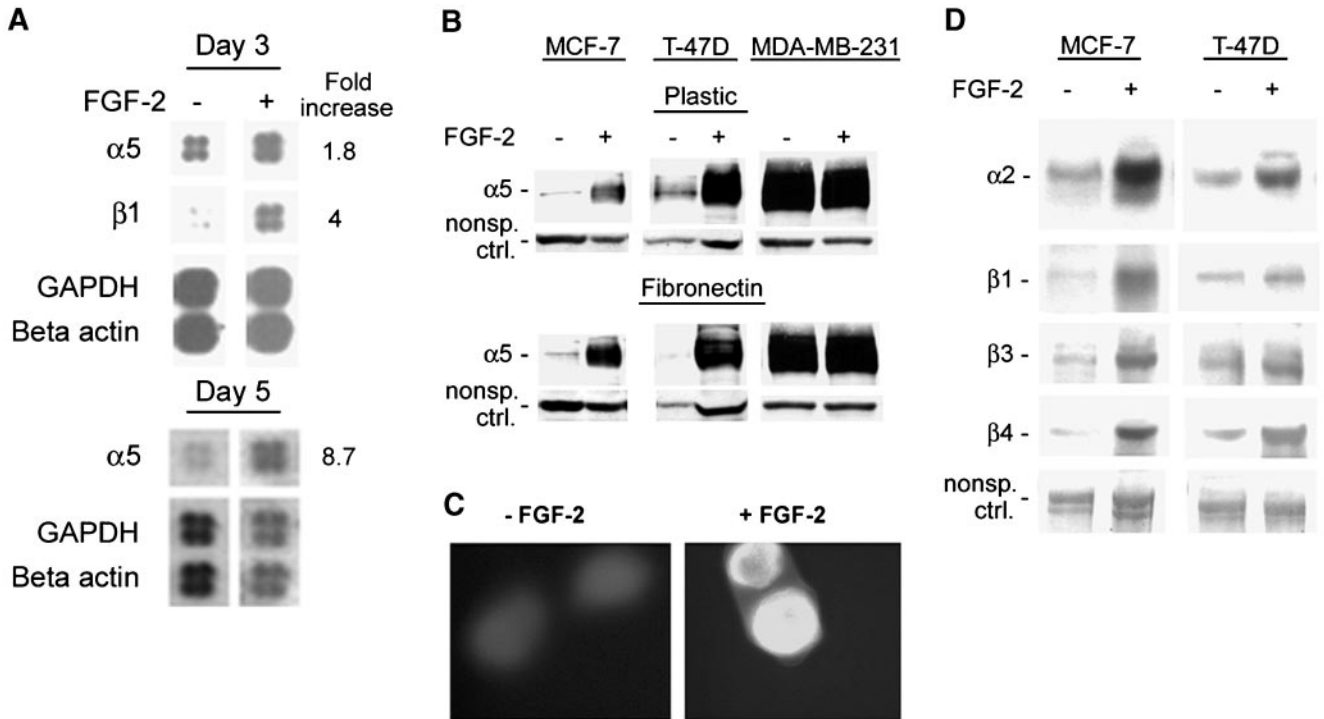


Fig. 3. FGF-2 regulates expression of integrins. *A*, gene chip analysis of integrin $\alpha 5$ and $\beta 1$ mRNA expression in MCF-7 cells incubated \pm FGF-2 for 3 or 5 days on fibronectin-coated plates. Densitometer quantitations normalized against glyceraldehyde-3-phosphate dehydrogenase and actin mRNA standards are shown. *B*, Western blots of integrin $\alpha 5$ from cells incubated \pm FGF-2 for 3 days on tissue culture- or fibronectin-coated dishes. *C*, indirect immunofluorescence of integrin $\alpha 5$ in T-47D cells on coverslips \pm FGF-2 10 ng/ml for 24 h. *D*, Western blots of integrins $\alpha 2$, $\beta 1$, $\beta 3$, and $\beta 4$ in MCF-7 and T-47D cells incubated \pm FGF-2 for 3 days. Nonspecific bands were used as loading controls.

distilled water, dried, and extracted with 10% acetic acid; and the A_{600} was measured.

Microarray Analysis. MCF-7 cells were cultured at clonogenic density on fibronectin-coated plates for 3 and 5 days with or without FGF-2. Total RNA

was isolated using Qiagen RNeasy Kit (Studio City, CA). Differential expression was analyzed using a Nonrad GE Array Q Series Human ECM and Adhesion Protein Chip (Super Array, Bethesda, MD) using manufacturer's instructions. Signals were quantified by densitometry and normalized to on-

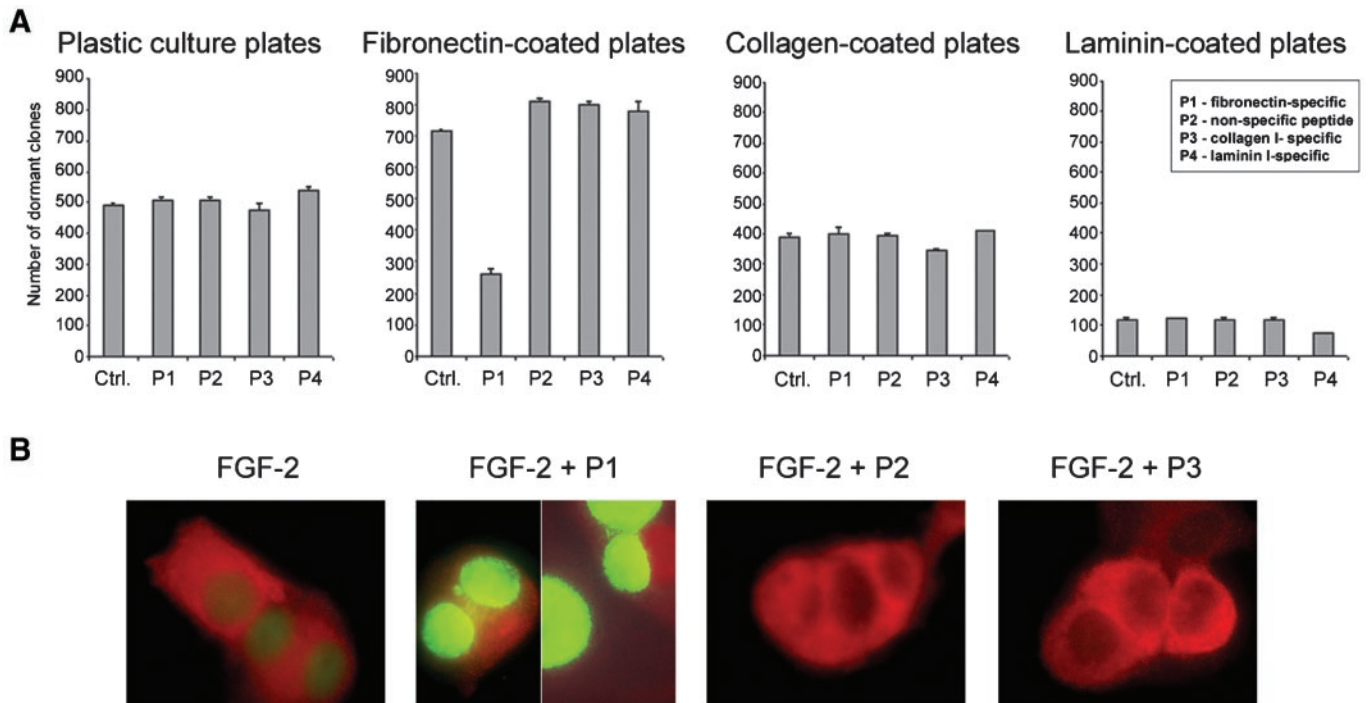


Fig. 4. Ligation of integrin $\alpha 5\beta 1$ provides specific protection from cell death in well-differentiated breast cancer cells. *A*, MCF-7 cells (and T-47D cells, not shown) were incubated with FGF-2 on variably coated plates. Blocking peptides were added after 3 days. Colonies with ≤ 10 cells were stained with crystal violet at 6 days and counted. Error bars indicate \pm SD. *B*, T-47D cells were incubated on fibronectin-coated plates with FGF-2, and blocking peptides were added after 3 days. Cells were probed 24 h later with anti-integrin $\alpha 5$ antibody and Texas Red-tagged secondary antibody and assayed by terminal deoxynucleotidyltransferase-mediated nick end labeling-FITC.

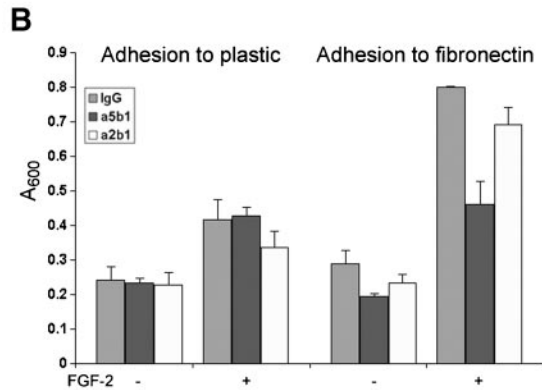
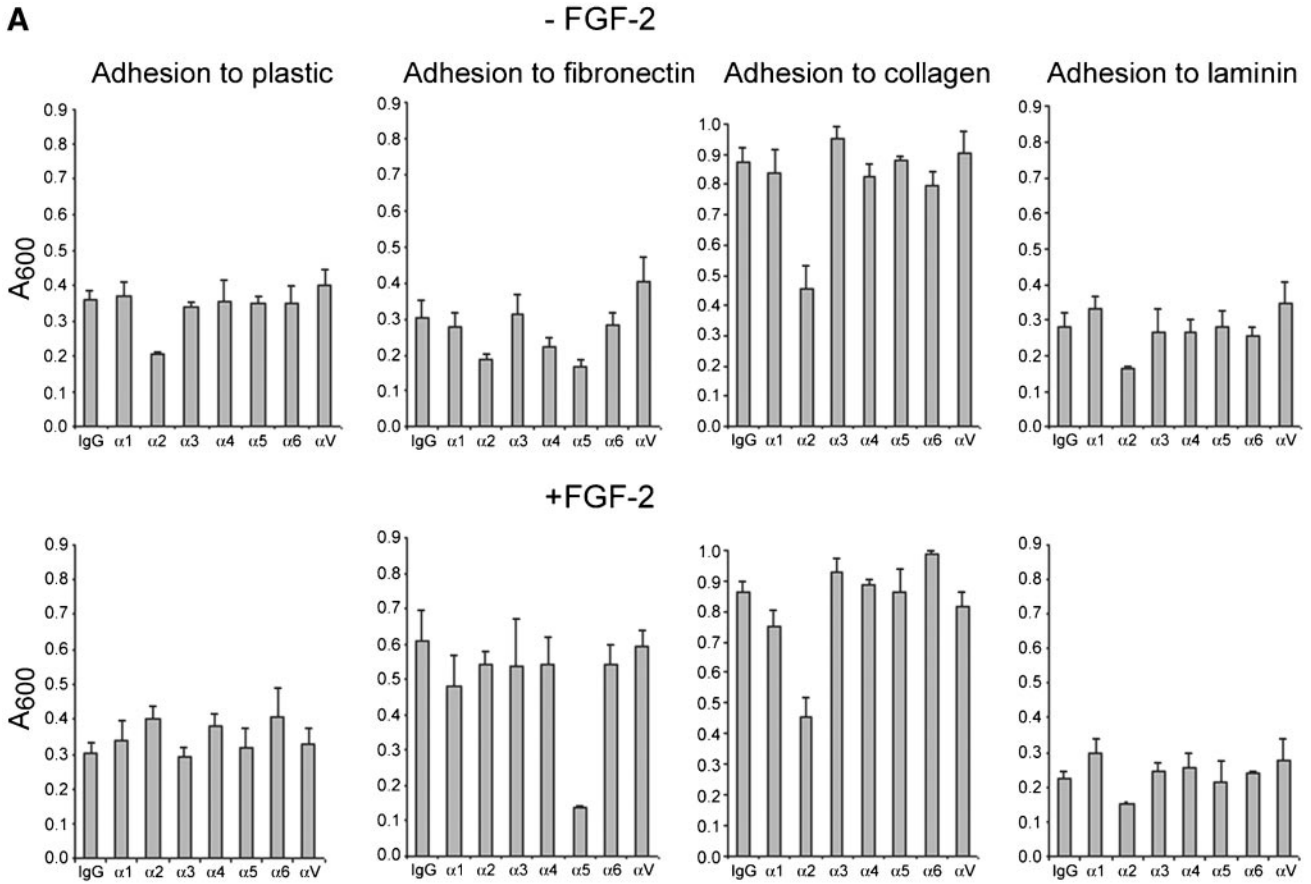


Fig. 5. Adhesion of breast cancer cells to stromal proteins. Both MCF-7 and T-47D cells were cultured \pm FGF-2 on tissue culture (A) or on fibronectin-coated plates (B), detached with cell dissociation solution, washed with PBS, and counted. Cells were incubated with 2 $\mu\text{g}/\text{ml}$ blocking monoclonal antibodies to the integrins or mouse IgG for 30 min at 37°C, and 50,000 cells were incubated in 24-well variably coated tissue culture plates for 45 min at 37°C. Attached cells were stained with crystal violet, and the A_{600} of the extracted dye was measured, as described. Results were similar for both cells. Shown are data for T-47D (A) and MCF-7 cells (B). Error bars indicate \pm SD.

chip controls consisting of glyceraldehyde-3-phosphate dehydrogenase, cyclophillin A, ribosomal L23, and β actin as positive controls and PUC18 plasmid DNA and blanks as negative controls.

Western Immunoblot Detection. Cells at clonogenic density were lysed in modified radioimmunoprecipitation assay buffer and analyzed by SDS-PAGE. Membranes were probed with antibodies to human integrins $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, and $\beta 4$; phospho-Akt; and total Akt (Santa Cruz Biotechnology, Santa Cruz, CA and Chemicon) and detected by ECL. Coomassie Blue-stained membranes were used to verify equal loading.

Bone Marrow Stroma Coculture. Bone marrow aspirates were obtained from normal volunteers under an Institutional Review Board-approved protocol. Stromal cultures were prepared in T75 tissue culture flasks, as described previously (26). The presence of FGF-2 was determined by Western blots of stromal cell lysates (27). After 4–5 weeks in culture, cells were trypsinized, distributed to 24-well plates at 20–50,000 cells/well, and grown to confluence. Cancer cells were seeded on the monolayers in DME/10% FCS at 1,000 cells/well for 6 days and stained with antibody to cytokeratin 19 and a horseradish peroxidase- or fluorescence-conjugated secondary antibody. Func-

tion-blocking peptides were added 3 days after seeding for an additional 3 days before assay.

RESULTS

To understand the effects of the bone marrow microenvironment on solitary well-differentiated breast cancer cells, we compared the clonal growth of MCF-7 and T-47D cells on fibronectin, collagen I, and collagen IV. These ECM proteins are abundant in the bone marrow and can act as ligands to integrins expressed on breast cancer cells. All three proteins caused an increase in the clonal growth of the cells above that of control plates after 6 days in culture (Fig. 1A). In addition to structural ligands to integrins, the bone marrow is rich in soluble growth factors deposited in the stromal matrix. One such growth factor with a role in mammary differentiation is FGF-2. In contrast to the effects of the integrin ligands, FGF-2 completely

inhibited the clonogenic potential of MCF-7 and T-47D cells, whereas another growth factor abundant in bone marrow, epidermal growth factor, had no such effect (Fig. 1B). Notably, however, FGF-2 did not eliminate the survival of a small number of morphologically distinct, dormant clones at the ≤ 10 -cell stage. These effects of FGF-2 were not evident on the growth potential of MDA-MB-231 cells, a highly de-differentiated cell line.

Because a small fraction of cells that metastasize to the bone marrow can survive, we investigated whether the ECM proteins that supported clonal growth in Fig. 1 would have an effect on FGF-2-arrested dormant clones. Indeed, fibronectin provided a distinct survival advantage to MCF-7 and T-47D clones of ≤ 10 cells in the presence of FGF-2 after 5 days in culture in contrast to collagen I and collagen IV (MCF-7 data shown in Fig. 2A). This preferential survival was sustained for up to 15 days in both MCF-7 and T-47D cells, with ratios of 249 and 305% for fibronectin, 55 and 57% collagen I and 32, and 99% for collagen IV, respectively, compared with normalized tissue culture-treated plates (Fig. 2B). The flattened morphology of FGF-2-treated dormant cells contrasted to growing cells on fibronectin underscores their altered status (Fig. 2C). To demonstrate that these dormant MCF-7 and T-47D cells retained their clonogenic potential, FGF-2 was removed from culture after 3 days, and growing clone formation was assessed 6 days later on day 9. Fig. 2D demonstrates that in the absence of FGF-2, there were no dormant clones of 10 or less cells after 9 days on fibronectin, but there were a large number of growing clones consisting of 29 or more cells with both MCF-7 and T-47D cells. In contrast, in the presence of FGF-2 for 9 days, there were no growing clones, but there were a significant number of dormant clones in both cell lines. When FGF-2 was removed on day 3, the number of dormant clones on day 9 persisted

and increased by about one-half. In addition, there were a small number of growing clones of 29 or more cells that began to appear in both cell lines. This suggested that the dormant clones had the capacity to begin proliferating once again upon removal of FGF-2. In parallel experiments on plastic tissue culture dishes, there were no observed growing clones on day 9 after removal of FGF-2 (not shown). This latter point further supported the contribution of fibronectin to the viability of the dormant cells.

The preferential long-term survival of FGF-2-treated dormant clones on fibronectin suggested initiation of active survival signaling and prompted a search for a potential mechanism. To determine the mechanism, we analyzed the effects of FGF-2 on changes in the expression patterns of integrin mRNAs in MCF-7 cells cultured at clonogenic density on fibronectin for 3 and 5 days using a gene chip microarray. Increases were observed in integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, and $\beta 4$ with either 3 or 5 days of incubation with FGF-2. Increases in the fibronectin receptor mRNA $\alpha 5$ and $\beta 1$ at 3 days and $\alpha 5$ at 5 days are shown in Fig. 3A. There were no significant differences in $\alpha 4$ mRNA, the other fibronectin receptor. FGF-2 induced marked increases in integrin $\alpha 5$ protein levels in both MCF-7 and T-47D cells (Fig. 3B) that were sustained for the 5 days assayed (not shown). MDA-MB-231 cells had constitutively high $\alpha 5$ levels that did not vary with FGF-2 treatment. The increases in integrin $\alpha 5$ by FGF-2 in T-47D cells (and MCF-7 cells; not shown) were confirmed by immunofluorescence micrographs (Fig. 3C). The increased protein levels of integrin $\beta 1$ were confirmed by Western blot in both MCF-7 and T-47D cells at 3 days (Fig. 3D) and 5 days (not shown). Increased protein levels of integrins $\alpha 2$, $\beta 3$, and $\beta 4$ were also observed (Fig. 3D), whereas protein levels of integrins $\alpha 3$, $\alpha 4$, and $\alpha 6$ did not vary significantly (not shown).

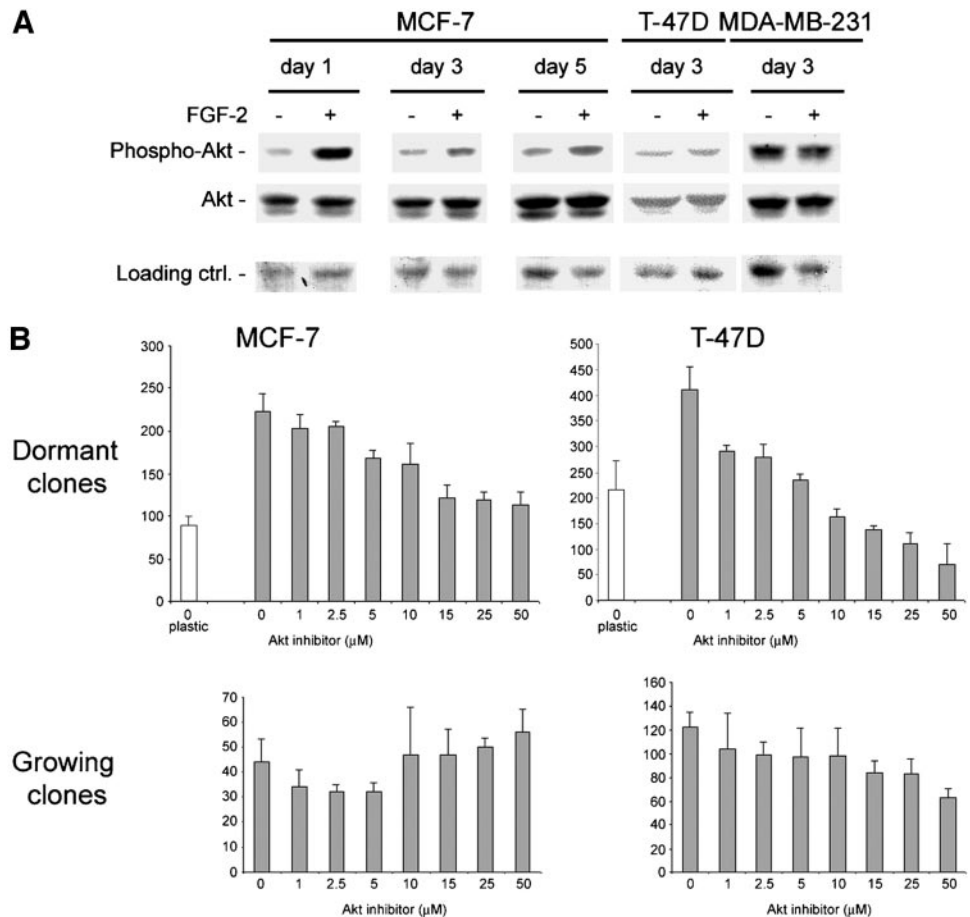


Fig. 6. Inhibition of activated Akt restricts dormant clone survival on fibronectin. **A**, Western blots of lysates from MCF-7, T-47D, and MDA-MB-231 cells incubated on fibronectin-coated plates with FGF-2 for up to 5 days were stained with antibody to phospho-Akt or total Akt. Blots show sustained phosphorylation of Akt by FGF-2 in MCF-7 and T-47D cells but no effect on constitutive Akt phosphorylation in MDA-MB-231 cells. No effect was noted on total Akt levels. Coomassie Blue-stained membrane was used as a loading control. **B**, MCF-7 and T-47D cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of Akt inhibitor were added after 3 days. Cells were stained with crystal violet at 6 days, and colonies with ≤ 10 cells in FGF-2-treated plates (*dormant clones*) and with ≥ 29 cells on cultures lacking FGF-2 (*growing clones*) were counted. Colonies on tissue culture plates are shown as baseline control for dormant clones. Error bars indicate \pm SD.

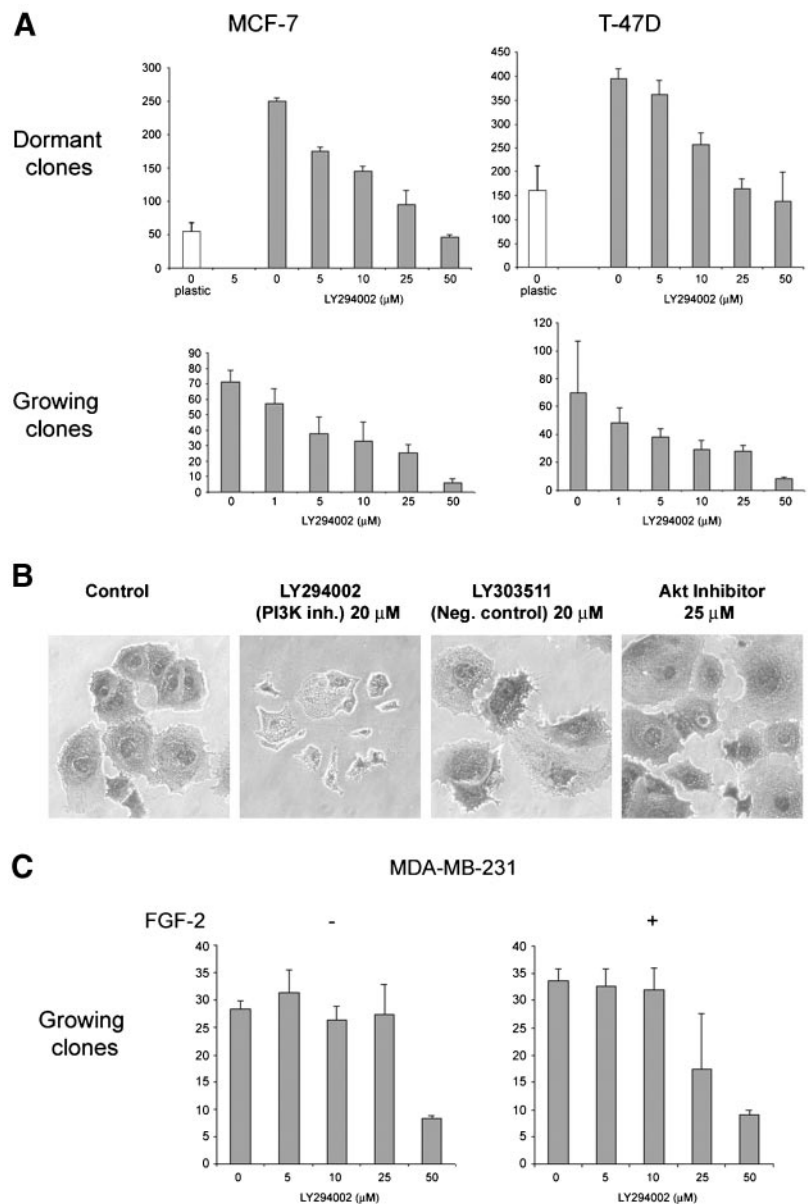
To demonstrate the functional specificity of fibronectin binding in rescuing survival of dormant clones, FGF-2-treated cells on fibronectin were incubated with blocking peptides to fibronectin, collagen I, laminin I, and a nonspecific control. Blocking peptide P1 to fibronectin specifically reversed the fibronectin-mediated rescue of dormant clones in FGF-2-treated MCF-7 cells by 63.6% ($P < 0.001$, Student's *t* test; Fig. 4A) and in T-47D cells by 50.1% ($P < 0.001$; not shown). Analogous experiments on tissue culture-, collagen I- or laminin I-coated plates showed no such effects with specific blocking peptides with either cell line. Blocking the ligation of integrin $\alpha 5\beta 1$ by peptide P1 induced terminal deoxynucleotidyltransferase-mediated nick end labeling staining in many of the clones, suggesting that the inhibitory effects of FGF-2 resulted in apoptosis rescuable by fibronectin ligation (Fig. 4B).

Control experiments demonstrated the capacity of both cell lines to adhere with specificity to all three stromal proteins. In T-47D (Fig. 5A) and MCF-7 (not shown) cells, inclusion of FGF-2 in day 3 cultures on plastic doubled re-adhesion to fibronectin but had no effect on adhesion to plastic, collagen, or laminin. Antibody to integrin $\alpha 5$ blocked adhesion to fibronectin in FGF-2 treated cells by 75%

but only inhibited untreated cell adhesion by a third. Blocking antibody to $\alpha 2$ decreased adhesion to collagen and laminin in both FGF-2-treated and untreated cells equally. Although adhesion to collagen surpassed adhesion to fibronectin, it did not support dormant clone survival (Fig. 4A). Similar specificity was seen in MCF-7 (Fig. 5B) and T-47D cells (not shown) cultured on fibronectin and re-adhered to fibronectin or plastic. Antibody to integrin $\alpha 5\beta 1$ blocked adhesion to fibronectin in FGF-2-treated cells, whereas antibody to integrin $\alpha 2\beta 1$ had no effect on fibronectin adhesion. Antibody to integrin $\alpha 5\beta 1$ had no effect on adhesion to plastic. These data are consistent with a specific survival effect derived from ligation to fibronectin in dormant cells.

To determine one possible mechanism contributing to this effect, we investigated the role of the PI3k/Akt signaling pathway. Fig. 6A demonstrates that FGF-2 induced the phosphorylation of Akt in MCF-7 cells on fibronectin. The effect was strongest after 24 h but remained sustained for the 5 days assayed. FGF-2 also induced sustained Akt phosphorylation in T-47D cells. GSK-3 α and β , downstream substrates of Akt, were phosphorylated on Ser-21/9 in both dormant MCF-7 and T-47D cells on fibronectin on day 6, as

Fig. 7. Inhibition of PI3k restricts dormant clone survival on fibronectin. A, MCF-7 and T-47D cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of LY294002 were added after 3 days. Cells were stained with crystal violet at 6 days, and colonies with ≤ 10 cells in FGF-2-treated plates (*dormant clones*) and with ≥ 29 cells on cultures lacking FGF-2 (*growing clones*) were counted. LY303511 50 μM was used as a negative control (not shown). Colonies on tissue culture plates are shown as baseline control for dormant clones. B, morphogenic appearance of dormant T-47D cells on fibronectin treated for 3 days with Akt inhibitor, LY294002, and LY303511 on day 6 of culture. C, MDA-MB-231 cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of LY294002 were added after 3 days. Cells were stained with crystal violet at 6 days, and colonies with ≥ 29 cells were counted (there were no dormant clones evident). Error bars indicate \pm SD.



determined by Western blots, indicating that phosphorylation of Akt resulted in its activation (data not shown). In MDA-MB-231 cells that do not undergo dormancy, Akt was phosphorylated at baseline on fibronectin and did not respond to FGF-2 treatment. To determine the significance of Akt phosphorylation in dormancy, the effects of the specific chemical Akt inhibitor I (Calbiochem) was investigated in the clonogenic assays. Addition of the inhibitor on day 3 resulted in a dose-dependent decrease of dormant clones on day 6, with an ED_{50} of $5 \mu M$, the reported K_d of the compound (Fig. 6B). The inhibitor was slightly more effective in T-47D cells than in MCF-7 cells, but did not eradicate dormant clones much past the level supported on plastic in either cell line, essentially reversing the survival effect afforded by fibronectin. The inhibitor did not have a significant effect on growing clones, a condition where Akt was not phosphorylated.

Inhibition of PI3k, the upstream activator of Akt, with LY294002 also inhibited dormant clone survival in a dose-dependent manner, with an ED_{50} of approximately $10 \mu M$ (Fig. 7A). However, dormant clones approximating in number those sustained on plastic were not eradicated by inhibition of PI3k either. In contrast to Akt inhibition, however, LY294002 inhibited proliferating clones in a dose-dependent manner with an ED_{50} of about $5 \mu M$, supporting the role of PI3k in their survival. The difference in blocking PI3k and its downstream target Akt was further demonstrated by the appearance of the surviving dormant clones on fibronectin (Fig. 7B). Whereas dormant cells surviving Akt inhibition remained characteristically large, spread out

with large cytoplasm to nucleus ratios, surviving cells after LY294002 treatment were small and dendritic and appeared distressed. As a control, LY294002 also inhibited the growing MDA-MB-231 clones equally with and without FGF-2 treatment but required a concentration about 10 times higher than that needed to inhibit growing MCF-7 or T-47D clones (Fig. 7C).

The significance of the results on fibronectin-coated dishes was tested in a bone marrow stromal coculture model. T-47D cells (and MCF-7 cells, not shown) were inhibited at the 4–8-cell stage at 6 days, whereas MDA-MB-231 cells proliferated freely without forming colonies (Fig. 8A). Fig. 8B demonstrates that cytokeratin 19-stained MCF-7 cells remained as primarily single cells or as clones made up of very few cells after 6 days on stromal coculture. The expression of FGF-2 in stromal cells was confirmed by Western blot (Fig. 8C). As in the model, blocking peptides to fibronectin specifically diminished the survival of ≤ 10 cell clones on the stromal monolayers in MCF-7 cells (Fig. 8D) and T-47D cells (not shown). The fibronectin blocking peptide did not affect the survival of stromal cells (not shown). To determine whether inhibition of PI3k will be useful as a potential therapeutic intervention in dormant clones in the bone marrow, stromal cells were treated with LY294002. Fig. 8E demonstrates that in contrast to the effects on dormant breast cancer cells, inhibition of PI3k did not decrease the survival of stromal cells. These experiments support this paradigm and support additional studies to block survival signaling to eradicate dormant breast cancer cells in the marrow.

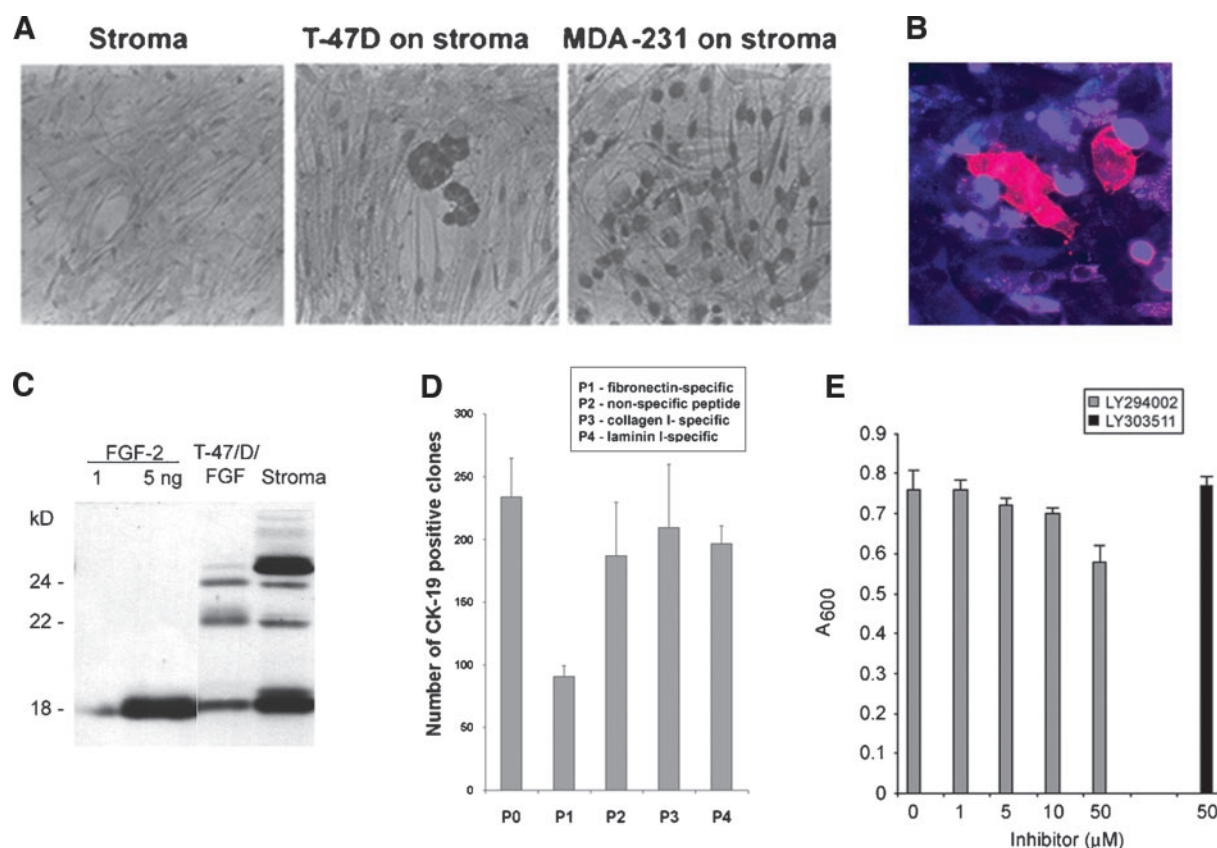


Fig. 8. Stroma restricts growth of well-differentiated T-47D breast cancer cells. *A*, confluent stromal cultures in 24-well plates seeded with 500 T-47D or MDA-MB-231 cells/well were cultured for 6 days. *B*, cytokeratin 19 immunofluorescence (red) staining of MCF-7 cells on stromal coculture (blue) demonstrated that MCF-7 cells remained as primarily single cells or as clones made up of very few cells after 6 days on stromal coculture. *C*, Western blots of stromal cell lysates ($100 \mu g$) with recombinant FGF-2 and lysates from T-47D cells transfected with a vector expressing M_r 18,000, 22,000, 22,500, and 24,000 FGF-2 isoforms. *D*, MCF-7 cells were seeded on stromal monolayers on 24-well plates (1,000 cells/well). Blocking peptides were added after 3 days. At 6 days, plates were stained with anticytokeratin 19 antibody and horseradish peroxidase-tagged secondary antibody and developed, and colonies of ≤ 10 cells were counted. *E*, lack of cytotoxic effects of LY294002 on stromal cells. Confluent stromal monolayers on 24-well plates were incubated with variable concentrations of LY294002 and $50 \mu M$ LY303511 for 3 days. The medium was aspirated; the cells were washed and fixed with freshly made crystal violet solution for 20 min, washed in distilled water, dried, and extracted with 10% acetic acid; and the A_{600} was measured. Error bars indicate \pm SD.

DISCUSSION

Our data support a potential mechanism for dormancy of well-differentiated breast cancer cells metastatic to the bone marrow. In the model, cancer cells encounter FGF-2, a growth factor implicated in mammary cell differentiation, and acquire a more differentiated phenotype. Newly acquired traits include growth arrest and altered expression of integrins, including up-regulation of integrin $\alpha 5\beta 1$. Most growth-arrested cells die, but some survive by binding fibronectin, which initiates survival signaling. The dormant cells retain their proliferative capacity, because removal of FGF-2 permits them to begin proliferating once again into growing clones. Dormant cells on fibronectin rely, at least in part, on activation of PI3k/Akt signaling to sustain their survival.

Prior studies provide support for all elements of this paradigm. FGF-2 is expressed and deposited in the marrow (16–20) and has a spectrum of effects on mammary cells. It is implicated in mammary duct morphogenesis (7), but its expression stops with malignant transformation (28–30). Enforced re-expression of FGF-2 in breast cancer cells induces a more differentiated phenotype, including formation of branched structures in Matrigel; decreased migration, invasion, and growth in soft agar; and tumor formation in nude mice (13, 14). FGF-2 inhibits proliferation of well-differentiated breast cancer cells in G_1 (9, 10, 31) through induced expression of $p21^{WAF1/Cip1}$ that results in inactivation of cdk2 and dephosphorylation of Rb (10). Here, FGF-2 inhibited growing clones in well-differentiated cells but had no effect in MDA-MB-231 cells, consistent with diminished dormancy rates of poorly differentiated cells. Whereas FGF-2 induces $p21^{WAF1/Cip1}$ in both MCF-7 and T-47D cells, MDA-MB-231 cells have constitutively high levels that are not affected by FGF-2.¹ Reports have shown that $p21^{WAF1/Cip1}$ is inactivated in cells with active Rac (32). Because MDA-MB-231 cells have mutant, constitutively active Ras, it is likely that Rac activity is also higher than baseline and may be inhibiting this cyclin-dependent kinase inhibitor. Aguirre-Ghiso *et al.* (15) have reported that dormancy in an epidermoid cancer cell line is modulated by the ratio of the activated levels of p38 and ERK 1/2. They demonstrated sustained ERK activation through transcriptional up-regulation of uPAR in cells that exited dormancy. In MCF-7 cells growth arrested by FGF-2, ERK was activated but the activation was quenched after 1 h (9). Follow-up studies will elucidate the state of p38 activation and its relationship to ERK activation in dormant and growing breast cancer cells.

Inhibition of the PI3k/Akt pathway resulted in inhibition of dormant clone survival. Although both inhibitors lowered the numbers of surviving clones to approximate those on plastic, the effects on the morphology of the remaining clones were quite different. Akt did not affect the appearance of the remaining dormant cells. They remained flattened and spread, suggesting that signaling pathways involved with morphological and cytoskeletal changes in the dormant cells remained unaffected. However, dormant clones that survived despite PI3k inhibition lost this characteristic appearance, suggesting that additional pathways, other than Akt, downstream of PI3k may affect the cytoskeletal changes of dormancy. These effects remain the subjects of ongoing investigations.

FGF-2 can modulate the expression of integrins, including $\alpha 5$ and $\beta 1$ (21, 22), whose ligation promotes survival in a variety of cell types (24, 25). Here, FGF-2 induced $\alpha 5\beta 1$ expression in well-differentiated breast cancer cells, an effect not mediated by feedback up-regulation through fibronectin (Fig. 3B). However, although $\alpha 5\beta 1$ induction may not have been mediated by fibronectin, it is possible that FGF-2-induced Akt phosphorylation may have been facilitated by $\alpha 5\beta 1$. Binding of integrin $\alpha 5\beta 1$ to fibronectin activates Akt signaling and

results in its Ser-473 phosphorylation (33). Integrin $\alpha 5\beta 1$ is only antiapoptotic when bound to integrins such as fibronectin and up-regulation of $\alpha 5\beta 1$ without ligation results in apoptosis (34). Binding of integrin $\beta 1$ by the ligand fibronectin significantly inhibits apoptosis in breast cancer cell lines MDA-MB-231 and MDA-MB-435 induced by paclitaxel and vincristine (35). Integrin modulation may be an element of differentiation that allows well-differentiated breast cancer cells to interact with the marrow microenvironment. Blocking appropriate ligation of integrin $\alpha 5\beta 1$ resulted in apoptosis. It is possible that this effect may serve to restrict the survival of well-differentiated breast cancer cells to fibronectin-rich compartments of the marrow.

The experiments presented here suggest a potential mechanism for dormancy and resistance to death in breast cancer cells in the bone marrow and present the integrin $\alpha 5\beta 1$ -fibronectin interaction and ensuing signaling as potential targets for disrupting this resistance. This paradigm of dormancy in the bone marrow microenvironment may differ from ones in other characteristic metastatic sites for breast cancer, such as lung and liver, where the microenvironment is different. The mechanisms of dormancy in these sites remain under investigation.

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¹ R. Wieder, unpublished observations.

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