AD_____

Award Number: DAMD17-03-1-0524

TITLE: Overcoming Bone Marrow Stroma-Mediated Chemoresistance in Metastatic Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Robert Wieder, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of New Jersey Medicine & Dentistry
Newark, NJ 07103

REPORT DATE: August 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2006	Final	7 JUL 2003 - 6 JUL 2006

4. TITLE AND SUBTITLE
Overcoming Bone Marrow Stroma-Mediated Chemoresistance in Metastatic Breast Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0524

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert Wieder, M.D., Ph.D.

E-Mail: wiederro@umdnj.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of New Jersey Medicine & Dentistry
Newark, NJ 07103

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
Mammary ductal integrity is lost during dedifferentiation to cancer. Ductal morphogenesis has been reported to depend on expression of FGF-2 and laminin 5. We demonstrate that the two proteins are coordinately lost during the progression to cancer and that FGF-2 may contribute to the deposition of Laminin 5. Breast cancer cells metastasize to the bone marrow early during cancer formation where they can remain dormant for years and are resistant to chemotherapy. We developed an *in vitro* model where FGF-2, a growth factor abundant in the marrow microenvironment, induces partial re-differentiation and integrin re-expression that permits ligation of bone marrow fibronectin in estrogen-dependent cells. Dormant cells require survival signaling through PI3K/Akt and Rho and combined disruption of PI3K and Rho can result in near complete elimination of dormant cells. Broad transcription inhibitors such as Flavopiridol have pleiotropic effects on key targets involved with survival of dormant breast cancer cells and may represent a useful approach to eliminating cells dependent on multiple signal pathways for survival that are resistant to taxanes. We developed a flow retardation device that represents the first application of a technology for assessing cell surface protein expression in single cancer cells without prior manipulation.

15. SUBJECT TERMS
Dormancy, integrins fibronectin, metastasis, Akt, PI3 kinase, all *trans*-retinoic acid (ATRA)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	30	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Cover……

SF 298…… 2

Introduction…….... 4

Body…… 4

Key Research Accomplishments…… 27

Reportable Outcomes…… 27

Conclusions…….. 29

References…… 30

INTRODUCTION

Mammary ductal epithelial cells de-differentiate to cancer through a series of mutational and epigenetically regulated steps where they acquire characteristics that can overcome control on proliferation, ductal morphogenesis and expression of cell adhesion molecules. Cells acquire the ability to invade, induce angiogenesis, metastasize, extravasate and survive at the metastatic site. The interaction at the metastatic site with the microenvironment induces a partial re-differentiation of the cancer cell, including inhibition of proliferation, re-induction of cellular adhesion molecules and initiation of survival signaling that provides a degree of resistance from chemotherapy. We have investigated putative mechanisms for loss of morphogenic differentiation during progression from normal ductal architecture to cancer, the induction of dormancy in the bone marrow microenvironment, methods of overcoming resistance to chemotherapy in dormant cells, potential survival signaling pathways responsible for chemoresistance and explored methods of characterizing surface receptor expression on cancer cells when few are available for diagnosis, such as the case of needle biopsies. The following are the specific objectives we investigated.

BODY

Objectives/Hypothesis:

A. Co-ordinate loss of FGF-2 and laminin 5 expression during neoplastic progression of mammary duct epithelium. (2006) Human Pathology (in press).

Branching morphogenesis in mammary ducts is associated with the expression of a number of proteins. These include laminin 5 and basic fibroblast growth factor (FGF-2). Both proteins are lost with malignant transformation of mammary epithelium and have causal roles in branching morphogenesis in breast cancer cells *in vitro*. We hypothesized that the expression of these two molecules is coordinately lost during the loss of branched structures during mammary ductal dedifferentiation and that there is a potential causal relationship between their expression and deposition.

B. Integrin $\alpha 5\beta 1$ promotes survival of growth-arrested breast cancer cells: an in vitro paradigm for breast cancer dormancy in bone marrow. *Cancer Research 64: 4514-4522.*

The mechanisms of long-term survival of occult breast cancer cells in the bone marrow microenvironment are not known. Breast cancer cell in the marrow are influenced by soluble and structural components of the microenvironment that bind to cancer cell surface receptors and modulate their behavior that includes re-differentiation and induction of dormancy that includes growth arrest, re-expression of integrins and survival signaling that contributes to resistance to chemotherapy. We hypothesize that FGF-2 in the marrow, a functional differentiation factor in mammary structural development, contributes to the re-differentiation, dormancy and chemoresistance of metastatic well-differentiated breast cancer cell in the marrow.

C. Flavopiridol blocks integrin-mediated survival in dormant breast cancer cells. Clinical Cancer Research 11:2038-2046.

Breast cancer cells that metastasize to the bone marrow can remain dormant and resistant to chemotherapy by virtue of non-proliferation and survival signaling initiated by integrin ligation by the microenvironment. We hypothesize that agents that inhibit this interaction and/or block survival signaling can eliminate these dormant cells.

D. Microfluidic techniques for single cell protein expression analysis. (2006) Clin. Chem. 52:1080-1088.

The analysis of single cells obtained from needle aspirates of tumors is constrained by the need for processing. To this end, we investigated two microfluidic approaches to measure the expression of surface proteins in single cancer cells, or in small populations (< 50 cells).

Study design:

A. We carried out indirect fluorescence staining on subsets of archived pathologic samples from 55 patients with a total of 140 pathologic entities, many with multiple stages of dedifferentiation present on the same cut, using antibodies to FGF-2, FGFR1 and laminin 5 to determine expression. We also used western blots to detect laminin 5 expression in MCF-7, T-47D and MDA-MB-231 cells transfected with vectors constitutively expressing FGF-2 and immunofluorescence staining of matrix proteins deposited by these cells to determine export and accumulation of laminin 5.

B. 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. Two well-differentiated breast cancer cell lines, MCF-7 and T-47D were used in the studies. Cells were incubated in DMEM/10% fetal calf serum (FCS) with FGF-2 10 ng/ml in fibronectin-coated, collagen-coated, laminin I-coated plates (Biocoat, Becton Dickinson, MA) or tissue culture-coated plates at clonogenic densities (1000 cells/well in 24 well plates for T-47D, 2000 cells/well for MCF-7 cells). At this plating density, cells do not come in contact with each other and primary interaction is with the substratum. After three days of culture, the medium was replaced or replaced and supplemented with a variety of inhibitors. Similar studies were carried out with human bone marrow-derived stromal cultures.

C. In an <u>in</u> <u>vitro</u> model of dormancy described in B, we investigated the effects of Taxotere, flavopiridol and MEK and p38 inhibitors on survival of dormant clones and that of flavopiridol on expression of integrins, adhesion strength and phosphorylation of Akt, ERK 1/2 and p38.

Two well-differentiated breast cancer cell lines, MCF-7 and T-47D were used in the studies. Cells were incubated in DMEM/10% fetal calf serum (FCS) with FGF-2 10 ng/ml in fibronectin-coated tissue culture plates (Biocoat, Becton Dickinson, MA) at clonogenic densities (1000 cells/well in 24 well plates for T-47D, 2000 cells/well for MCF-7 cells). At this plating density, cells do not come in contact with each other and primary interaction is with the substratum. After three days of culture, the medium was replaced with DMEM/5% charcoal stripped FCS and all trans-retinoic acid (ATRA) was added at concentrations of 0 (DMSO 1:10000 control), 10^{-9}, 10^{-8} and 10^{-7} M for an additional three or six days. Cells were fixed and stained with crystal violet solution containing sodium borate and ethanol. Colonies of 2-10 cells (dormant clones) were counted. In parallel experiments, after three day in culture or an additional three days in ATRA, cells were collected and lysates were prepared and analyzed by Western blot.

D. One approach involved indirect fluorescence labeling of cell surface proteins and channeling of cells in a microfluidic device past a fluorescence detector for signal quantitation and analysis. A second approach channeled cells in a microfluidic device over detection zones coated with ligands

to surface proteins, and measured rates of passage and of retardation based on transient interactions between surface proteins and ligands.

BODY

A. FGF-2 and laminin 5 expression were found throughout benign and atypical dedifferentiation in mammary tissue samples and were lost primarily with transformation to invasive cancer. FGFR1 was expressed in all cell types. Cancer cells enforced to express FGF-2 did not have detectable laminin 5 on western blot but matrix proteins deposited in culture did stain positive, suggesting accumulation of exported laminin 5.

Figure 1. Figure 2.

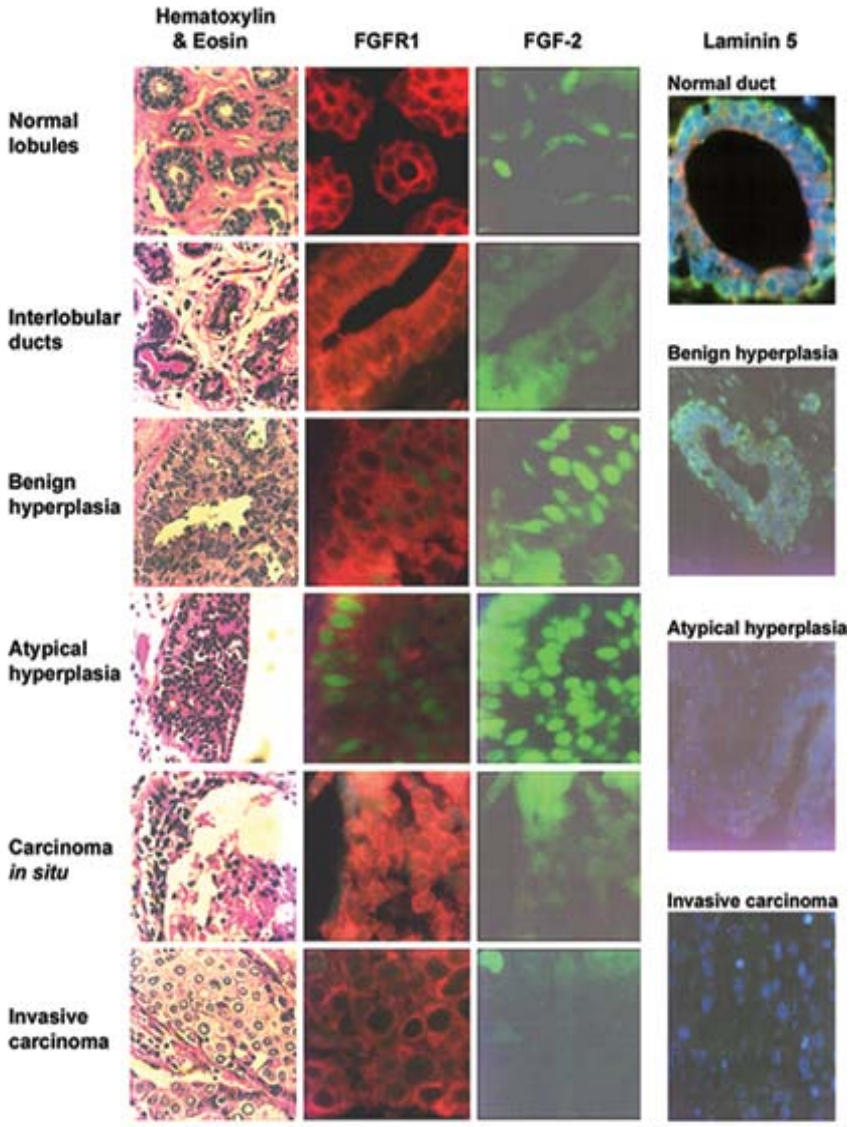

Figure 1. Hematoxylin and eosin (H&E) and immunofluorescence staining of sample tissue specimens from normal lobules, interlobular ducts, benign hyperplasia, atypical hyperplasia, carcinoma *in situ* and invasive cancer. Slides mounted with 4 micron thick tissue samples were either stained with H&E or with primary rabbit antiserum to FGFR1 or monoclonal anti-FGF-2 antibody and FITC-labeled anti-mouse or anti-rabbit secondary antibody. Cells were photographed at 100X magnification (H&E) and 400 X magnification (immunofluorescence slides) using an Olympus BX40 fluorescence photomicroscope. Fluorescence staining for FGF-2 is evident within the cells of the normal or hyperplastic cellular portions of the magnified corresponding ducts shown in the H&E-stained images in the left hand panels. Minimal or background staining is evident in the cells of

carcinoma *in situ* or invasive cancer samples shown. Some non-specific, non-cellular staining is also evident.

Figure 2. Indirect immunofluorescence staining of illustrative samples from tissue specimens containing normal ducts, benign hyperplasia, atypical hyperplasia and invasive cancer with antibody to laminin 5 γ2 and a secondary FITC-labeled fluorescence antibody and photographed at 400X magnification.

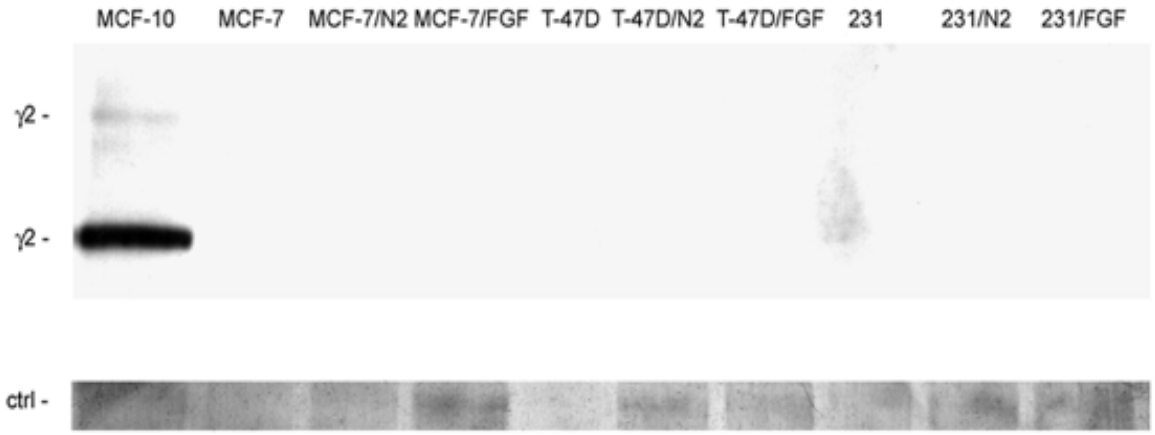

Figure 3. Western immunoblot of MCF-10A nontransformed mammary epithelial cells, MCF-7, T-47D and MDA-MB-231 cancer cells or the three cancer cell lines transfected with a constitutively expressing FGF-2 vector (FGF) or the backbone vector (N2) stained with a goat anti-laminin 5 γ2 antibody and a secondary horseradish peroxidase-linked donkey anti-goat antibody. A non-specific band from the Coomasie blue-stained membrane was used as a loading control.

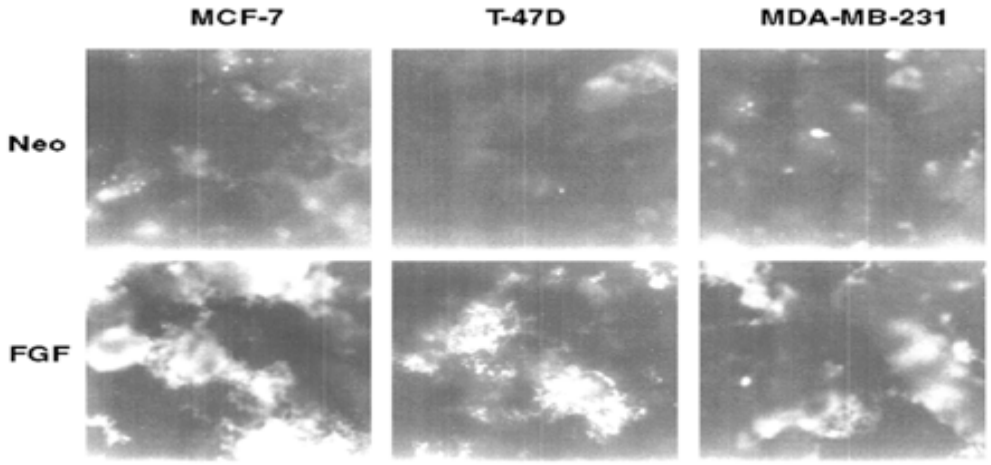

Figure 4. Increased expression of laminin 5 in breast cancer cells enforced to express FGF-2. Neo-transfected (upper panel) and FGF-2-expressing cells (lower panel) were grown to confluence on tissue culture-treated plastic plates, washed off with PBS, and the deposited matrix was fixed and probed with an antibody to the γ2 chain of laminin 5, stained with an immunofluorescence-tagged secondary antibody and photographed at 100X magnification, as described in the Materials and Methods section.

Table 1. Immunofluorescence detection of FGF-2 in mammary cells

Pathology	number of samples	number FGF-2 positive (% positive)
normal duct/lobule	43	37 (86)
fibrocystic duct	20	14 (70)
benign hyperplasia	22	22 (100)
atypical hyperplasia	11	9 (82)
carcinoma *in situ*	14	10 (71)
invasive carcinoma	23	7 (30)
fibroadenoma	6	2 (33)
phylloides tumor	1	1 (100)

Table 2. Immunofluorescence detection of FGFR in mammary cells

Pathology	number of samples	number FGFR positive
normal lobule	7	7
normal duct	10	10
fibrocystic duct	1	1
benign hyperplasia	2	2
atypical hyperplasia	2	2
carcinoma *in situ*	6	6
invasive carcinoma	14	14

Table 3. Immunofluorescence detection of laminin 5 in mammary cells

Pathology	number of samples	number laminin 5 positive (% positive)
normal duct/lobule	27	27 (100)
hyperplasia (all types)	25	22 (88)
invasive carcinoma	22	0 (0)

B. Basic fibroblast growth factor (FGF-2), a mammary differentiation factor abundant in the bone marrow stroma, induced growth arrest of relatively well-differentiated breast cancer cells, induced a spread appearance and enhanced their survival on fibronectin by upregulating integrin $\alpha5\beta1$. Most of the FGF-2-arrested cells failed to establish optimal ligation to fibronectin and underwent cell death. Cells that did attach to fibronectin, another major constituent of the bone marrow microenvironment, stayed alive and growth-arrested for many weeks. While 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 showed a specific contribution of $\alpha5\beta1$-fibronectin interaction in maintaining survival of growth-arrested cells, potentially by negatively modulating apoptotic response via signaling pathways. Blocking of phosphoinositol 3-kinase and Akt inhibits survival of dormant clones, demonstrating this as one of those pathways. Experiments with human bone marrow stroma co-cultures confirm the role of fibronectin ligation in maintaining survival of dormant clones.

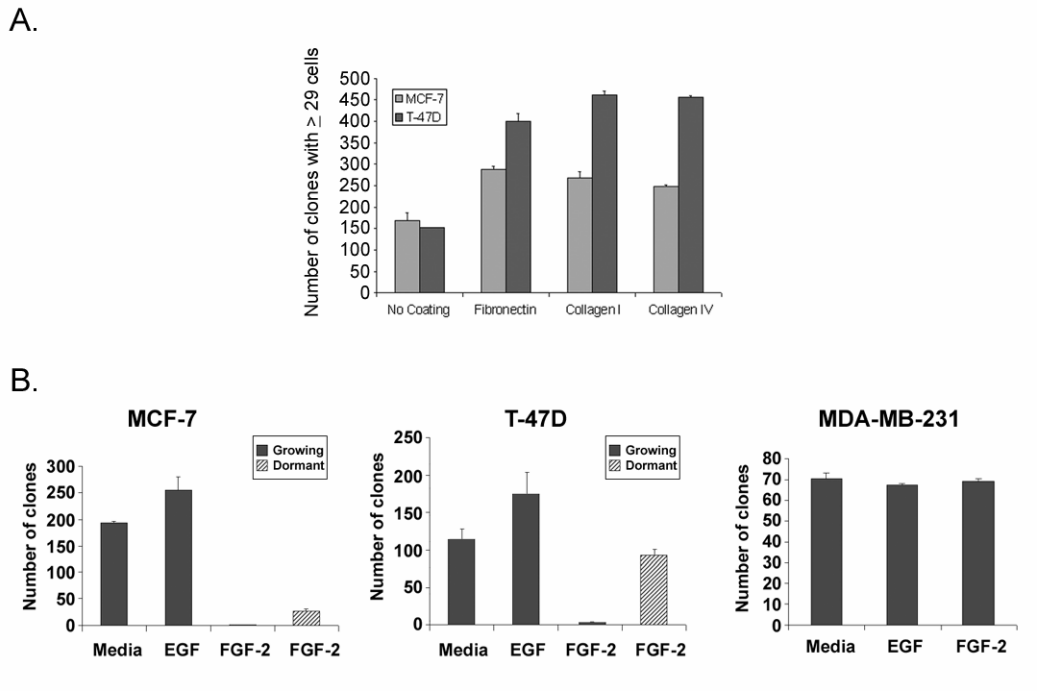

Figure 1. A. Effects of ECM proteins on the clonogenic efficiency of MCF-7 and T-47D cells. Cells were incubated on uncoated 24 well plates or plates coated with fibronectin, collagen I or collagen IV at clonogenic densities of 1,000 cells per well. Crystal violet-stained colonies containing at least 29 cells were counted after 6 days in culture. B. Effects of EGF 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 per well) were incubated in 24 well plates ± 10 ng/ml EFG or FGF-2 for 6 days, 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, ± S.D.

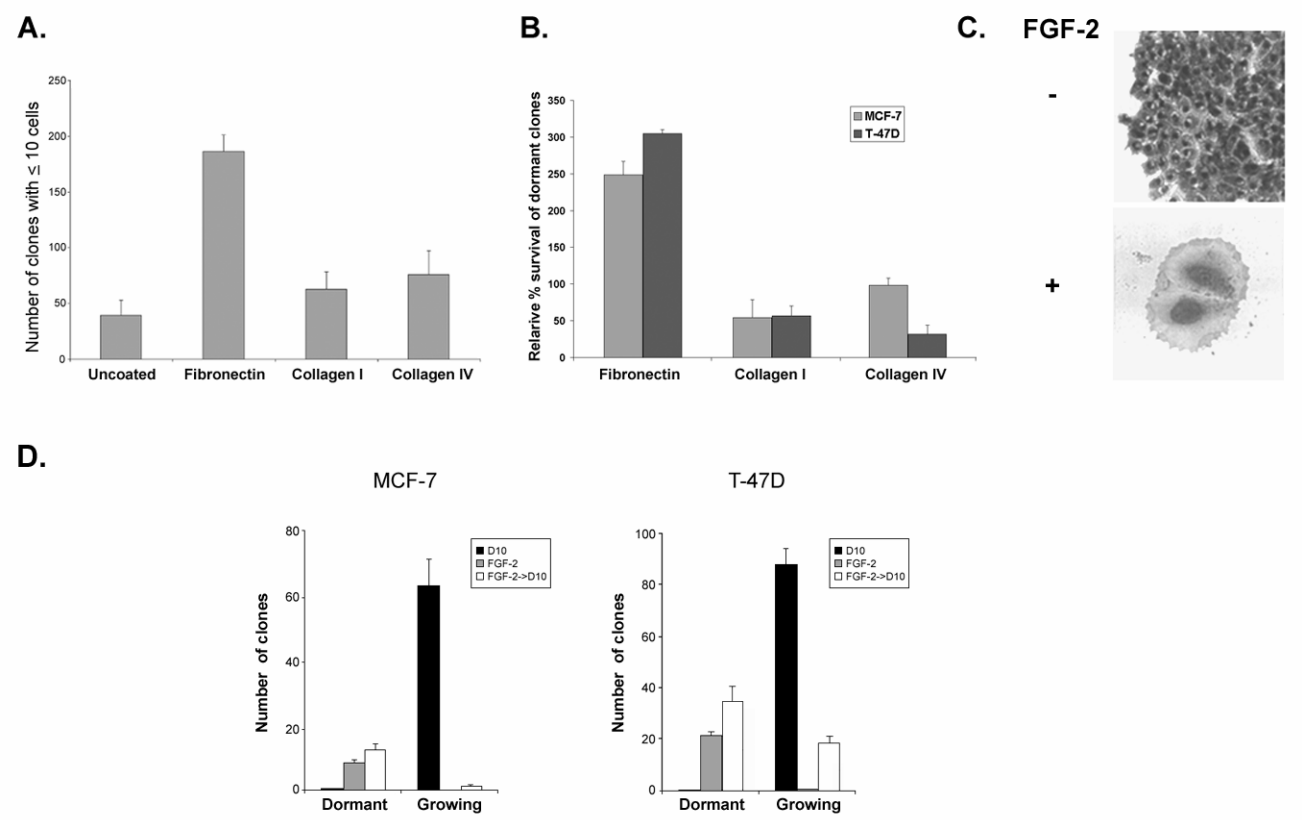

Figure 2. Fibronectin rescues the survival of FGF-2-treated dormant MCF-7 and T-47D cells. Cells were incubated in 6 well variably coated plates (5,000 cells/well) with FGF-2 10 ng/ml for 5 days (A) (MCF-7 cell data are shown) to 15 days, stained with crystal violet and colonies of ≤ 10 cells counted. 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; 200X magnification. D. Growth potential of dormant cells after removal of FGF-2. MFC-7 and T-47D cells were incubated on fibronectin coated plates with or without FGF-2 (D10). After three days, FGF-2 was replenished in 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 nine days. Error bars, ± S.D.

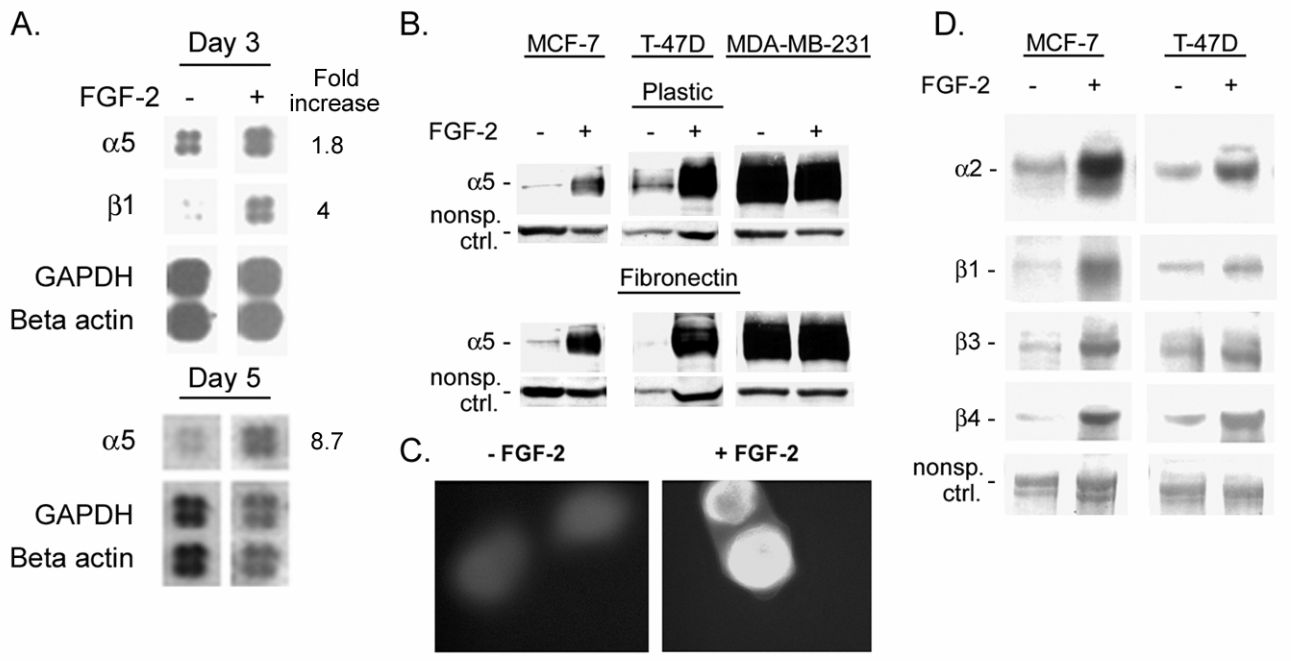

Figure 3. FGF-2 regulates expression of integrins. A. Gene chip analysis of integrin α5 and β1 mRNA expression in MCF-7 cells incubated ± FGF-2 for 3 or 5 days on fibronectin-coated plates. Densitometer quantitations normalized against GAPDH and actin mRNA standards are shown. B. Western blots of integrin α5 from cells incubated ± FGF-2 for 3 days on tissue culture- or fibronectin-coated dishes. C. Indirect immunofluorescence of integrin α5 in T-47D cells on cover slips ± FGF-2 10 ng/ml for 24 hours. D. Western blots of integrins α2, β1, β3 and β4 in MCF-7 and T-47D cells incubated ± FGF-2 for 3 days. Nonspecific bands were used as loading controls.

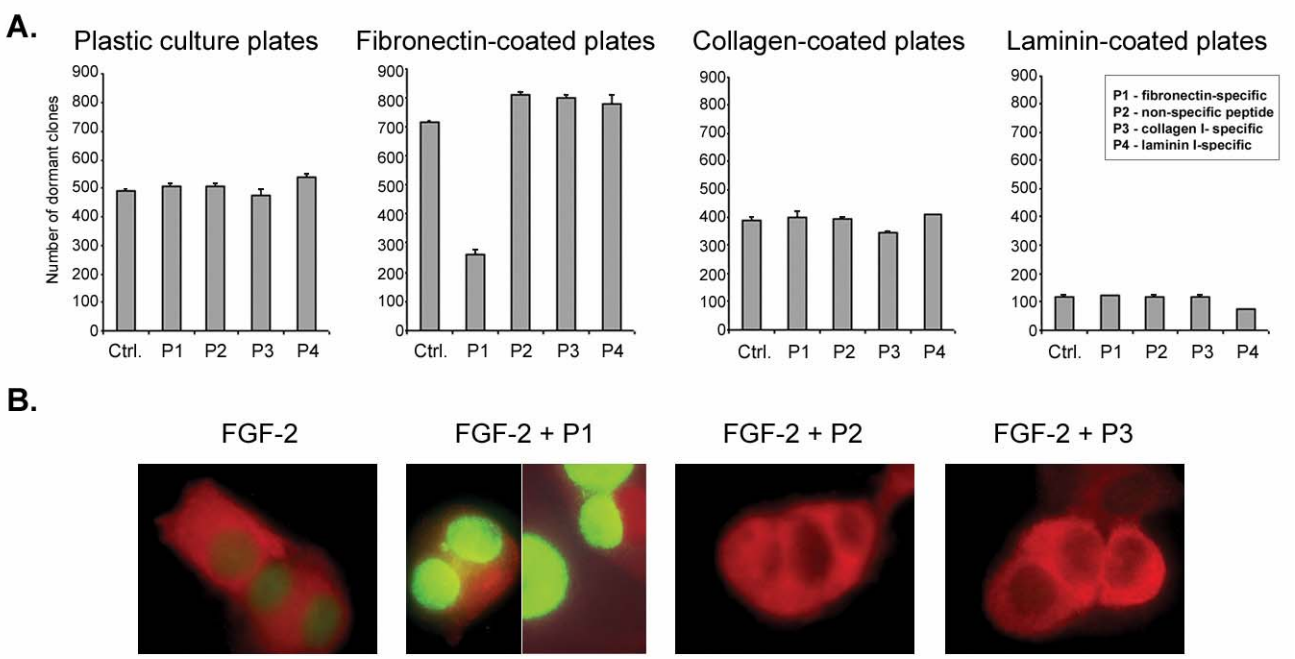

Figure 4. Ligation of integrin $\alpha5\beta1$ provides specific protection from cell death in well-differentiated breast cancer cells. A. MCF-7 cells (and T-47D cells, not shown) were incubated with FGF-2 on variably coated plates. Blocking peptides were added after 3 days. Colonies with ≤10 cells were stained with crystal violet at 6 days and counted. Error bars, ± S.D. 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 hours later with anti-integrin α5 antibody and Texas Red-tagged secondary antibody and assayed by TUNEL-FITC.

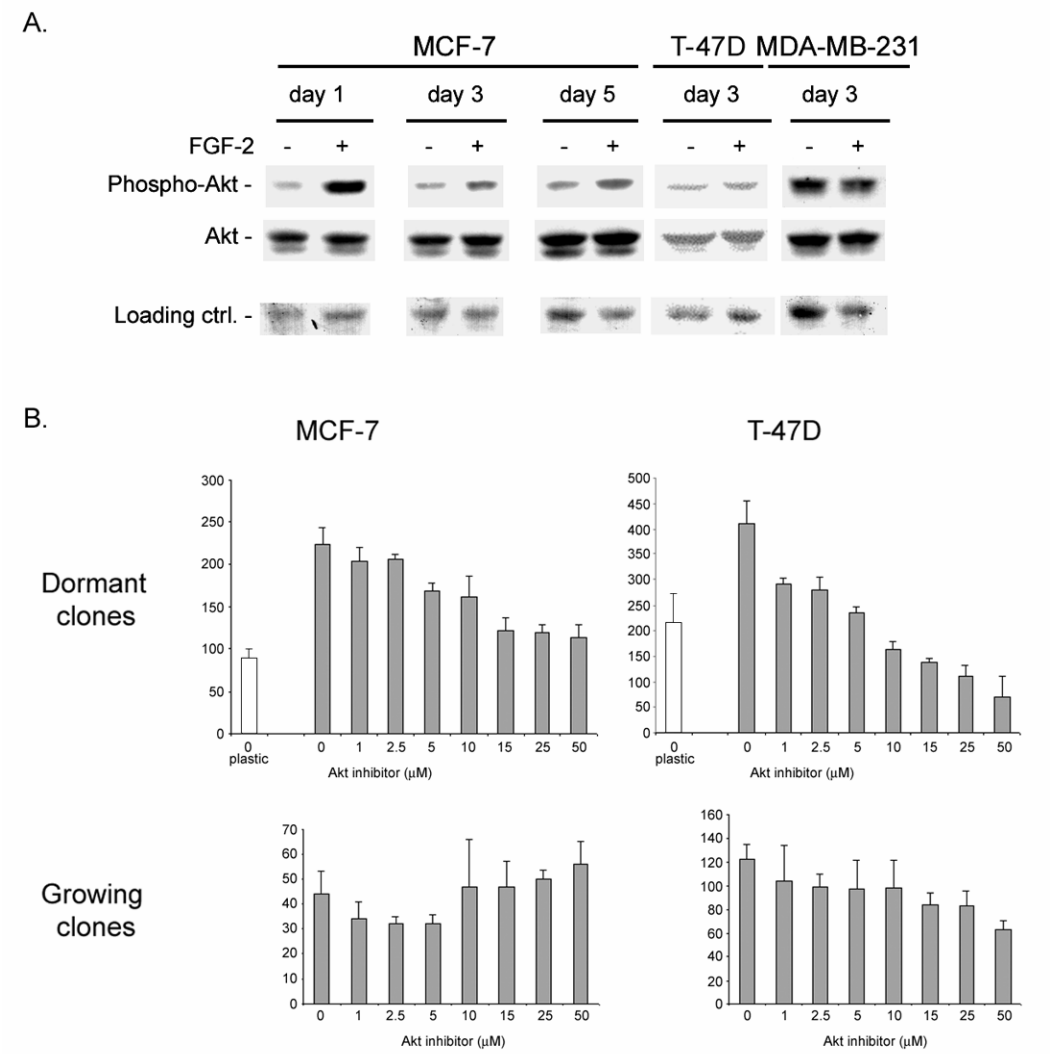

Figure 5. Inhibition of activated Akt restricts dormant clone survival on fibronectin. A. Western blots of lysates from MCF-7, T-47D and MDA-MB-231 cells incubated on fibronectin-coated plates with FGF-2 for up to 5 days were stained with antibody to phospho-Akt or total Akt. Blots show sustained phosphorylation 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. Coomasie 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 $\leq$10 cells in FGF-2-treated plates (dormant clones) and with $\geq$ 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, $\pm$ S.D.

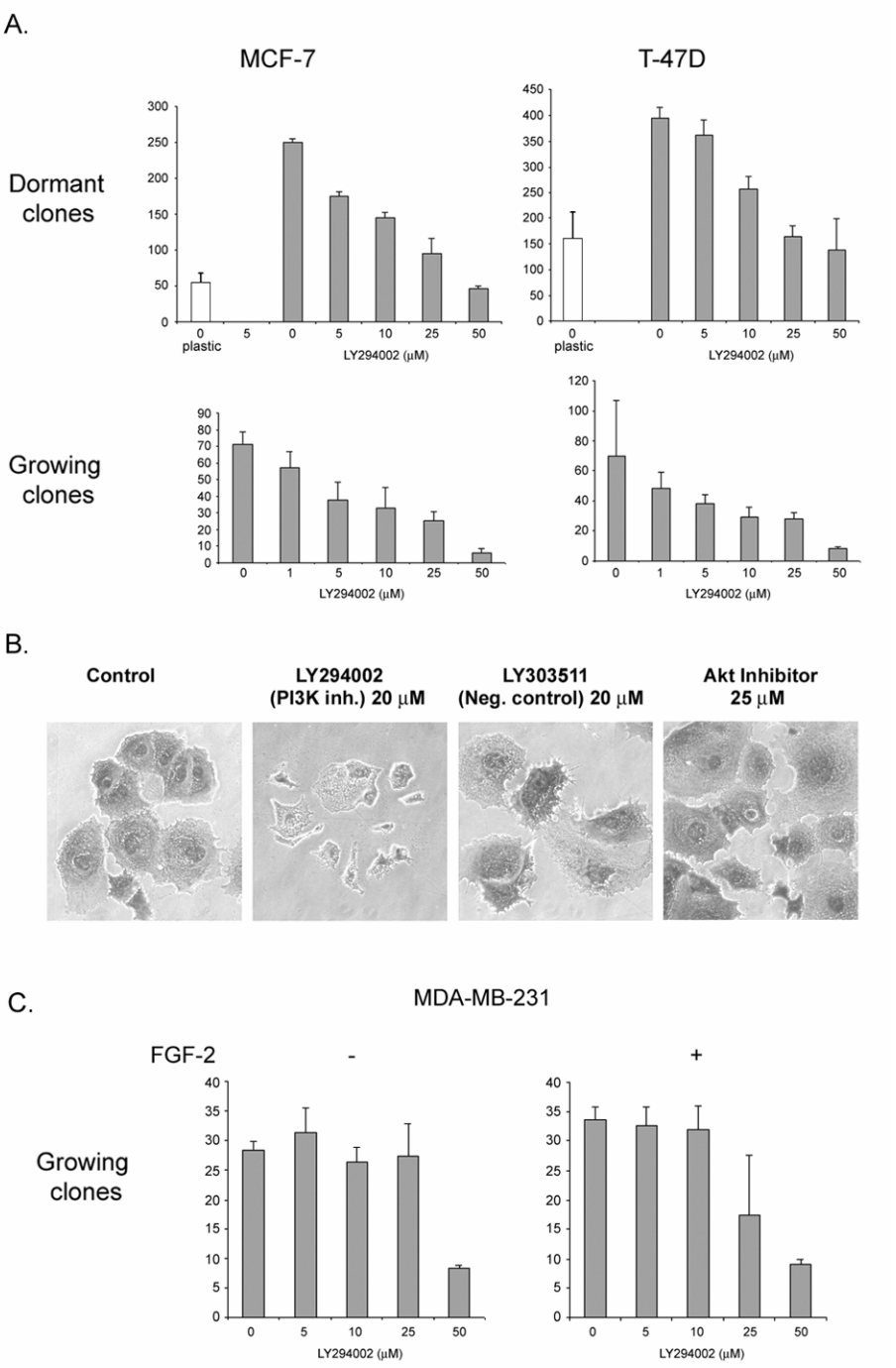

Figure 6. Inhibition of PI3K restricts dormant clone survival on fibronectin. A. MCF-7 and T-47D cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of LY294002 were added after 3 days. Cells were stained with crystal violet at 6 days and colonies with $\leq$10 cells in FGF-2-treated plates (dormant clones) and with $\geq$ 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 $\geq$ 29 cells were counted (there were no dormant clones evident). Error bars, $\pm$ S.D.

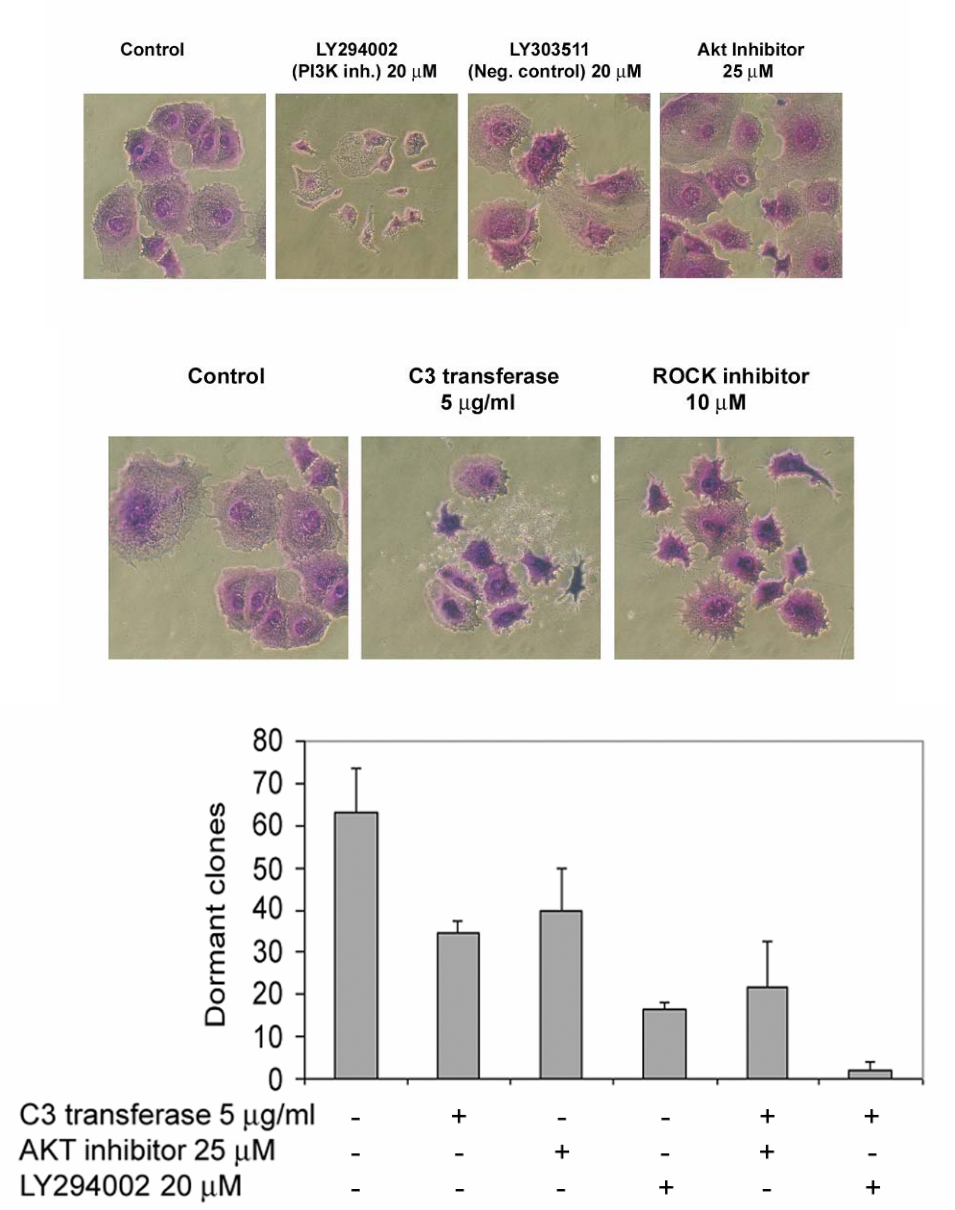

Figure 7. A. The appearance of surviving dormant T-47D clones after treatment with PI3K inhibitor LY294002, negative control LY303511 and Akt inhibitor (Calbiochem), C3 transferase and ROCK inhibitor Y27632 (Santa Cruz). B. Combined effects of RhoA inhibitor and Akt inhibitor or PI3 kinase inhibitor on survival of dormant T-47D clones. T-47D cells were incubated at clonogenic density of 1000 cells per well on 24 well fibronectin-coated plates with FGF-2 10 ng/ml for 6 days. C3 transferase 5 μg/ml, Akt inhibitor 25 μM and LY294002 20 μM were added individually or in combination on day 3 and dormant clones were counted on day 6.

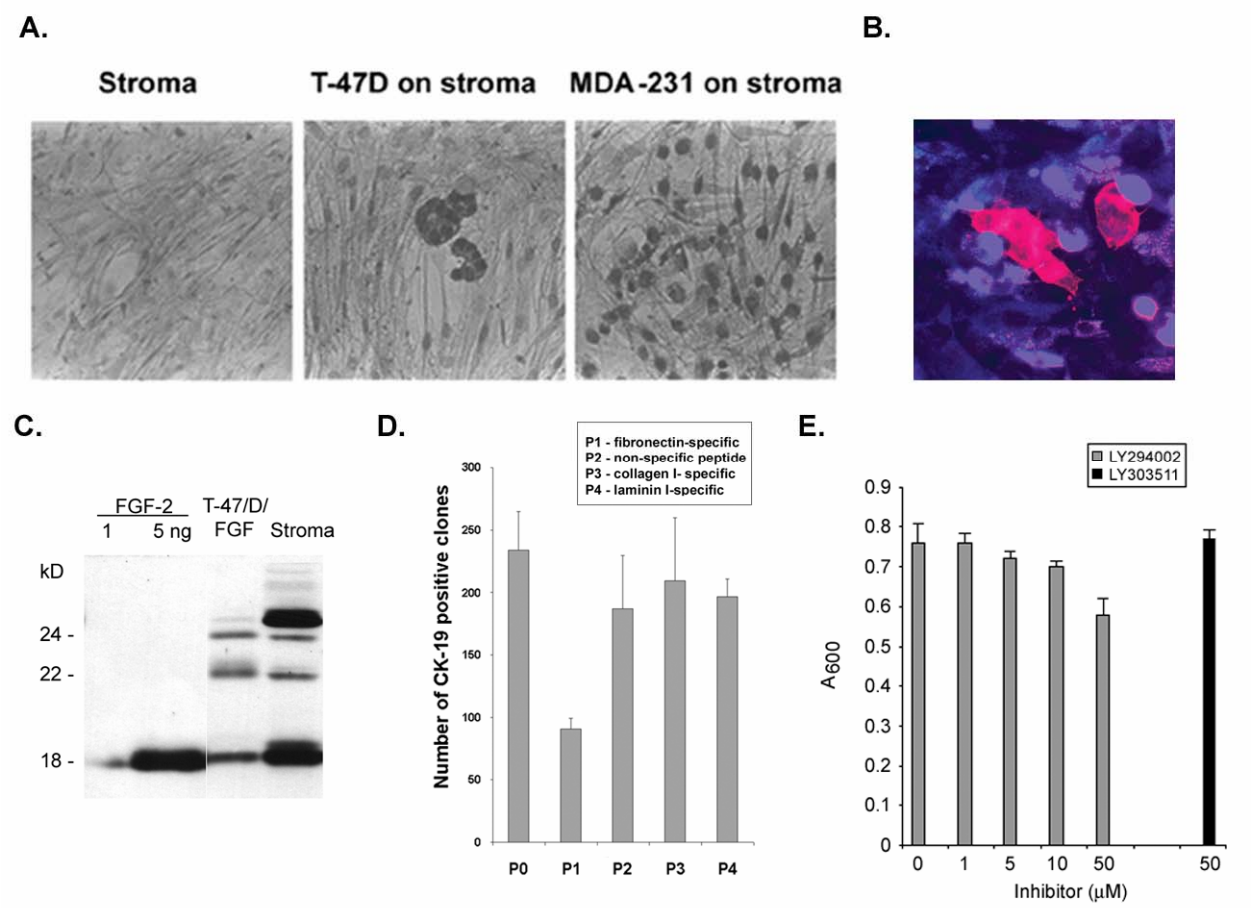

Figure 8. Stroma restricts growth of well-differentiated T-47D breast cancer cells. A. *Confluent stromal cultures in 24 well plates seeded with 500 T-47D or MDA-MB-231 cells/well were cultured for 6 days. B. Cytokeratin 19 immunofluorescence (red) staining of MCF-7 cells on stromal co-culture (blue background) demonstrated that MCF-7 cells remained as primarily single cells or as clones made up of very few cells after 6 days on stromal co-culture. C. Western blots of stromal cell lysates (100 μg) with recombinant FGF-2 and lysates from T-47D cells transfected with a vector expressing 18, 22, 22.5 and 24 kD 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 anti-cytokeratin 19 antibody and horseradish peroxidase-tagged secondary antibody, developed and colonies of ≤10 cells 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 μM LY303511 for 3 days. The medium was aspirated, the cells were washed and fixed with freshly made crystal violet solution for 20 minutes, washed in distilled water, dried, extracted with 10% acetic acid and the A_{600} measured. Error bars, ± S.D.*

C. Dormant MCF-7 and T-47D cell clones were resistant to Taxotere® concentrations 10-fold higher than needed to eliminate growing clones but were almost completely eradicated by 200 nM flavopiridol. Flavopiridol caused a decrease in FGF-2-induced expression of integrins, including α5 and β1, and decreased FGF-2-induced specific adhesion to fibronectin. It diminished Akt phosphorylation, but re-expression of active Akt was not sufficient to reverse dormant clone inhibition. Flavopiridol did not affect phosphorylation of ERK 1/2 and p38 but diminished total protein levels. Chemical inhibition of these pathways partially abrogated dormant clone survival.

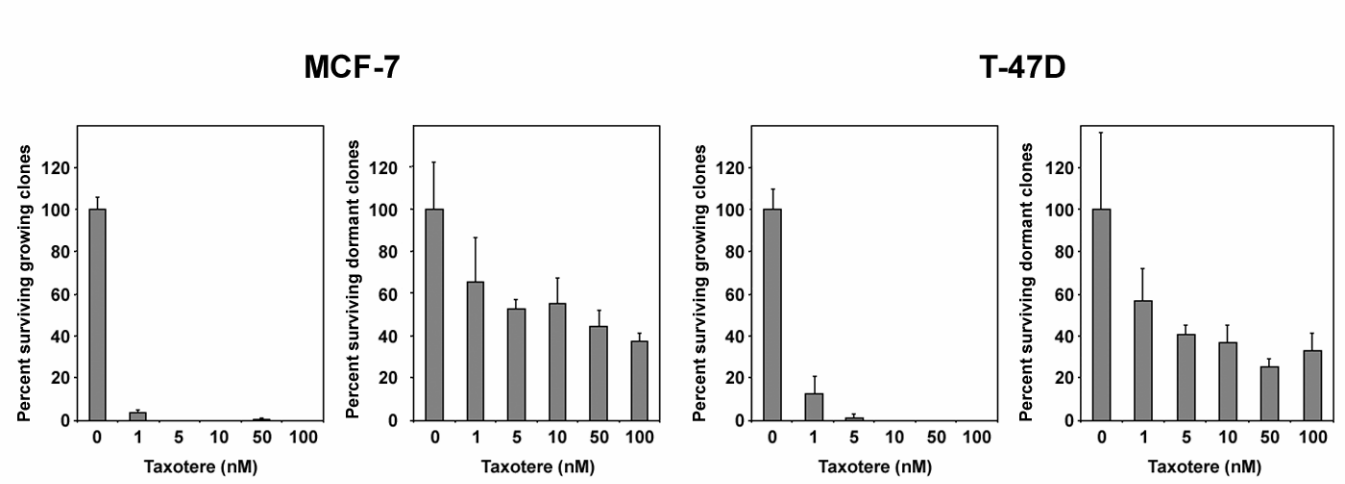

Figure 1. Taxotere® eradicates growing but not dormant breast cancer cell clones. A total of 2,000 MCF-7 cells/well or 1,000 T-47D cells/well were incubated on fibronectin-coated 24 well plates without (growing clones) or with (dormant clones) 10 ng/ml FGF-2 (day 0). The media and FGF-2 were changed and Taxotere® was added on day 3. Cells were fixed and stained on day 6 and growing and dormant clones were counted as described in Methods. The data was normalized to the untreated controls. Error bars are $\pm$ S.D.

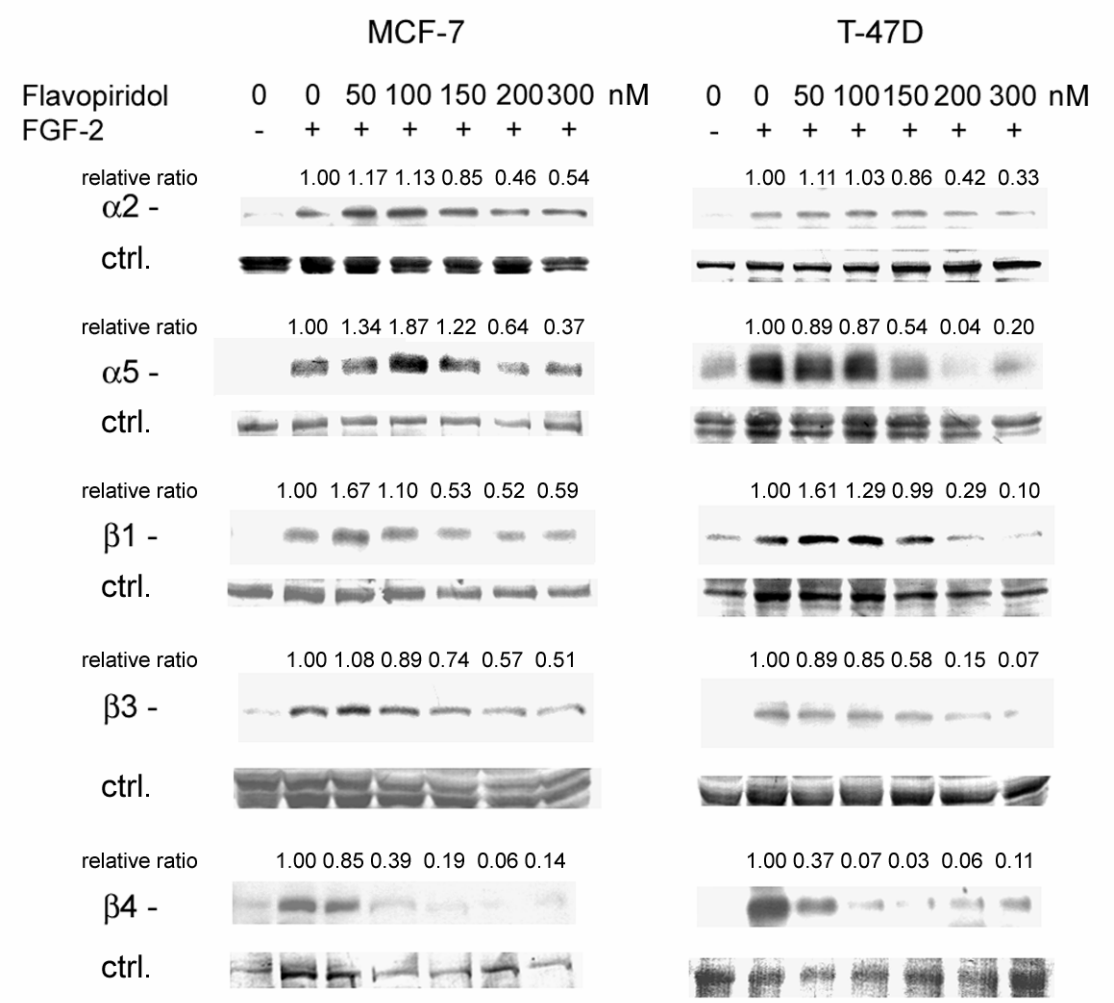

Figure 2. Flavopiridol inhibits expression of integrins α2, α5, β1, β3 and β4 upregulated by FGF-2 on fibronectin. MCF-7 and T-47D cells were incubated at low density on fibronectin-coated tissue culture plates in standard media (day 0). On day 1, the media was replaced with fresh media with and without FGF-2. On day 2, flavopiridol was added at the variable concentrations indicated along with fresh media and FGF-2. On day 4, lysates were prepared and analyzed by western blot with antibodies to the integrins shown. Nonspecific bands from Coomasie blue-stained membranes were used to verify equal loading. Band intensities were measured using a densitometer. The ratios of the integrin band density to that of the control band density were normalized to the FGF-2-treated, no flavopiridol treatment control ratios (relative ratios).

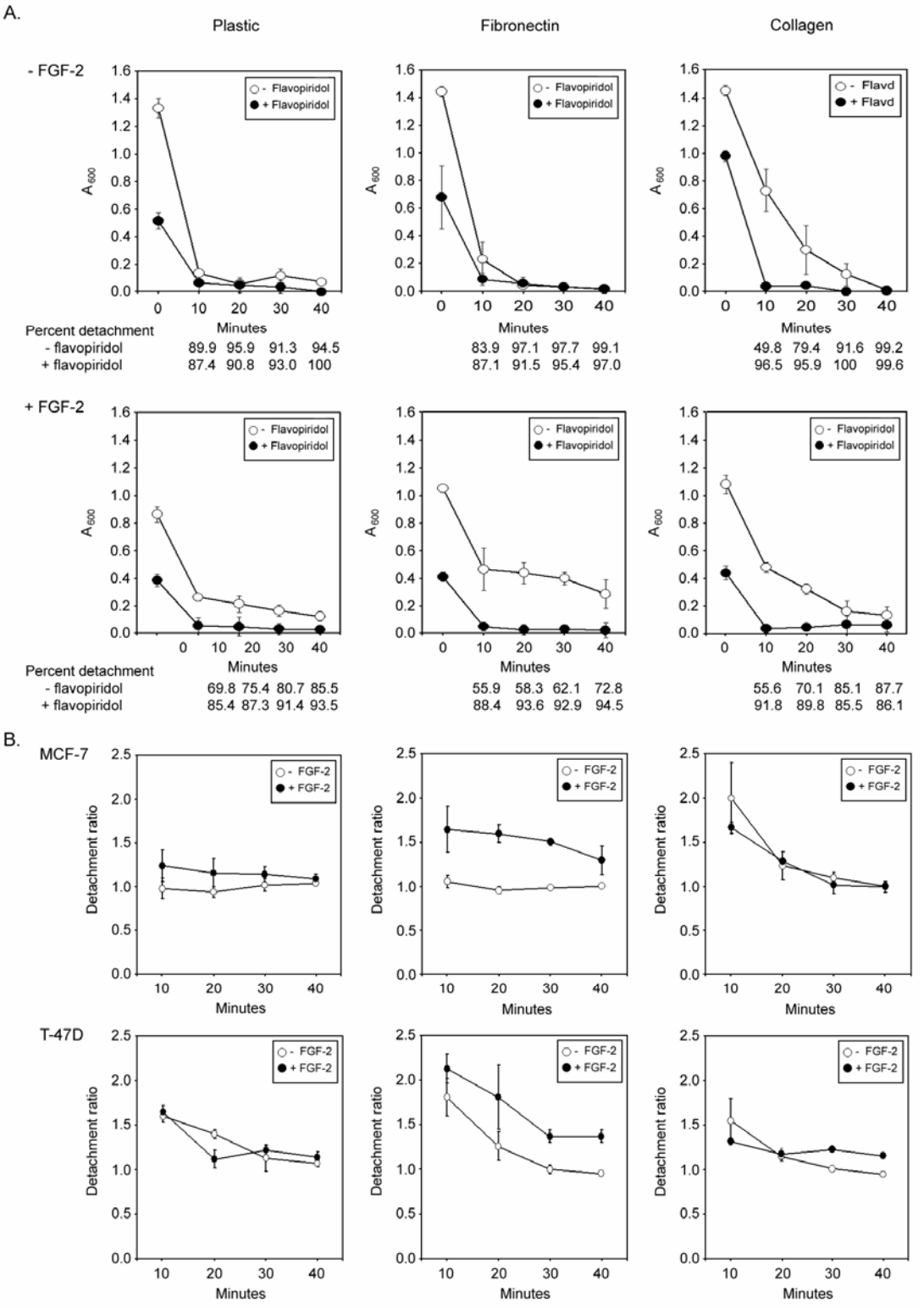

Figure 3. Flavopiridol reverses FGF-2-induced cell attachment in dormant cells. Cells were cultured on tissue culture-coated (plastic), fibronectin-coated or collagen I-coated 24 well plates at 20,000 MCF-7 cells/well or 15,000 T-47D cells/well $\pm$ FGF-2 10 ng/ml (day 0). The media and FGF-2 were replaced on day 3 and flavopiridol 200 nM was added. On day 4, cells were washed and incubated with 0.25 ml Cell Dissociation Solution (Sigma, St. Louis, MO) at 37^{o}C 5% CO_2 for variable times, washed once with PBS and stained with 0.1% crystal violet solution, as described. The dye was extracted with 10% acetic acid and the A_{600} was measured. Background absorbance obtained from wells with no cells remaining, usually at 50 minutes for each substratum type without FGF-2, was subtracted from the absorbance values for graphing. A. One typical experiment with MCF-7 cells

demonstrating differential effects of flavopiridol on growing compared to dormant FGF-2-treated cells. Percent detachment was calculated as $1 - A_{600}t/A_{600}t_o$ where t is the time of incubation with Cell Dissociation Solution and t_o is the 0 time point and A_{600} is the corrected absorbance. B. Accentuated difference in the detachment ratio (percent detachment + flavopiridol/percent detachment – flavopiridol) between FGF-2 treated and rapidly growing cells on fibronectin compared to plastic or collagen I in the experiment shown in A with MCF-7 cells and a comparable experiment with T-47D cells. All experiments were done in quadruplicate and carried out at least twice. Error bars are ± S.D.

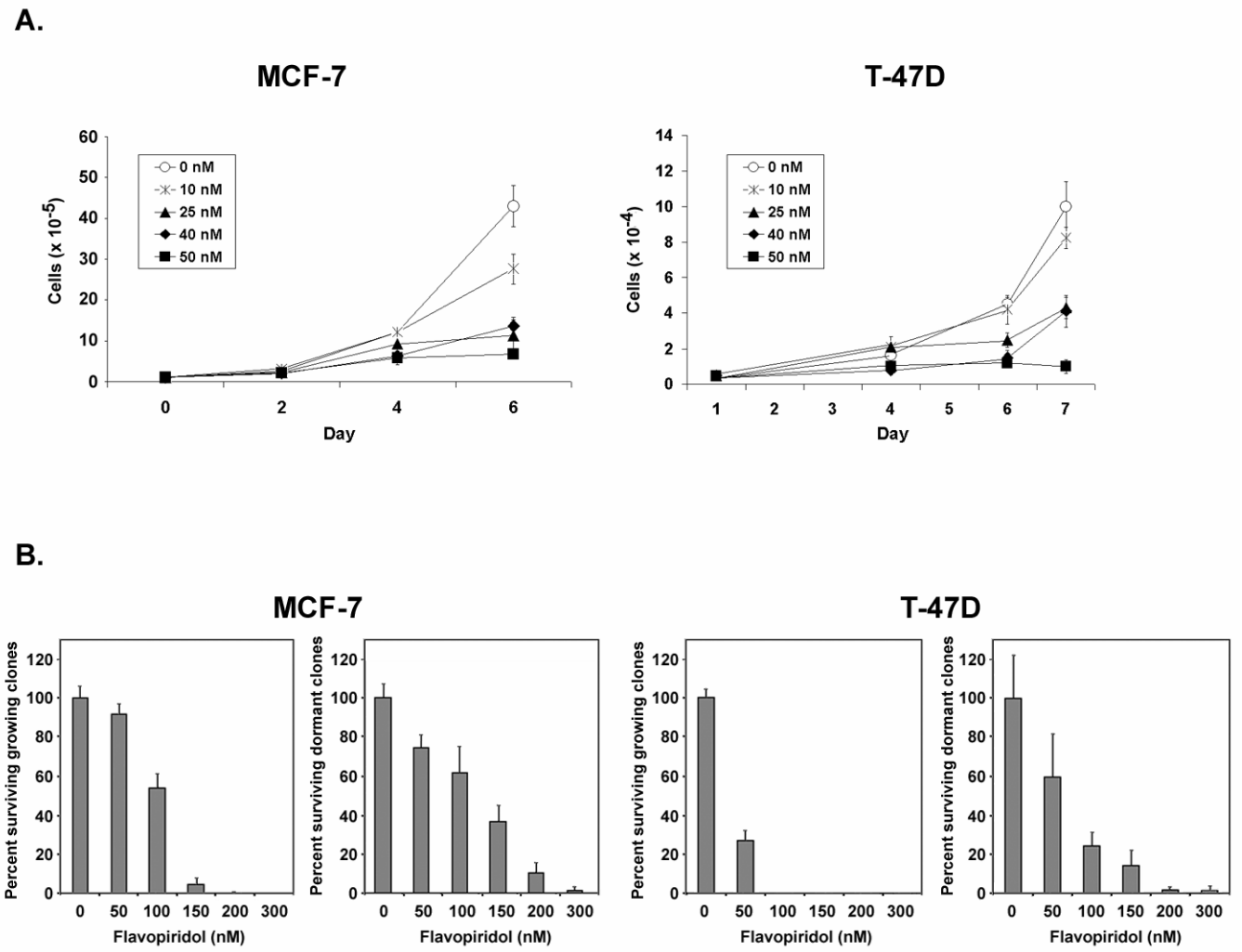

Figure 4. Flavopiridol inhibits survival of growing and dormant breast cancer cells. A. A total of 50,000 MCF-7 cells/plate or 5,000 T-47D cells/plate were incubated on triplicate tissue culture-coated plates with variable concentrations of flavopiridol and proliferation was measured by counting cells in 0.2% trypan blue at the variable times indicated. Experiments are representative of at least three carried out at different times. (B) Clonogenic assays of MCF-7 and T-47D cells on fibronectin without (growing clones) or with (dormant clones) 10 ng/ml FGF-2 incubated with variable concentrations of flavopiridol. MCF-7 cells (2,000 cells/well) and T-47D cells (1,000 cells/well) were incubated with and without FGF-2 in 24 well fibronectin-coated plates (day 0). The media and FGF-2 were replaced and flavopidirol was added on day 3. Cells were fixed and stained on day 6. Growing and dormant clones were counted as described in Methods. The data was normalized to the untreated controls. Error bars are ± S.D.

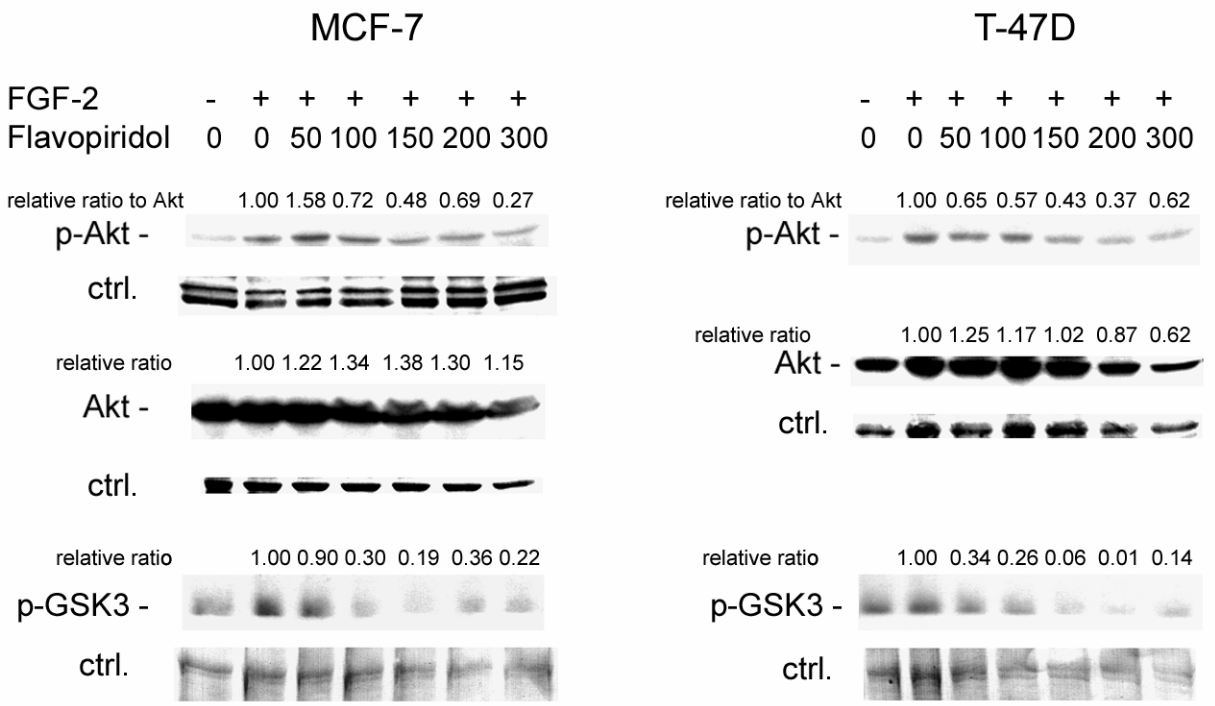

Figure 5. Flavopiridol inhibits Akt activation induced by FGF-2 on fibronectin. Cells were incubated at low density on fibronectin-coated plates (day 0). Fresh media with and without 10 ng/ml FGF-2 were added on day 1. Flavopiridol was added on day 2. Lysates were prepared on day 4 and analyzed by western blot with anti phospho-Akt and total Akt antibodies and with antibody to phosphorylated Ser21/9 of GSK-3alpha/beta isoforms. Nonspecific bands from Coomasie blue-stained membranes were used to verify equal loading. Band intensities were measured using a densitometer. The ratios of phospho-Akt/ control band densities to total Akt/control band densities in MCF-7 cells (different PVDF membranes) or the ratio of the phospho-Akt /total Akt densities for T-47D (same re-stained membrane) were normalized to the FGF-2-treated, no flavopiridol treatment controls (relative ratios to Akt). The ratios of total Akt/control bands or P-GSK3/control bands were also normalized to untreated controls (relative ratios).

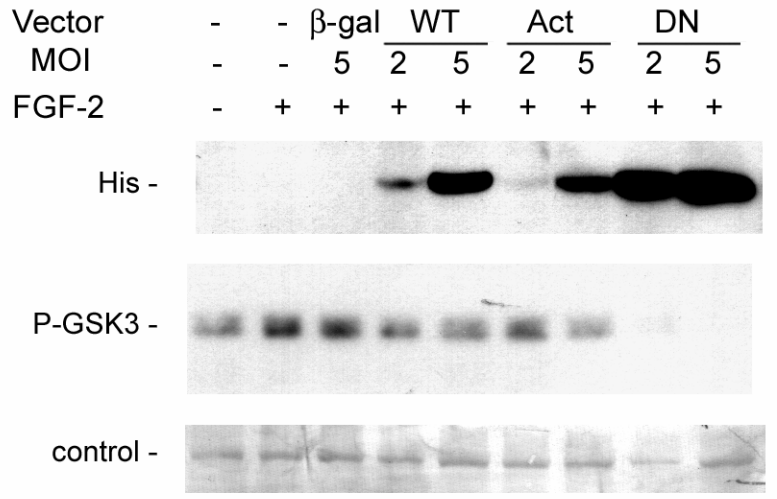

Figure 6. Enforced expression of adenoviral mediated Akt mutants in MCF-7 cells. Cells were incubated with FGF-2 on fibronectin-coated plates. On day 3, fresh media was added containing FGF-2 and adenoviral vectors at the MOI's shown. Lysates were collected 24 and 72 hours later. Shown is the western blot for His and phospho-GSK3 at 72 hours. Non-specific bands on Coomasie blue-stained gels were used to determine loading.

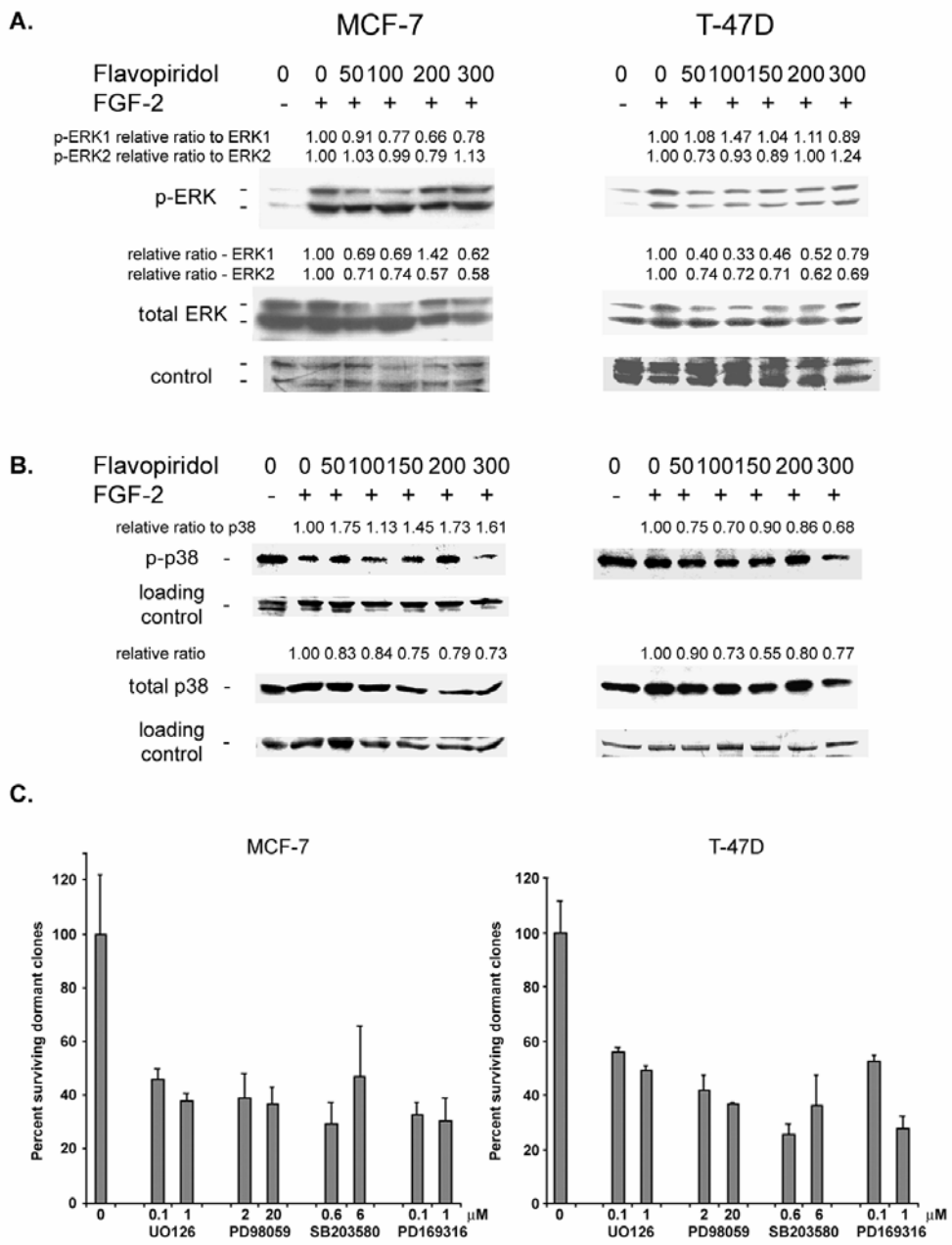

Figure 7. Effects of flavopiridol on the activation state of MAP kinases (A) ERK and (B) p38. Cells were incubated on fibronectin with and without FGF-2 at low density. On day 3, flavopiridol was added at the variable concentrations shown. On day 4, lysates were prepared and analyzed by western blot with antibodies to phosphorylated and total ERK and p38. Non-specific bands on Coomasie blue-stained gels were used to determine loading. The ratios of phospho-ERKs/total ERKs and phospho-p38/total p38 (T-47D cells) or phospho-p38/control to total p38/control (MCF-7 cells) densities were normalized to the FGF-2-treated, no flavopiridol treatment controls (relative ratios to ERK and relative ratios to p38, respectively). The ratios of total ERKs/control and total p38/control bands were also normalized to untreated controls (relative ratios). C. Effect of MEK inhibitors and p38 inhibitors on survival of dormant clones. MCF-7 cells and T-47D cells were incubated with FGF-2 on fibronectin for three days, media was changed and supplemented with variable concentrations of MEK inhibitors UO126 or PD98059 and p38 inhibitors SB203580 or PD169316 and fresh FGF-2, and incubated for an additional three days. Control cells were incubated in 10 μM DMSO, the solvent used for the inhibitors. Dormant clones were counted after crystal violet staining on day 6. Error bars are ± S.D.

D. The fluorescence device detected the expression of integrin $\alpha 5$ induced by FGF-2 treatment in MCF-7 cells and that of Her2/*neu* in SK-BR-3 cells compared with controls. Experiments measuring passage retardation demonstrated significant differences in passage rates between FGF-2-treated and untreated MCF-7 cells over reaction regions coated with fibronectin and antibody to integrin $\alpha 5\beta 1$ compared with control regions. Blocking peptides reversed the retardation demonstrating specificity.

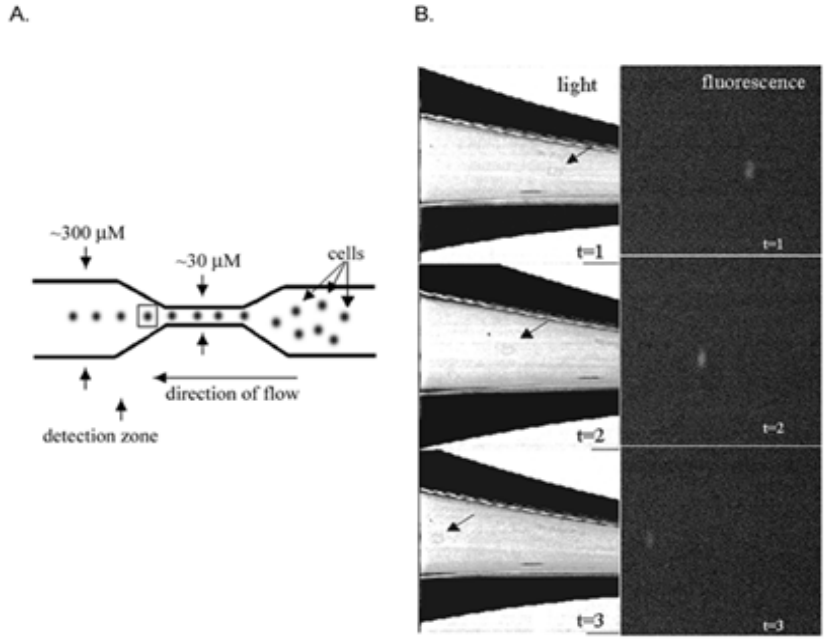

Figure 1. A. Schematic of the microfluidic device. Fluorescence-labeled cells in suspension are introduced into a microcapillary tube by a Hamilton syringe and propelled by a syringe pump. The inner diameter of the tube of ~300 μm is narrowed to ~30 μm where cells assume a single file. As the cells exit the constriction, their flow rate slows considerably, and are queried by the 40x objective of an IX 70 Olympus microscope hooked to a photomultiplier. B. Light and fluorescence micrographs of a phallacidin-stained cell 0.2 seconds apart as it exits the constricted portion of the microcapillary (boxed cell in A).

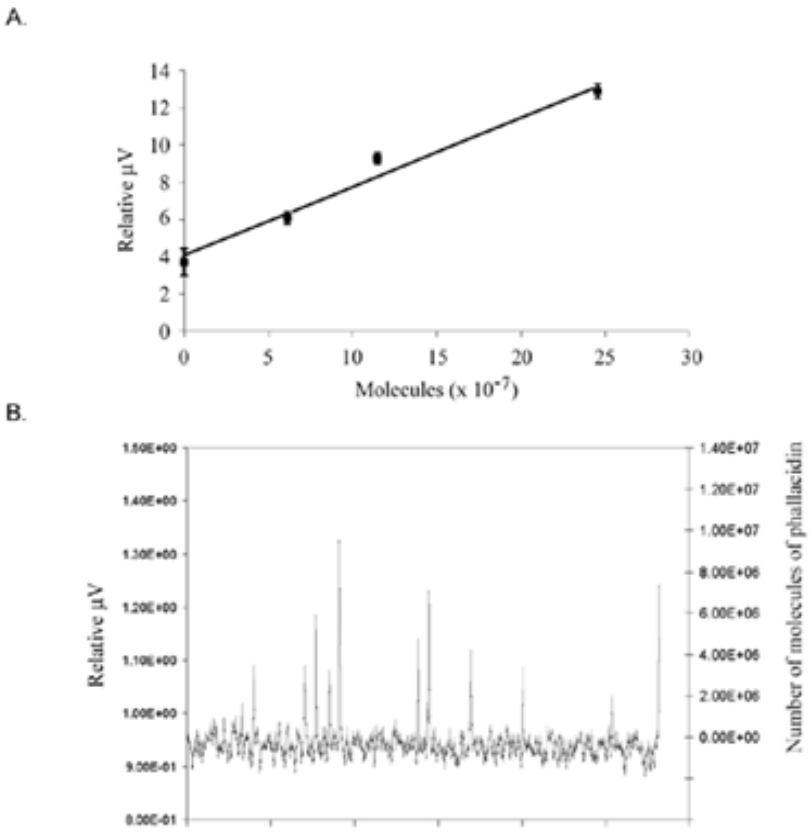

Figure 2. Voltage intensity corresponding to number of phallacidin molecules in the acquisition field. Solutions containing variable dilutions of phallacidin were placed in an orthogonal tube with 75 μm x 75 μm edges in its cross section (y and z dimension) with 133.35 μm of its length (x dimension) within the field of the 40X objective. Multiple measurements of the voltage generated at each concentration were obtained and correlated with the voltage obtained with the phallacidin concentration. The graph depicts changes in voltage intensity with respect to the number of phallacidin molecules. B. Voltage peaks generated in the photomultiplier by MCF-7 cells labeled with BODIPY FL phallacidin passing though the microfluidic device.

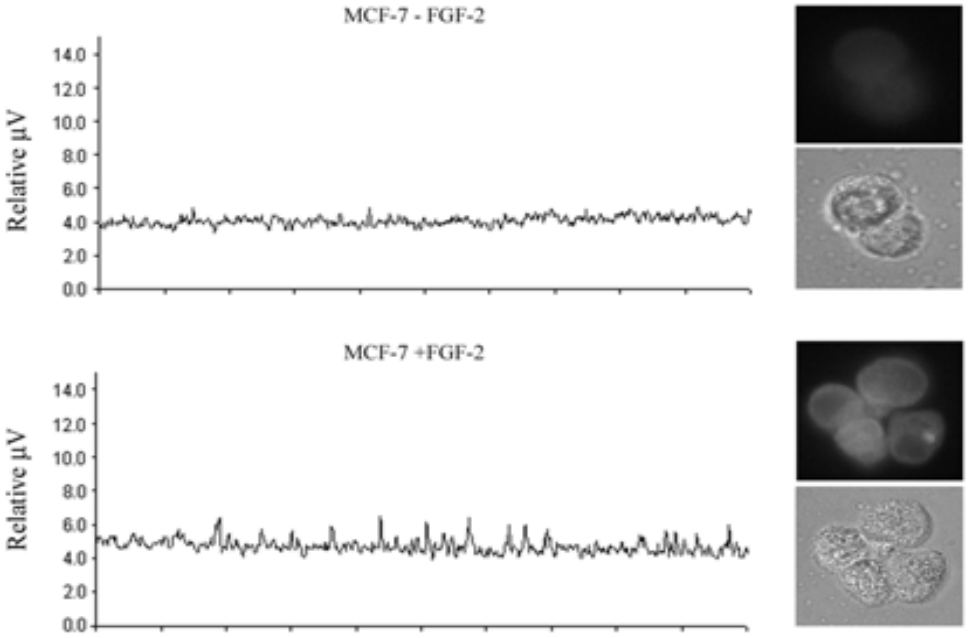

Figure 3. Detection of indirect immunofluorescence signal from anti-integrin α5 antibody-labeled MCF-7 cells. Cells were treated with media with or without FGF-2 10 μg/L for 24 hours and labeled

with an anti-integrin α5 rabbit polyclonal antibody and a goat anti-rabbit Texas Red-tagged secondary antibody. Immunofluorescence and light microscopy demonstrates labeling of FGF-2-treated MCF-7 cells but only background fluorescence in untreated MCF-7 cells. Cells assayed by the microfluidic device are recognized as peaks in the tracing as they pass the objective linked to the photomultiplier.

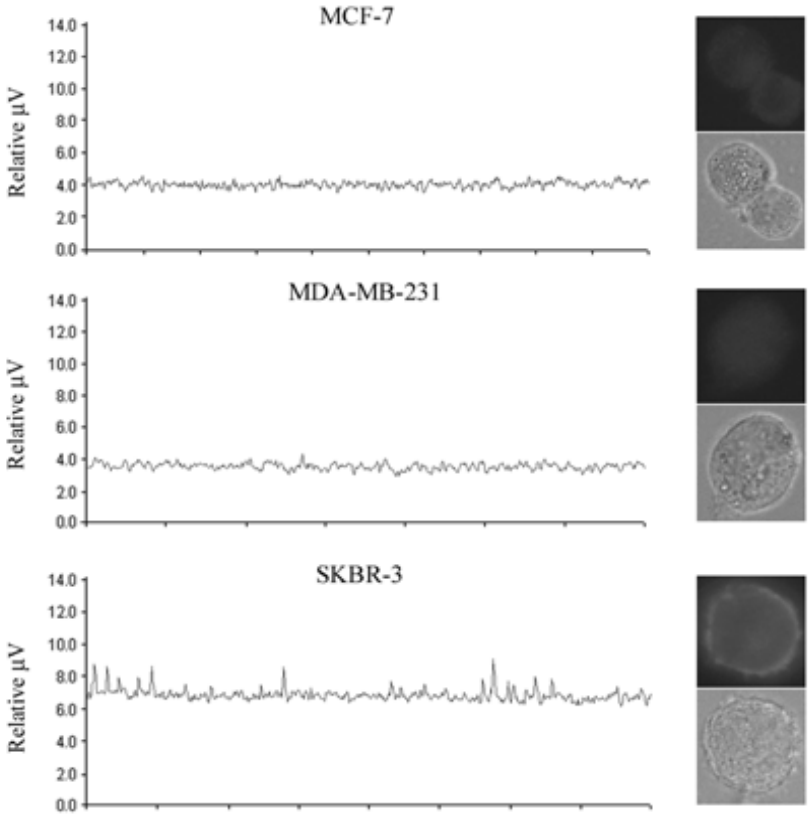

Figure 4. Indirect immunofluorescence signal from MCF-7, MDA-MB-231 and SK-Br-3 cells labeled with anti-Her2/*neu* antibody. Cells were labeled with mouse monoclonal antibody to Her2/*neu* and with a rabbit anti-mouse Texas Red-tagged secondary antibody. Immunofluorescence and light microscopy demonstrates labeling of Sk-Br-3 cells but only background fluorescence in MCF-7 and MDA-MB-231 cells. Cells assayed by the microfluidic device are recognized as peaks in the tracing as they pass the objective linked to the photomultiplier.

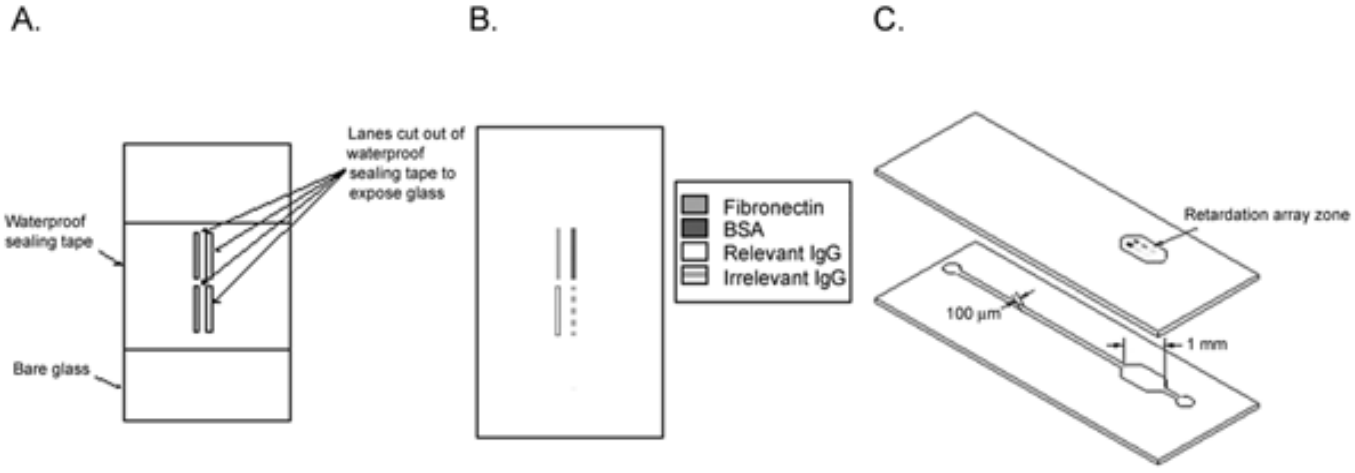

Figure 5. Schematic design of an etched glass channel with a demountable glass top cover with fabricated protein-binding surfaces. A. Slide covered with waterproof tape mask with thin rectangular cutouts representing the flow lanes to be coated. B. Flow lanes coated with different ligands illustrated after removal of waterproof tape. C. Flow cell with demountable slide coated on the flow retardation array zone. The channel dimensions are 50-100 microns x 50-100 microns.

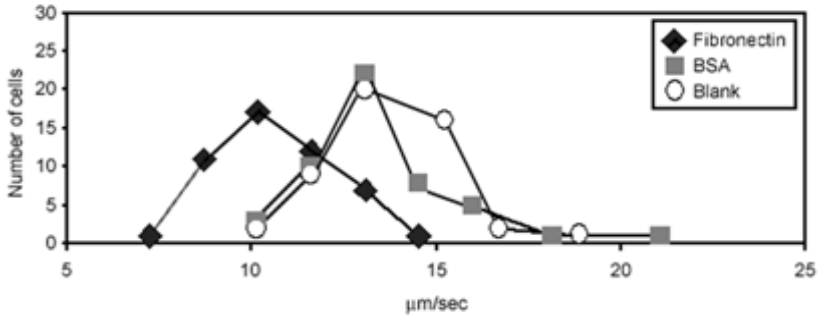

Figure 6. Flow rate distributions of MCF-7 cells treated with FGF-2 10 μg/L for 24 hours over channels coated with fibronectin, BSA and poly-L-lysine (blank).

Table 1. Signal generated by indirect immunofluorescence

Cells	*Antibody*	*Baseline + SD* relative μV	*Signal + SD* relative μV
MCF-7	integrin α5	4.09 ± 0.20 (n = 1000)	4.55 ± 0.25* (n = 11)
MCF-7 + FGF-2	integrin α5	4.51 ± 0.14 (n = 1000)	5.71 ± 0.42* (n = 24)
MCF-7	Her2/*neu*	4.05 ± 0.14 (n = 1000)	4.65 ± 0.38* (n = 11)
MDA-MB-231	Her2/*neu*	3.63 ± 0.15 (n = 1000)	4.07 ± 0.12* (n = 11)
SK-Br-3	Her2/*neu*	6.88 ± 0.24 (n = 1000)	8.16 ± 1.10* (n = 23)

*p< 0.001 compared with baseline

Table 2. FGF-2 treatment induces selective integrin $\alpha5\beta1$-specific flow retardation of MCF-7 cells on fibronectin

a.

Coating	*MCF-7* Velocity (μM/sec)	*MCF-7 + FGF-2* Velocity (μM/sec)
Blank (poly-lysine)	23.0 ± 3.4 (n = 5)	18.8 ± 1.3 (n 8)
Fibronectin	18.7 ± 3.3* (n = 5)	13.2 ± 0.9** (n = 11)

*p< 0.01 compared with blank slide; **p< 0.001 compared with blank slide

b.

Coating	*Velocity* (μM/sec)
BSA	14.4 ± 1.5 (n = 7)
Control IgG	15.5 ± 2.4 (n = 6)
Anti-integrin $\alpha5\beta1$ IgG	12.1 ± 1.6* (n = 7)
Fibronectin	11.0 ± 1.3* (n = 7)

*p< 0.001 vs. BSA, p< 0.001 vs. control IgG

c.

Coating	Control peptide (GRGESP) Velocity (μM/sec)	Blocking peptide (GRGDSP) Velocity (μM/sec)
BSA	15.5 ± 1.6 (n = 11)	15.5 ± 1.8 (n = 11)
Fibronectin	12. 8 ± 1.4* (n = 11)	15.0 ± 2.4** (n = 11)

*p< 0.001 compared with BSA-coated slide; **p< 0.1 compared with BSA-coated slide

KEY RESEARCH ACCOMPLISHMENTS

A. Data suggest roles for FGF-2 and laminin 5 in ductal integrity during mammary carcinogenesis, with loss of expression corresponding to loss of ductal structure. *In vitro* data suggest FGF-2 as causal in laminin 5 expression and export. Downregulation of FGF-2 during transformation may contribute to loss of laminin 5 expression.

B. FGF-2, a growth factor important in ductal morphogenesis and abundantly-deposited in the bone marrow, can induce dormancy and re-differentiation in estrogen-dependent breast cancer cells in an *in vitro* model. These cells re-express integrins, and specifically integrin $\alpha5\beta1$ provides a reversible survival advantage. The cells depend on PI3K, Akt and Rho signaling for survival and combined inhibition of PI3K and Rho or its downstream target ROCK can nearly completely eliminate dormant clones on fibronectin. Similar behavior was confirmed on human bone marrow stromal co-cultures. This model for a targeted inhibition of interconnected relevant survival pathways may represent a useful approach to eliminating dormant micrometastases.

C. Flavopiridol has pleiotropic effects on key targets involved with survival of dormant breast cancer cells and may represent a useful approach to eliminating cells dependent on multiple signal pathways for survival that are resistant to taxanes.

D. Immunofluorescence detection in a microfluidic channel demonstrates the potential for assaying surface protein expression in a few individual cells and will permit the development of future iterations not requiring cell handling. The flow retardation device represents the first application of this technology for assessing cell surface protein expression in cancer cells and may provide a way for analyzing expression profiles of single cells without prior manipulation.

REPORTABLE OUTCOMES

Manuscripts

Korah R, Choi L, Barrios J and Wieder R. (2004) Constitutive expression of FGF-2 abrogates focal adhesion signaling in MDA-MB-231 breast cancer cells. Breast Cancer Research and Treatment 88: 17-28 (Erratum – color photos (2005) 89: 319 – 322).

Korah R, Boots M, and Wieder R. (2004) Integrin $\alpha5\beta1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. Cancer Research 64: 4514-4522.

Najmi S, Korah R, Chandra R, Abdellatif M, Wieder R. (2005) Flavopiridol blocks integrin-mediated survival in dormant breast cancer cells. Clinical Cancer Research 11:2038-2046.

Wieder R. Insurgent micrometastases: sleeper cells and harboring the enemy. (2005) J. Surgical Oncology 89:207-210.

Fitzpatrick E, McBride S, Yavelow J, Najmi S, Zanzucchi P and Wieder R. Microfluidic techniques for single cell protein expression analysis. (2006) Clin. Chem. 52:1080-1088.

Korah R, Das K, Lindy ME, Hameed M and Wieder R. Co-ordinate loss of FGF-2 and laminin 5 expression during neoplastic progression of mammary duct epithelium. (2006) Human Pathology (in press).

Abstracts

A. Shah, R. Korah and R. Wieder. FGF-2 suppresses EGF-mediated T-47D breast cancer cell motility. Proceedings of the American Association for Cancer Research 2003, v. 44, 2nd ed., p. 869, #4384.

Ankoor Shah, Reju Korah and Robert Wieder. FGF-2 inhibits motility response to EGF in T-47D breast cancer cells. (2003) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 68, # P59.

Mike Lindy, Reju Korah, Monika Boots, and Robert Wieder. Role of RhoA in survival of dormant breast cancer cells by basic fibroblast growth factor. Proceedings of the American Association for Cancer Research 2004, v. 45, #2435 p. 563 (oral presentation).

Michael E. Lindy, Reju Korah, Monika Boots, and Robert Wieder. Survival of dormant breast cancer cells depends on PI3 kinase and Rho signaling. (2004) The National Student Research Forum, The University of Texas Medical Branch, Galveston, TX. (selected for oral presentation).

Joseph, V, Wieder, R. All trans-retinoic acid modulates survival signaling in dormant breast cancer cells. Program Proceedings of American Society of Clinical Oncology, 2004, v. 23. # 422.

Korah R, Lindy M, Boots M, B. Benn and Wieder R. Interactions with the bone marrow microenvironment contribute to survival of breast cancer cells in a dormancy paradigm. Keystone Symposia on Microenvironment in tumor induction, Banff Centre, Alberta, Canada, February, 2005, p. 74, #333.

Robert Wieder, Saltanat Najmi, Rachna Chandra, Maha Abdellatif, Reju Korah. Flavopiridol disrupts adhesion and survival signaling in taxane-resistant dormant breast cancer cells. Proceedings of the American Association for Cancer Research 2005, v. 46, #5927, p.1394.

S Najmi, R Korah, M Abdellatif, R Wieder. Use of flavopiridol to disrupt adhesion and survival in dormant breast cancer cells. (2005) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, #116.

R. Wieder, M. Lindy, M. Boots, R. Korah . Survival signaling in dormant breast cancer cells. The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope", June 2005, Philadelphia, p. 416, #P59-22.

Ethan Fitzpatrick, Jonathan Yavelow, Sterling McBride and **Robert Wieder.** Development of a microfluidic system for single cell analysis of breast cancer cells (2003) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 96, # P61.

Ethan Fitzpatrick, Jonathan Yavelow, **Robert Wieder**, Peter Zanzucchi, Sterling McBride. Flow retardation of MCF-7 cells by immobilized ligands to cell surface receptors. Proceedings of the American Association for Cancer Research 2004, v. 45, # 2745 p. 633.

Korah R, Lindy M, Boots M, B. Benn and **Wieder R.** Interactions with the bone marrow microenvironment contribute to survival of breast cancer cells in a dormancy paradigm. Keystone Symposia on Microenvironment in tumor induction, Banff Centre, Alberta, Canada, February, 2005, p. 74, #333.

Robert Wieder, Saltanat Najmi, Rachna Chandra, Maha Abdellatif, Reju Korah. Flavopiridol disrupts adhesion and survival signaling in taxane-resistant dormant breast cancer cells. Proceedings of the American Association for Cancer Research 2005, v. 46, #5927, p.1394.

S Najmi, R Korah, M Abdellatif, R Wieder. Use of flavopiridol to disrupt adhesion and survival in dormant breast cancer cells. (2005) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, #116.

R. Wieder, M. Lindy, M. Boots, R. Korah . Survival signaling in dormant breast cancer cells. The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope", June 2005, Philadelphia, p. 416, #P59-22.

Amadio WJ, Wang Q, Yavelow J, Wieder R. Cluster analysis of gene expression profiles of MCF-7 cells treated with all *trans*-retinoic acid (ATRA), paclitaxel or the two compounds in combination. Proceedings of the American Association for Cancer Research 2006, v. 47, #4931, p 1158.

Barrios J and Wieder R. Regulation of Cytoskeletal Aberrations and Cellular Survival in Dormant Differentiated Breast Cancer Cells. (2006) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, #P48.

Amadio WJ, Wang Q, Yavelow J, and Wieder R. Modulation of distinct differentiation and survival signaling revealed by gene expression profiles of MCF-7 cells treated with all *trans*-retinoic acid (ATRA), paclitaxel or the combination. (2006) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, #P23.

Fitzpatrick E, McBride S, Yavelow J, Najmi S, Zanzucchi P, and. Development of Microfluidic Techniques for Single Cell Protein Expression Analysis. (2006) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, Session D: Molecular Mechanisms of Tumor Growth II, Oral Presentation.

CONCLUSIONS

1. FGF-2 and Laminin 5 are coordinately lost with ductal integrity during mammary epithelial cell transformation, and laminin 5 expression and deposition may be regulated by FGF-2.

2. FGF-2, a growth factor abundantly-deposited in the bone marrow, may contribute to the initiation of dormancy, re-differentiation and chemoresistance of estrogen sensitive breast cancer cells in the bone marrow microenvironment though modulation of integrin $\alpha 5\beta 1$ expression and initiation of survival signaling through PI3K, Akt and Rho.

3. Flavopiridol has pleiotropic effects on key targets involved with survival of dormant breast cancer cells and may represent a useful approach to eliminating cells dependent on multiple signal pathways for survival that are resistant to taxanes.

4. We developed the first example and proof of principle for measuring the expression of surface receptors on breast cancer cells without prior labeling by measuring transient interactions with potential ligands immobilized in a microfluidic channel that result in a diagnostic flow retardation. This may provide a way for analyzing expression profiles of single cells without prior manipulation.

REFERENCES

Korah R, Boots M, and Wieder R. (2004) Integrin $\alpha 5\beta 1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. Cancer Research 64: 4514-4522.

Najmi S, Korah R, Chandra R, Abdellatif M, Wieder R. (2005) Flavopiridol blocks integrin-mediated survival in dormant breast cancer cells. Clinical Cancer Research 11:2038-2046.

Wieder R. Insurgent micrometastases: sleeper cells and harboring the enemy. (2005) J. Surgical Oncology 89:207-210.

Fitzpatrick E, McBride S, Yavelow J, Najmi S, Zanzucchi P and Wieder R. Microfluidic techniques for single cell protein expression analysis. (2006) Clin. Chem. 52:1080-1088.

Korah R, Das K, Lindy ME, Hameed M and Wieder R. Co-ordinate loss of FGF-2 and laminin 5 expression during neoplastic progression of mammary duct epithelium. (2006) Human Pathology (in press).