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Specific extracellular matrix (ECM) proteins and their cellular receptors (integrins) are required for normal mammary gland morphogenesis and differentiation, while their expression is dramatically altered during tumorigenesis. It is not clear how ECM proteins affect breast tumor growth, however. We have examined the effects of five ECM proteins on two estrogen (E) regulated events, proliferation and induction of progesterone receptor (PgR), using estrogen receptor positive (MCF-7, T47D) or negative (MDA MB231) breast cancer cells. In the presence of serum, E enhanced proliferation of MCF-7 or T47D cells on collagen I, collagen IV, fibronectin, and vitronectin, but was less effective on laminin. MDA MB231 cells were not E-responsive on any ECM protein in the presence or absence of serum. MCF-7 cell PgR induction was enhanced by E on all ECM proteins in the presence or absence of serum, though response on laminin was weaker. In the absence of serum, MCF-7 and T47D cells proliferated in response to E maximally on collagen I, followed by fibronectin or vitronectin. However, the growth factors, IGF-I and EGF, were required for E-induced proliferation and PgR induction in the absence of serum. These results have demonstrated that laminin can inhibit, while collagen I, fibronectin or vitronectin may enhance E-mediated cell proliferation and PgR induction. Since specific ECM proteins may confer and others inhibit E-responsiveness in breast cancer cells, therapeutic strategies may be designed to alter the ECM or integrin-ECM adhesion in the tumor microenvironment to prevent tumor growth and spreading.

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Terry Woodward 10/20/97

PI - Signature Date

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INTRODUCTION

Breast cancers are often divided into those that can be regulated by ovarian steroids and those which are not responsive to endocrine therapy, i.e. estrogen independent. While more than 60% of breast cancers are estrogen-receptor positive, only one third of estrogen receptor positive tumors respond to endocrine therapy (Martin et al., 1994). Many researchers are currently trying to understand the mechanisms involved in the loss of estrogen responsiveness, since estrogen responsive tumors have a better prognosis and are less likely to become metastatic and invasive when compared to steroid non-responsive tumors (Garcia et al., 1992; Moghazy et al., 1992.

Acquisition of estrogen-responsiveness in the normal mammary gland is also poorly understood. Estrogen stimulates mammary development directly, independent of endocrine influences, but has not been shown to stimulate the proliferation of isolated normal mammary epithelial cells, in vitro. Epithelial cells do proliferate in response to estrogen, however, when cocultured with stromal cells. Additionally, Haslam and Counterman (1991) and Cunha and coworkers (1997) have demonstrated an absolute requirement for stromal-epithelial interactions in estrogen induced mammary gland development. Stromal cells are largely thought to influence estrogen induced epithelial cell proliferation by 2 major mechanisms, 1) alteration of growth factor expression, and 2) alteration of extracellular matrix expression (Woodward et al., 1998). Extracellular matrix proteins bind to cell surface receptors, including integrins, which may stimulate intracellular pathways to induce apoptosis, differentiation, or proliferation, similar to growth factor-intracellular pathways. Recently, our laboratory has demonstrated that normal murine mammary epithelial cells can proliferate in response to progesterone in serum-free media when cultured on specific ECM proteins.

Progesterone, like estrogen, has been shown to stimulate normal mammary epithelial cell proliferation and mammary gland development (Skarda et al., 1989; Wang et al., 1990; Haslam and Counterman, 1991). Progesterone, however, does not stimulate mammary gland development until the acquisition of epithelial progesterone receptors, at 7-weeks of age in the mouse. These progesterone receptors are E-inducible. Similarly, others have demonstrated that

breast cancer cell lines, including MCF-7 cells, also contain E-inducible PgR (Katzenellenbogen and Norman, 1990). Progesterone may decrease proliferation of uterine epithelial cells. In fact, P has been added to E in hormone replacement therapies to reduce uterine cancer risk (Mason, 1994). It is not well understood how P may influence breast cancer risk, though one report has suggested that P added to E in HRT does not reduce and may actually increase breast cancer risk (Colditz et al., 1995).

The current studies were designed to exam the role of ECM proteins, on steroidal responsiveness of breast cancer cells. Specifically, we have examined the effect of E on breast cancer cell proliferation, when cells were plated on plastic, poly-L-lysine, collagen I, collagen IV, laminin, fibronectin, or vitronectin in charcoal stripped fetal calf serum. Next, minimal conditions were established for the culture of MCF-7 and T47D cells in serum-free media. Plating efficiencies on all ECMs were also measured. It was also determined that growth factor (EGF, IGF-I) additions to serum-free media were necessary to support E-induced proliferation. Cell proliferation in response to E was reassessed on all ECMs in serum-free media with optimal growth factor additions. In both serum-free and serum containing media, proliferation of the ER-negative MDA MB-231 cells was also examined.

Estrogen-induction of PgR was determined in stripped serum on all ECM proteins for the MCF-7 cells. T47D cells had high constitutive expression of PgR on various ECMs, and levels were not affected by E. PgR-induction in serum-free media was also determined for the MCF-7 cells on all ECM proteins.

These experiments, therefore, will examine the role that ECM proteins have in E-induced events in breast cancer cells, and determine which specific ECM proteins may influence E-action. Identification of either acquisition or loss of E-action by specific ECM proteins, should shape future research in determining how ECM may influence normal mammary growth and tumor growth.

BODY

Experimental Methods:

Materials

IMEM and RPMI 1640 with or without phenol red were obtained from Biofluids Inc. (Rockville MD). HBSS and Phenol-red free DME/F12 was purchased from Sigma Chemical Co., (St. Louis, MO). All hormones, growth factors and media supplements were purchased from Sigma, except IGF-I (Gropep Pty. Ltd., Adelaide, Australia), Non-essential amino acids and insulin (Life Technologies, Grand Island, NY). Corning (Corning, NY) dishes and multiwell plates were used. R5020 and 3H-R5020 were supplied by New England Nuclear Corp., (Boston, MA). Charcoal/Dextran Treated FBS (DCC-FBS) from HyClone (Logan, UT) was used in all serum-containing experiments. Purified rat collagen I, mouse collagen IV, ultrapure mouse laminin, and human vitronectin were purchased from Collorabative Biomedical Division of Becton Dickinson (Bedford, MA). Poly-L-lysine was from Sigma and human plasma fibronectin from ICN (Aurora, OH).

Cell lines

T47D cells that were growth responsive to E were supplied by Dr. Barbara Vonderhaar (NCI, Bethesda, MD) and MDA MB231 cells were obtained from Dr. William Helferich (Michigan State University, East Lansing, MI). Routine culture of MCF-7 and MDA MB231 cells was in IMEM +FBS+1nM E2. T47D cells were also grown in FBS+1nM E2, but in RPMI 1640 media containing 25 mM HEPES and 500 ng/ml insulin. Serum-free media for all cells was phenol-red free DME/F12 +250 ng/ml insulin + 6 ug/ml transferrin + non essential amino acids.

Cell Proliferation Assays

Serum containing experiments. MCF-7 cells ($6X10^3$ cells/cm^2) or MDA MD 231 or T47D cells ($1X10^4$ cells/cm^2) were plated in normal 5% FBS containing media for 24 h. Optimal plating densities for each cell line in serum-containing and serum-free (see below) media were determined previously. Cells were rinsed 2-times and media was replaced with 5% DCC-FBS

media. Cells were incubated for 2 d and then estrogen (10 nM) was added. Media was changed after 2 d and experiments were harvested for DNA assay after a total of 4 d following E-addition.

Serum-free experiments. Cells were removed by trypsinization, trypsin was inactivated with FBS-containing media and cells were pelleted by centrifugation (200 X g for 3 minutes). Pellet was resuspended and centrifuged 2-times in serum-free media and cells plated in (500 ul/2 cm^2 well) serum-free media. Serum-free plating densities were $3.5X10^4/cm^2$ for MCF-7 cells and $4X10^4 cells/cm^2$ for T47D or MDA MB231 cells. IGF-I (25 ng/ml) and EGF (5 ng/ml) with or without estrogen (10 nM) were added 2 d after plating. Treatments were changed 2 d later and cells were harvested.

DNA assay.

DNA content was determined fluorometrically as previously described (Xie and Haslam,1997)

PgR assays

Serum-containing PgR experiments. All cells were plated at $5X10^4$ cells/cm^2 in serum containing media as for DNA assays for 24 h, then cells were rinsed and switched to 5% DCC-FBS. After 24 h, fresh media with 5% DCC-FBS with or without 10 nM estrogen was added. Cells were cultured for 2 d and estrogen containing media was changed. After 24 h (total 72 h E-treatment), cells were assayed for PgR content.

Serum-free PgR experiments. Cells were plated at $2.5X10^5$ cells/cm^2 in serum-free media for 1d. Control or estrogen treatments were added for 2 d, and E-containing media was changed. Media was removed and cells rinsed with HBSS. All media and rinse media was transferred to 15 ml tube, and cells were treated with 1 mM EDTA (37 C 5-10 minutes). Cells were added to 15 ml tube, and plates were rinsed with HBSS to remove all remaining cells. Cells were centrifuged at 100 X g for 3 minutes and resuspended in serum-free media. Cells were assayed as previously described (Katzenellenbogen and Norman, 1990).

PgR assay. PgR content was determined by ligand binding assay as previously described (Xie and Haslam,1997). Briefly, cells were incubated with 8 nM 3H-R5020 (Sp Act. 84.8 Ci/mmol; New England Nuclear, Boston, MA) plus 500-fold excess dexamethasone (to suppress R5020 binding to glucocorticoid binding sites) or 500-fold excess unlabeled R5020. The specific binding was calculated by subtracting non-specific binding, in the presence of excess unlabeled R5020, to total binding, in the presence of dexamethosone. Additional wells were assayed in parallel for DNA content.

Results

E-induced proliferation on ECMs

Serum containing experiments

Estrogen-increased total DNA of MCF-7 cells and T47D cells by 1.6 to 1.9-fold on collagen I, collagen IV, fibronectin or vitronectin, following 4 d of culture (Figures 1 A,B). MCF-7 cells plated on laminin grew slower in the absence of E, and were growth stimulated only 1.5-fold by E, which was less than on any other ECM. T47D cells did not proliferate in response to E when cultured on laminin. MBA MD231 cells also exhibited reduced growth on laminin. The ER-negative MBA MD231 cells did not show a proliferative response to E on any ECM. (Figure 1C).

Attachment in Serum-free media to ECM

MCF-7 cells had an attachment percentage of 70-80% on all ECMs except for laminin (55% attachment) by 16 h post plating (Figure 2A). T47D cells (Figure 2B) had low attachment on collagen IV, laminin, fibronectin, and vitronectin (15-20%). T47D cell attachment was high on both poly-L-lysine and collagen I (80-83%).

Culture conditions in serum-free media

Since we and others have found substantially reduced responses to steroids in the absence of growth factors, growth factor responsiveness in serum-free media without BSA or fetuin was examined. MCF-7 cells showed a proliferative response to EGF at 5 ng/ml, and maximal response was found at 100 ng/ml (Figure 3A). T47D cells proliferated at lower concentrations of

EGF, 1ng/ml, and were also maximally stimulated at lower (5ng/ml) concentrations (Figure 3B). IGF-I, on the other hand, was able to induce proliferation in both MCF-7 and T47D cells at 10 ng/ml IGF-I, with maximal stimulation at 200 ng/ml (Figure 4 A,B). Lack of BSA or fetuin may have contributed to the apparent need for high concentrations of the growth factors, since growth factors are more likely to nonspecifically bind tissue culture plastic and ECM proteins in the absence of BSA or fetuin.

From these dose-response experiments we choose levels of IGF-I and EGF that stimulate proliferation of cells, submaximally. Submaximal concentrations were used since maximal concentrations of both IGF-I and EGF were less effective than submaximal concentrations in potentiating E effects (data not shown). EGF was used at 5 ng/ml and IGF-I at 25 ng/ml for both T47D and MCF-7 cells. IGF-I or EGF alone stimulated proliferation up to 35% in MCF-7 cells plated on collagen I, and E was ineffective alone (Figure 5 A). T47D cells were growth stimulated approximately 15% by EGF, IGF-I or by E alone (Figure 5B). When E was combined with either IGF or EGF in either cell line, there was a synergistic response. The E response was maximal when both growth factors were added with E.

These studies indicated the need for growth factors to obtain E-induced proliferation. Therefore, IGF-I and EGF were added to E and growth response tested on all ECMs. MCF-7 cell basal proliferation was highest on collagen I, and nearly equal on all other ECMs. MCF-7 cells were not responsive to E when plated on plastic, poly-L-lysine, collagen IV or laminin (Figure 6A). Collagen I= vitronectin> fibronectin were able to stimulate proliferation in response to E. T47D cells had the lowest basal proliferation on plastic, collagen IV, and laminin (Figure 6B). Like MCF-7 cells, T47D cells were not E-responsive on plastic, collagen IV, or laminin. E-induced cell proliferation was obtained on Poly-L-lysine, collagen I, fibronectin and vitronectin. MDA MB231 cells did not exhibit significant proliferation on any ECM in serum-free media, and no E response was obtained (Figure 6C).

E-induced PgR expression on ECMs

Another measure of E-specific responsiveness is PgR induction. MCF-7 cells, but not T47D or MDA MB231 cells, contain E-inducible PgR. MCF-7 cells cultured in the presence of stripped serum, PgR was increased on Plastic, Poly-L-lysine, collagen I, fibronectin and vitronectin (Figure 7A). Very little increase in PgR was obtained on laminin. T47D cells had high constitutive expression of PgR that was not regulated by E or ECM (data not shown). When MCF-7 cells were plated in serum-free media, PgR was increased by E in all groups except plastic and laminin (Figure 7B).

Discussion

Acquisition and maintenance of steroidal responsiveness is critical for normal breast development, whereas lack of steroidal responsiveness is the hallmark of the most aggressive breast cancers. Previous reports have indicated growth factors are necessary for certain steroid mediated responses in breast epithelial cells, such as expression of PgR in response to E (Katzenellenbogen and Norman, 1990). In our experiments, we have determined that ECM proteins can also regulate E-induced proliferation and PgR expression in breast cancer epithelial cells. In the presence of serum, and hence serum-derived growth factors, MCF-7 cells and T47D cells have enhanced proliferation in response to E, when plated on ECM proteins, except laminin. Similar to previous reports, E did not induce proliferation in the absence of growth factors. In the presence of IGF-I and EGF, however, E could stimulate proliferation of both MCF-7 and T47D cells when plated on collagen I, and less markedly on fibronectin and vitronectin. Similarly, PgR was induced by E when cells were plated on collagen I, and fibronectin or vitronectin, but E was least effective in inducing PgR when cells were cultured on laminin, in serum-containing or serum-free (with growth factors) media.

Thus we have made the novel finding that laminin inhibits several E-mediated events, since 1) MCF-7 cells plated in the presence of serum have E-inducible PgR when plated on plastic, but lose responsiveness when plated on laminin and 2) T47D cells plated in the presence of serum

proliferate in response to E on all ECM proteins (including plastic) except laminin. Therefore, laminin may repress at least 2 measures of E-responsiveness. Laminin may induce these responses by several mechanisms. First, laminin may nonspecifically inhibit E action by preventing cell attachment. However, in serum containing media, cell attachment is equally high on all ECM proteins for both MCF-7 and T47D cells. Therefore, lack of attachment to a substratum, which can prevent cell proliferation (Zhu et al., 1996) does not appear to explain the effects of laminin. Laminin does, however, appear to alter cell shape and cell proliferation in the presence or absence of E. For example, when MCF-7 cells, T47D cells or MDA MB231 cells were cultured in serum containing media in the absence of E, all cell lines exhibit reduced proliferation when plated on laminin when compared to any other ECM. Therefore, general cytoskeletal or metabolic pathways may be altered by laminin binding, which may directly or indirectly effect E action. Alternatively, laminin may switch cells from a proliferating state to a differentiating state, since laminin appears to be necessary for mammary epithelial cell differentiation (Streuli et al., 1995). Thus, laminin binding may inhibit growth independent of E and also block or significantly downregulate E-dependent events.

Although these studies with breast cancer cells have identified laminin as a potential negative regulator of E induced responses, we have previously found that other ECM molecules may positively regulate P effects in normal mammary epithelial cells (Xie and Haslam, 1997). In serum-free cultures, fibronectin and collagen IV were required for normal mammary epithelial cell (from nulliparous animals) proliferation in response to progesterone when cells were cultured in serum-free media. Interestingly, no P responses were obtained on any ECM when cells were obtained from pregnant animals, indicating that integrin and P cooperative signaling is altered by state of the gland. Similar to other normal mammary epithelial cells in culture, these cells did not proliferate in response to E, regardless of ECM composition. This is in contrast to many breast cancer cell lines, including the MCF-7 and T47D lines, that are responsive to E. Our cancer cell studies do have some similarities with Xie and Haslams' results, however. Both studies have found that certain ECM proteins can regulate acquisition of steroidal responsiveness. Additionally, laminin was not effective in mediating steroid induced proliferation, while fibronectin and collagen I or collagen IV were. It will be of interest to

continue to determine the similarities and differences between normal and cancer breast epithelial cells in ECM regulated acquisition of steroidal responsiveness. Currently, there is no information about the integrins and intracellular pathways involved in these ECM regulated events.

Recently, several researchers have demonstrated that ECM proteins can regulate proliferation, and differentiation when signaling through specific extracellular matrix cell surface receptors, integrins. Wary and colleagues (1996) have demonstrated that proliferation of human vascular endothelial cells by fibroblast growth factor (FGF) is acutely regulated by ECM/integrin interaction. Endothelial cells did not proliferate in response to FGF-2 when cultured on laminin-1, but were growth stimulated by FGF when cultured on fibronectin or vitronectin. Furthermore, the authors found that integrins that were associated with Shc ($\alpha 1\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$) could mediate activation of a Shc-Grb2-MAP kinase pathway. They have postulated that integrins that are not associated with Shc ($\alpha 2\beta 1$ and possibly $\alpha 6\beta 1$) may promote differentiation or apoptosis. Therefore, these researchers have found that laminin and two of its receptors (the $\alpha 2\beta 1$ and the classical laminin receptor $\alpha 6\beta 1$) do not potentiate proliferation by growth factors, whereas fibronectin (classical receptor=$\alpha 5\beta 1$) and vitronectin (classical receptor=$\alpha v\beta 3$) and their receptors do. These results are in agreement with results presented herein, that laminin may inhibit cell proliferation, while fibronectin and collagens mediate E-induced proliferation. A single mammary cell study by Elliot and colleagues (1992) have found similar ECM requirements for growth factor induced proliferation in murine mammary tumor cells. PDGF and FGF-2 induced maximal proliferation of a murine mammary tumor cell line when cultured on fibronectin, but not on collagen I.

Adhesion to substratum is required for cell cycle progression through G1 and into S phase for nearly all non-transformed cells (Zhu et al., 1996). Furthermore, Giancotti and Mainiero (1994) have found that optimal cellular proliferation and migration occur when integrin-ECM adhesion is 'moderate', either weak adhesion or strong adhesion may prevent proliferation. While, Ingber (1990) has determined that ECM induced cell proliferation only occurs when ECM is rigidly attached to a substrate and when cells have ample ECM integrin receptors to attach to the ECM.

We have demonstrated that attachment in the presence of serum is not different on laminin, but laminin does alter E-induced proliferation. We have not determined whether laminin receptors (laminin-specific integrins), however are in lower numbers than other integrins for collagens, fibronectin or vitronectin. Interestingly, Shi and coworkers (1993), however, have found that laminin receptors increase following E or P treatment of T47D cells. Therefore, if cell attachment to laminin was weak in the absence of E, one would expect E to enhance attachment and perhaps then facilitate proliferation. This was not the case in our studies, therefore, it is unlikely that attachment or degree of attachment regulate these E-induced responses.

These results indicate that specific ECM proteins may potentiate, while others may E-mediated events in breast cancer cells. We have not determined whether ECM proteins directly alter ER activity, or how laminin may alter the cell cycle in the presence or absence of E. Current and future experiments will examine whether ECM proteins directly regulate ERE activity. Furthermore, we will use ^{3}H-thymidine or cell cycle analyses to 1) increase the sensitivity of our serum-free growth assays, and 2) determine whether ECM proteins are altering specific phases of cell cycle progression. In addition to more fully understanding how specific ECM regulate the cell cycle and ER responsiveness, we are interested in determining which integrins are involved in these responses, as outline below. Finally, we have began to analyze non-transformed breast epithelial cell proliferation on the different ECM proteins (as outlined in the grant) and compare these results with the breast cancer results. These results should give us a comprehensive understanding of how ECM-integrin interactions effect E-responsiveness of breast cells and how these events are altered during breast tumorigenesis.

Recommendations to Statement of Work

We have completed experiments to examine growth responsiveness and PgR induction by E for both T47D and MCF-7 cells in the presence of serum on all outlined ECM proteins. We have also determined optimal conditions for these cell lines to measure cell proliferation and/or PgR induction by E in the absence of serum. Data from these experiments indicate that both MCF-7 cell and T47D cell proliferation in response to E in serum-free conditions is low, regardless of ECM. However, we have consistently gotten E-induced responses on certain ECM proteins

(collagen I), while other ECM proteins (laminin) have not facilitated E-responsiveness. We have determined that the sensitivity of our DNA assay is too low to accurately assess the effects of steroids in serum-free conditions.

Increase sensitivity of growth assays

To increase sensitivity, we will measure E-responsiveness by ^{3}H-thymidine incorporation and/or by cell cycle analysis. The disadvantage of using a DNA assay to determine growth of cells, which are dividing slowly, is that your background DNA will be high, and cells that are slowly dividing in response to E will only be marginally higher. Both cell cycle and ^{3}H-thymidine incorporation analyses have the advantage that background will be very low, i.e. very few cells are proliferating. Therefore, any increase in proliferation will be more easily noticed. We have recently found, in similar serum-free experiments, that cells treated with E increased ^{3}H-thymidine incorporation by up to 80%, whereas total DNA was only stimulated 10-15%. It is not possible to lengthen the treatment time (4 d) in order to increase the sensitivity of DNA assays, since epithelial cells can lay down their own ECM proteins and respond to these proteins by 6-8 d of culture. Moreover, propidium iodine/florescence activated cell sorting, will provide us with more information on ECM effects by allowing us to examine cell cycle regulation on different ECMs, before and after E-treatment.

Direct Measurement of estrogen receptor activity

Since we have found that E is less effective in inducing PgR and in stimulating cell proliferation when cells are cultured on laminin, we are interested in determining estrogen response element activity. Using ERE-reporter constructs (ERE-CAT or ERE-luciferase), we will determine the effect of E on ERE activity when MCF-7, T47D or MDA MB231 cells are plated on various ECM proteins. The effect of growth factor additions to ECM may also be measured by these assays. These experiments should allow us to determine if laminin inhibits E-mediated events by non-specifically inhibiting proliferation (or inducing differentiation) or whether laminin directly inhibits ERE activity.

Determine which integrins are involved and whether they are absolutely needed for E-responsiveness or lack of E-responsiveness.

Additionally, we are interested in determining which integrin(s) are responsible for signal transduction. We will obtain function blocking antibodies to specific integrins, including laminin ($\alpha6\beta4$ or $\alpha6\beta1$) and fibronectin ($\alpha5\beta1$) integrins. These antibodies will be used to determine if 1) laminin inhibits E function by specific integrin binding, and 2) fibronectin influences E-function by its classical integrin ($\alpha5\beta1$). It would also be of interest to determine the effects of blocking fibronectin binding to the $\alpha5\beta1$ in the presence of serum on T47D and MCF-7 cell responsiveness to E, since a major attachment factor in serum is fibronectin.

CONCLUSIONS

These studies have determined that E-responsiveness is regulated by ECM composition. Specifically, laminin inhibits E-mediated cell proliferation and E-induced PgR responsiveness in the presence of serum. In the absence of serum, E-mediated cell proliferation is strongest when cells are plated on collagen I, followed by fibronectin or vitronectin. Laminin, poly-L-lysine, and collagen IV have little or no E-induced proliferation in MCF-7 or T47D cells. PgR induction in MCF-7 cells is least on laminin in the absence of serum. E-induced proliferation in serum-free assays is significantly lower than in the presence of serum. Other more sensitive techniques for measuring E-regulated events in breast cancer cells will be explored, including 3H-thymidine incorporation, cell cycle analysis, and ERE-reporter assays. We have made the novel finding that laminin may negatively regulate E-responsiveness of breast epithelial cells, while other ECM proteins (including collagen I and fibronectin) may sensitize epithelial cells to the effects of E. These result suggest that therapeutic strategies may be designed to alter ECM composition or ECM-integrin interactions in the tumor microenvironment to prevent tumor growth and metastasis.

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FIGURE LEGENDS

Figure 1
DNA content of MCF-7 (A), T47D (B) and MDA MB231 (C) cells following 4 d culture in estrogen containing media in the presence of 5% charcoal stripped fetal bovine serum. White bars=control treatment, Black bars=10 nM estrogen. Cells were plated on plastic (Plas), poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Standard error bars, from 3-8 experiments are included in parts A and B, Figure C are results from a single experiment. Note: MDA MB231 cells are estrogen receptor negative and represented a negative control.

Figure 2
MCF-7 (A) and T47D (B) cell attachment following 18 h of culture in serum-free media on plastic (Plas), poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Attachment was measured by the DNA content of attached cells per well as a percent of the total DNA content in a well (attached and not attached).

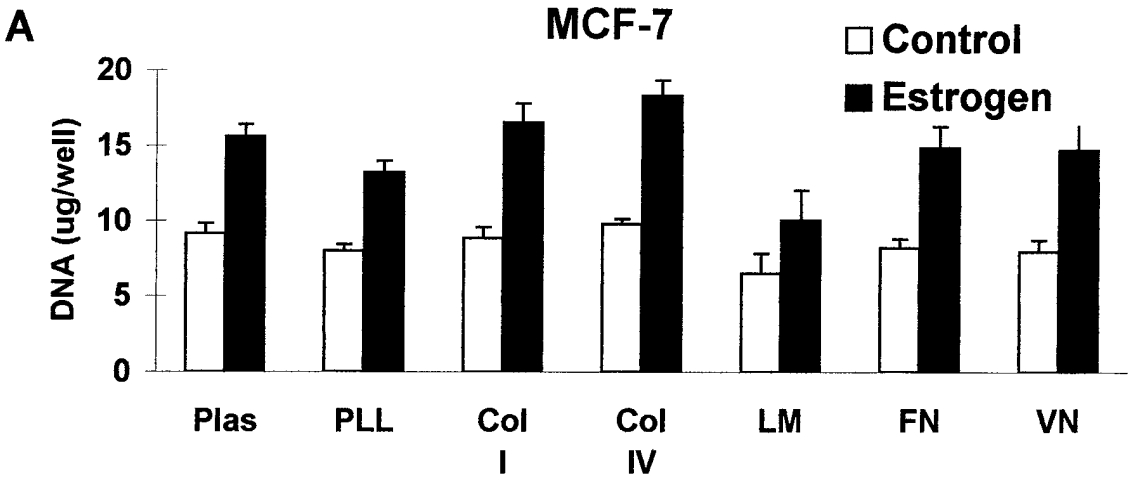
Figure 3
Epidermal growth factor (EGF) dose response of MCF-7 (A) and T47D (B) cells as measure by DNA content following 4 d culture in EGF containing serum-free media. Media did not contain albumin or fetuin. Cells were plated in the absence of serum on collagen I for 2 d and switched to EGF treatments. DNA content as a percentage of no treatment (no EGF) was determined after 4 d culture in EGF containing media.

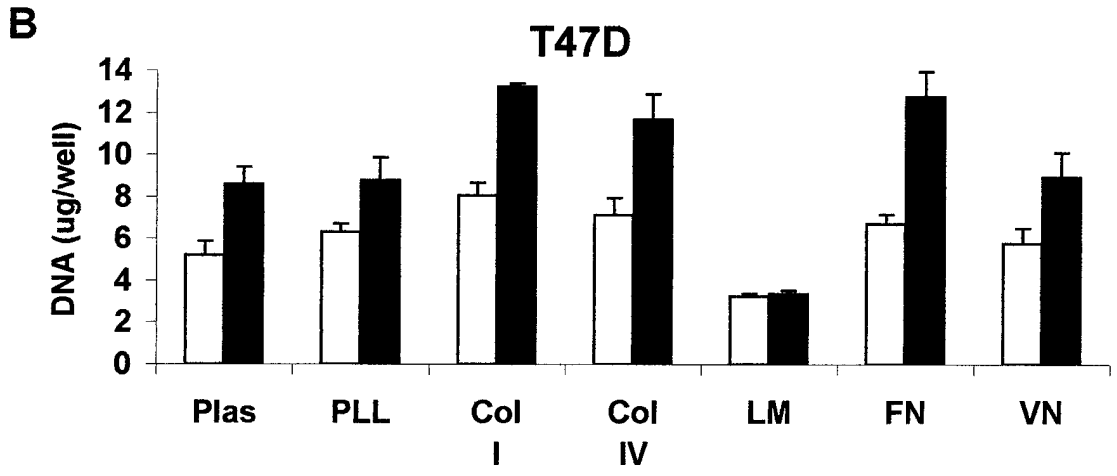
Figure 4
Insulin-like growth factor-I (IGF-I) dose response of MCF-7 (A) and T47D (B) cells as measure by DNA content following 4 d culture in IGF-I containing serum-free media. Media did not contain albumin or fetuin. Cells were plated in the absence of serum on collagen I for 2 d and switched to IGF-I treatments. DNA content as a percentage of no treatment (no IGF-I) was determined after 4 d culture in IGF-I containing media.

Figure 5
Synergistic effects of insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and estrogen (E) on DNA content of MCF-7 (A) and T47D (B) cells following 4 d culture in treatments. Media did not contain albumin or fetuin. Cells were plated in the absence of serum on collagen I for 2 d and switched to growth factor/steroid treatments. DNA content as a percentage of no treatment was determined after 4 d culture in steroid/growth factor containing media.

Figure 6
DNA content of MCF-7 (A), T47D (B) and MDA MB231 (C) cells following 4 d culture in estrogen containing media in serum-free media. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I, but no albumin or fetuin. White bars=control treatment, Black bars=10 nM estrogen. Cells were plated on plastic (Plas), poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Standard error bars, from 3 experiments are included in parts A and B, Figure C are results from a single experiment. Note: MDA MB231 cells are estrogen receptor negative and represented a negative control.

Figure 7
Specific ^{3}H-R5020 binding in MCF-7 cells in the presence of 5% charcoal stripped fetal bovine serum (5% DCC-FBS) (A) or in the absence of serum (B). . Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I, but no albumin or fetuin. White bars=control treatment, Black bars=10 nM estrogen. Cells were plated on plastic (Plas), poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Standard error bars, from 3 experiments are included in parts A and B. Cells were cultured in the presence of estrogen for 3 d. Progesterone receptor (PgR) was determined by specific ^{3}H-R5020 and normalized to DNA content.


A
MCF-7
Control
Estrogen
DNA (ug/well)
20
15
10
5
0
Plas
PLL
Col I
Col IV
LM
FN
VN


B
T47D
DNA (ug/well)
14
12
10
8
6
4
2
0
Plas
PLL
Col I
Col IV
LM
FN
VN

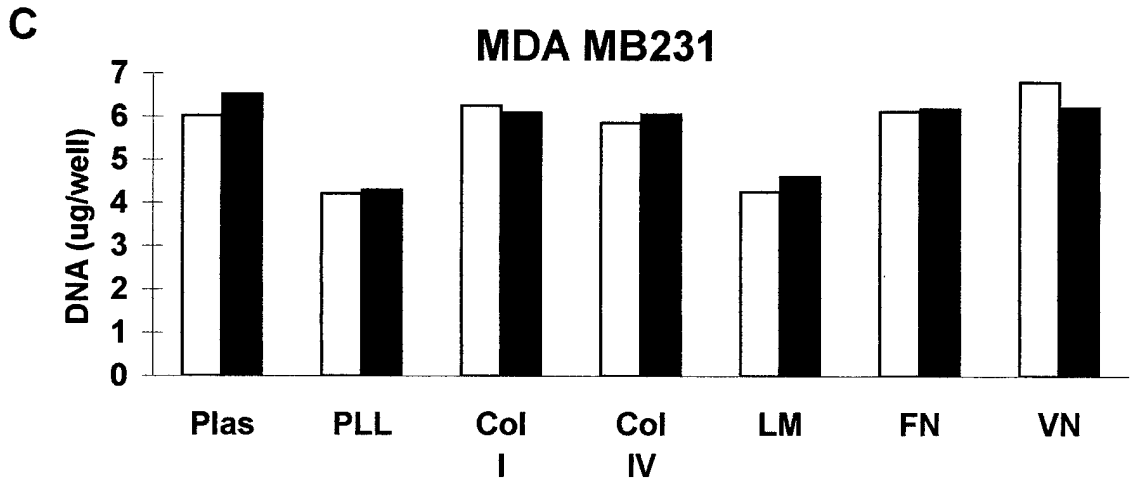

C
MDA MB231
DNA (ug/well)
7
6
5
4
3
2
1
0
Plas
PLL
Col I
Col IV
LM
FN
VN

Figure 1

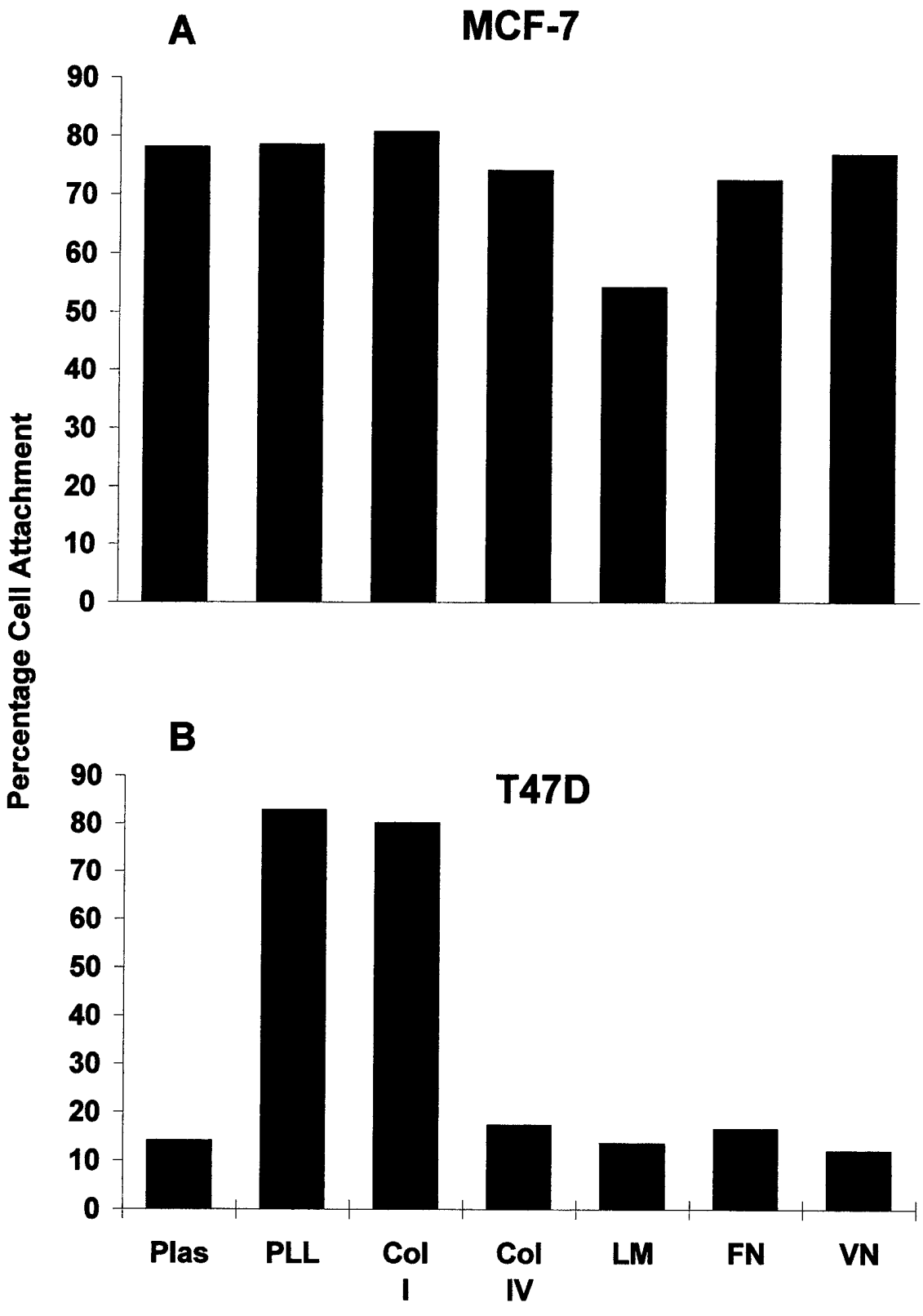

A
MCF-7
Percentage Cell Attachment
90
80
70
60
50
40
30
20
10
0
B
T47D
90
80
70
60
50
40
30
20
10
0
Plas
PLL
Col I
Col IV
LM
FN
VN

Figure 2

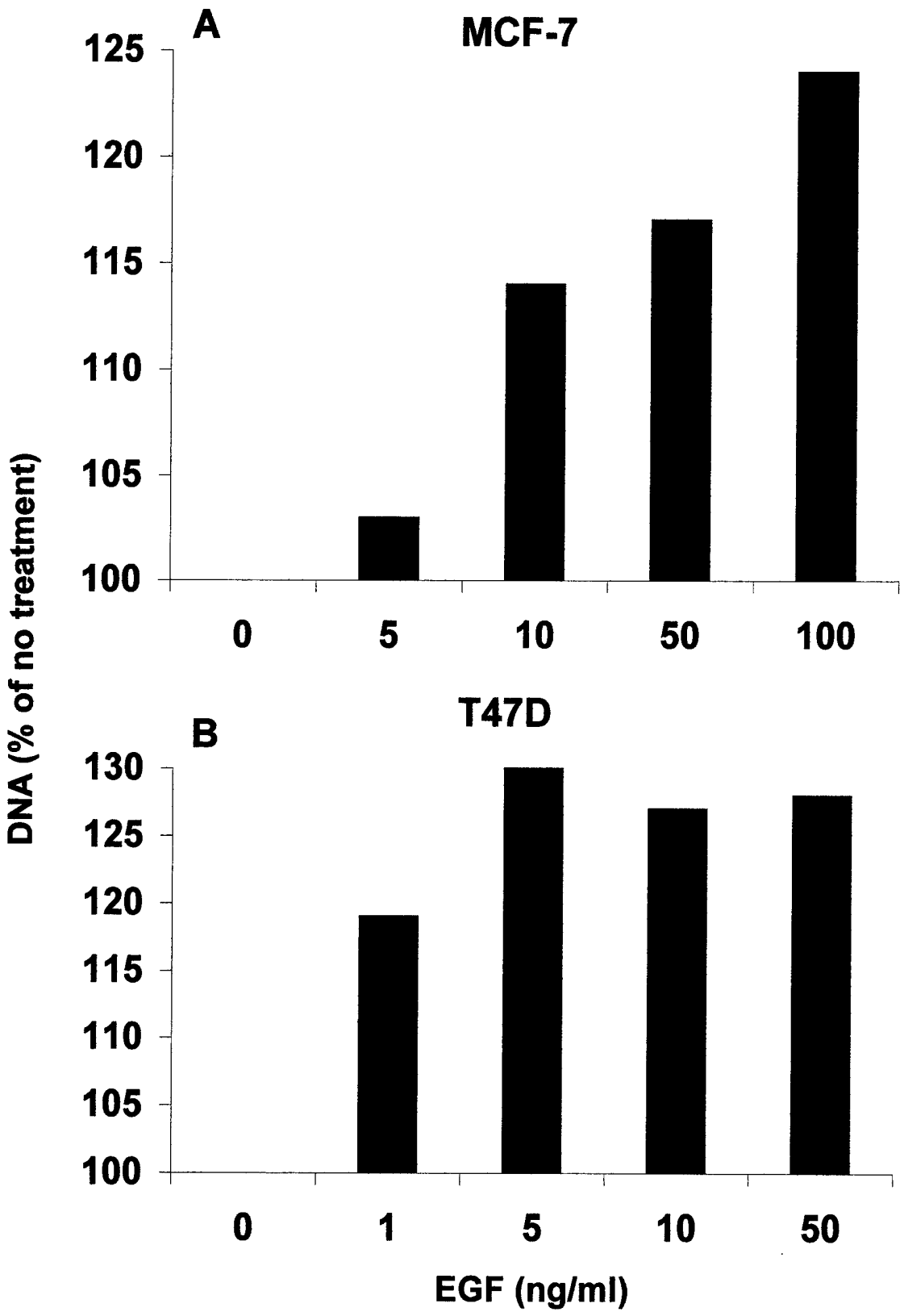

A
MCF-7
125
120
115
110
105
100
0
5
10
50
100
B
T47D
130
125
120
115
110
105
100
0
1
5
10
50
DNA (% of no treatment)
EGF (ng/ml)

Figure 3

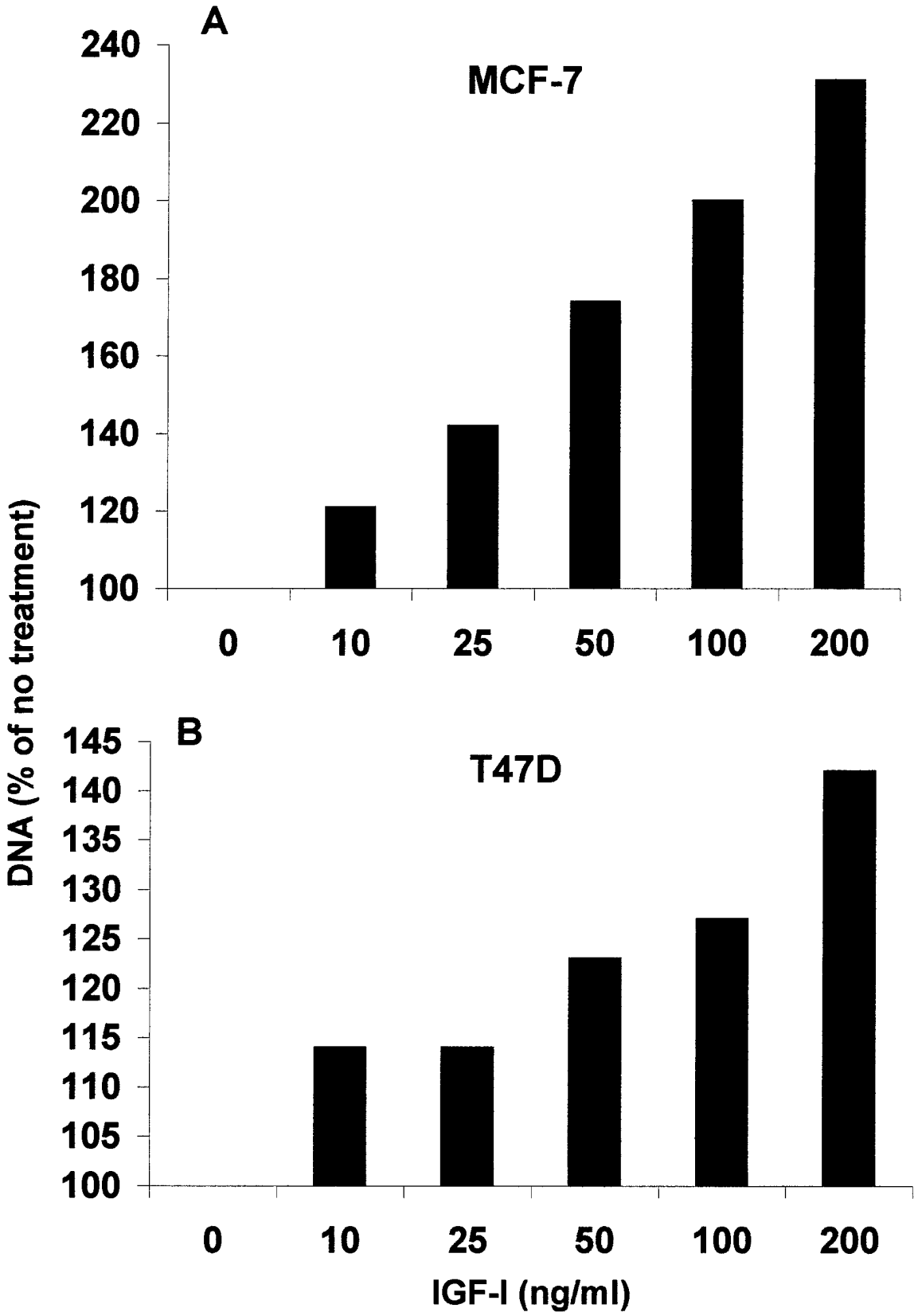

A
MCF-7
240
220
200
180
160
140
120
100
0
10
25
50
100
200
B
T47D
145
140
135
130
125
120
115
110
105
100
0
10
25
50
100
200
DNA (% of no treatment)
IGF-I (ng/ml)

Figure 4

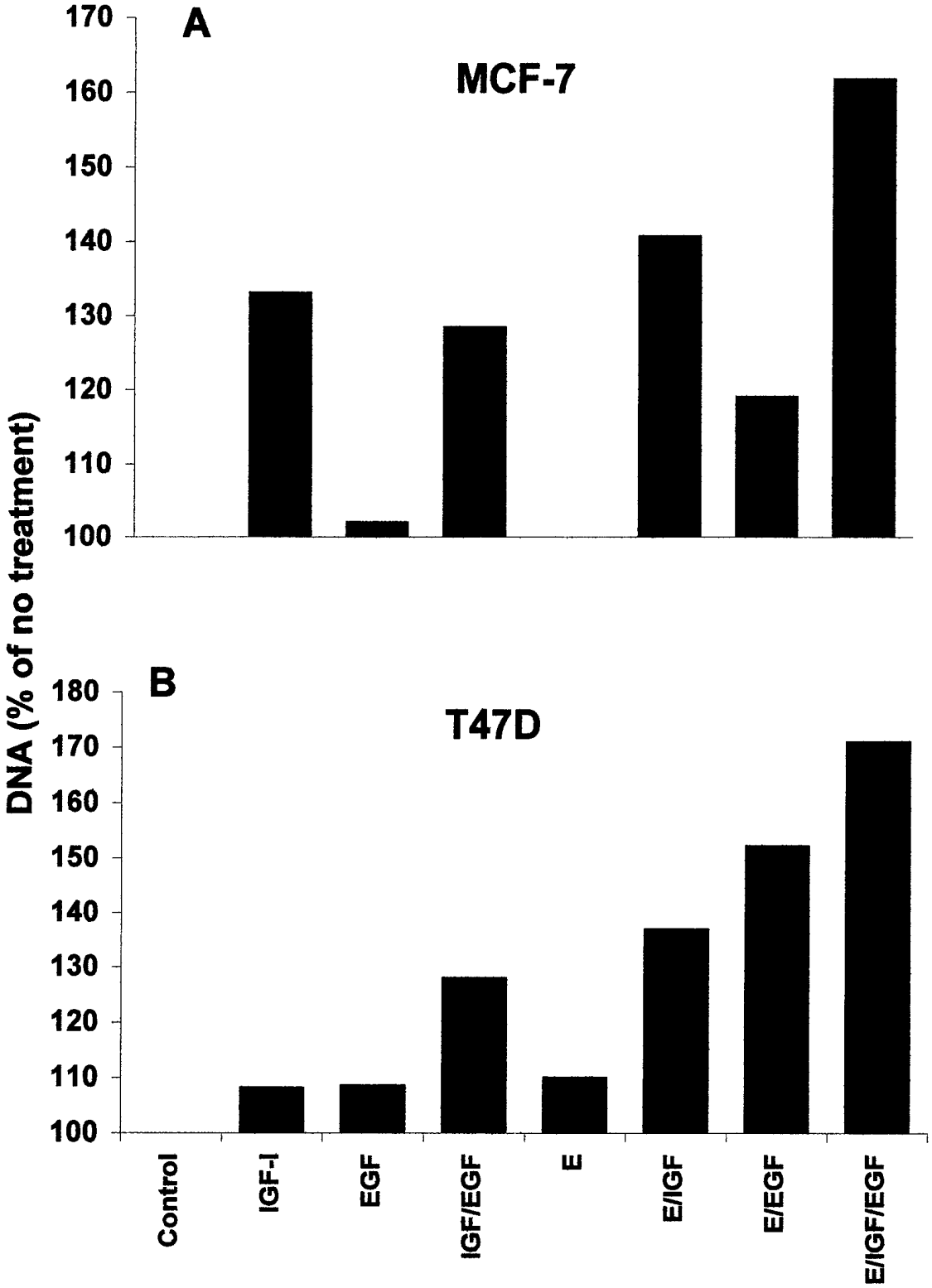

A
MCF-7
170
160
150
140
130
120
110
100
B
T47D
180
170
160
150
140
130
120
110
100
DNA (% of no treatment)
Control
IGF-I
EGF
IGF/EGF
E
E/IGF
E/EGF
E/IGF/EGF

Figure 5

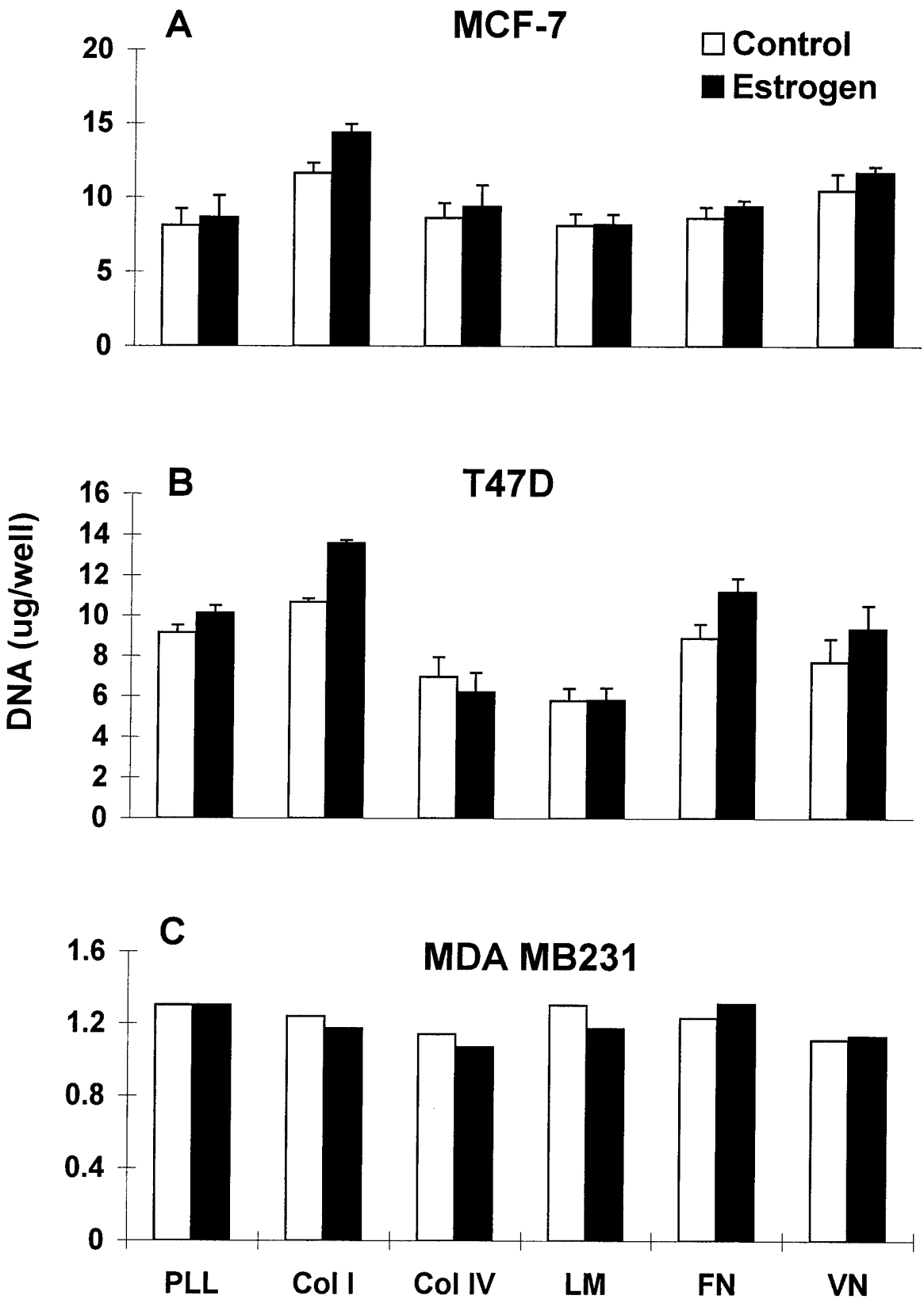

A
MCF-7
Control
Estrogen
20
15
10
5
0
B
T47D
DNA (ug/well)
16
14
12
10
8
6
4
2
0
C
MDA MB231
1.6
1.2
0.8
0.4
0
PLL
Col I
Col IV
LM
FN
VN

Figure 6

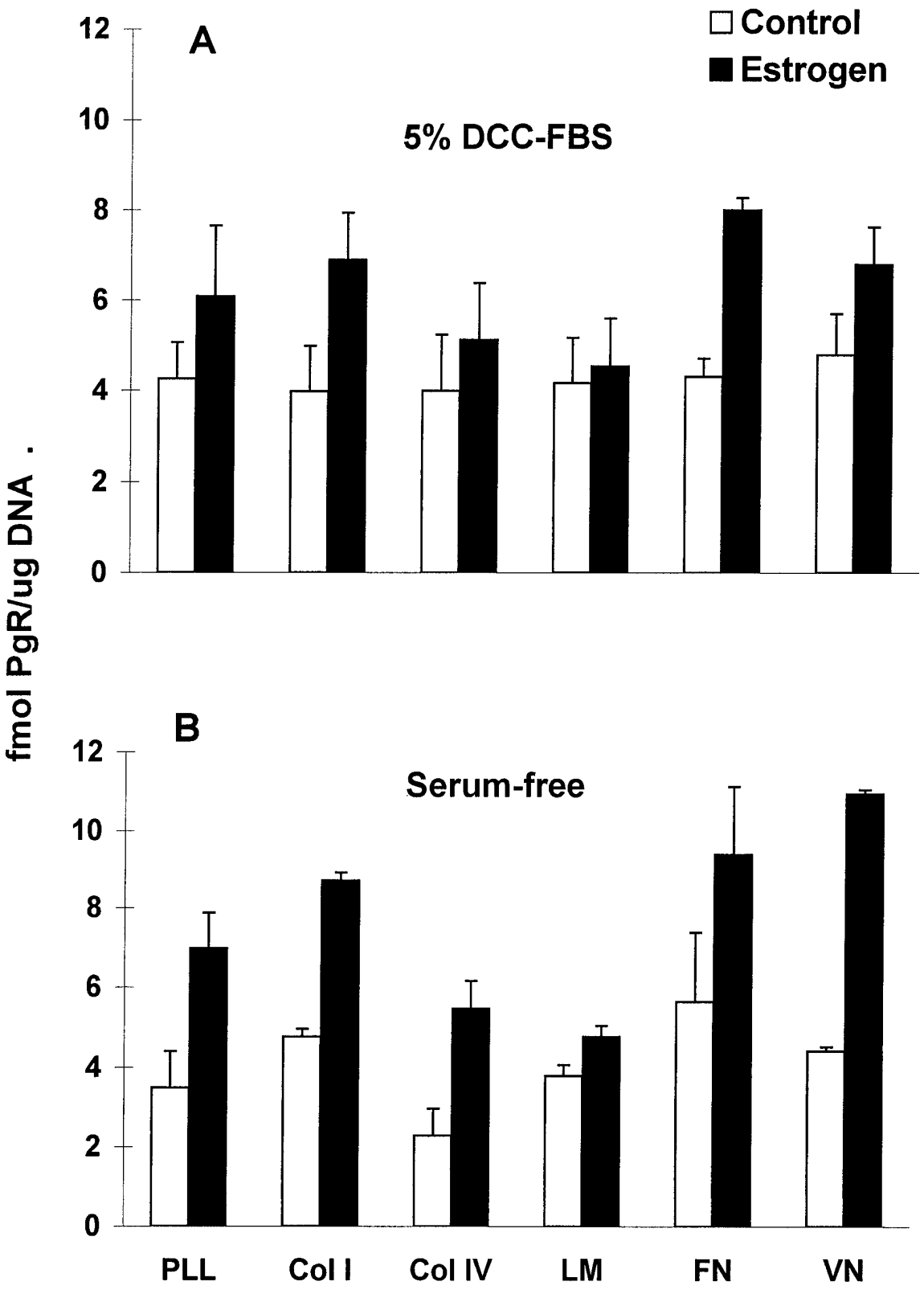

A
Control
Estrogen
5% DCC-FBS
fmol PgR/ug DNA
12
10
8
6
4
2
0
B
Serum-free
12
10
8
6
4
2
0
PLL
Col I
Col IV
LM
FN
VN

Figure 7