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Collagen-IV and laminin-1 regulate estrogen receptor α expression and function in mouse mammary epithelial cells

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Summary

The expression level and functional activity of estrogen receptor α is an important determinant of breast physiology and breast cancer treatment. However, it has been difficult to identify the signals that regulate estrogen receptor because cultured mammary epithelial cells generally do not respond to estrogenic signals. Here, we use a combination of two- and three-dimensional culture systems to dissect the extracellular signals that control endogenous estrogen receptor α. Its expression was greatly reduced when primary mammary epithelial cells were placed on tissue culture plastic; however, the presence of a reconstituted basement membrane in combination with lactogenic hormones partially prevented this decrease. Estrogen receptor α expression in primary mammary fibroblasts was not altered by these culture conditions, indicating that its regulation is cell type specific. Moreover, estrogen receptor-dependent reporter gene expression, as well as estrogen receptor α levels, were increased threefold in a functionally normal mammary epithelial cell line when reconstituted basement membrane was added to the medium. This regulatory effect of reconstituted basement membrane was reproduced by two of its components, collagen-IV and laminin-1, and it was blocked by antibodies against α2, α6 and β1 integrin subunits. Our results indicate that integrin-mediated response to specific basement membrane components, rather than cell rounding or cell growth arrest induced by reconstituted basement membrane, is critical in the regulation of estrogen receptor α expression and function in mammary epithelial cells.

Keywords

Basement membrane; Extracellular matrix; Mammary fibroblasts; Integrins

Introduction

In the mammary gland, estrogen receptor α and β (ERα and ERβ) act as hormone-dependent transcriptional regulators controlling many aspects of cell proliferation and differentiation (reviewed by Cunha et al., 2000; Gustafsson and Warner, 2000). ERα is believed to be responsible for the majority of estrogenic effects in the mammary ductal development, and its deregulation correlates with the progression of mammary neoplasia to invasive carcinoma (Sommer and Fuqua, 2001). However, when primary mammary epithelial cells are placed in monolayer cultures, they rapidly lose their functional and morphological characteristics, including expression of ERα and growth response to estrogen (reviewed by Ronnov-Jessen et al., 1996; Anderson et al., 1998). Thus, it is clear that the expression and function of ERα

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require additional functions of molecules not present under these culture conditions. Isolated mammary epithelial cells in culture recover growth response to estrogen when co-cultured with stromal cells (reviewed by Haslam and Woodward, 2001). Under these conditions, the cells could elaborate a basement membrane (BM), a specialized form of extracellular matrix (ECM), and thus can recapitulate certain aspects of the epithelial microenvironment (reviewed by Bissell et al., 1999). In this regard, it has been shown that the expression of ERα is maintained at higher levels in normal mouse and human mammary epithelial cells cultured in three-dimensional collagen-I gels (Edery et al., 1985; Yang et al., 2000), a culture system that allows primary cells to produce their own BM (Streuli and Bissell, 1990).

In the normal mammary gland, BM is a continuous deposit that separates epithelial cells from the surrounding stroma. It is rich in laminins and collagen-IV, and also contains entactin, proteoglycans and other glycoproteins (reviewed by Aumailley and Gayraud, 1998). Signals from the BM regulate epithelial cell morphology, growth, functional differentiation and apoptosis in mammary cells (Streuli et al., 1991; Boudreau et al., 1995; Muschler et al., 1999; Weaver et al., 2002). Cultivated in three-dimensional cultures in the presence of a reconstituted BM (rBM) and lactogenic hormones, mammary epithelial cells arrest growth and reorganize into tissue-like structures (acini) that secrete milk proteins into a central lumen (Barcellos-Hoff et al., 1989). Using Scp2, a clonal mammary epithelial cell line established in our laboratory that is unable to produce and organize its own BM (Desprez et al., 1993), we found previously that two distinct signals control β-casein expression in response to laminin-1. These include a morphogenic signal that leads to changes in cell shape (cell rounding) and most probably involves dystroglycan (Muschler et al., 1999; Muschler et al., 2002), and a subsequent biochemical signal that involves integrin activation (Roskelley et al., 1994).

Given that cell contact with the BM constitutes a crucial regulator of cell structure and gene expression, we hypothesized that a cause of deregulation of ER expression and function in culture may be the result of loss of BM integrity. Here, we have used non-malignant primary mammary epithelial cells to investigate whether and how the signals from the BM may be required to maintain ERα expression. We find that biochemical signals from collagen-IV and laminin-1, transmitted through α2, α6 and β1 integrins, are required to maintain the majority of ERα expression. We also use Scp2 cells to show that these same signals and signaling molecules can increase the expression of ERα in cultured mammary epithelial cells.

Materials and Methods

Antibodies and reagents

The function-blocking integrin antibodies against α1 (Ha31/8), α2 (HMα2), α6 (GoH3) and β1 (Ha2/5) subunits were purchased as sodium azide- and endotoxin-free reagents from PharMingen (San Diego, CA). The anti-ERα polyclonal antibody (MC-20) was purchased from Santa Cruz (Santa Cruz, CA) and the anti-ERα monoclonal antibody (NCL-ER-6F11) was purchased from NovoCastra (Newcastle, UK). The monoclonal anti-rat β-casein antibody was a gift from C. Kaetzel (Case Western Reserve University, Cleveland, OH). The anti-E-cadherin antibody (C20820) was purchased from Transduction Laboratories (Lexington, KY). Reconstituted basement membrane (rBM; Matrigel), purified laminin-1, purified collagen-IV and MatriSperse Cell Release Solution were purchased from Collaborative Biomedical Products (Bedford, MA). Collagen-I was purchased from Vitrogen (Palo Alto, CA). Poly(2-hydroxyethyl methacrylate) (polyHEMA) and fibronectin were purchased from Sigma Chemical. Collagenase type A and 5-bromo-2′-deoxy-uridine (BRdU) were purchased from Boehringer-Mannheim (Indianapolis, IN). Alamar blue was purchased from Accumed International (Westlake, OH).

Cell culture

Primary cultures—Primary mammary epithelial cells were obtained by a procedure slightly modified from Kittrell et al. (Kittrell et al., 1992). Briefly, after removal of the 4th inguinal mammary glands from nulliparous 12-week-old virgin BALB/c mice, the glands were minced by chopping with two razor blades in parallel. Mammary cells were dissociated by collagenase type A (2 mg/ml) in the presence of 5 μg/ml insulin (Sigma Chemical) and with antibiotics [600 U/ml nystatin (Sigma Chemical), 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin (Gibco, Rockville, MA)] in DMEM/F12 medium for 3 hours at 37°C with constant shaking (100 rpm). The resulting suspension was centrifuged at 1000 ***g*** for 10 minutes, and the pellet resuspended in 4 ml DMEM/F12 containing 2 U/ml DNase (Sigma Chemical). After gently shaking for 2 minutes the DNase was diluted by adding 4% fetal bovine serum (FBS) in 4 ml of DMEM/F12 medium, and the final suspension (containing 2% FBS in DMEM/F12) was centrifuged again at 1000 ***g*** for 10 minutes. The resulting pellet was resuspended in phosphate-buffered saline (PBS) containing 5% adult bovine serum (Atlanta Biologicals, Norcross, GA), and this procedure was repeated six times at 1500 ***g*** for 15 seconds each time to remove stromal cells. This protocol yielded 90% or greater purity of epithelial cells (mostly as organoids of approximately 100 cells) as determined by immunofluorescence for keratin (data not shown). Each fraction, pellet (epithelial cells) and supernatant (mostly fibroblast cells, according to vimentin staining) was resuspended in growth medium (indicated below). The day of the isolation from the gland was considered time 0 in the culture period.

Cell lines—Scp2 is a functionally normal mouse mammary epithelial cell line established in our laboratory (Desprez et al., 1993). The Scp2-ERE-TK-CAT cell line is a derivative of Scp2 that has been stably transfected by cotransfecting 30 μg of the pA2(−331/−87)tk-CAT8+ plasmid and 3 μg of pSV2neo plasmid. pA2(−331/−87)tk-CAT8+ contains the chloramphenicol acetyltransferase (CAT) enzyme as a reporter gene, under the control of a minimal thymidine kinase (TK) promoter containing an upstream consensus estrogen-response element (ERE). The ERE corresponds to the region −331 to −87 of the *Xenopus vitellogenin A2* gene (Klein-Hitpass et al., 1986). The resulting SCp2-ERE-TK-CAT cells were obtained by pooling neomycin-resistant colonies. They were selected under 400 μg/ml G418 (Gibco, Rockville, MA) and maintained under 40 μg/ml G418. These cells were used at passage 6-8 after transfection/selection.

Scp2, Scp2-ERE-TK-CAT and primary mammary cells were cultured at a density of ~50,000 cells/cm^2 or ~100,000 cells/cm^2 (for cultures on top rBM and on polyHEMA, see below) in DMEM/F12 medium containing 50 μg/ml gentamycin, 5 μg/ml of insulin, 1 μg/ml of hydrocortisone and 3 μg/ml of prolactin (Sigma Chemical). For primary cultures, the growth medium was supplemented with epidermal growth factor (EGF, 5 ng/ml; Sigma), linoleic acid (5 μg/ml; Sigma) and bovine serum albumin (BSA, 5 mg/ml; Sigma). Attachment and spreading of the cells to the covered-glass chamber slide (for immunofluorescence) or the plastic dish were performed for 24 hours of culture in the presence of 2% FBS. The cells were then grown for the period indicated in each case with fresh serum-free medium containing insulin, hydrocortisone and prolactin, with or without addition of ECM components (see below). In experiments where the ER activity was measured (CAT reporter assays) we used charcoal-treated FBS (HyClone, Logan, Utah) and phenol red-free DMEM/F12 medium to avoid interference from exogenous estrogens. When indicated, 10^{-8} M of 17β-estradiol (Sigma Chemical), 10^{-7} M of the antagonist ICI 182,780 (Tocris Cookson, Ellisville, MO) or the same volume of ethanol (vehicle) were added to the medium for the last 48 hours.

Culture substrata

The culture conditions for cell lines or primary cells consisted of untreated tissue culture plastic or plastic covered by a thick layer (50 μl/cm^2) of growth factor-reduced rBM derived from

Englebreth-Holm-Swarm tumor (Matrigel). For this last condition, the cells were seeded on top of the gel (on top rBM) and covered with the corresponding serum-free medium (see above). Matrigel was previously allowed to solidify at 37°C for 40 minutes. For assays in pre-rounded cells, primary or Scp2 cells were cultured in suspension by placing ~100,000 cells/cm^2 in a culture dish coated with the nonadhesive substratum polyHEMA in serum-free medium. PolyHEMA-coated dishes were prepared using a solution of 6 mg/ml polyHEMA in 95% ethanol added to culture plates at 0.05 ml/cm^2 and allowed to evaporate to dryness.

For the 'dripping' conditions, soluble rBM or purified ECM components laminin-1, collagen-I, collagen-IV or fibronectin were diluted in the culture medium, and were added as an overlay to previously attached and spread cells in the case of Scp2 cells or immediately after isolation from the gland in the case of primary cultures. In the case of polyHEMA cultures, when indicated, rBM was mixed in the medium with the cells. For rBM we tested 1%, 2% and 5% dilution from a 10 mg/ml protein concentration of Matrigel. Because the most effective dilution was 2%, we estimated the final concentration for the ECM components corresponding to their relative proportion in 2% Matrigel. The final concentrations used were: 150 μg/ml of laminin-1, 20 μg/ml of collagen-I, 20 μg/ml of collagen-IV and 10 μg/ml fibronectin. Under these conditions, the components form a precipitate covering the cultured cells.

Cellular lysis

Cells were treated for the required number of days (as indicated in results and legends to figures), with one change of medium every 2 days, and at the end of the culture period, cells were lysed and extracted for protein or RNA analysis. For lysis and extraction, cells were rinsed once with PBS; for protein extraction, we used the protein extraction reagent for mammalian cells (M-PER; Pierce, Rockford, IL) and for total RNA extraction, we used the RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's directions. For cells growing on top of rBM, cells were removed from the gel by using enzymatic digestion with MatriSperse for 1 hour on ice, followed by a centrifugation for 5 minutes at 1000 ***g***. The resulting pellet was then subjected to protein or RNA extraction. For cells growing on polyHEMA-coated dishes, they were transferred to Eppendorf tubes, centrifuged and lysed.

Integrin blocking

Scp2 cells or primary mammary epithelial cells were grown on plastic or in the presence of rBM, collagen-IV or laminin-1 for 3 days in the presence of 10 μg/ml of mouse IgG (control, c) or in the presence of 10 μg/ml of α1, α2 or α6 or 5 μg/ml of β1 integrin blocking antibodies. The antibodies were diluted in the corresponding medium at the time of plating the cells on top of rBM, or after 24 hours of plating under other conditions to let them attach and spread. At the end of the experiment, cell lysis followed by protein extraction was performed as described above. Cell viability using Alamar blue vital dye assay was carried out in parallel cultures according to manufacturer's instructions.

Immunofluorescence for ERα

For ERα detection, cells were fixed with −20°C methanol:acetone (1:1) solution for 5 minutes, air dried for 10 minutes, rehydrated in PBS, blocked with Super Block Blocking Buffer in PBS (Pierce) and incubated with ERα monoclonal antibody (NCL-ER-6F11) followed by FITC-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA), mounted and observed under fluorescence microscopy. Before mounting, 4′,6-diamidino-2-phenylindole (DAPI, Sigma) was used to stain DNA. Control experiments were carried out omitting the primary antibody. Images were captured using Spot RT camera and software (Technical Instruments, Burlingame, CA). Cellular labeling indices for ERα were determined by counting at least 100 cells from randomly selected visual fields and calculating the intensity of labeling

in the cells by using Simple PCI imaging software (Compix Imaging Systems, Cranberry Township, PA).

Cell proliferation assay

To study the influence of cell proliferation on ERα expression, Scp2 cells cultured for 2 days on plastic or in the presence of rBM were treated with increasing amounts of insulin (between 0 and 10 μg/ml). The cells were maintained for another 24 hours, including a 6 hours labeling period with 10 μM BrdU (BrdU labeling and detection kit to measure DNA synthesis) according to the manufacturer's instructions. Nuclear labeling indices were determined by counting at least 100 cells from randomly selected visual fields and calculating the percentage of cells with labeled nuclei. Parallel experiments were performed to detect ERα levels by western blot.

CAT assay

We used the nonradioactive FLASH CAT Assay kit (Stratagene, La Jolla CA) to measure the CAT activity in cell lysates from Scp2-ERE-TK-CAT cells. Briefly, we mixed 5 μg protein/sample quantified by protein assay (Protein Assay DC, Bio Rad, Hercules, CA) with the fluorescent derivative of chloramphenicol BODIPY (borondipyrromethane difluoride fluorophore). This substrate is converted to a single monoacetylated product by CAT that is separated from the substrate by thin layer chromatography (TLC). The TLC plates were scanned using STORM fluoroimager (Amersham Biosciences, Sunnyvale, CA) and quantitation was performed using ImageQuant (Amersham).

Western blot for ERα and β-casein detection

Equal amounts of protein (20 μg of cellular extracts) were treated with reducing protein sample buffer and were size-fractionated in a 10% SDS-PAGE gel. The resulting gel slabs were electrotransferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH), and the membranes incubated for 2 hours at room temperature in blocking buffer containing 5% non-fat milk in 0.1% Tween-PBS, pH 7.5. The blots were incubated with specific primary antibodies for 1 hour at room temperature. The antibody used for loading control recognizes the 120 kDa transmembrane glycoprotein E-cadherin. To detect ERα, we used a polyclonal antibody raised against the C-terminus of the protein, MC-20 (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes a band of ~67 kDa. The monoclonal antibody used to detect β-casein recognizes a band of ~30 kDa. The blots were washed appropriately with 0.1% Tween-PBS followed by the addition of the appropriate horseradish-peroxidase-conjugated secondary antibody. After 1 hour of incubation and appropriate washes, the signal was detected using the SuperSignal West Dura detection kit (Pierce, Rockford, IL). The intensity of each band was quantified using the ChemiImager (Alpha Innotech Corporation, San Leandro, CA) scanning densitometry equipment.

Quantitative PCR

cDNA was prepared with 2 μg of total RNA using M-MLV reverse transcriptase and oligo-dT primer (Gibco Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Quantification was done using LightCycler and the DNA Master Syber Green I kit (Roche, Indianapolis, IN). The set of primers used in the PCR (forward primer 5′ AGACCGCCGAGGAGGGAGAATGTT 3′ and reverse primer 5′ GGAGCGCCAGACGAGACCAATC 3′) amplify the region between +783 and +1197 of *ERα* mRNA corresponding to the C-terminus of the protein. To normalize the values of *ERα* we performed a quantification of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, forward primer 5′ CCCCTGGCCAAGGTCATCCATGAC 3′ and reverse primer 5′

CATACCAGGAAATGAGCTTGACAAAG 3′) in the same samples. The reactions for both amplifications were carried out for 40 cycles with an annealing temperature of 59°C.

Statistics

All values presented in this study are means±the standard error of the mean (s.e.m.) of at least three independent experiments. Comparisons between groups were performed employing one-way analysis of variance, and differences between means were determined by a Student-Newman-Keuls multiple comparison test.

Results

A reconstituted basement membrane (rBM) stabilizes ERα expression levels in primary mammary epithelial cells

To determine to what extent the loss of ERα expression in culture is caused by disruption of signals from the endogenous BM, we cultured mouse mammary epithelial organoids (Fig. 1Aa) in the presence or absence of rBM (Fig. 1Ab,c). Twenty-four hours after plating, almost 70% of the organoids had attached and spread on plastic, and by 2 days almost all of them had formed monolayers (Fig. 1Ab). There was a significant reduction of ERα protein levels in epithelial cells cultured on tissue culture plastic as early as 1 day after isolation (Fig. 1Ba), and an additional steady decrease throughout the rest of the culture period (9 days). When the organoids were cultured on rBM, however, their morphology was retained to day 10, the longest time tested (Fig. 1Ac), and the early loss of ERα levels was significantly attenuated (Fig. 1Bb,c). To rule out differential adhesiveness of ERα-negative cells to tissue culture plastic, we measured ERα protein levels in cells and organoids that failed to attach to tissue culture plastic during the first 24 hours of culture. ERα protein levels were similar in attached and detached cells (data not shown). As a measure of functional integrity of the organoids, we measured β-casein synthesis and, as expected, cells grown on plastic did not synthesize β-casein (Fig. 1Ba), even in the presence of lactogenic hormones, hydrocortisone and prolactin. But appreciable expression of this milk protein was found after 4 days of culture on rBM (Fig. 1Bb), and its expression continued to increase throughout 10 days of culture. Furthermore, we observed that in the absence of lactogenic hormones, the regulatory effect of rBM on ERα expression disappeared (Fig. 1Bd). For this reason, the next series of experiments were all performed in the presence of insulin, hydrocortisone and prolactin.

ERα is expressed in both the epithelial and stromal components of the mammary gland (Shyamala et al., 2002). To determine which of the two cell types loses ERα expression in culture, we separated them by differential centrifugation of collagenase-dissociated mammary tissue. The stromal component thus obtained contained mainly fibroblasts as determined by vimentin staining (data not shown). We measured ERα levels in mouse primary fibroblasts immediately after isolation (time 0), and after 2 and 6 days of culture in the presence or absence of rBM. Primary fibroblasts cultured on plastic adopted the characteristic spindle-shaped morphology (Fig. 1Ca), but aggregated when cultured on rBM (Fig. 1Cb). The level of ERα in fibroblasts was significantly lower than that in epithelial cells at the time of isolation, time 0 (Fig. 1D, t0), and this level did not change either during the course of the culture or after the addition of rBM (Fig. 1D). These results indicate that loss of BM is partially responsible for the selective loss of ERα in the epithelial component of the mammary gland.

A functionally normal mammary epithelial cell line can be used to dissect the BM response

To dissect the molecular mechanisms involved in the response of mammary epithelial cells to BM and to determine whether BM only reduces degradation of ERα or whether it can also induce ERα de novo, we used a clonal mouse mammary epithelial cell line, Scp2 (Desprez et al., 1993). These cells synthesize little or no BM components in culture. Scp2 cells cultured

on plastic adopted the typical flattened morphology (Fig. 2Aa). BM components were provided by culturing the cells either on top of a rBM gel ('top cultures'; Fig. 2Ab) or by the addition of rBM to the medium of Scp2 cells cultured on plastic (Fig. 2Ac,d,e). An effect on cell morphology (cell rounding) was evident 6 hours after the addition of rBM. The technique of adding a diluted rBM solution to the medium, 'rBM dripping', enables a cleaner dissection of biochemical signals from exogenous ECM components (Roskelley et al., 1994; Streuli et al., 1995b).

The addition of 2% dripped rBM produced a significant increase in the basal ERα expression level in Scp2 cells, although to a lesser extent than in cells plated on top of rBM (Fig. 2Ba). We found that the ERα level was upregulated as early as 12 hours after culturing Scp2 cells in the presence of 2% rBM and reached its maximum level by 24 hours (Fig. 2Bb). Note that, as cells reach high densities, there is endogenous production of BM components that raises the base line of ERα levels on tissue culture plastic as would be expected from our data above. Our experiments also suggested that the effect of rBM on ERα expression was not directly related to the effect of rBM on cellular morphology, given that addition of 5% rBM to the medium had no additional effect in regulating ERα levels (Fig. 2Ba) in spite of its more profound effect on cell morphology (Fig. 2Ac,d,e).

In the mammary gland, ERα is expressed in a fraction of the luminal epithelial cell population (Shyamala et al., 2002). The effect of rBM on ERα levels in culture could be the consequence of increased levels of ERα expression in every cell or an increase in the fraction of cells expressing ERα. To distinguish between these possibilities, we determined by immunofluorescence the percentage of Scp2 cells expressing ERα as well as the level of expression per cell, in the presence or absence of rBM. We found that dripping rBM on the cells increased the percentage of ERα-expressing cells from 21% (on plastic) (Fig. 2Ca,b) to 53% (drip rBM) (Fig. 2Cc,d,e). However, the intensity of fluorescence per cell (ERα level/cell) was not significantly affected by rBM (Fig. 2Ce). These data suggest that rBM can both protect against ERα loss as seen in primary cultures and also induce endogenous expression of ERα in previously silent cells as seen in Scp2 cultures.

BM regulates ER-mediated transcriptional activity in a ligand-independent manner

The function of estrogen involves the binding of ER dimers to target gene promoters that contain a palindromic estrogen-response element (ERE). To determine whether the regulation of ERα levels by rBM is reflected in an increase in the activity of the receptor, we transfected Scp2 cells with an ERE-response element attached to a reporter gene (Klein-Hitpass et al., 1986), generating the Scp2 ERE-TK-CAT cell line. These cells underwent the characteristic morphological differentiation when cultured on top of rBM (Fig. 3Aa,b), analogous to the nontransfected Scp2 cells (Fig. 2Aa,b), and in the presence of lactogenic hormones they functionally differentiated and produced β-casein (data not shown). When exposed to rBM, the ERE-TK-CAT cells exhibited higher reporter activity than cells cultured on plastic (Fig. 3B). This increase in the ER-mediated response reflected the increment in the level of ER expression when the cells were cultured in the presence of rBM (Fig. 2B). However, the effect of rBM on CAT activity was independent of the presence of 17β-estradiol. When estradiol was added to the medium, the proportional increase in reporter activity was similar between plastic and rBM (Fig. 3B). The addition of the ER antagonist ICI 182,780 blocked the increase in CAT activity induced by estradiol but only partially blocked the increase induced by rBM. These results suggest that rBM signals can partially replace the downstream function of estrogen, and stimulate ER transcriptional activity in mouse mammary epithelial cells.

BM upregulates *ERα* mRNA levels

To determine whether BM regulates ERα expression at the mRNA or protein levels, we measured *ERα* mRNA level by quantitative RT-PCR. *ERα* mRNA in cultured primary mammary organoids decreased during the initial 24 hour culture period (data not shown). The decrease was less pronounced when the cells were cultured in the presence of rBM and was similar to the decrease of ERα protein levels (Fig. 1Bc). After the first 24 hours, however, *ERα* mRNA levels remained significantly higher when cells were cultured in the presence of rBM (Fig. 4A). Similarly, when *ERα* mRNA levels were evaluated in Scp2 cells, we found a significant increase in cells grown for 3 days in the presence of rBM compared with cells grown on plastic (Fig. 4B). These results indicate that the regulatory effect of rBM on ERα expression in mammary epithelial cells is exerted, at least in part, at the mRNA level.

The rBM-induced increase in ERα levels is due neither to reduced proliferation nor to changes in cell shape

Shoker et al. have shown mutual exclusion between ER and Ki67 antigen in luminal epithelial cells in normal breast (Shoker et al., 1999). We had shown previously that rBM inhibits the growth of cultured mammary epithelial cells (Petersen et al., 1992; Weaver et al., 1997). Therefore, the increase in ERα elicited by rBM could be due to a reduced growth rate. To test this hypothesis, we cultured Scp2 cells for 3 days in the presence of different levels of insulin to modulate cellular proliferation (Srebrow et al., 1998). Growth of Scp2 cells was stimulated by insulin and attenuated by rBM as shown by BrdU incorporation (Fig. 5A). There was no correlation between ERα expression in the cell population (Fig. 5Ba,b) and the proliferation status of the cells (Fig. 5A), regardless of the presence or absence of rBM. Whether the proliferating cells can express ERα while dividing in culture remains to be seen. In any case, our results show that the upregulatory effect of rBM on ERα expression is not due to a differential proliferation rate.

Another effect of adding rBM to epithelial cells is the change from ‘flat’ to ‘rounded’ cell morphology (Fig. 1A, Fig. 2A), and this change itself has been shown to induce alterations in gene expression (Roskelley et al., 1994;Close et al., 1997). To determine whether cell rounding per se was responsible for the regulation of ERα by rBM, we cultured primary mammary epithelial cells and Scp2 cells on polyHEMA-coated dishes, which prevents cell attachment and forces cells to remain in suspension as rounded-aggregates (Fig. 6Aa-d). This type of culture reproduces some aspects of the morphological changes induced by rBM, including cell rounding (Roskelley et al., 1994;Muschler et al., 1999). Culture on polyHEMA did not alter ERα protein levels either in primary organoids (Fig. 6Ba) or in Scp2 cells (Fig. 6Bb). Adding rBM to these cultures increased ERα expression (Fig. 6Ba,b) without affecting the level of cellular aggregation further (Fig. 6Ae,f). These observations indicate that, unlike the expression of milk protein genes (Roskelley et al., 1994;Close et al., 1997), morphological changes per se are not required for regulation of ERα levels by BM.

Collagen-IV and laminin-1 are the BM components responsible for the regulation of ERα levels

To establish which components of the rBM regulated ERα, we treated primary mammary epithelial and Scp2 cells with purified ECM components at a final concentration equivalent to that in 2% rBM (see Materials and Methods). The presence of ECM components did not affect the attachment and spreading of isolated mammary epithelial organoids (Fig. 7Aa-e). Collagen-I was ineffective in maintaining ERα levels, but collagen-IV and laminin-1 attenuated the reduction of ERα levels (Fig. 7Af). However, laminin-1 and collagen-IV in combination did not synergize, suggesting that the intracellular signals elicited by these molecules may converge on the same downstream targets.

The same ECM components were tested also on Scp2 cells. Collagen-I or -IV had no obvious effect on cell shape (Fig. 7Ba,b) but as expected, laminin-1 (Fig. 7Bc) or rBM (Fig. 2Ac,d,e) induced cell rounding. As in primary cultures, collagen-IV was the most effective BM component tested in increasing ERα protein levels in Scp2 cells; laminin-1 was effective to a lesser degree, and collagen-I or fibronectin had no effect (Fig. 7Bd; data not shown). The fact that collagen-IV increased ERα levels without involving discernable cell rounding, together with the previous observations that culture on polyHEMA had no effect on the regulation of ERα, further indicate that, unlike many other changes in gene expression induced by rBM, ERα regulation is most probably independent of mechanochemical changes in the cytoskeleton. Because the effect of collagen-IV and laminin-1 on ERα levels were comparable to the increase observed after dripping rBM in Scp2 cells (Fig. 2Ba), we conclude that the major components responsible in the BM are collagen-IV and laminin-1.

The BM-induced regulation of ERα expression can be abrogated by integrin-blocking antibodies

The specificity of two individual BM components to reproduce the regulatory effect of rBM on ERα expression and lack of response by other components indicated the involvement of specific ECM receptors. We therefore used blocking antibodies to identify which ECM receptors transduce the signals to the cell nucleus. Cells in the mammary gland express α1β1, α2β1, α3β1, α6β1 and α6β4 heterodimers, all of which may potentially bind to laminin (for reviews, see Alford and Taylor-Papadimitriou, 1996; Mercurio et al., 2001), although α1β1 and α2β1 mainly serve as collagen receptors (Zutter and Santoro, 1990). We analyzed the effect of blocking antibodies directed specifically against −α1, −α2, −α6, or −β1 integrin subunits. These antibodies did not affect cell viability during the assay period as determined by the Alamar blue assay [(Lochter et al., 1999); data not shown]. Primary mammary cells cultured on plastic, or in the presence of collagen-IV or laminin-1 (Fig. 8A) and Scp2 cells cultured on plastic, or in the presence of collagen-IV or rBM (Fig. 8B) were treated with the specific integrin-blocking antibodies. In both cell types, α2, α6, and β1 antibodies partially blocked the effect of rBM on ERα protein levels, whereas α1 blocking antibody had no effect. Specifically, α2 and β1 antibodies blocked the regulatory effect of collagen-IV on ERα levels, whereas α2, α6 and β1 antibodies effectively abolished the regulatory effect of laminin-1. These results indicate that integrin-mediated cell adhesion to BM components and the subsequent activation of integrin signaling pathways are involved in the regulation of ERα levels in mammary epithelial cells.

Discussion

We show here that a culture system in which appropriate cell-BM interactions are re-established can be used to restore appreciable ERα expression and function in non-malignant mammary epithelial cells. We found that a reconstituted BM exerts this regulatory effect on ERα through signaling induced by two of the individual matrix components, collagen-IV and laminin-1. These signals were due neither to changes in cell shape nor inhibition of cell proliferation. When cell-BM interactions were disrupted by function-blocking antibodies against α2, α6 or β1 integrin subunits, the regulatory effect of rBM was abolished. Furthermore, our data suggest that the increase in the level of ERα in normal mammary epithelial cells in response to rBM is due, at least in part, to the induction of ERα expression in previously nonexpressing cells within the population.

BM is a regulator of ERα expression in primary cultures and in a mammary epithelial cell line

The action of rBM on ERα expression in both primary mammary epithelial cells and Scp2 could be largely replaced by BM components collagen-IV and laminin-1. The regulatory effect on ERα expression is not coupled to the cytoskeletal reorganization induced by rBM because

collagen-IV reproduced the ERα-enhancing effect without inducing morphological alterations, and mammary epithelial cells maintained in suspension as rounded-aggregates on polyHema-coated dishes did not alter ERα levels or function.

Differences between our results and previous investigations on the role of specific ECM components on ERα regulation are informative. The study by Woodward et al. on MCF-7 and T47-D human breast tumor cell lines showed downregulation of the ER-mediated response without affecting ERα levels when cultured on laminin gels (Woodward et al., 2000), whereas our results showed an upregulation of ERα levels and of an ER-mediated response in non-malignant mouse mammary epithelial cells under similar conditions. These differences suggest that tumor cells, which often have a higher level of ERα in culture, may have become independent of the ECM response. Our preliminary data with a mouse tumor cell line are consistent with this hypothesis (data not shown). Edery et al. reported that the expression of ERα was maintained by embedding mammary epithelial cells within collagen-I gels (Edery et al., 1985), whereas we found that ERα levels remained low when collagen-I was added to the medium. Primary cultures are a mixed population of epithelial, myoepithelial and stromal fibroblasts. The latter two cell types are known to contribute to BM production. We have shown previously (Streuli and Bissell, 1990) that when primary cells were cultured in collagen-I gels and allowed to contract and form three-dimensional structures, they could synthesize and deposit their own BM. It is therefore most likely that the effect of collagen-I reported by Edery et al. is due to the newly synthesized BM, rather than signaling by collagen-I per se (Edery et al., 1985). This conclusion is supported by the fact that in the clonal cell line Scp2, which is unable to produce and organize its own BM, collagen-IV and laminin-1 were able to up-modulate ERα, whereas collagen-I was ineffective. However, the initial drop in ERα expression that occurs even in the presence of rBM after isolating mammary epithelial cells from their surrounding myoepithelial, adipocyte and fibroblast cells, suggests that other aspects of the stromal-epithelial interaction could also participate in the regulation of ERα expression.

Ligand-independent regulation of ER activity by BM

Several groups have reported ligand-independent activation mechanisms for ER and other steroid hormone receptors in a number of cell types (Kato et al., 1998; Weigel and Zhang, 1998). One such mechanism involves the association of ER with the steroid receptor coactivator (SRC) family. The binding of estrogen to ER induces a conformational change that leads to exposure of the activation function (AF) 2 domain of ERα, which contains a binding site for SRC-1. In the absence of hormone, the activation of the ER could occur through a third protein that recruits the coactivators to ER (Bernards, 1999). Cyclin D1 might be one of these ‘bridges’, as it is capable of binding ER and SRC-1 simultaneously and its overexpression induces an ER-mediated response in the absence of estrogen in several cell lines, including Scp2 (Neuman et al., 1997; Zwijsen et al., 1997; Zwijsen et al., 1998). Furthermore, D-type cyclins have previously been implicated as downstream targets of ECM signaling pathways (Buckley et al., 1997; Yu et al., 2001), and Neuman et al. have shown that ECM increases cyclin D1 levels (Neuman et al., 1997). Other evidence supporting the notion that the cellular microenvironment modulates ER function through cyclin D1 in mammary epithelial cells comes from Lamb et al., who showed in MCF-7 cells that the association between ER and cyclin D1 is enhanced when cells are cultured in the presence of lactogenic hormones and pre-adipocytes (Lamb et al., 2000). Our results showing that a portion of the BM-induced upregulation of ER-mediated transcriptional response was independent of the presence of estradiol and that it was not completely blocked by the antagonist ICI 182,780 support the above data. However, we cannot rule out other signaling events that also lead to a ligand-independent transcriptional activity of the ER; for example, the binding to different coactivators that have intrinsic histone acetylase activity, or the phosphorylation of the receptor by the Ras-MAPK (mitogen-activated protein kinase) or the PI3k/Akt pathways in response to growth factors (for reviews, see McDonnell

and Norris, 2002; Ali and Coombes, 2002). The study of these pathways is crucial for the understanding of the mechanisms that result in anti-estrogen resistance of ER-positive breast tumors, which constitutes a significant clinical problem. However, until now, none of these mechanisms had been studied in the presence of rBM. It is clear from our data that BM regulates ER function at multiple levels, and the overall increase in ERα levels may be a cumulative response.

Hormonal status and ERα expression

We observed that the presence of lactogenic hormones, insulin, hydrocortisone and prolactin, is required for the regulatory effect of rBM on ERα levels, as primary mammary epithelial cells could not maintain ERα expression in media lacking these hormones, even when rBM was added. Using medium lacking lactogenic hormones, Xie et al. reported that ERα levels were not maintained in nulliparous mouse-derived cells cultured on laminin, fibronectin, collagen-I, collagen-IV or tenasin (Xie et al., 1997). However, ERα levels were maintained when cultures were prepared from pregnant animals. Taken together with our data, these results suggest that a pretreatment of mammary epithelial cells with high prolactin levels, such as those found in pregnant animals, is necessary for the cells to be responsive to BM-induced ERα expression. Interestingly, certain properties of ER (acidity, molecular weight, DNA binding capacity, responsiveness to estrogen) are different in the lactating vs. non-lactating (nulliparous) mammary gland (Gaubert et al., 1986; Shyamala et al., 1992) (for a review, see Shyamala et al., 2002), providing further support for the idea that the hormonal status of the animal during pregnancy and lactation alters ER function and level. The fact that ER regulation by BM is different under different hormonal conditions suggests that ER expression is part of the broader process of the mammary gland differentiation. This process has been shown to require interactions between prolactin and ECM involving STAT5 (signal transducer and activator of transcription factor 5) (Streuli et al., 1995a; Myers et al., 1998). However, we observed that collagen-IV could induce ERα expression without inducing β-casein production (data not shown), indicating that regulation of ERα and β-casein are independent processes, despite being prolactin- and BM-dependent.

The regulation of ERα by BM is mediated by integrins: relevance to tumorigenesis

We have found that integrin-activated signal transduction pathways are responsible for the regulation of ERα levels by BM and its components. However, the involvement of other nonintegrin receptors, such as dystroglycan, cannot be ruled out (Muschler et al., 2002). The role of integrins in the regulation of mammary gland development and gene expression is crucial for understanding tumor progression. Alterations in the microenvironment or altered signaling through the receptors that sense the microenvironment can cause normal cells to display tumorigenic behaviour, and vice versa (for a review, see Bissell and Radisky, 2001). We had previously shown that cultivation of human breast cancer cell lines within either a rBM or a collagen-I gel led to differences in the levels of specific integrins (Howlett et al., 1995). Furthermore, altered expression of α2-, α3-, α6-, β1- and β4-integrins has been reported in breast cancer cell lines and in mammary tumor tissue sections (Natali et al., 1992; Gui et al., 1995; Zutter et al., 1995). These changes in integrin expression may result in altered cell surface ratios of individual integrins, which could, in turn, affect tissue organization and lead to tumor progression via altered intracellular signaling. We showed in this report that laminin-1 exerts its effect via α2, α6 and β1 integrins, whereas collagen-IV induces ERα expression via α2 and β1 integrins. Taking the previous literature and our present report, it is possible that changes in the composition of the ECM leads to changes in the ECM receptor profiles of these cells, which, in turn, could alter the ERα levels.

The α2 gene promoter contains estrogen-response elements (EREs) (Zutter et al., 1994), suggesting that estrogen may play a role in the regulation of integrin expression, and therefore

in tumor cell invasion. It is possible that collagen type IV signaling and ER are connected by a positive feedback loop in mammary epithelial cells: collagen type IV, through its receptor α2β1, increases ERα levels, and in turn, ERα stimulates the expression of α2 integrin subunit. In this regard, ER gene expression has been positively correlated with α2β1 integrin and collagen-IV expression in breast carcinomas. Ductal carcinomas that lack ER also lack α2β1 expression and are more invasive (Maemura et al., 1995; Lanzafame et al., 1996).

In conclusion, we have determined that adhesion to particular components of the BM upregulates ERα both in primary cultures of normal mammary epithelial cells and in an established mammary epithelial cell line, but not in mammary fibroblasts. Thus, context-dependent regulation of ERα activity appears to be a fundamental and specific property of mammary epithelial cells. These data may provide a possible explanation for the loss of ER expression that occurs during breast tumor progression. We have shown previously that malignant mammary epithelial cells are irresponsive to adhesive clues from the BM (Petersen et al., 1992) (for reviews, see Werb et al., 1996; Bissell and Radisky, 2001). The presence of significant amounts of ERα in breast tumors is an indication of hormone responsiveness and it is a critical determinant of the prognosis and therapeutic management of breast cancer patients (Lapidus et al., 1998; Sommer and Fuqua, 2001). Breast tumors that acquire hormone independence usually display a less differentiated and more aggressive phenotype. The concepts developed in this paper may be applicable to the study of breast cancer cells to decipher why some breast cancers remain hormone-sensitive and ER-positive, whereas others develop into hormone-resistant ER-negative tumors.

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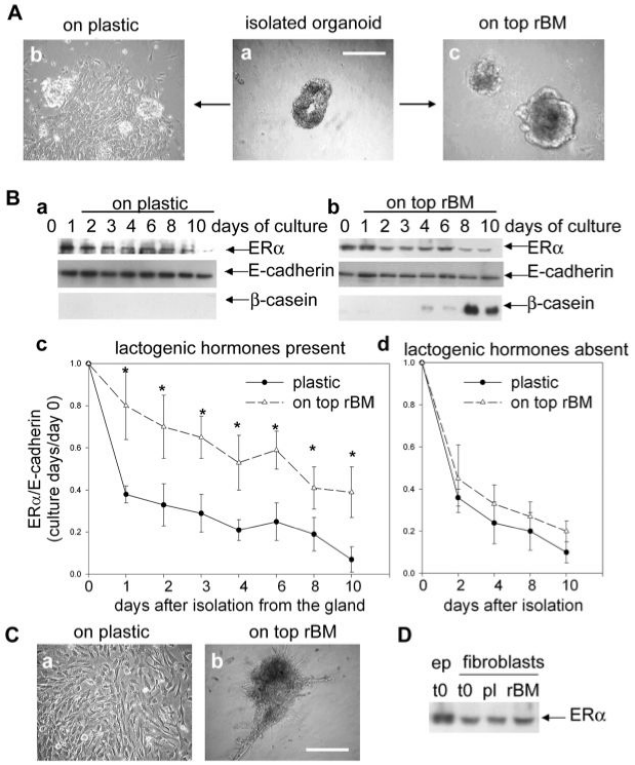

Fig. 1.

Reconstituted basement membrane (rBM) allows partial maintenance of ERα protein levels in primary mammary epithelial cells, but has no effect in primary fibroblasts. (A) Morphology of (a) freshly isolated mammary organoids derived from mouse mammary glands (time 0); (b) primary epithelial cells grown for 2 days on plastic; or (c) on top rBM. Bar, 100 μm. (B) Representative western blots showing ERα and β-casein protein expression in cell extracts derived from primary mammary epithelial cells grown in the presence of lactogenic hormones for 10 days on plastic (a) or on top of rBM (b). Total E-cadherin levels do not change in these experiments and were used for loading control. Quantification was by densitometry of ERα protein levels from mammary epithelial cells grown on plastic (solid line) or on top of rBM (dotted line) in the presence (c) or absence (d) of lactogenic hormones. ERα was expressed in relative units (day of isolation of the organoids, time 0, corresponds to 1). Results are mean and s.e.m. of five different primary culture preparations using a pool of bilateral 4th inguinal mammary glands from ten nulliparous Balb/c mice in each preparation. *P<0.05 vs. plastic. (C) Morphology of mammary fibroblasts grown for 6 days on plastic (a) or on top of rBM (b). Bar, 100 μm. (D) ERα protein expression in epithelial cells (ep) and fibroblasts just after isolation from the gland (t0), and in fibroblasts grown for 6 days on plastic (pl) or on top of rBM (rBM).

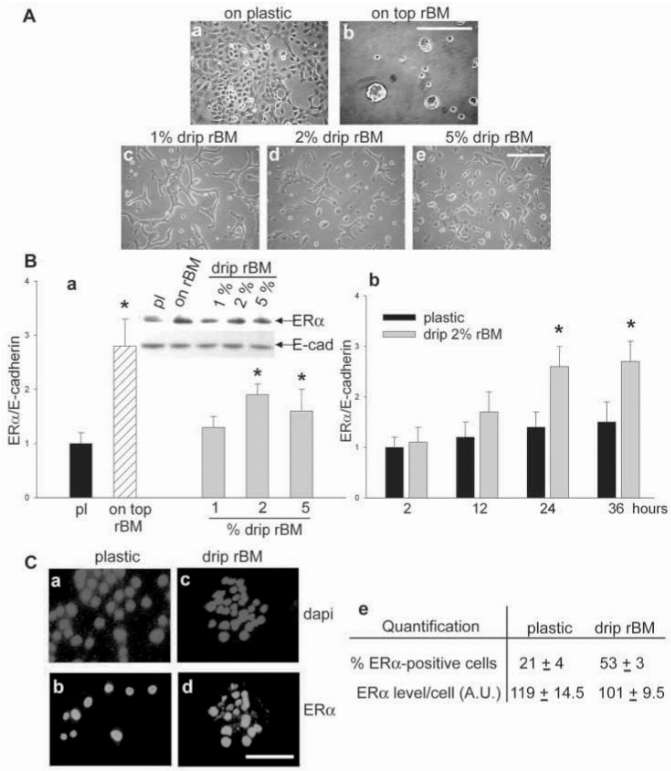

Fig. 2.

rBM increases ERα protein levels in a clonal mammary epithelial cell line.

(A) Morphological changes in Scp2 cells. Cells grown on plastic formed a flattened monolayer (a), whereas cells grown on top of rBM aggregated and formed acinus-like structures (b). Bar, 100 μm. They progressively become rounded when cultured in the presence of increasing concentrations of rBM (c-e). Bar, 100 μm. (B) In Scp2 cells grown as indicated in A for 3 days, ERα protein levels were determined by western blot (a, representative gel) quantified by densitometry and expressed relative to the values measured on plastic (a, bar graph). In Scp2 cells grown for the indicated hours after the addition of serum-free medium containing lactogenic hormones in the presence (grey bars) or absence (black bars) of 2% rBM (b), ERα protein levels were determined by western blot and expressed relative to the values measured on plastic 2 hours after the addition of the new medium. n=5; *P<0.05. (C) Immunofluorescence of ERα in Scp2 cells grown for 2 days on plastic (a,b) or in the presence of 2% rBM (c,d). Note that not all the cells (DAPI staining of DNA; blue) stained for ERα (green), and that the staining for ERα was predominantly nuclear. Bar, 20 μm. The table (e) shows mean ± s.e.m. corresponding to percentages of ERα-positive cells and to ERα expression levels quantified in single cells grown on plastic or in the presence of rBM. Intensity of fluorescence was expressed as arbitrary units (A.U.). At least 100 cells were analyzed for each culture condition. The proportion of ERα-positive cells was higher in the presence of rBM.

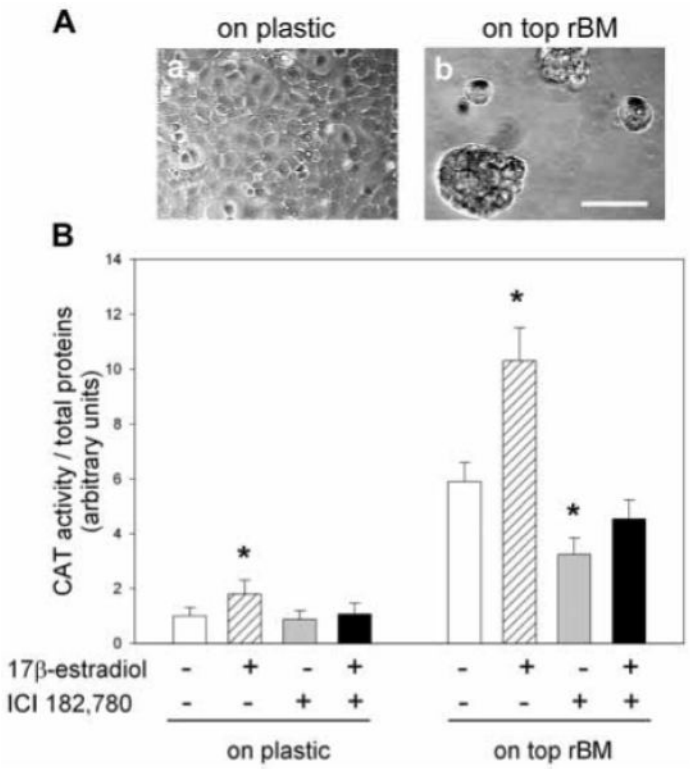

Fig. 3.

rBM increases ER-mediated response independently of estradiol. (A) Morphological changes in Scp2-ERE-TK-CAT cells grown on plastic (a) or on top rBM (b). Bar, 25 μm. (B) ER-mediated transcriptional activity (measured using CAT as reporter gene) was quantified in cells grown for 3 days on plastic or on top rBM, in the presence or absence of 17β-estradiol 10^{-8} M, or the antagonist ICI 182,780 10^{-7} M for the last 48 hours in culture. The reporter activity was expressed relative to values measured in cells grown on plastic in the presence of the vehicle ethanol. n=4; *P<0.01 versus ethanol for each substratum.

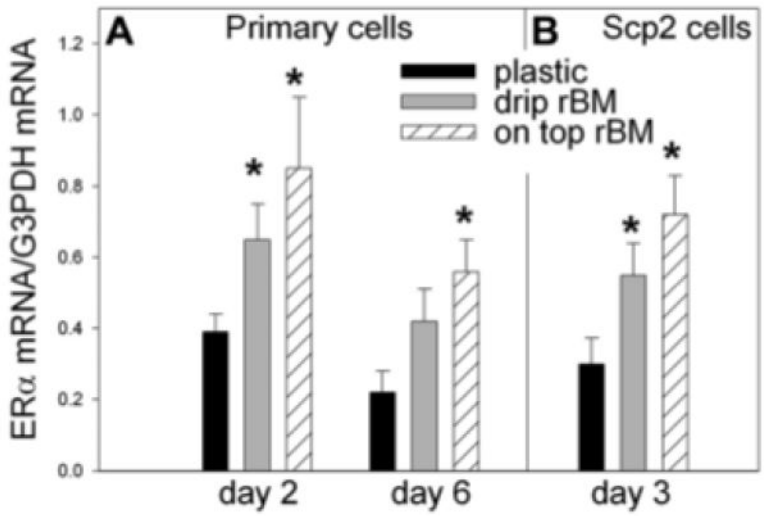

Fig. 4.

rBM increases ERα mRNA levels in mammary epithelial cells. ERα mRNA was analyzed by quantitative RT-PCR 2 and 6 days after isolating primary cells from the mammary gland (A), or in Scp2 cells cultured for 3 days (B) on plastic or in the presence of rBM (dripped or on top). Values were normalized with respect to GAPDH mRNA. Three independent experiments comprising different primary culture preparations or Scp2 cultures were used to create the graph. *P<0.05 vs. plastic for each condition.

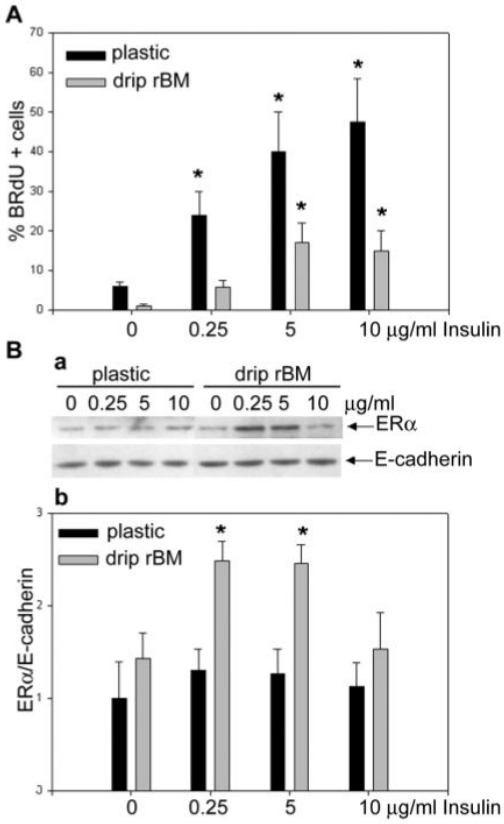

Fig. 5.

The regulatory effect of rBM on ERα expression is not due to changes in growth rate. (A) Proliferation of Scp2 cells grown for 3 days on plastic or in the presence of rBM after adding different concentrations of insulin (in the absence of serum or other growth factor). Proliferation was determined as the percentage of BrdU-labeled cells by immunofluorescence. (B) ERα protein levels in Scp2 cells grown in the presence of different insulin concentrations as indicated in A were evaluated by western blot (a, representative gel) and quantified by densitometry (b). ERα expression did not correlate with the proliferation status of the cells, regardless of the substrata. n=4; *P<0.05 versus no insulin.

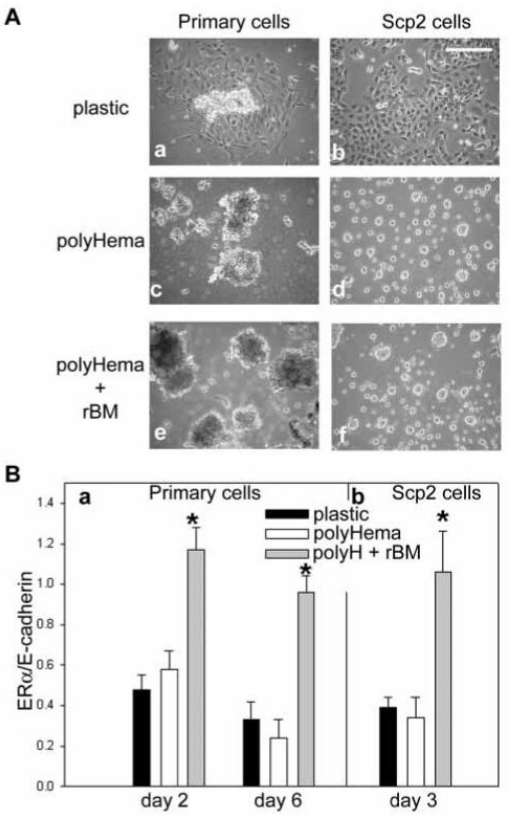

Fig. 6.

The regulatory effect of rBM on ERα levels is independent of cell shape changes. (A) Primary cells (a) and Scp2 cells (b) form typical flattened monolayers on plastic. Morphological change (cell rounding and aggregation) can be induced by the non-adherent substratum polyHEMA in primary cells (c) and in Scp2 cells (d). The addition of rBM on polyHEMA did not affect this cell rounding (e,f). Bar, 100 μm. (B) The densitometric analysis of ERα protein levels determined by western blot in primary mammary epithelial cells at days 2 or 6 after isolation (a) and in Scp2 cells at day 3 in culture (b) shows that addition of rBM to pre-rounded cells was necessary to upregulate ERα over basal levels detected on plastic. n=3; *P<0.05.

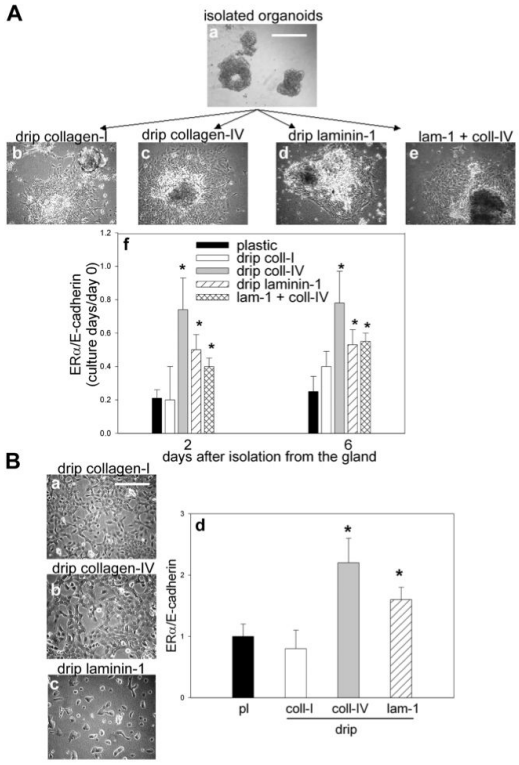

Fig. 7.

The regulatory effect of rBM on ERα levels is reproduced by purified collagen-IV and laminin-1.

(A) Isolated mouse mammary epithelial organoids (a) were grown in the presence of different matrix components dripped into the medium: collagen-I (b), collagen-IV (c), laminin-1 (c) or a mixture of laminin-1 and collagen-IV (e). In all cases the organoids were attached and spread on the plastic after 2 days in culture. Bar, 100 μm. ERα protein levels were measured in these different culture conditions by western blot at days 2 and 6 after culture, and expressed as relative units to levels at time 0 (f). Results are the average of four different primary culture preparations. **P*<0.05 vs. plastic. (B) Scp2 cells formed monolayers after dripping collagen-I (a) or collagen-IV (b), and adopted a rounded morphology when laminin-1 was dripped into the medium (c). Bar, 100 μm. ERα protein levels were determined by western blot at day 3 in culture, quantified by densitometry and were expressed relative to the values measured on plastic (d). *n*=5; **P*<0.05.

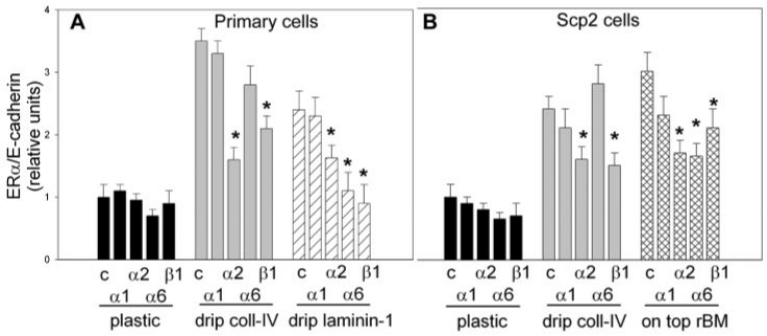

Fig. 8.

The regulatory effect of collagen-IV and laminin-1 on ERα levels is mediated by α2, α6 and β1 integrin subunits. ERα protein levels were determined by western blot in primary mammary epithelial cells (A) and in Scp2 cells (B) after 3 days adding mouse IgG (control, c), or 10 μg/ml of α1, α2 or α6 or 5 μg/ml of β1 integrin blocking antibodies to cells grown on plastic or in the presence of collagen-IV, laminin-1 or rBM. n=3; *P<0.05 versus control (IgG addition).