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Interactions between normal mammary epithelial cells (HMECs) and extracellular matrix (ECM) are important for mammary gland homeostasis and loss of ECM-sensitivity is an early event in mammary carcinogenesis. The purpose of this grant is to investigate how the CREBP-binding protein (CBP) might target the elimination of damaged HMECs. We have observed that 1) suppression of CBP results in apoptosis-resistance through impaired laminin expression and 2) CBP promotes induction of interferon-regulated genes during apoptosis. These findings will provide novel targets for chemoprevention and are being used to develop markers for response to current prevention strategies.

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INTRODUCTION:

Interactions between normal mammary epithelial cells (HMECs) and extracellular matrix (ECM) are important for mammary gland homeostasis; loss of ECM-sensitivity is thought to be an early event in mammary carcinogenesis. The CREBP binding protein (CBP) is known to regulate both proliferation and apoptosis but the role of CBP in ECM-signaling is poorly characterized. **The purpose of this grant is to investigate how CBP might regulate apoptosis in HMECs. Major findings and progress in fulfillment of Specific Aim I:** We investigated the relationship between CBP expression and sensitivity to prepared ECM (rECM)-induced growth arrest and apoptosis in an *in vitro* model of early mammary carcinogensis. Suppression of CBP expression in HMECs by antisense oligonucleotides (ODNs) resulted in loss of rECM-mediated growth regulation, polarity, and apoptosis. Chromatin immunoprecipitation studies (ChIP) and reporter studies demonstrated that inhibition of CBP protein expression resulted in 1) loss of CBP-occupancy of the *LAMA3A* promoter and 2) a decrease in *LAMA3A* promoter activity. rECM-resistance correlated with 1) loss of CBP occupancy of the *LAMA3A* promoter, 2) decreased *LAMA3A* promoter activity, and 3) loss of laminin-5 α3-chain mRNA and protein expression. These observations suggest a critical role for CBP in rECM-mediated growth regulation, polarity, and apoptosis through modulation of *LAMA3A* activity and laminin-5 α3-chain expression. **Major findings and progress in fulfillment of Specific Aim II:** CBP is a known regulator of interferon (IFN)-signaling and dysregulated expression of IFN-signal transduction genes has been observed during mammary carcinogeneis. We hypothesize that the level of CBP expression is critical for the induction of IFN-activated transcription during rECM-mediated apoptosis in HMEC-E6 cells. The role of CBP expression in modulating IFN-signaling is currently being tested in apoptosis-sensitive HMEC-E6 cells. We observed that CBP regulates induces expression of interferon regulated genes in the absence of interferon release.

BODY:

SPECIFIC AIM I: Does the level of the CREBP binding protein (CBP) protein expression in normal human mammary epithelial cells (HMECs) determine sensitivity to prepared extracellular matrix (rECM)-mediated apoptosis? [See appended manuscript Dietze et al.]

1a) Suppression of CBP in early passage HMECs by antisense oligonucleotides (ODNs). Antisense ODNs were utilized to suppress CBP protein expression in HMECs to test whether the level of CBP protein expression might be important for rECM-sensitivity. Relative levels of CBP protein expression were tested by Western analysis. Early passage HMEC vector controls (HMEC-LXSN) and early passage apoptosis-sensitive HMECs transduced with human papillomavirus-16 E6 protein (HMEC-E6) treated with the active, CBP-specific ODN,

A3342V, exhibited a 65% and 72% respective decrease in CBP protein expression relative to untreated controls. Cells treated with the inactive CBP ODN, scrA3342V, did not exhibit a significant decrease in CBP protein expression.

1b) Suppression of CBP enhances proliferation in rECM. Treatment of early passage HMEC-LXSN controls and HMEC-E6 cells with CBP-specific ODNs resulted in enhanced proliferation in rECM-culture as measured by 1) physical growth parameters and 2) Ki-67 staining. Both early passage HMEC-LXSN controls and HMEC-E6 cells treated with active CBP-specific ODNs (A33243V) demonstrated a continued increase in sphere diameter from Day 7-9 in rECM culture. In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive ODNs (scrA33243V) did not exhibit an increase in sphere diameter after Day 7. Treatment of early passage HMEC-LXSN and HMEC-E6 cells with CBP-specific ODNs (A33243V) resulted in continued Ki-67 staining at 9 and 11 days in rECM culture. In contrast, Ki-67 staining at 9 and 11 days were markedly reduced in HMEC-LXSN and HMEC-E6 cells treated with inactive ODNs (scrA33423V). These observations show that suppression of CBP protein expression in early passage HMEC-E6 cells and HMEC-LXSN controls resulted in enhanced proliferation in rECM culture.

1c) Suppression of CBP protein results in altered localization and expression of biochemical markers of polarity. Early passage HMEC-E6 cells and HMEC-LXSN controls treated with active CBP ODNs (A33423V) exhibited a loss of epithelial polarity as evidenced by dispersed and intracellular staining of 1) E-cadherin and 2) the tight junction-associated protein, ZO-1. In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive CBP ODNs (scrA33423V) demonstrated expression of E-cadherin and ZO-1 at the cell-cell junction, consistent with a correctly polarized epithelium. These observations indicate that suppression of CBP protein expression in HMECs by antisense ODNs promotes a loss of epithelial polarity in rECM culture.

1d) Suppression of CBP inhibits apoptosis in rECM culture. Early passage HMEC-E6 cells were treated with CBP-specific antisense ODNs to test whether suppression of CBP protein expression blocked apoptosis in rECM culture. Early passage HMEC-E6 treated with CBP-specific antisense ODNs (A33423V) formed large irregular clusters in rECM and did not undergo apoptosis as assessed by either electron microscopy or TUNEL-staining. In contrast, early passage HMEC-E6 cells treated with inactive CBP ODNs underwent apoptosis on Day 7 as assessed by either morphologic criteria or TUNEL-staining. Similar to early passage HMEC-E6 cells, early passage HMEC-LXSN cells treated CBP-specific, antisense ODNs (A33423V) formed large irregular clusters in rECM. Early passage HMEC-LXSN controls treated with inactive ODN (scrA33423V) formed a morphologically organized, ascinus-like

structure and did not undergo apoptosis consistent with what has been previously observed for early passage HMEC-LXSN untreated controls. These observations demonstrate that suppression of CBP protein expression in early passage HMEC-E6 cells by antisense ODNs blocks apoptosis in rECM culture.

A second HMEC strain, AG11134, was tested to ensure that these observations were not HMEC strain-specific. Similar to observations made in HMEC strain AG11132 above, 1) early passage AG11134-E6 cells treated with inactive CBP ODN (scrA33423V) were sensitive to rECM-growth regulation and underwent apoptosis at Day 7, 2) early passage AG1134-LXSN controls treated with antisense-CBP ODN (A99424V) were resistant to rECM growth arrest and did not undergo apoptosis at Day 7-9, and 3) early passage AG11134-LXSN controls treated with CBP-specific antisense ODNs were resistant to rECM-mediated growth regulation and did not undergo apoptosis (data not shown).

1e) Laminin-5 expression is decreased in rECM resistant, late passage HMEC-E6 cells. We previously observed that sensitivity to rECM-apoptosis in early passage HMEC-E6 cells required polarized expression of α3/β1-integrin. Differential gene expression studies, semi-quantitative RT-PCR, and Western analysis were performed to test whether the loss of sensitivity to rECM-mediated growth regulation, polarity, and apoptosis observed in late passage HMEC-E6 cells correlated with altered expression of laminin-5 and/or α3/β1-integrin mRNA. Differential gene expression studies demonstrated decreased expression of all three laminin-5 chains (α3, β3, and γ2) in apoptosis-resistant, late passage HMEC-E6 cells relative to early passage HMEC-LXSN controls and early passage HMEC-E6 cells grown in rECM. Semi-quantitative RT-PCR confirmed a 98% decrease in laminin-5 α3-chain ($p \leq 0.01$), an 88% decrease in laminin-5 β3-chain ($p \leq 0.01$), and a 75% decrease in laminin-5 γ2-chain ($p \leq 0.01$) mRNA expression relative to early passage HMEC-LXSN controls. There was no significant change in the level of α3/β1-integrin mRNA expression. Western analysis similarly demonstrated an 85% ($p < 0.001$) decrease in laminin-5 α3-chain protein expression in apoptosis-resistant, late passage HMEC-E6 cells relative to early passage HMEC-LXSN controls. Expression of laminin-5 α3-chain protein did not significantly vary between early passage HMEC-LXSN cells and early passage HMEC-E6 cells (Figure 6 *d*). There was a 130% ($p < 0.002$) increase in laminin-5 α3-chain protein in late passage HMEC-LXSN controls relative to early passage HMEC-LXSN cells. These observations demonstrate that the presence of rECM-resistance in late passage HMEC-E6 cells correlates with a loss of laminin-5 mRNA and protein expression.

1f) Lack of polarized expression of laminin-α3 and integrin-α3 proteins in late passage HMEC-E6 cells grown in rECM. Early and late passage HMEC-LXSN controls and HMEC-

E6 cells were grown in rECM and tested for 1) laminin-5 α3-chain and 2) α3- and β1-integrin expression by immunohistochemistry (clones P5H10, P1F2, and P4C10, respectively). Early and late passage HMEC-LXSN controls and early passage HMEC-E6 cells exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins. In contrast, late passage, CBP-"poor" HMEC-E6 cells grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of both laminin-5 α3-chain and α3-integrin. As predicted by differential gene expression studies and Western analysis, there was also a qualitative decrease in laminin-5 α3-chain expression in late passage HMEC-E6 cells relative to controls. Late passage HMEC-E6 cells grown in rECM exhibited polarized basal β1-integrin expression but had an increase in the amount of cytosolic expression relative to early passage cells. These observations demonstrate a loss of polarized expression of laminin-5 α3-chain and α3-integrin in rECM-resistant late passage HMEC-E6 cells.

1g) Suppression of CBP expression in HMECs alters both laminin-α3 and integrin-α3 protein expression in rECM culture. We observed that late passage HMEC-E6 cells grown in rECM culture exhibit 1) reduced levels of CBP protein expression and 2) disorganized expression of both laminin-5 α3-chain and α3-integrin. This observation led us to hypothesize that suppression of CBP in HMECs would alter laminin-5 α3-chain and α3-integrin expression and/or distribution. CBP protein expression was suppressed in early passage HMEC-E6 cells and HMEC-LXSN controls by treatment with CBP-specific, antisense ODN (A99424V). HMECs with suppressed CBP expression exhibited disorganized plasma membrane and cytosolic distribution of laminin-5 α3-chain and α3-integrin. β1-integrin expression was observed at the basal surface. In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive CBP ODN (scrA99424V) exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins . These observations demonstrate that suppression of CBP protein expression in HMECs alters the distribution of both laminin-5 α3-chain and α3-integrin.

1h) Decreased CBP expression in rECM-resistant late passage HMEC-E6 cells correlates with decreased LAMA3A promoter activity. We tested whether the observed decrease in CBP and laminin-5 α3-chain expression in late passage HMEC-E6 cells correlated with decreased LAMA3A promoter activity. Early and late passage HMEC-E6 and passage-matched HMEC-LXSN controls were transiently transfected with a CAT reporter coupled to the *LAMA3A* promoter sequence (1403 bp, GenBank Accession Number AF279435) and grown in rECM culture. rECM-sensitive early passage HMEC-E6 cells and early and late passage HMEC-LXSN controls exhibited a similar level of *LAMA3A* activity. In contrast, rECM-resistant, late passage HMEC-E6 cells with decreased CBP and laminin-5 α3-chain expression exhibited a 91% decrease in *LAMA3A* promoter activity relative to early passage HMEC-E6 cells ($p \leq 0.01$).

These experiments demonstrate in HMECs a positive correlation between 1) the level of CBP and laminin-5 α3-chain protein expression and 2) *LAMA3A* promoter activity.

1i) LAMA3A promoter activity in HMECs with suppressed CBP protein expression. We next tested whether suppression of CBP protein expression resulted in decreased *LAMA3A* promoter activity. LAMA3A-CAT reporter activity was compared in early passage HMEC-LXSN and HMEC-E6 cells treated with either CBP-specific antisense ODNs (A33423V) or inactive ODNs (scrA33423V). A 92% and 89% decrease ($p<0.01$) in *LAMA3A* promoter activity was observed, respectively, in early passage HMEC-LXSN and HMEC-E6 cells grown in rECM and treated with CBP-specific ODNs (A33423V) relative to cells treated with inactive ODNs (scrA33423V). No significant difference in *LAMA3A* promoter activity was observed in HMECs treated with or without inactive ODNs. These observations demonstrate that suppression of CBP expression in HMECs results in a reduction in *LAMA3A* promoter activity.

1j) Lack of CBP occupancy of the human laminin 5 (LAMA3A) promoter correlates with rECM-resistance. The human *LAMA3A* promoter contains three AP-1 sites at positions –387, –185, and –127 (Miller et al., 2001). The AP-1 site, at position –185, has been previously shown to be critical for basal activity in mammary epithelial cells. Chromatin immunopreciptation (ChIP) was performed in rECM-resistant, CBP-"poor" late passage HMEC-E6 cells and controls to test whether the observed 1) decrease in laminin-5 α3-chain expression and 2) loss of *LAMA3A* activity correlated with a lack of CBP binding to the 277 bp AP-1-"rich" site of the *LAMA3A* promoter (position -402 to –125). Early and late passage HMEC-LXSN control cells and rECM-sensitive, early passage HMEC-E6 cells grown in rECM demonstrated CBP binding to the AP-1-"rich" site of the *LAMA3A promoter.* In contrast, rECM-resistant, late passage HMEC-E6 cells, with decreased CBP and laminin-5 α3-chain expression, failed to demonstrate CBP binding. These observations suggest that a decrease in CBP expression might promote loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter.

1k) Suppression of CBP expression in HMECs results in loss of CBP occupancy of the LAMA3A promoter. Early passage HMEC-E6 cells were treated with active CBP ODNs and tested by ChIP to determine whether suppression of CBP protein expression resulted a loss of CBP occupancy of the AP-1-"rich" region of the *LAMA3A* promoter. Early passage HMEC-E6 cells treated with CBP-specific ODNs, and grown in rECM, did not demonstrate CBP occupancy of the *LAMA3A* promoter. In contrast, early passage HMEC-E6 controls, treated with inactive ODNs, and grown in rECM demonstrated CBP-occupancy (Figure 10 b). These observations demonstrate that suppression of CBP expression in HMEC-E6 cells by antisense ODNs results in a loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter.

Since the AP-1 site, at position –185, is critical for basal activity in mammary epithelial cells, these observations provide a mechanism by which loss of CBP expression might promote loss of *LAMA3A* promoter activity and laminin-5 α3-chain expression in HMECs.

SPECIFIC AIM II: Does CBP modulate interferon (IFN)-signal transduction during rECM-mediated apoptosis in HMEC-E6 cells?

2a) Microarray analysis of induced gene transcripts. To investigate the molecular mechanism of rECM-induced apoptosis, we analyzed the expression profiles of HMEC-E6 cells and passage matched HMEC-LXSN controls treated with and without rECM for 6 hrs. Analysis was performed using Hu6800 cDNA microarrays (Affymetrics). Twenty-four interferon-response genes (IRGs) were significantly upregulated in treated HMEC-E6 cells but not in treated HMEC-LXSN controls. Differential expression was confirmed by quantitative RT-PCR in triplicate, normalized to actin. The upregulated genes included IFI 9-27, interferon regulatory factor-1 (IFR-1), ISG15, ISG-54, MX-A, interferon gamma inducible protein 16, STAT-1 alpha, STAT-1 beta, IFI-6-16, and ISG12. Based on these observations, we hypothesized that IRGs 1) may participate in or 2) may be a marker for rECM- induced apoptosis of HMEC-E6 cells.

2b) Interferon-alpha, -beta, and –gamma are not induced by rECM. Assays for interferon-alpha, -beta, and –gamma were performed in HMEC-E6 cells and HMEC-LX controls treated with rECM to determine whether rECM induced interferon production in HMEC-E6 cells correlated with the induction of apopotosis. ELISA assays determined that interferon release was not observed 15 mins-24 hrs following tamoxifen treatment of HMEC-E6 cells (data not shown). These data show that rECM-induced apoptosis in HMEC-E6 cells results in induction of IRGs in the absence of interferon release.

2c) Interferon regulatory factor-1 (IRF-1) is induced by rECM. IRF-1 is a transcriptional regulator that has been shown to promote apoptosis following DNA-damage and is critical for mammary gland involution. Differential gene expression studies demonstrated that IRF-1 mRNA was induced by rECM in HMEC-E6 cells at 6 hrs. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and western analysis was performed to determine the kinetics of IRF-1 mRNA and protein induction. We observed that IRF-1 mRNA and protein was induced by rECM in HMEC-E6 cells but not in HMEC-LXSN controls. IRF-1 mRNA induction in HMEC-E6 cells was first observed at 30 min (5-fold) and was maximally induced at 3 hrs (7-fold). IRF-1 protein induction was first observed at 30 min (1.5-fold) and was maximally induced at 3 hrs (2.3-fold). These observations demonstrate that IRF-1 mRNA and protein are induced by rECM starting at 30 min.

KEY RESEARCH ACCOMPLISHMENTS:

1) Demonstration that suppression of the CREBP binding protein (CBP) results in loss of cellular polarity and apoptosis resistance.
2) Evidence that CBP regulates expression of laminin-5 through binding to the laminin 5 promoter (*LAMA3A*).
3) Demonstration that prepared extracellular matrix (rECM) promotes induction of interferon regulated genes (IRGs), including interferon regulatory factor-1 (IRF-1) in the absence of interferon production.

Reportable Outcomes:

1) **Manuscripts:** Dietze, E.C., Bowie, M.L., Troch, M.M., and Seewaldt, V.L. CBP Modulates Reconstituted Extracellular Matrix-Growth Regulation, -Polarity, and -Apoptosis in Human Mammary Epithelial Cells. Submitted to Journal of Cell Biology.

2) **Funding applied for:** AVON/NCI Developing markers of breast cancer risk in breast fine needle aspiration.

Conclusions:

In this report we demonstrate that suppression of the CREBP binding protein (CBP) results in apoptosis-resistance and loss of epithelial polarity and that CBP is critical for laminin-5 expression. These observations are important for 1) identifying improved targets for breast cancer prevention and 2) developing novel markers to test for response to chemoprevention agents. Information gained in this report is currently being translated to benefit women at risk for breast cancer prevention in our multi-institutional cohort.

References:

None.

Appendices

1) Dietze, E.C., Bowie, M.L., Troch, M.M., and Seewaldt, V.L. CBP Modulates Reconstituted Extracellular Matrix-Growth Regulation, -Polarity, and -Apoptosis in Human Mammary Epithelial Cells. Submitted to Journal of Cell Biology.

CBP Modulates Reconstituted Extracellular Matrix-Growth Regulation, -Polarity, and -Apoptosis in Human Mammary Epithelial Cells

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ABSTRACT

Interactions between normal mammary epithelial cells (HMECs) and extracellular matrix (ECM) are important for mammary gland homeostasis; loss of ECM-sensitivity is thought to be an early event in mammary carcinogenesis. The CREBP binding protein (CBP) is known to regulate both proliferation and apoptosis but the role of CBP in ECM-signaling is poorly characterized. We investigated the relationship between CBP expression and sensitivity to prepared ECM (rECM)-induced growth arrest and apoptosis in an *in vitro* model of early mammary carcinogensis. Suppression of CBP expression in HMECs by antisense oligonucleotides (ODNs) resulted in loss of rECM-mediated growth regulation, polarity, and apoptosis. Chromatin immunoprecipitation studies (ChIP) and reporter studies demonstrated that inhibition of CBP protein expression resulted in 1) loss of CBP-occupancy of the *LAMA3A* promoter and 2) a decrease in *LAMA3A* promoter activity. rECM-resistance correlated with 1) loss of CBP occupancy of the *LAMA3A* promoter, 2) decreased *LAMA3A* promoter activity, and 3) loss of laminin-5 α3-chain mRNA and protein expression. These observations suggest a critical role for CBP in rECM-mediated growth regulation, polarity, and apoptosis through modulation of *LAMA3A* activity and laminin-5 α3-chain expression.

Introduction

Breast tissue is composed of mammary epithelial cells that rest on extracellular matrix (ECM)[1]. Interactions between epithelial cells and ECM regulate normal growth, polarity, and apoptosis (Folkman and Moscona, 1978; Petersen, et al. 1992; Strange, et al., 1992; Zutter, et al., 1995; Ilic et al., 1998; Farrelly, et al., 1999). Loss of ECM-signaling is thought to be an early event in mammary carcinogenesis and is postulated to promote some of the phenotypic changes observed during malignant progression (Petersen, et al., 1992; Howlett, et al., 1995; Farelly, et al., 1999; Mercurio, et al., 2001). Carcinogenesis is hypothesized to be a multistep process resulting from the progressive accumulation of genetic damage. While loss or mutation of specific tumor suppressor genes such as *TP53* promotes mammary carcinogenesis, not all damaged epithelial cells progress to malignancy and many are thought to be eliminated by apoptosis (Fabian, et al. 1996; Ashkenazi and Dixit, 1998; Rohan, et al. 1998). Mammary gland homeostasis requires a coordinated balanced between proliferation and programmed cell death. ECM-signaling is thought to play an important role in regulating this balance (Petersen, et al., 1992; Howlett, et al., 1995; Farelly, et al., 1999; Mercurio, et al., 2001). Loss of ECM-signaling is thought to result in apoptosis-resistance and may promote mammary carcinogenesis by preventing the apoptotic elimination of damaged mammary epithelial cells.

Laminins are ECM glycoproteins that promote mammary gland homeostasis through regulating cell adhesion, migration, proliferation, differentiation, and angiogenesis (Aberdam et al., 2000). Laminins have three distinct protein subunits, designated α, β, and γ. Laminin-5 (α3A, β3, and γ1) is the most abundant ECM glycoprotein produced by mammary epithelial cells (D'Ardenne, et al., 1991). Laminin-5 functions as a ligand for α3β1- and α6β4-integrins to regulate adhesion, migration, and morphogenesis (Stahl, et al., 1997). Breast cancers frequently demonstrate loss of laminin-5 expression and disruption of α3β1- and α6β4-integrins receptors, associated with loss of tissue organization, growth regulation, and polarity (D'Adenne, et al., 1991; Natali et al., 1992; Koukoulis, et al., 1993; Zutter, et al., 1995; Weaver, et al., 1999; Henning, et al. 1999; Shaw, 1999; Simpson-Haidaris and Rybarczyk, 2001; Seewaldt, et al., 2001*a*, Weaver, et al., 2002). Loss of laminin-5 α3- and γ2-chain expression is observed in malignant breast lesions while benign ductal and lobular epithelial cells demonstrate continuous laminin-5 staining at the

epithelial-stromal interface (Henning, et al., 1999). These observations suggest that loss of laminin-5-signaling may be important for breast cancer progression.

We previously developed an *in vitro* model of early mammary carcinogenesis to investigate the potential role of ECM-signaling in eliminating acutely "damaged" HMECs (Seewaldt, et al., 2001*a*). Acute cellular damage was modeled by either 1) retroviral-mediated expression of the Human Papillomavirus Type-16 (HPV-16) E6 protein (HMEC-E6) or 2) treatment with p53-specific antisense oligonucleotides (ODNs) (p53(-) HMEC-AS). We observed that while HMEC controls grown in rECM underwent growth arrest on Day 7, HMEC-E6 and p53(-) HMEC-AS cells underwent apoptosis (Seewaldt, et al., 2001*a*). While the acute expression of either HPV-16 E6 or suppression of p53 in HMECs promoted sensitivity to rECM-mediated apoptosis, HMEC-E6 cells passaged in non-rECM culture rapidly acquired resistance to both rECM-mediated growth arrest and apoptosis associated with 1) loss of genetic material from chromosome 16 and 2) loss of polarized expression of the laminin-5 receptor, α3β1-integrin (Seewaldt, et al., 2001*a*). These observations lead us to hypothesize that 1) 16p might harbor a gene(s) whose loss and/or rearrangement may promote rECM-resistance and 2) laminin-5/α3β1-integrin-growth regulation and -polarity signals may be critical for targeting the elimination of acutely "damaged" HMECs.

In these studies, HPV-16 E6 was expressed in HMECs as a model of acute cellular damage. E6 interacts with a large number of cellular proteins critical for normal growth regulation and apoptosis (O'Connor, 2000). HPV-16 E6 binds to p53 and targets it for ubiquitin-mediated degradation and also abrogates p53 transcriptional activity (Zimmermann, et al., 1999; O'Connor, 2000; zur Hausen, 2000). E6 has been shown to interact with the CREBP-binding protein, CBP, at the C/H1 and C/H3 domains as well as sequences near the carboxy-terminus (O'Connor, 2000; Patel, et al. 1999). HPV-16 E6 protein also activates telomerase (Klingelhurtz, et al., 1996), binds to Bak (Thomas and Banks, 1998), physically interacts with the focal adhesion proteins, paxillin and fibulin-1 (Tong and Howley, 1997), and interacts with the transcriptional regulator, interferon regulatory factor-3 (Ronco, et al. 1998). Taken together, these observations provide evidence that expression of HPV-16 E6 in HMECs leads to marked cellular damage.

Detailed cytogenetic analysis performed in this report indicate that chromosome 16p13 is the critical area whose loss and/or rearrangement promotes rECM-resistance. CBP, is a nuclear protein located at chromosome band 16p13.3 that regulates proliferation, differentiation, and apoptosis (Yao, et al., 1998; Giles, et al., 1997*a*). CBP is a key integrator of diverse signaling pathways including those regulated by retinoids, p53, estrogen, and BRCA1 (Kawasaki, et al., 1998; Robyr, et al., 2000). Chromosomal loss at 16p13 has been reported to occur in a majority of benign and malignant papillary neoplasms of the breast and loss or amplification of 16p is frequently observed in premalignant breast lesions (Lininger, et al., 1998; Tsuda, et al., 1998; Aubele, et al., 2000). Taken together, these observations suggest that loss of CBP expression might promote mammary carcinogenesis.

Prior studies indicated that laminin-5/α3β1-integrin-growth regulation and -polarity signals might be critical for targeting the elimination of acutely damaged HMECs (Seewaldt et al., 2001*a*). Little is known about the regulation of laminin-5 gene transcription in normal mammary epithelial tissue, nor about the molecular mechanism underlying the loss of laminin-5 expression observed in early breast carcinogenesis (Miller, et al., 2000). The human *LAMA3A* promoter is known to contain three binding sites of the dimeric transcription factor, activating protein-1 (AP-1) (Virolle, et al., 1997; Miller, et al., 2001). It has been recently observed that the second AP-1 binding site present in the human *LAMA3A* promoter at position –185 base pairs is critical for baseline transcription of laminin-5 α3-chain (Miller, et al., 2001). CBP is known to interact with AP-1 response elements (Benkoussa, et al., 2002). However, the relationship between CBP and laminin-5 expression in mammary epithelial cells has not been studied.

This report describes a novel role for CBP in mediating sensitivity to rECM-growth regulation, -polarity, and –apoptosis through induction of laminin-5 α3-chain expression. Observations in our model system have important implications as they predict a critical role for CBP in regulating mammary homeostasis and targeting the elimination of acutely "damaged" HMECs through a laminin-5 α3-signaling pathway.

Materials and Methods

Cell Culture and Media

Normal human mammary epithelial cell (HMEC) strains AG11132 and AG1134 (M. Stampfer #172R/AA7 and #48R, respectively) were purchased from the National Institute of Aging, Cell Culture Repository (Coriell Institute) (Stampfer, 1985). HMEC strains AG11132 and AG11134 were established from normal tissue obtained at reduction mammoplasty, have a limited life span in culture, and fail to divide after approximately 20 to 25 passages. HMECs exhibit a low level of estrogen receptor staining characteristic of normal mammary epithelial cells. HMECs were grown in Mammary Epithelial Cell Basal Medium (Clonetics, San Diego, CA) supplemented with 4 µl/ml bovine pituitary extract (Clonetics #CC4009), 5 µg/ml insulin (Sigma, St. Louis, MO), 10 ng/ml epidermal growth factor (UBI Lake Placid, NY), 0.5 µg/ml hydrocortisone (Sigma), 10^{-5}M isoproterenol (Sigma), and 10 mM HEPES buffer (Sigma) [Standard Media]. Cells were cultured at 37°C in a humidified incubator with 5% CO_2/95% air. Mycoplasma testing was performed as previously reported (Seewaldt et al., 1997*a*).

Retroviral Transduction

The LXSN16E6 retroviral vector containing the HPV-16 E6 coding sequence was provided by D. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) (Demers et al., 1996). HMECs (passage 8) were plated in four T-75 tissue culture flasks (Corning, Corning, NY) in Standard Medium and grown to 50% confluency. Transducing virions from either the PA317-LXSN16E6 or the control PA317-LXSN (without insert) retroviral producer line were added at a multiplicity of infection at 1:1 in the presence of 4 µg/ml Polybrene (Sigma) to log-phase cells grown in T-75 flasks (Seewaldt, et al., 1995). The two remaining T-75 flasks were not infected with virus. After 48 hours, two flasks containing transduced cells and one flask with untransduced cells were passaged 1:3 (passage 9) and selected with Standard Media containing 300 µg/ml G418. Cells were grown in Standard

Media containing 300 μg/ml G418 (Gibco, Grand Island, NY) for four to seven days, until 100% of control, untransduced cells were dead. Cells were passaged 1:3 at the completion of selection (passage 10), and were maintained in the absence of selection before immediately proceeding to apoptosis experiments. The fourth flask of unselected, untransduced parental control cells was passaged in parallel with the selected, transduced experimental and vector control cells. Parental AG11132 cells were designated HMEC-P. Transduced AG11132 cells expressing the HPV-16 E6 construct were designated HMEC-E6 and vector control clones were designated HMEC-LXSN. All cells were maintained in Standard Media after transfection in the absence of G418 selection to ensure that any observed chromosomal abnormalities or apoptosis-resistance was not due to continued exposure to G418. All experiments were performed on mass cultures. Mycoplasm testing was as previously reported (Seewaldt, et al., 1997*b*)

Cytogenetic Analysis of Early and Late Passage HMECs

Spectral analyses (SKY) of HMEC-LXSN controls (passages 10 and 16) and HMEC-E6 cells (passages 10 and 18) were performed as previously described (Mrózek, et al., 1993; Schröck, et al., 1996; Seewaldt, et al., 2001*a, b*).

Western Blotting

Preparation of cellular lysates and immunoblotting were performed as previously described (Seewaldt, et al. 1997*b;* Seewaldt, et al. 1999*b*). For p53 expression, the membrane was incubated with a 1:100 dilution of mouse anti-human p53 (Oncogene Science Ab-2). For CBP expression, the blocked membrane was incubated with 1:200 dilution of the CBP C20 antibody (Santa Cruz Biotechnology). For laminin 5 expression the membrane was incubated with a 1:100 dilution of the C-19 antibody to the laminin-5 α3-chain (Santa Cruz). Loading control was provided by 1:200 dilution of the I-19 antibody to beta-actin (Santa Cruz). The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software (Kodak, Rochester, NY).

HMEC Culture in rECM

HMECs were grown in rECM as previously described (Seewaldt et al., 2001*a*). 100 µl of rECM (Growth Factor Depleted Matrigel™, Collaborative Research, Bedford, MA) were added per well to a 48 well plate and allowed to gel at 37°C for 20 min. Transduced HMECs were trypsinized, counted, and pelleted in a sterile microcentrifuge tube. Approximately 1 x 10^4 cells were resuspended in 100 µl rECM on ice, gently overlaid on the initial undercoating of extracellular matrix, and allowed to gel at 37° C for 20 min. Standard Media was then added and wells were inspected to ensure there was an equal distribution of cells in each well. Cells were grown for 5 to 13 days in culture.

Cell Growth and Proliferation in rECM Culture

Cell growth in rECM culture was determined by the following criteria: the size of growing spherical cell colonies was measured with an eye-piece equipped with a micrometer spindle. The 20 largest colonies were measured. Proliferation was assessed by Ki67 staining index as follows: five micron sections were immunostained with antibody directed against Ki-67. Cells were scored visually (100-500 cells) for immunopositive nuclei. The proliferation index was calculated by dividing the number of immunopositive cells as a percentage of the total number of cells scored.

Detection of Apoptosis by In Situ *TUNEL*

HMECs were grown in rECM for 5 to 11 days and prepared for TUNEL staining as previously described (Seewaldt, et al., 2001*a*). Two hundred cells were scored. The apoptotic index was determined by expressing the number of TUNEL positive cells as a percentage of the total number of cells scored.

Transmission Electron Microscopy

HMECs were grown in contact with rECM as described above. Electron microscopy was

as previously described (Seewaldt, et al., 1999*a*; Dietze, et al., 2001). Fifty colonies were scored for the presence of apoptosis by morphologic criteria that included 1) margination of chromatin, 2) nuclear condensation, 3) cell shrinkage, and 4) formation of apoptotic bodies (Majno and Joris, 1995).

Immunostaining

HMECs were grown in rECM as described above and embedded in O.C.T. (Miles), snap frozen, and 7 μ sections obtained. For E-cadherin staining: sections were fixed with 3.7% formaldehyde in PBS for 30 min at RT and were blocked with 0.5% heat-denatured bovine serum albumin (HD-BSA) in PBS for 1 hr at RT. Sections were then incubated for 30 min with mouse anti-human E-cadherin antibody (BD Signal Transduction Laboratories) diluted in PBS with 0.5% HD-BSA for 30 min at RT and then washed 6 times with PBS at RT. For integin and laminin immunostaining: cells were fixed for 20 min at RT with 2% formaldehyde in 0.1 M sodium cacodylate, and 0.1 M sucrose at pH 7.2, permeabilized with 0.1% Triton X-100 for 10 min at RT, and blocked with 0.5% HD-BSA in PBS for 1 hr at RT. Cells were incubated with a primary antibody diluted in PBS with 0.5% HD-BSA for 1 hr at RT and washed 6 times with PBS at RT. Antibodies against integrin subunits α3 (P1F2, P1B5), and β1 (P4C10) were a generous gift of William Carter and have been previously described (Carter, et al., 1990*a*, *b*; Wayner and Carter, 1987; Wayner, et al., 1988). Monoclonal antibodies P5H10 directed against the α3 chain of laminin-5 were a generous gift of William Carter. For immunoflurorescence, cells were incubated with either FITC- or Rhodamine-conjugated goat anti-mouse antibody at a 1:200 antibody dilution (Santa Cruz) in PBS with 0.5% HD-BSA for 30 min at RT and washed. Sections were mounted in 30% glycerol in PBS and visualized for immunofluorescence using a Zeiss LSM 410 fluorescence microscope (Carl Zeiss, Jena, Germany).

Suppression of CBP Expression

Nine antisense oligonucleotides (ODNs) to human CBP were generated by the PAS

program (Ugai, et al., 1999). The CBP antisense A3342V ODN (24-mer, nucleotide position 3342-3363) was initially chosen on the basis of selective inhibition of CBP protein expression in MCF-7 cells (data not shown); suppression was confirmed in HMECs. Inactive CBP ODN A2172Z (26 mer, nucleotide position 2172-2197) was 1) chosen to be the scrambled sequence of the antisense ODNs to ensure identical nucleotide content and minimize differences potentially attributable to nucleic acid content and 2) selected based on lack of suppression of CBP in MCF-7 and HMECs. See Table 1 for a list of ODNs. The first and last three nucleotides of all ODNs were phosphorothioate modified to increase their stability *in vitro*. Early passage HMEC-LXSN controls and HMEC-E6 cells were plated in T-75 plates in Standard Media. After allowing 24 hr for attachment, cell cultures were treated for 72 hr with either active or inactive ODNs (0.001 to 0.1μM final concentration). Every 24 hr the culture media was replaced by new Standard Media containing fresh ODNs. Western analysis was performed to confirm 1) suppression of CBP expression as described above and 2) lack of suppression of the related co-activator p300. The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software.

CBP-Suppression in rECM Culture

Early passage HMEC-LXSN controls and HMEC-E6 cells were trypsinized and approximately 1 x 10^4 cells were resuspended in 100 μl rECM containing either active or inactive CBP-specific ODNs (0.01 to 0.1 μM final concentration) on ice. rECM cultures were prepared as above. rECM cultures were overlayed with Standard Media containing active or inactive CBP-specific ODNs (0.01 to 0.1 μM final concentration). Overlay media were changed every 24 hours to ensure a fresh supply of ODNs. The diameter of the growing colonies was determined and cells were prepared for electron microscopy and immunostaining as described above.

Large-Scale rECM Culture

Large-scale rECM culture was utilized to prepare total RNA or protein lysate for analysis utilizing techniques previously developed by the laboratory of Minna Bissell (Roskelly, et al., 1994). Early and late passage HMEC-E6 cells and HMEC-LXSN controls were plated in T-75 flasks, previously treated with poly(2-hydroxyethyl methacrylate) (Poly-HEME). Cells were grown in Standard Media with 5% (v:v) rECM.

Differential Gene Expression Studies

Cells were grown in rECM utilizing large-scale rECM culture techniques as described above. Isolation of total RNA was as previously described (Seewaldt, et al., 1995). RNA integrity was confirmed by electrophoresis, and samples were stored at –80°C until used. All RNA combinations used for array analysis were obtained from cells that were matched for passage number, cultured under the identical growth conditions, and harvested at identical confluency. cDNA synthesis and probe generation for cDNA array hybridization were obtained by following the standardized protocols provided by Affymetrix™ (Affymetrix, Santa Clara, California).

Expression data for approximately 5,600 full-length human genes was collected using Affymetrix GeneChip HuGeneFL™ arrays, following the standardized protocols provided by the manufacturer. Data was collected in triplicate using independent biological replicates (Baldi and Long, 2001). Array images were processed using Affymetrix MAS 5.0 software, where we filtered for probe saturation, employed a global array scaling target intensity of 1000, and collected the signal intensity value for each gene. For each set of replicate measurements, gene signal intensity values were averaged and an array-level normalization was performed relative to the β-actin averaged value. Normalized values were $\log_2$ transformed and an additional gene-level normalization was performed using the LXSN-ECM data set. Data was imported into *Cluster/TreeView* software (http://rana.lbl.gov/EisenSoftware.htm), where heat maps were generated.

Semi-Quantitative RT-PCR

To confirm microarray data, relative transcript levels were analyzed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Five micrograms of total RNA was used in first-strand cDNA synthesis with Superscript II reverse transcriptase (Invitrogen). PCR reaction conditions were optimized for integrin-α3 (*ITGA3*), integrin-β1 (*ITGB1*), laminin-α3 (*LAMA3*), laminin-β3 (*LAMB3*), and laminin-γ2 (*LAMC2*). Primer sequences were obtained from published sources as follows: ITGA3 (Hashida, et al., 2002), ITGB1 (Hsu, et al., 2001), LAMA3 (forward primer, Virolle, et al., 2002), LAMB3 and LAMC2 (Manda, et al., 2000). A 50 µl reaction was set up containing 100 nM forward primer, 100 nM reverse primer, 250 µM of each dNTP, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, 2.5 units Taq polymerase, and 2.0 µl cDNA. Reaction conditions for beta-actin were 300 nM forward primer, 300 nM reverse primer, 250 µM of each dNTP, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH = 8.3, 2.5 units Taq polymerase, and 2.0 µl cDNA in a total volume of 50 µl. Products were amplified with Perkin Elmer GeneAmp PCR system 2400. Preliminary reactions were preformed to determine the PCR cycle number of linear amplification for each primer set. The primer sets, cycling conditions, and cycle numbers used are indicated in Table 2. Ten microliters of PCR product were analyzed by electrophoresis in 1.2-1.5% agarose gels containing ethidium bromide and visualized under UV light and quantitated. All samples were performed in triplicate and normalized to beta-actin control.

LAMA3A Reporter Studies

A 1403 bp region of the laminin-5 α3 (*LAMA3*) promoter corresponding to GenBank Accession Number AF279435 was amplified with PCR primers, sense 5'-AAG CTT AAG TTT TCC CAT CCG CAA C-3' and antisense 5'-TCT AGA GCT GAC CGC CTC ACT GC-3' (Miller, et al., 2001). The PCR product was cloned into pCRII

(Invitrogen), digested out with HindIII and BamHI, and cloned into the reporter plasmid pBLCAT5 (ATCC). Cells were transfected with the resultant pBLLAMA3aCAT5 reporter plasmid using previously published transfection conditions and controls (Seewaldt et al., 1997*a*). Transfected cells were plated in Standard Media in T-25 flasks pre-treated with Poly-HEME, treated with 5% (v:v) rECM for 24 hr, and harvested for CAT activity assays as previously described (Seewaldt, et al. 1997*a*).

Occupancy of the AP-1-"rich" region of *LAMA3A* promoter from positions –387 to –127 was tested by chromatin immunopreciptiation (ChIP). ChIP was performed by published methods with some modifications (Yahata, et al., 2001). Early and late passage HMEC-E6 cells and HMEC-LXSN controls were plated in T-25 flasks treated with Poly-HEME and grown in Standard Media with 5% (v:v) rECM. Preliminary experiments were run to determine optimal sonication and formaldehyde cross-linking time. Once optimized, cells were harvested, pelleted, and treated with 1% formaldehyde for 15-20 minutes to cross-link cellular proteins. The formaldehyde was quenched by adding 1.0 ml of 250 mM glycine followed by a 5 min RT incubation. Cells were then rinsed twice in ice cold PBS containing protease inhibitors, pelleted, and resuspended in Lysis Buffer [1% SDS, 10 mM EDTA, 50mM Tris-HCl at pH 8.1, 1x Protease Inhibitor Cocktail (4 μg/ml epibestatin hydrochloride, 2 μg/ml calpain inhibitor II, 2 μg/ml pepstatin A, 4 μg/ml mastoparan, 4 ug/ml leupeptin hydrochloride, 4 μg/ml aprotinin, 1 mM TPCK, 1 mM phenylmethylsulfonyl fluoride, and 100 μM TLCK)]. Samples were then sonicated 3 x 15 seconds each with a 1 min incubation on ice in between pulses on a Branson sonifier model 250 at 50% duty and maximum mini probe power. Supernatants were diluted (1:10) in Dilution Buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1, 1x Protease Inhibitor Cocktail], and precleared with 2 μg of sheared salmon sperm DNA, 20 μl normal human serum, and 45 μl of protein A-sepharose [50% slurry in 10 mM

Tris-HCl at pH 8.1, 1 mM EDTA]. Human anti-CBP antibody (A22, Santa Cruz) was added to the precleared lysate, and placed on a shaker at 4 ^{0}C, followed by the addition of 45 µl of protein A-sepharose and 2.0 µg sheared salmon sperm DNA, and an additional 1 hour incubation on a shaker at 4 ^{0}C. Sepharose beads were then collected and washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 500 mM NaCl), and buffer III (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1). Beads were washed once with TE buffer and DNA eluted with 100 µl of 1% SDS-0.1 M $NaHCO_3$. Eluate was heated at 65°C overnight to reverse the formaldehyde cross-linking. DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation, and then amplified by using PCR primers, sense 5'-AAG CTT AAG TTT TCC CAT CCG CAA C-3' and antisense 5'-TCT AGA GCT GAC CGC CTC ACT GC-3'. Thirty microliters of PCR product were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide and visualized under UV light. All samples were performed in triplicate.

Results

Late passage HMEC-E6 cells acquire resistance to rECM-induced apoptosis associated with rearrangement of the CBP locus at chromosome 16p13.

Previous cytogenetic analysis suggested that chromosome 16p harbored a gene(s) whose loss and/or rearrangement might play a role in resistance to rECM-mediated growth control and apoptosis (Seewaldt, et al. 2001*a*). In this study, detailed SKY-based cytogenetic analysis was performed on rECM-resistant, late passage HMEC-E6 cells to identify gene rearrangements that might pinpoint the chromosomal location of the gene of interest. Chromosomal rearrangements and deletions involving 16p were surveyed in 35 unique late passage HMEC-E6 cells. A majority of chromosomal changes involving 16p were whole chromosome or whole chromosome arm deletions, however, 1) one rECM-resistant cell with a 16p deletion retained material proximal to 16p12 while, 2) a second rECM-resistant cell exhibited an unbalanced translocation der(16)t(13;16)(q1?2;p13) that affected band 16p13 (Figure 1). These observations indicated that the gene of importance was located in the distal region of 16p, at 16p13. Chromosomal band 16p13 is the locus of the CBP gene (Giles, et al., 1997*a,b*; Yao, et al., 1998). Since CBP is known to play a role in growth regulation and apoptotic signaling, we hypothesized that loss of CBP protein expression might promote rECM-resistance in HMEC-E6 cells.

Resistance to rECM-mediated growth regulation and apoptosis correlates with a decrease in CBP protein expression.

Western analysis tested whether rECM-resistant late passage HMEC-E6 cells exhibited decreased expression of CBP. CBP protein expression was markedly decreased in late passage, rECM-resistant HMEC-E6 cells relative to early passage, rECM-sensitive HMEC-E6 cells and HMEC-LXSN controls. Late passage HMEC-E6 cells exhibited a 41% ($p \leq 0.01$) and 72% ($p \leq 0.01$) respective decrease in CBP protein expression relative to early passage HMEC-LXSN controls and early passage HMEC-E6 cells (Figure 2 *c*). In contrast, there was no significant decrease in CBP protein expression in late passage

HMEC-LXSN controls relative to early passage cells (Figure 2 *c*). These observations in late passage HMEC-E6 cells 1) demonstrate that rECM-resistance correlates with decreased CBP protein expression and 2) are consistent with cytogenetic analysis demonstrating loss or rearrangement of the CBP locus at 16p13.

Suppression of CBP in early passage HMECs by antisense ODNs.

Antisense ODNs were utilized to suppress CBP protein expression in HMECs to test whether the level of CBP protein expression might be important for rECM-sensitivity. Relative levels of CBP protein expression were tested by Western analysis. Early passage HMEC-LXSN control and early passage HMEC-E6 cells treated with the active, CBP-specific ODN, A3342V, exhibited a 65% and 72% respective decrease in CBP protein expression relative to untreated controls (Figure 2 *d*). Cells treated with the inactive CBP ODN, scrA3342V, did not exhibit a significant decrease in CBP protein expression (Figure 2 *d*).

Suppression of CBP enhances proliferation in rECM.

Treatment of early passage HMEC-LXSN controls and HMEC-E6 cells with CBP-specific ODNs resulted in enhanced proliferation in rECM-culture as measured by 1) physical growth parameters and 2) Ki-67 staining. Both early passage HMEC-LXSN controls and HMEC-E6 cells treated with active CBP-specific ODNs (A33243V) demonstrated a continued increase in sphere diameter from Day 7-9 in rECM culture (Figure 3 *a, b*). In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive ODNs (scrA33243V) did not exhibit an increase in sphere diameter after Day 7 (Figure 3 *a, b*). Treatment of early passage HMEC-LXSN and HMEC-E6 cells with CBP-specific ODNs (A33243V) resulted in continued Ki-67 staining at 9 and 11 days in rECM culture (Figure 3 *c, d*). In contrast, Ki-67 staining at 9 and 11 days were markedly reduced in HMEC-LXSN and HMEC-E6 cells treated with inactive ODNs (scrA33423V) (Figure 3 *c, d*). These observations show that suppression of CBP protein expression in early passage

HMEC-E6 cells and HMEC-LXSN controls resulted in enhanced proliferation in rECM culture.

Suppression of CBP protein results in altered localization and expression of biochemical markers of polarity.

Early passage HMEC-E6 cells and HMEC-LXSN controls treated with active CBP ODNs (A33423V) exhibited a loss of epithelial polarity as evidenced by dispersed and intracellular staining of 1) E-cadherin and 2) the tight junction-associated protein, ZO-1 (Figure 4). In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive CBP ODNs (scrA33423V) demonstrated expression of E-cadherin and ZO-1 at the cell-cell junction, consistent with a correctly polarized epithelium (Figure 4). These observations indicate that suppression of CBP protein expression in HMECs by antisense ODNs promotes a loss of epithelial polarity in rECM culture.

Suppression of CBP inhibits apoptosis in rECM culture.

Early passage HMEC-E6 cells were treated with CBP-specific antisense ODNs to test whether suppression of CBP protein expression blocked apoptosis in rECM culture. Early passage HMEC-E6 treated with CBP-specific antisense ODNs (A33423V) formed large irregular clusters in rECM and did not undergo apoptosis as assessed by either electron microscopy or TUNEL-staining (Figure 5 *b, d*). In contrast, early passage HMEC-E6 cells treated with inactive CBP ODNs underwent apoptosis on Day 7 as assessed by either morphologic criteria or TUNEL-staining (Figure 5 *c, d*). Similar to early passage HMEC-E6 cells, early passage HMEC-LXSN cells treated CBP-specific, antisense ODNs (A33423V) formed large irregular clusters in rECM (Figure 5 *a*). Early passage HMEC-LXSN controls treated with inactive ODN (scrA33423V) formed a morphologically organized, ascinus-like structure and did not undergo apoptosis consistent with what has been previously observed for early passage HMEC-LXSN untreated controls (Figure 5 *e* and data not shown) (Seewaldt, et al., 2001*a*). These observations demonstrate that

suppression of CBP protein expression in early passage HMEC-E6 cells by antisense ODNs blocks apoptosis in rECM culture.

A second HMEC strain, AG11134, was tested to ensure that these observations were not HMEC strain-specific. Similar to observations made in HMEC strain AG11132 above, 1) early passage AG11134-E6 cells treated with inactive CBP ODN (scrA33423V) were sensitive to rECM-growth regulation and underwent apoptosis at Day 7 (data not shown), 2) early passage AG1134-LXSN controls treated with antisense-CBP ODN (A99424V) were resistant to rECM growth arrest and did not undergo apoptosis at Day 7-9 (data not shown), and 3) early passage AG11134-LXSN controls treated with CBP-specific antisense ODNs were resistant to rECM-mediated growth regulation and did not undergo apoptosis (data not shown).

Laminin-5 expression is decreased in rECM resistant, late passage HMEC-E6 cells.

We previously observed that sensitivity to rECM-apoptosis in early passage HMEC-E6 cells required polarized expression of α3/β1-integrin (Seewaldt et al., 2001*a*). Differential gene expression studies, semi-quantitative RT-PCR, and Western analysis were performed to test whether the loss of sensitivity to rECM-mediated growth regulation, polarity, and apoptosis observed in late passage HMEC-E6 cells correlated with altered expression of laminin-5 and/or α3/β1-integrin mRNA. Differential gene expression studies demonstrated decreased expression of all three laminin-5 chains (α3, β3, and γ2) in apoptosis-resistant, late passage HMEC-E6 cells relative to early passage HMEC-LXSN controls and early passage HMEC-E6 cells grown in rECM (Figure 6 *a*). Semi-quantitative RT-PCR confirmed a 98% decrease in laminin-5 α3-chain ($p \leq 0.01$), an 88% decrease in laminin-5 β3-chain ($p \leq 0.01$), and a 75% decrease in laminin-5 γ2-chain ($p \leq 0.01$) mRNA expression relative to early passage HMEC-LXSN controls (Figure 6 *b, c*). There was no significant change in the level of α3/β1-integrin mRNA expression (Figure 6 *a, b, c*). Western

analysis similarly demonstrated an 85% ($p<0.001$) decrease in laminin-5 α3-chain protein expression in apoptosis-resistant, late passage HMEC-E6 cells relative to early passage HMEC-LXSN controls (Figure 6 *d*). Expression of laminin-5 α3-chain protein did not significantly vary between early passage HMEC-LXSN cells and early passage HMEC-E6 cells (Figure 6 *d*). There was a 130% ($p<0.002$) increase in laminin-5 α3-chain protein in late passage HMEC-LXSN controls relative to early passage HMEC-LXSN cells. These observations demonstrate that the presence of rECM-resistance in late passage HMEC-E6 cells correlates with a loss of laminin-5 mRNA and protein expression.

Lack of polarized expression of laminin-α3 and integrin-α3 proteins in late passage HMEC-E6 cells grown in rECM.

Early and late passage HMEC-LXSN controls and HMEC-E6 cells were grown in rECM and tested for 1) laminin-5 α3-chain and 2) α3- and β1-integrin expression by immunohistochemistry (clones P5H10, P1F2, and P4C10, respectively). Early and late passage HMEC-LXSN controls and early passage HMEC-E6 cells exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins (Figure 7 *a-i*). In contrast, late passage, CBP-"poor" HMEC-E6 cells grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of both laminin-5 α3-chain and α3-integrin (Figure 7 *j, l*). As predicted by differential gene expression studies and Western analysis, there was also a qualitative decrease in laminin-5 α3-chain expression in late passage HMEC-E6 cells relative to controls (Figure 7 *l*). Late passage HMEC-E6 cells grown in rECM exhibited polarized basal β1-integrin expression but had an increase in the amount of cytosolic expression relative to early passage cells (Figure 7 *k*). These observations demonstrate a loss of polarized expression of laminin-5 α3-chain and α3-integrin in rECM-resistant late passage HMEC-E6 cells.

Suppression of CBP expression in HMECs alters both laminin-α3 and integrin-α3

protein expression in rECM culture.

We observed that late passage HMEC-E6 cells grown in rECM culture exhibit 1) reduced levels of CBP protein expression and 2) disorganized expression of both laminin-5 α3-chain and α3-integrin. This observation led us to hypothesize that suppression of CBP in HMECs would alter laminin-5 α3-chain and α3-integrin expression and/or distribution. CBP protein expression was suppressed in early passage HMEC-E6 cells and HMEC-LXSN controls by treatment with CBP-specific, antisense ODN (A99424V). HMECs with suppressed CBP expression exhibited disorganized plasma membrane and cytosolic distribution of laminin-5 α3-chain and α3-integrin (Figure 8 *a, b, i, j*). β1-integrin expression was observed at the basal surface (Figure 8 *e, f*). In contrast, early passage HMEC-LXSN controls and HMEC-E6 cells treated with inactive CBP ODN (scrA99424V) exhibited polarized basal expression of laminin-5 α3-chain and α3- and β1-integrins (Figure 8 *c, d, g, h, k, l*). These observations demonstrate that suppression of CBP protein expression in HMECs alters the distribution of both laminin-5 α3-chain and α3-integrin.

Decreased CBP expression in rECM-resistant late passage HMEC-E6 cells correlates with decreased LAMA3A promoter activity.

We tested whether the observed decrease in CBP and laminin-5 α3-chain expression in late passage HMEC-E6 cells correlated with decreased *LAMA3A* promoter activity. Early and late passage HMEC-E6 and passage-matched HMEC-LXSN controls were transiently transfected with a CAT reporter coupled to the *LAMA3A* promoter sequence (1403 bp, GenBank Accession Number AF279435) and grown in rECM culture. rECM-sensitive early passage HMEC-E6 cells and early and late passage HMEC-LXSN controls exhibited a similar level of *LAMA3A* activity (Figure 9 *a*). In contrast, rECM-resistant, late passage HMEC-E6 cells with decreased CBP and laminin-5 α3-chain expression exhibited a 91% decrease in *LAMA3A* promoter activity relative to early passage HMEC-E6 cells ($p \leq 0.01$) (Figure 9 *a*). These experiments demonstrate in HMECs a positive correlation between 1)

the level of CBP and laminin-5 α3-chain protein expression and 2) *LAMA3A* promoter activity.

LAMA3A promoter activity in HMECs with suppressed CBP protein expression.

We next tested whether suppression of CBP protein expression resulted in decreased *LAMA3A* promoter activity. *LAMA3A*-CAT reporter activity was compared in early passage HMEC-LXSN and HMEC-E6 cells treated with either CBP-specific antisense ODNs (A33423V) or inactive ODNs (scrA33423V). A 92% and 89% decrease ($p<0.01$) in *LAMA3A* promoter activity was observed, respectively, in early passage HMEC-LXSN and HMEC-E6 cells grown in rECM and treated with CBP-specific ODNs (A33423V) relative to cells treated with inactive ODNs (scrA33423V) (Figure 9 *b*). No significant difference in *LAMA3A* promoter activity was observed in HMECs treated with or without inactive ODNs. These observations demonstrate that suppression of CBP expression in HMECs results in a reduction in *LAMA3A* promoter activity.

Lack of CBP occupancy of the LAMA3A promoter correlates with rECM-resistance.

The human *LAMA3A* promoter contains three AP-1 sites at positions –387, –185, and –127 (Miller et al., 2001). The AP-1 site, at position –185, has been previously shown to be critical for basal activity in mammary epithelial cells (Miller, et al., 2001). Chromatin immunopreciptation (ChIP) was performed in rECM-resistant, CBP-"poor" late passage HMEC-E6 cells and controls to test whether the observed 1) decrease in laminin-5 α3-chain expression and 2) loss of *LAMA3A* activity correlated with a lack of CBP binding to the 277 bp AP-1-"rich" site of the *LAMA3A* promoter (position -402 to –125) (Miller, et al., 2001). Early and late passage HMEC-LXSN control cells and rECM-sensitive, early passage HMEC-E6 cells grown in rECM demonstrated CBP binding to the AP-1-"rich" site of the *LAMA3A* promoter. In contrast, rECM-resistant, late passage HMEC-E6 cells, with decreased CBP and laminin-5 α3-chain expression, failed to demonstrate CBP binding

(Figure 10*a*). These observations suggest that a decrease in CBP expression might promote loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter.

Suppression of CBP expression in HMECs results in loss of CBP occupancy of the LAMA3A promoter.

Early passage HMEC-E6 cells were treated with active CBP ODNs and tested by ChIP to determine whether suppression of CBP protein expression resulted a loss of CBP occupancy of the AP-1-"rich" region of the *LAMA3A* promoter. Early passage HMEC-E6 cells treated with CBP-specific ODNs, and grown in rECM, did not demonstrate CBP occupancy of the *LAMA3A* promoter (Figure 10 *b*). In contrast, early passage HMEC-E6 controls, treated with inactive ODNs, and grown in rECM demonstrated CBP-occupancy (Figure 10 *b*). These observations demonstrate that suppression of CBP expression in HMEC-E6 cells by antisense ODNs results in a loss of CBP occupancy of the AP-1-"rich" site of the *LAMA3A* promoter. Since the AP-1 site, at position –185, is critical for basal activity in mammary epithelial cells (Miller, et al., 2001), these observations provide a mechanism by which loss of CBP expression might promote loss of *LAMA3A* promoter activity and laminin-5 α3-chain expression in HMECs.

Discussion

ECM has been shown to provide signals critical for mammary epithelial cell survival; in their absence cells undergo apoptosis (Streuli, et al., 1991; Strange, et al., 1992; Pullan, et al., 1996; Ilic, et al., 1998; Boudreau, et al., 1998; Bissell, et al., 1999). However, there is also evidence that growth arrest and survival signals promote apoptosis in genetically damaged cells (Seewaldt, et al., 1995; Wahl, et al., 1996; Hong and Sporn, 1997; Mancini, et al., 1997; Seewaldt, et al., 1997*b*; Seewaldt et al. 2001*a*). We previously demonstrated that rECM-derived growth regulation and polarity signals promoted apoptosis in our model of early breast carcinogenesis. Based on these observations we hypothesized that resistance to rECM-regulated growth arrest and polarity would promote apoptosis-resistance.

In this report we show that partial suppression of CBP protein expression in HMECs 1) results in loss of growth regulation and polarity in rECM culture and 2) blocks apoptosis in acutely damaged HMEC-E6 cells. This is the first demonstration that CBP-regulated growth and polarity signaling may be important for regulating apoptosis in "acutely damaged" HMECs. CBP is a tightly regulated transcription factor that regulates proliferation, differentiation, and apoptosis. Current models suggest that CBP is present in limiting amounts and transcriptional regulation may be, in part, achieved through competition for this cofactor, as only partial suppression of CBP is required for a phenotype in the CBP heterozygote "knock out" mouse (Kawasaki, et al., 1998; Shang, et al., 2000; Yao, et al., 1998). Consistent with observations in our *in vitro* system, partial suppression of CBP protein levels in virgin CBP(+/-) heterozygote mice results in a 90% incidence of severe mammary gland hyperplasia and hyperlactation (Yao, personal communication). Taken together these observations provide evidence that partial suppression of CBP protein expression promotes 1) loss of growth regulation and polarity and 2) apoptosis resistance.

In this report, we demonstrate that suppression of CBP protein expression results in loss of laminin-5 expression. Laminin-5 is a major component of mammary gland extracellular matrix and loss of laminin-5 immunostaining at the epithelial-stromal interface has been observed in premalignant breast lesions (Henning, et al., 1998, Mercurio, et al., 2001). However, the regulation of laminin-5 gene transcription in normal mammary epithelial tissue and subsequent loss of laminin-5 expression during mammary carcinogenesis is poorly understood (Miller, et al., 2001).

AP-1 response elements are *cis*-acting DNA sequences known to regulate a wide range of cellular processes, including proliferation, apoptosis, survival, and differentiation (Shaulian and Karin, 2002). Despite extensive study, the target genes regulated by AP-1 that mediate these activities have not been completely characterized (Shaulian and Karin, 2002). The transcriptional activity of AP-1 is extremely complex and is thought to be regulated by several different mechanisms. First, AP-1 activity is determined by the composition of the AP-1 heterodimer. Different AP-1 heterodimers display different affinities for a given response element (Metz, et al., 1994; McBride and Damer, 1998; Cook, et al., 1999). Second, posttranslational modifications exert a strong control on AP-1 activity. Notably, phosphorylation of c-jun at Ser 63 and Ser 73 increases its affinity for CBP (Bannister, et al., 1995). Third, AP-1 activation/deactivation may depend on interactions with specific transcriptional co-activators and co-repressors (Karin, 1995; Karin, et al., 1997; Pessah, et al., 2001; Benkoussa, et al. 2002).

CBP is known to interact with the AP-1 response element (Horvai, et al., 1997; Benkoussa, et al. 2002). Several independent approaches, involving co-transfection assays or microinjection of anti-CBP antibodies, have demonstrated that CBP participates in activation of AP-1 (Kwok, et al., 1994; Arias, et al., 1994). Activation of nuclear receptors during steroid/thyroid hormone signaling results in loss of AP-1 activity through recruitment of CBP to the nuclear receptor co-activator complex (Kamei, et al., 1996). It has also been

recently shown that retinoic acid receptors inhibit AP-1 activity through regulating extracellular signal-regulated kinase and CBP recruitmentment to an AP-1-resposive promoter (Benkoussa, et al. 2002).

The relationship between CBP, AP-1 activity, and laminin-5 expression is poorly defined. Both the mouse and human *LAMA3A* promoter contain an AP-1-"rich" region (Virolle, et al., 1998; Miller, et al., 2001). The second AP-1 binding site present in both the mouse and human *LAMA3A* promoter is critical for baseline transcription of laminin-5 α3-chain (Virolle, et al., 1998; Miller, et al., 2001). Here we show that suppression of CBP results in loss of *LAMA3A* promoter activity and laminin-5 α3-chain expression and 2) blocks the apoptotic elimination of acutely damaged HMEC-E6 cells. This decreased production of laminin 5-α3 correlates with loss of CBP occupancy of the AP-1-"rich" region of the *LAMA3A* promoter. Taken together, these observations suggest that 1) CBP occupancy of the *LAMA3A* promoter promotes laminin-5 α3-chain expression and 2) loss of CBP occupancy inhibits laminin-5 α3-chain expression and may promote survival of acutely "damaged" HMECs.

In contrast to our observation that suppression of CBP inhibits laminin-5 expression in HMECs, it has been previously observed that overexpression of the related co-activator, p300, inhibits laminin-5 production in MCF-10A cells (Miller, et al., 2000). One potential explanation for these seemingly divergent results may lie in differences in cell type. MCF-10A is an immortalized human breast epithelial cell line that exhibits complex chromosomal rearrangements (Yoon, et al., 2002). Our CBP suppression studies were performed in either early passage HMEC-LXSN control cells or in early passage HMEC-E6 cells. These transduced cell strains are not immortalized and previous cytogenetic analysis demonstrates the absence of chromosomal rearrangements in early passage transduced HMECs (Seewaldt, et al., 2001*a*). It is also possible that the difference between these previous studies and our results can be accounted for by differences between CBP and p300

activities. While p300 and CBP have many overlapping functions, there is ample evidence that they also have distinct activities. For example, CBP and p300 play a distinct role during retinoic acid-induced differentiation in F9 cells (Kawasaki, et al., 1998; Ugai, et al., 1999) and p300, but not CBP, has been shown to be transcriptionally regulated by BRCA1 in breast cancer cell lines (Fan, et al., 2002).

In summary, observations in our model system predict that a partial reduction of CBP expression results in 1) loss of CBP occupancy of the AP-"rich" region of the *LAMA3A* promoter, 2) decreased *LAMA3A* promoter activity, and 3) reduced expression of laminin-5 α3-chain protein. We also observe that loss of CBP/laminin 5-α3 expression blocks rECM-growth regulation, -polarity, and -apoptosis *in vitro* and thereby may promote the clonal expansion of "damaged" HMECs *in vivo*. These observations have potential clinical implications and suggest that suppression of CBP may promote mammary hyperplasia and also may increase the risk of subsequent breast cancer.

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Footnotes:

[1] Abbreviations: **ECM**, extracellular matrix; **rECM**, reconstituted extracellular matrix; **HMEC**, human mammary epithelial cells; **ER**, estrogen receptor; **HPV-16**, human papillomavirus type 16; **PBS**, phosphate buffered saline; **ECL**, enhanced chemiluminescent detection; **FACS**, fluorescent activated cell sorting; **RT**, room temperature; **SKY**, spectral karyotyping; **DAPI**, 4,6-diamino-2-phenyliodole; **Ab,** antibody; **ODN**, oligonucleotides; **CBP,** CREBP binding protein.

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Table 1: CBP-specific antisense ODN sequences

Target gene	Sequences	Size	Status
CBP			
A3342V	5'-CACTTCAGGTTTCTTTTCATCC -3'	22 bp	Active
A2172Z	5'-CTTCTAGTTCTTTTTGTATCTTGTAG-3'	26 bp	Inactive
scrA3342V	5'- ATTCTCATCATCGTCTTCGTTC-3'	22 bp	Inactive

The first and last three base pairs of each ODN sequence were phosphorothiolate modified.

Table 2: Laminin and integrin primers

Gene	Primer set	Cycle conditions	PCR cycle number
ITGA3	F: 5'-AAGCCAAGTCTGAGACT -3' R: 5'-GTAGTATTGGTCCCGAGTCT -3'	94°C 3 min. 94°C 30 sec. 60°C 1 min. 72°C 1 min. 72°C 7 min.	22
ITGB1	F: 5'-GCGAAGGCATCCCTGAAAGT -3' R: 5'-GGACACAGGATCAGGTTGGA -3'	94°C 3 min. 94°C 30 sec. 54°C 30 sec. 72°C 1 min. 72°C 7 min.	19
LAMA3	F: 5'-TGTGGATCTTTGGGGCAG-3' R: 5'-TTGCCATAGTAGCCCTCCTG -3'	94°C 3 min. 94°C 30 sec. 58°C 30 sec. 72°C 1 min. 72°C 7 min.	20
LAMB3	F: 5'-TGAGGTTCAGCAGGTACTGC -3' R: 5-TAACTGTCCCATTGGCTCAG -3'	95°C 3 min. 95°C 1 min. 55°C 1 min. 72°C 1 min. 72°C 7 min.	23
LAMC2	F: 5'-CTGAGTATGGGCAATGCCAC -3' R: 5'-GCTCTGGTATCAACCTTCTG -3'	95°C 3 min. 95°C 1 min. 55°C 1 min. 72°C 1 min. 72°C 7 min.	22
Beta-actin	F: 5'-GCTCGTCGTCGACAACGGCTC-3' R: 5'-CAAACATGATCTGGGTCATCTTCTC-3' (Invitrogen)	94°C 2 min. 94°C 15 sec. 55°C 30 sec. 72°C 30 sec. 72°C 7 min.	18

Figure 1: Partial karyotypes of a representative late passage HMEC-E6 (passage 20) mitotic cells demonstrate two copies of an unbalanced translocation between chromosomes 13 and 16 involving 16p13 (arrows). *(a)* SKY in display colors (blue, chromosome 13 material; bluish gray, chromosome 16 material). *(b)* SKY in classification colors (red, chromosome 13 material; orange, chromosome 16 material). *(c)* Inverted and contrast-enhanced DAPI image of the same metaphase cels.

Figure 2: *(a)* Expression of endogenous p53 and exogenous HPV-16 E6 mRNA in HMECs. Passage 10 and 18 HMEC-P parental cells **(Parental)**, HMEC-LXSN controls **(LXSN)**, and HMEC-E6 cells **(E6)** were analyzed for p53 and HPV-16 E6 mRNA expression. Ten micrograms of RNA were loaded per lane. 36B4 served as a loading control.

(b) Expression of p53 protein is suppressed in HMEC-E6 cells. Passage 10 and 18 HMEC-P parental cells **(Parental)**, HMEC-LXSN controls **(LXSN)**, and HMEC-E6 cells **(E6)** were analyzed for p53 protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin serves as a loading control.

(c) CBP protein expression is decreased in apoptosis-resistant late passage HMEC-E6 cells. Early and late passage HMEC-LXSN vector controls **(LXSN)** (passages 11 and 16) and HMEC-E6 cells **(E6)** (passages 11 and 18) were analyzed for CBP protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin was used as a loading control.

(d) CBP protein expression is suppressed by antisense ODNs. HMEC-LXSN vector controls **(LXSN)** (passage 12) and early passage HMEC-E6 cells **(E6)** (passage 12) were cultured in the presence of **(1)** no treatment, **(2)** active CBP-specific ODN (A3342V), and **(3)** inactive CBP ODN (scrA3342V). Resultant cells were analyzed for CBP protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin was used as a loading control.

Figure 3: Inhibition of CBP expression in HMECs by antisense ODNs results in enhanced proliferation in rECM. The mean diameter of spheres formed by early passage HMEC-LXSN vector controls (passage 10) *(a)* and early passage HMEC-E6 cells (passage 11) *(b)* treated with either CBP antisense ODN (A3342V) **(CBP-as)** or inactive CBP ODN (scrA3342V) **(CBP-scr)** were plotted as a function of days in culture. Cells were plated in rECM on Day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. Ki-67 *(c, d)* staining indices in early passage HMEC-LXSN cells (passage 10) *(c)* and early passage HMEC-E6 cells (passage 11) *(d)*. Two hundred cells were surveyed per time point and indices were calculated from an average of 3 separate experiments. *Error bars* show standard error.

Figure 4: Suppression of CBP expression in HMECs inhibits polarized expression of E-cadherin and ZO-1 in rECM culture. Localization of E-cadherin and ZO-1 in HMECs treated with CBP antisense ODNs using immunofluorescence microscopy. Frozen section of early passage HMEC-LXSN vector controls (passage 11) *(a, c, e, g)*, HMEC-E6 cells (passage 11) *(b, d, f, h)*, treated with either CBP antisense ODN (A3342V) *(a, b, e, f)* or inactive CBP ODN (scrA3342V) *(c, d, g, h)* grown in rECM for 6 days, cryosectioned, and stained with a monoclonal antibody to E-cadherin *(a-d)* or ZO-1 *(e-h)* as described in Materials and Methods. E-cadherin was localized primarily at points of cell-cell contact in HMEC-LXSN and HMEC-E6 cells treated with inactive CPB ODNs (scrA3342V) *(c, d, g, h* arrows*)*. In contrast, HMEC-LXSN and HMEC-E6 cells treated with antisense CPB ODNs (A3342V) showed dispersed membrane and intracellular staining of both E-cadherin and ZO-1 *(a, b, e, f,* arrowheads*)*.

Figure 5: Inhibition of CBP in early passage HMECs by antisense ODNs blocks apoptosis in rECM culture. Electron micrographs of early passage HMEC-LXSN control cells (passage 11) *(a)* and early passage HMEC-E6 cells (passage 11) *(b)* treated with CBP antisense (A3342V) ODN and grown in rECM for 9 days. Cells formed large, dense, irregularly shaped multicellular colonies with no central lumen *(a, b)*. In contrast, early passage HMEC-E6 cells (passage 10) treated with inactive CBP ODN (scrA3342V) *(c)* underwent apoptosis when grown in rECM for 7 days as evidenced by 1) nuclear condensation **(n)**, 2) cell shrinkage and separation, and 3) margination of chromatin **(mr)**. Percent of apoptotic cells in early passage HMEC-E6 cells (passage 11) *(d)* and early passage HMEC-LXSN controls (passage 11) *(e)* treated either with active (A3342V) or inactive (scrA3342V) CBP-specific ODNs. Apoptosis was measured by TUNEL-staining as described in Materials and Methods. Apoptotic index was measured by calculating the percentage of TUNEL-staining cells relative to the total number of cells surveyed. Data represents an average of three separate experiments. *Error bars* show standard error.

Figure 6: Laminin-5 α3-chain mRNA and protein expression is decreased in rECM-resistant, CBP-"poor" late passage HMEC-E6 cells.

(a) Analysis of differential gene expression in early **(E6E)** and late **(E6L)** passage HMEC-E6 cells (passage 10 and 18) relative to early passage HMEC-LXSN controls (passage 10) **(LXSN)**. Cells were grown in contact with rECM and harvested for differential gene expression as described in Materials and Methods. All RNA combinations used for array analysis were obtained from cells that were matched for passage number, cultured under the identical growth conditions, and harvested at identical confluency. Data was collected in triplicate using independent biological replicates. Array images were processed using Affymetrix MAS 5.0 software as described in Materials and Methods. Pair-wise "treatment vs control" comparisons were made employing CyberT (Baldi, et al., 2001), a Bayesian t-statistic algorithm derived for microarray analysis. Color-coding: green, downregulation of gene expression; red, induction; black, no significant change; grey, no data available.

(b) Semiquantitative RT-PCR analysis of integrin and laminin-5 mRNA expression in early and late passage HMEC-E6 cells (passage 10 and 18) and HMEC-LXSN controls (passage 10 and 16). Expression was normalized to beta-actin. These data are representative of three separate experiments.

(c) Quantitation of RT-PCR expression data. Expression studies were performed in triplicate. The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software. Expression was normalized to beta-actin.

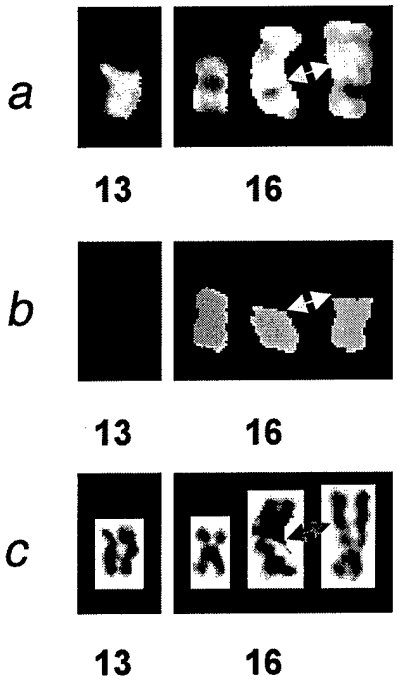
(d) Laminin-5 α3-chain protein expression is decreased in rECM-resistant, late passage HMEC-E6 cells (passage 18) relative to rECM-sensitive, early passage HMEC-E6 cells (passage 10) and early and late passage HMEC-LXSN controls (passage 11 and 16). Western analysis was performed as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin serves as a loading control.

Figure 7: Immunofluorescence characterization of α3/β1-integrin and laminin-5 expression in rECM-sensitive and –resistant cells. Frozen section of early passage HMEC-LXSN controls (passage 10) *(a-c),* late passage HMEC-controls (passage 16) *(d-f),* early passage HMEC-E6 cells (passage 10) *(g-i),* and late passage HMEC-E6 cells (passage 18) *(j-l)* grown in rECM for 6 days, cryosectioned, and immunostained for localization of α3-integrin *(a, d, g, j),* β1-integrin *(b, e, h, k),* and laminin-5 α3-chain *(c, f, i, l).* α3-integrin, β1-integrin, and laminin-5 α3-chain expression was primarily localized at the basal surface of early and late passage HMEC-LXSN and early passage HMEC-E6 cells *(a-i).* In contrast, apoptosis resistant, late passage HMEC-E6 cells showed dispersed membrane and intracellular staining of α3-integrin and laminin-5 α3-chain (arrowheads).

Figure 8: Immunofluorescent characterization of α3β1-integrin and laminin-5 expression in HMECs treated with CBP antisense ODNs. Frozen section of early passage HMEC-LXSN vector controls (passage 11) and HMEC-E6 cells (passage 11) treated either with CBP antisense ODN (A3342V) or inactive CBP ODN (scrA3342V). Cells were grown in rECM for 6 days, cryosectioned, and immunostained for either α3-integrin *(a-d)*, β1-integrin *(e-h)*, or laminin-5 α3–chain *(i-l)* as described in Materials and Methods. α3- and β1-integrin and laminin-5 α3-chain expression was primarily localized at the basolateral surface in HMEC-LXSN and HMEC-E6 cells treated with inactive CBP ODNs *(arrow heads)*. In contrast, HMEC-LXSN and HMEC-E6 cells treated with antisense CBP ODNs showed cells demonstrated disorganized membrane and cytosolic staining of α3-integrin and laminin-5 α3-chain *(arrows)*.

Figure 9: Suppression of CBP expression results in a decrease in *LAMA3A* promoter activity in cells grown in contact with rECM. *(a) LAMA3A* promoter activity was measured in 1) early and late passage HMEC-LXSN controls (passage 11 and 16) and 2) early passage HMEC-E6 cells (passage 10) and compared to rECM-resistant, CBP-"poor" late passage HMEC-E6 cells (passage 18). *(b) LAMA3A* promoter activity is measured in early passage HMEC-LXSN controls (passage 11) or HMEC-E6 cells (passage 11) treated with either 1) CBP-specific antisense ODNs (A3342V) or 2) inactive ODNs (scrA3342V) and grown in rECM. *LAMA3A* promoter activity was measured as described in Materials and Methods. Data represent two experiments performed in triplicate. *Error bars* show standard error.

Figure 10: Suppression of CBP in HMECs grown in rECM promotes decreased occupancy of the 277 bp AP-1-"rich" region of the *LAMA3A* promoter. *(a)* ChIP was performed in 1) early and HMEC-LXSN controls (passage 11 and 16) and 2) early passage HMEC-E6 cells (passage 11) and compared with rECM-resistant, CBP-"poor", late passage HMEC-E6 cells (passage 18). *(b)* Early passage HMEC-E6 cells (passage 10) treated with CBP-specific ODNs, and grown in contact with rECM, were tested by ChIP to determine whether suppression of CBP expression resulted in a loss of CBP-binding AP-1-"rich" site of the *LAMA3A* promoter. ChIP was performed as described in Materials and Methods. Input controls test the integrity of the DNA samples. These data are representative of three separate experiments.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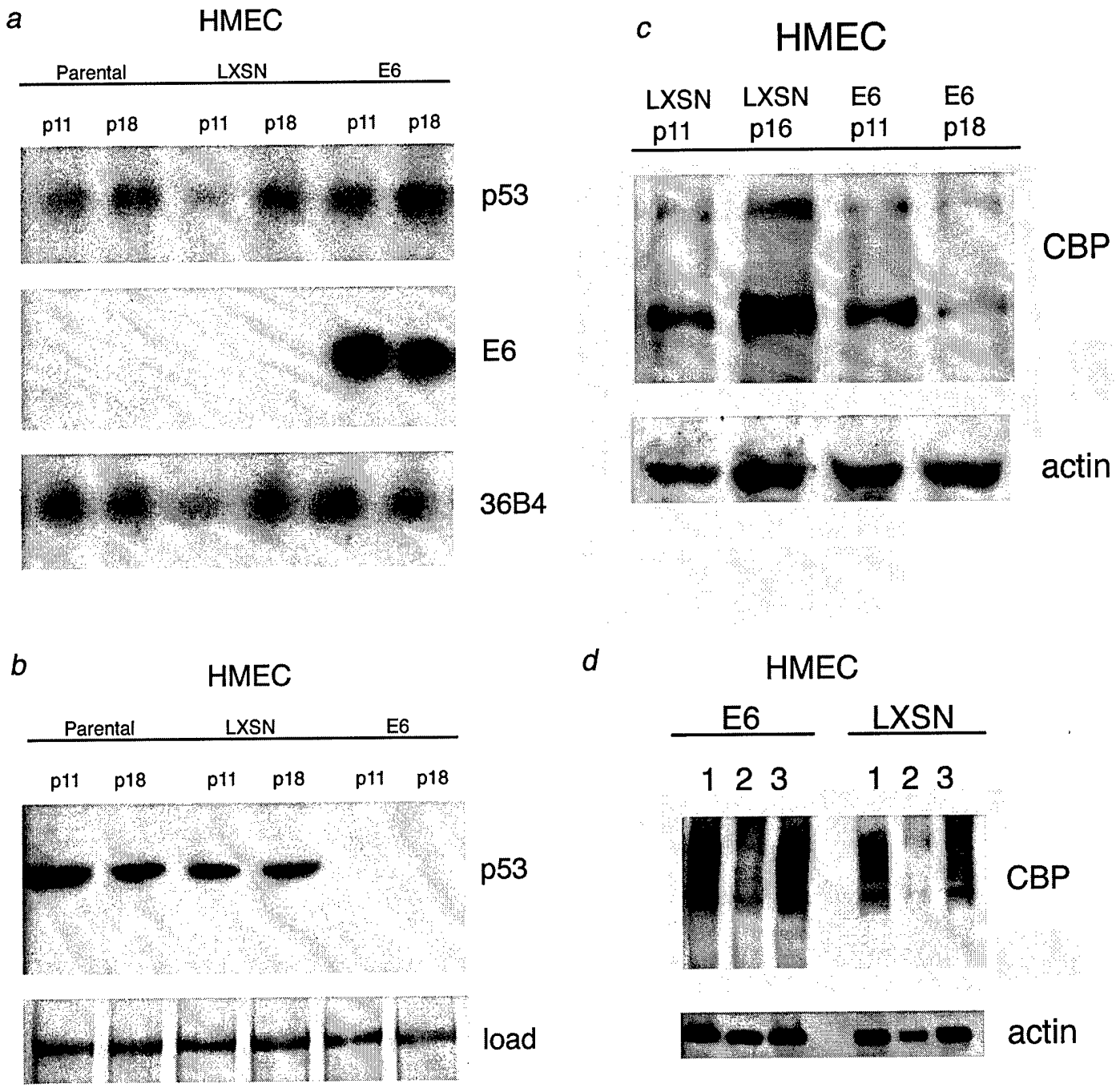
16
b
13
16
c
13
16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p53
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HMEC
Parental
LXSN
E6
p11
p18
p11
p18
p11
p18
p53
load
c
HMEC
LXSN p11
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LXSN
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CBP
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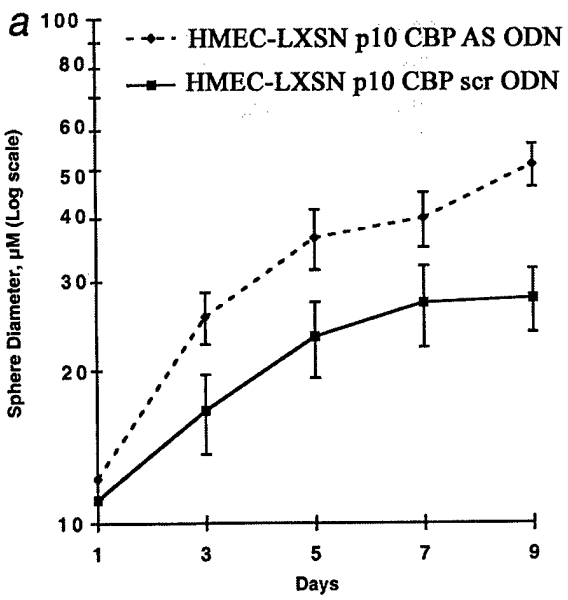
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HMEC-LXSN p10 CBP scr ODN
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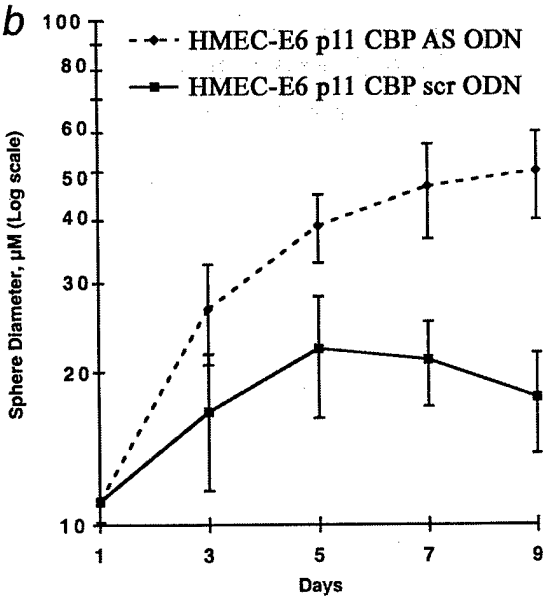
40
30
20
10
1
3
5
7
9
Days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HMEC-E6 p11 CBP scr ODN
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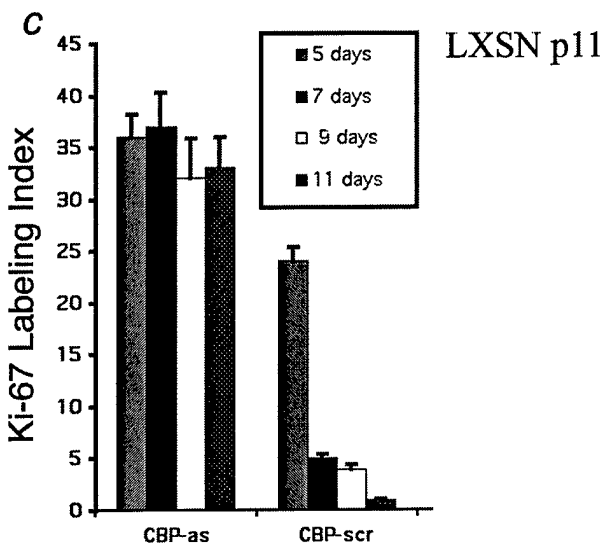
30
20
10
1
3
5
7
9
Days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5 days
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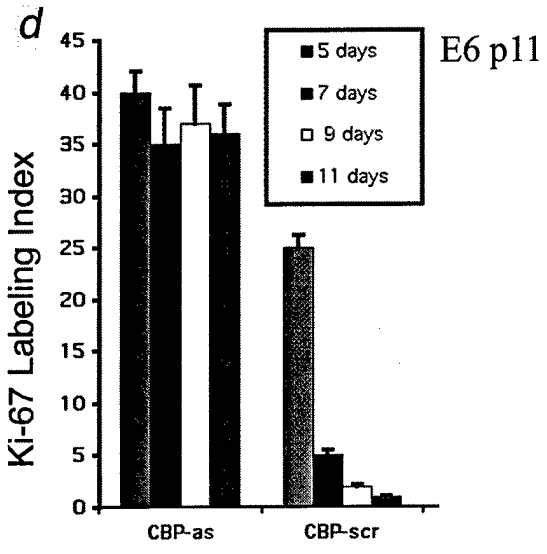
CBP-as
CBP-scr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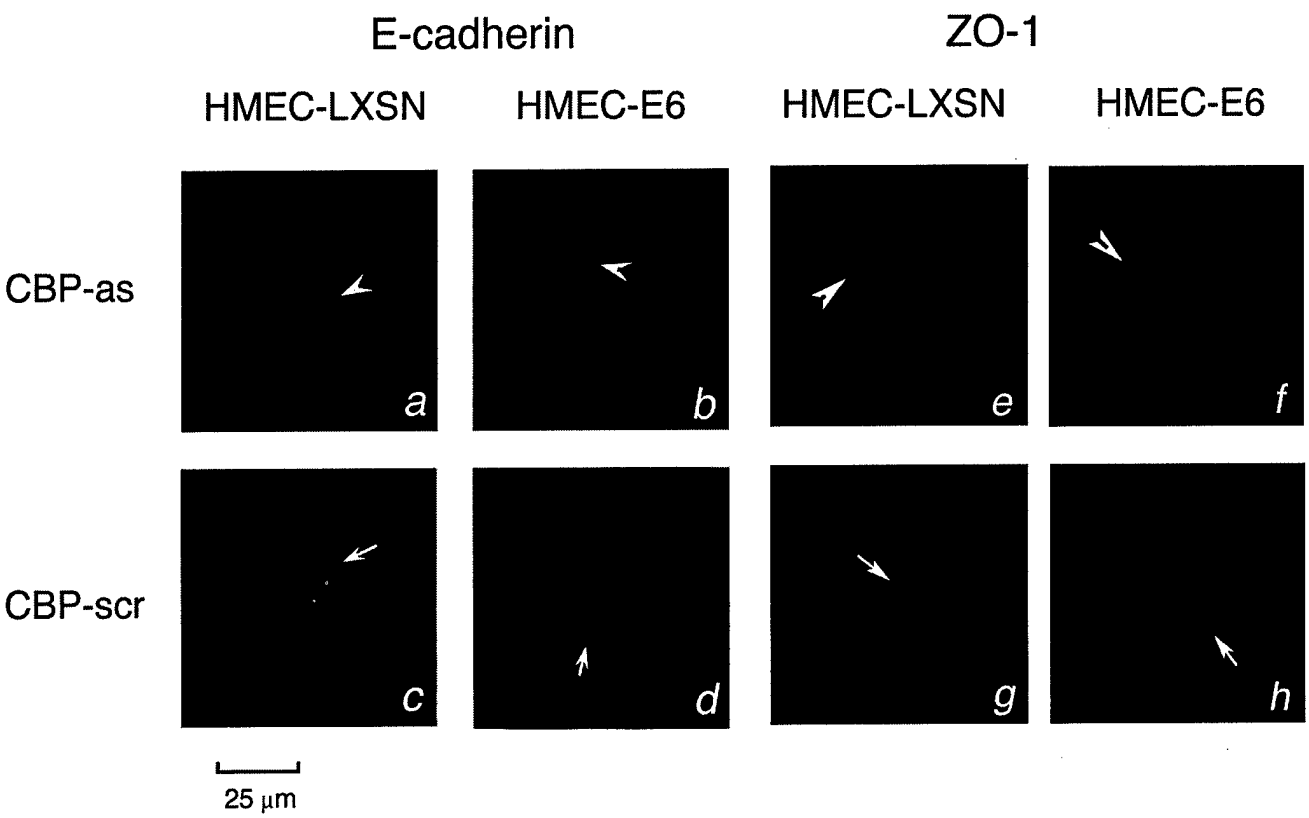
CBP-scr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ZO-1
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HMEC-E6
HMEC-LXSN
HMEC-E6
CBP-as
CBP-scr
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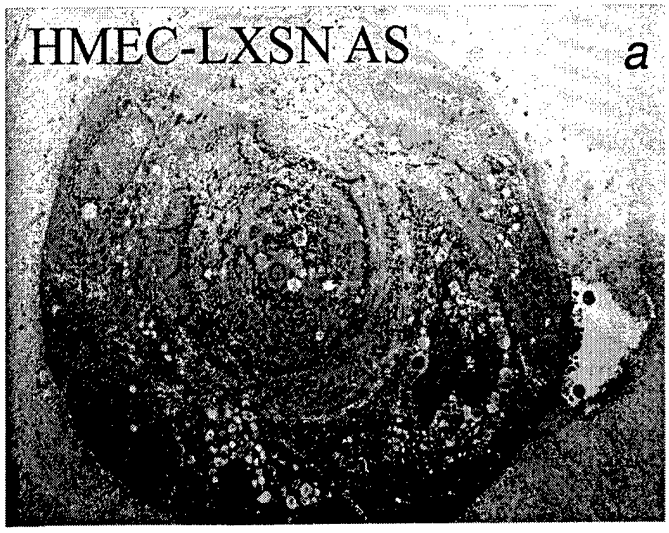
b
e
f
c
d
g
h
25 μm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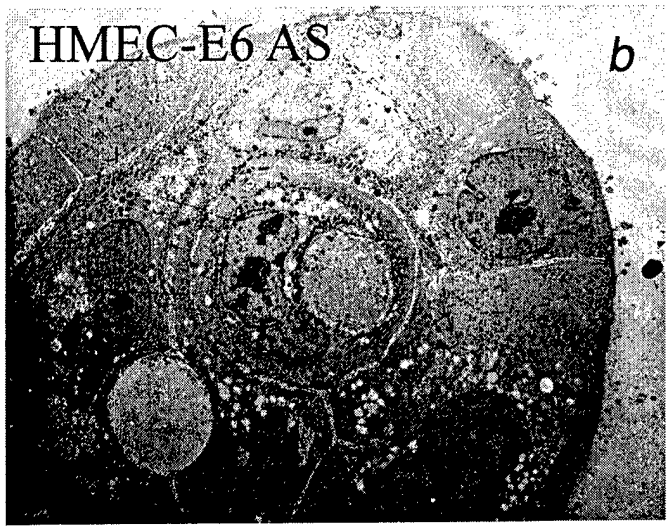
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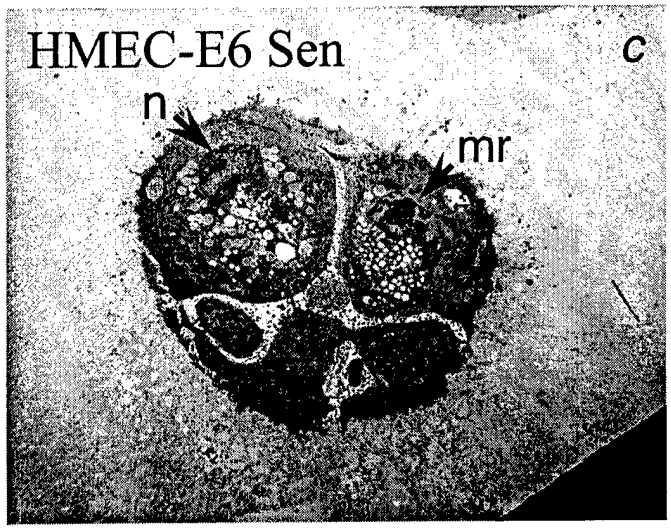
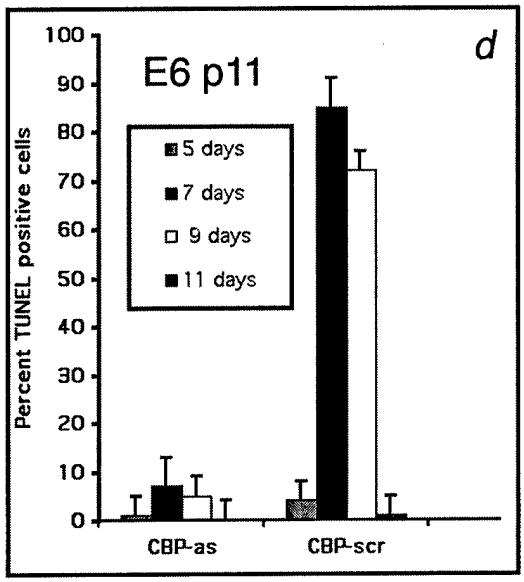

HMEC-E6 Sen
c
n
mr

10 μm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E6 p11
Percent TUNEL positive cells
100
90
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5 days
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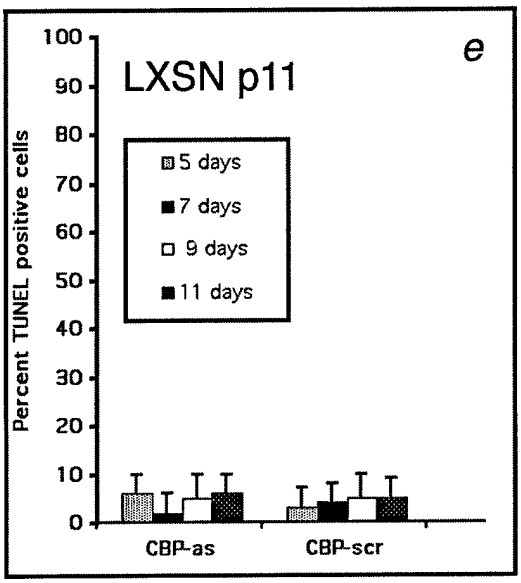
11 days
CBP-as
CBP-scr


e
LXSN p11
Percent TUNEL positive cells
100
90
80
70
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5 days
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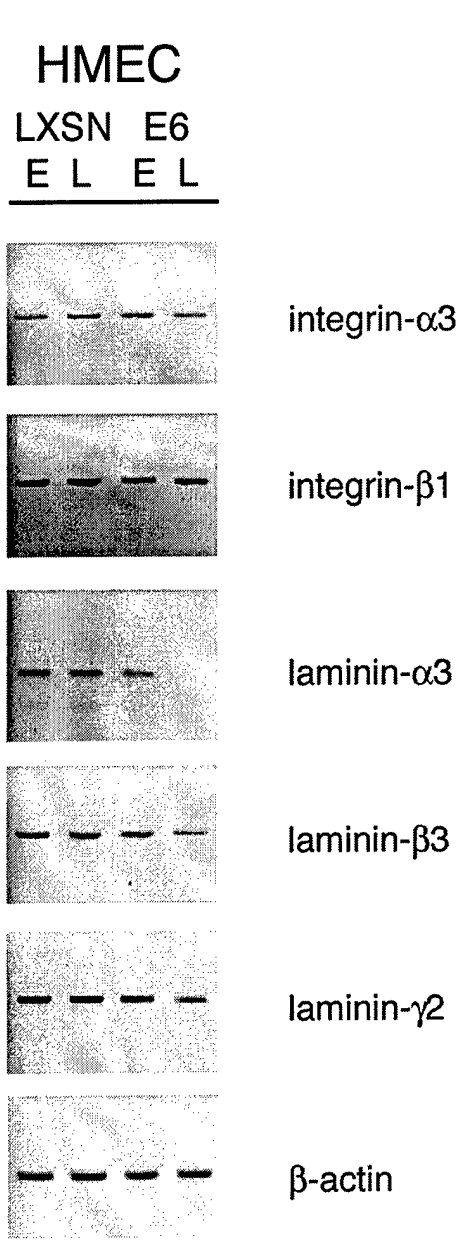
11 days
CBP-as
CBP-scr

a.

M10277. Human cytoplasmic beta-actin gene, complete cds
M35198. Human integrin B-6 mRNA, complete cds
U31201. Human laminin gamma2 chain gene (LAMC2). Human laminin gamma2 chain
L34155. Homo sapiens laminin-related protein (LamA3) mRNA, complete cds
X53587. Human mRNA for integrin beta 4
U17760. Human laminin S B3 chain (LAMB3) gene
U31201. Human laminin gamma2 chain gene (LAMC2). Human laminin gamma2 chain
M14648. Human cell adhesion protein (vitronectin) receptor alpha subunit
J03925. Human Mac-1 gene encoding complement receptor type 3, CD11b
X53002. Human mRNA for integrin beta-5 subunit
J05633. Human integrin beta-5 subunit mRNA, complete cds
U40279. Human beta-2 integrin alphaD subunit (ITGAD) gene, exons 25-30
S80335. Integrin beta 7 subunit [human, mRNA, 2798 nt]
X68742. H.sapiens mRNA for integrin, alpha subunit
X64072. H. sapiens CD18 exon 2
M34189. Integrin Beta 1
L25851. Homo sapiens integrin alpha E mRNA, complete cds
M34344. Human platelet glycoprotein IIb (GPIIb) gene
M14199. Human laminin receptor (2H5 epitope) mRNA, 5 end
X07979. Human mRNA for fibronectin receptor beta subunit
U40282. Human integrin-linked kinase (ILK) mRNA, complete cds
D25303. Human mRNA for integrin alpha subunit, complete cds
L36531. Homo sapiens integrin alpha 8 subunit mRNA, 3 end
Y00796. Human mRNA for leukocyte-associated molecule-1 alpha subunit
X16983. Human mRNA for integrin alpha-4 subunit
X02761. Human mRNA for fibronectin (FN precursor)
M61916. Human laminin B1 chain mRNA, complete cds
M59911. Human integrin alpha-3 chain mRNA, complete cds
S70348. Integrin beta 3 (alternatively spliced, clone beta 3C)
X79683. H.sapiens LAMB2 mRNA for beta2 laminin.
X74295. H.sapiens mRNA for alpha 7B integrin
M55210. Human laminin B2 chain (LAMB2) gene
Z26653. H.sapiens mRNA for laminin M chain (merosin)
M15395. Human leukocyte adhesion protein (LFA-1/Mac-1/p150,95 family)
J02963. Human platelet glycoprotein IIb mRNA, 3 end
X02761. Fibronectin, Alt. Splice 1
X06256. Human mRNA for fibronectin receptor alpha subunit
S78569. laminin alpha 4 chain [human, fetal lung, mRNA, 6204 nt]
U33880. Human beta 1 integrin isoform D (ITGB1) gene, partial cds.
X53586. Integrin alpha 6 (or alpha E) protein gene extracted from Human mRNA
U43901. Human 37 kD laminin receptor precursor/p40 ribosome associated protei
M73780. Human integrin beta-8 subunit mRNA, complete cds
M35999, Human platelet glycoprotein IIIa (GPIIIa) mRNA, complete cds

b.


HMEC
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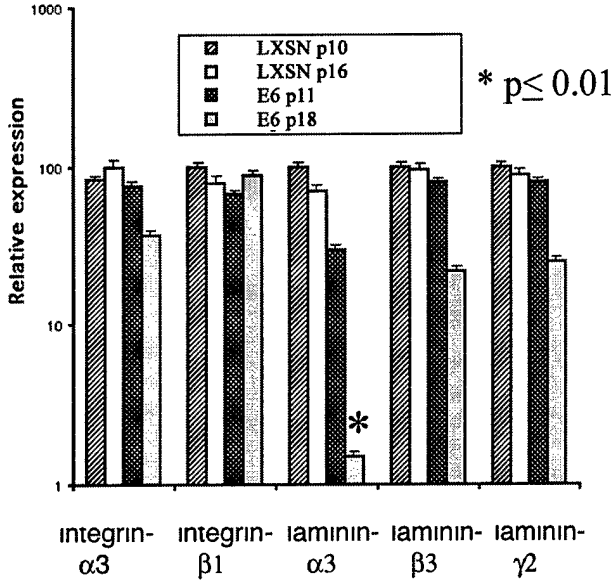
integrin-β1
laminin-α3
laminin-β3
laminin-γ2
β-actin

c.


1000
100
10
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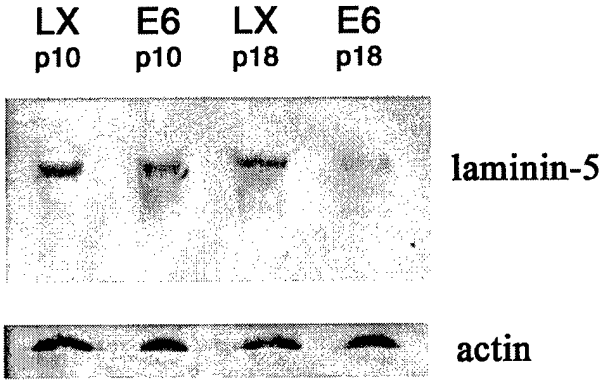
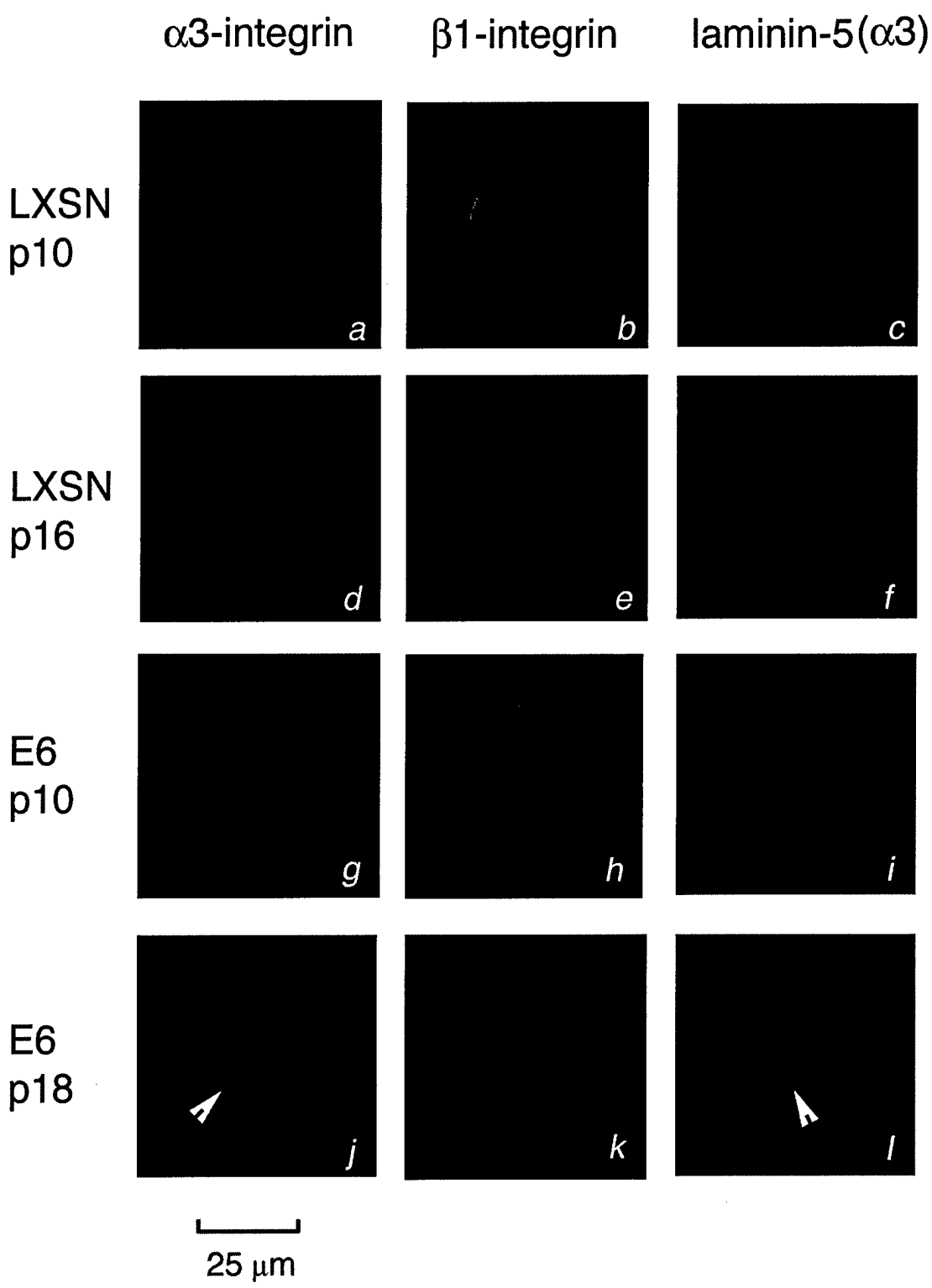
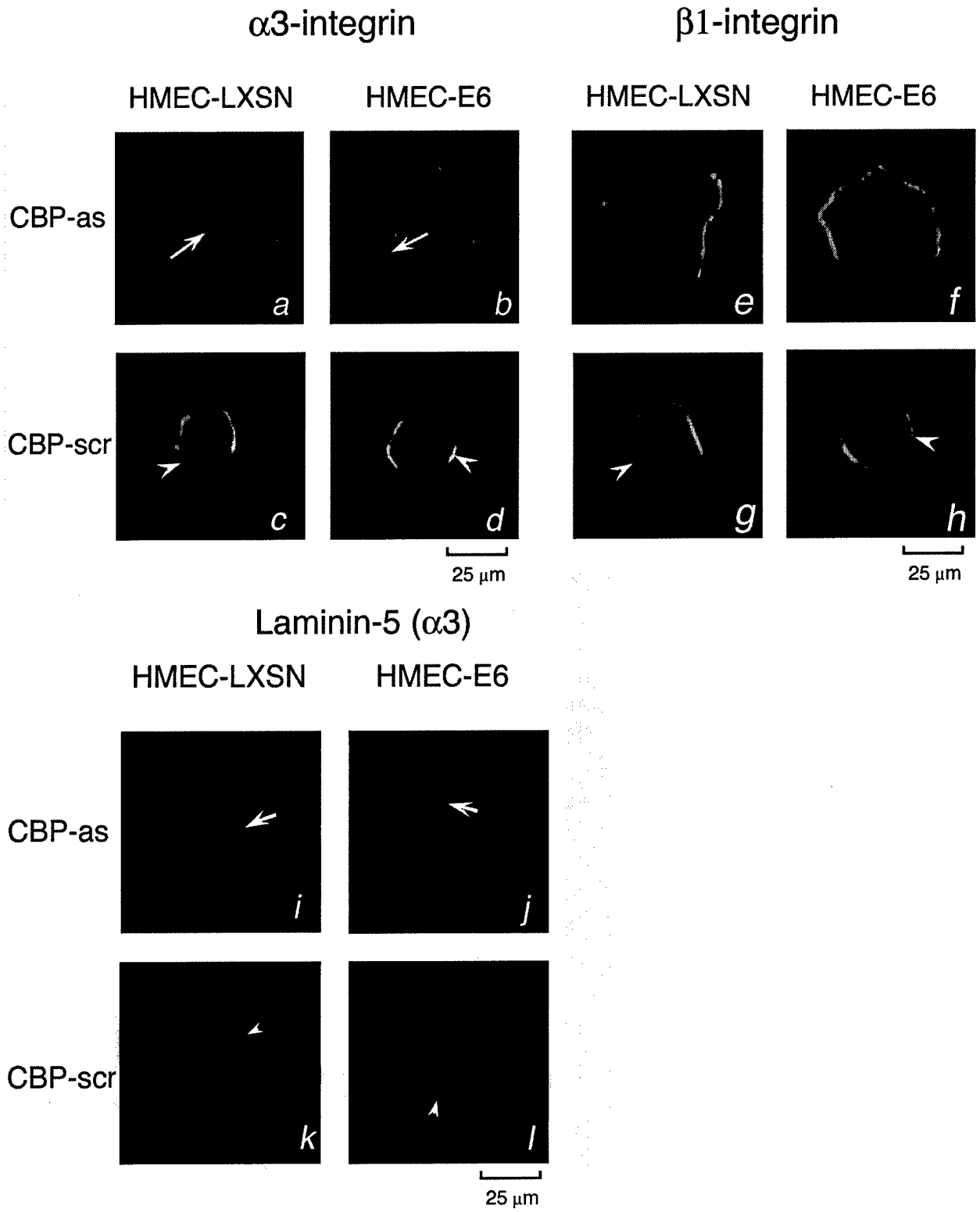
Relative expression
LXSN p10
LXSN p16
E6 p11
E6 p18
* p≤ 0.01
*
integrin-α3
integrin-β1
laminin-α3
laminin-β3
laminin-γ2

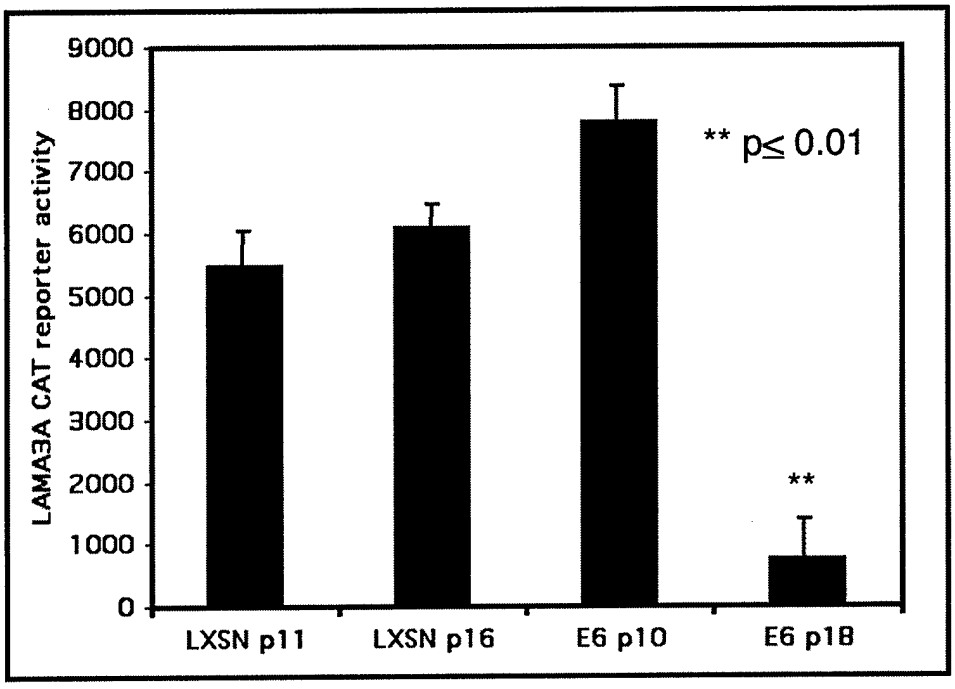
d.


LX p10
E6 p10
LX p18
E6 p18
laminin-5
actin

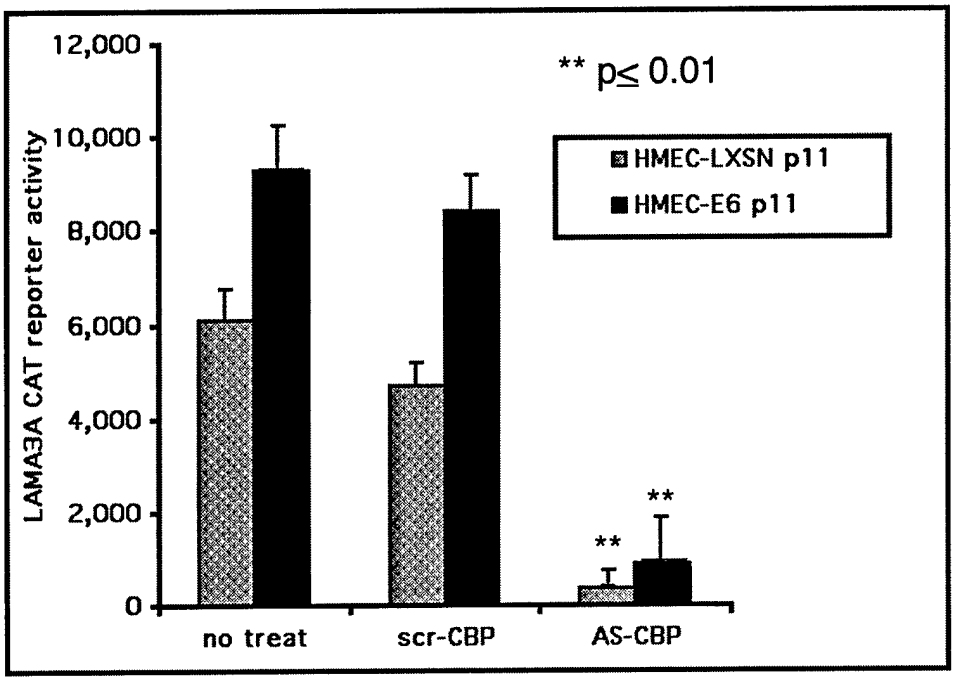

α3-integrin
β1-integrin
laminin-5(α3)
LXSN p10
LXSN p16
E6 p10
E6 p18
a
b
c
d
e
f
g
h
i
j
k
l
25 μm


α3-integrin
β1-integrin
HMEC-LXSN
HMEC-E6
HMEC-LXSN
HMEC-E6
CBP-as
CBP-scr
a
b
c
d
e
f
g
h
25 μm
25 μm
Laminin-5 (α3)
HMEC-LXSN
HMEC-E6
CBP-as
CBP-scr
i
j
k
l
25 μm

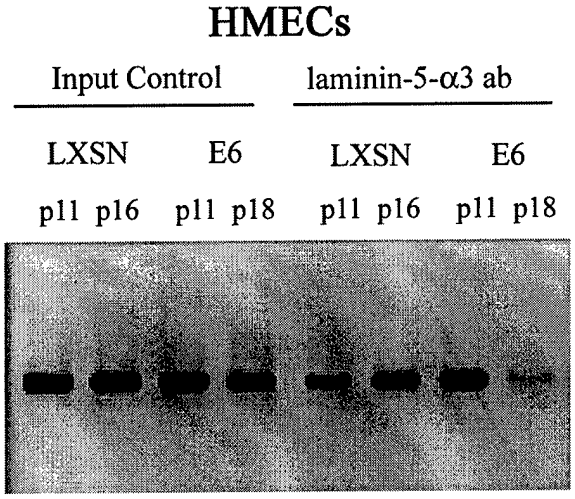
a


LAMA3A CAT reporter activity
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
** p≤ 0.01
**
LXSN p11
LXSN p16
E6 p10
E6 p18

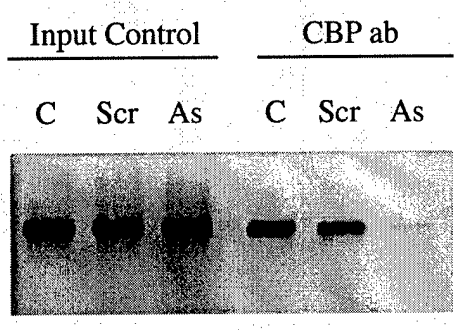
b


LAMA3A CAT reporter activity
12,000
10,000
8,000
6,000
4,000
2,000
0
** p≤ 0.01
HMEC-LXSN p11
HMEC-E6 p11
**
**
no treat
scr-CBP
AS-CBP

a


HMECs
Input Control
laminin-5-α3 ab
LXSN
E6
LXSN
E6
p11 p16 p11 p18 p11 p16 p11 p18

b


HMEC-E6
Input Control
CBP ab
C Scr As C Scr As