CREB-binding protein regulates apoptosis and growth of HMECs grown in reconstituted ECM via laminin-5

Eric C. Dietze1, Michelle L. Bowie1, Krzysztof Mrózek2, L. Elizabeth Caldwell3, Cassandra Neal3, Robin J. Marjoram1, Michelle M. Troch1, Gregory R. Bean1, Kazunari K. Yokoyama4, Catherine A. Ibarra1 and Victoria L. Seewaldt1,5,*

1Division of Medical Oncology, Duke University, Durham, NC 27710, USA
2Division of Hematology and Oncology, Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA
3Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
4RIKEN, Tsukuba Institute, Ibaraki 305-0074, Japan
5Department of Pharmacology and Cancer Biology, Duke University, Durham, NC 27710, USA

*Author for correspondence (e-mail: seewa001@mc.duke.edu)

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Summary
Interactions between normal mammary epithelial cells and extracellular matrix (ECM) are important for mammary gland homeostasis. Loss of interactions between ECM and normal mammary epithelial cells are thought to be an early event in mammary carcinogenesis. CREB-binding protein (CBP) is an important regulator of proliferation and apoptosis but the role of CBP in ECM signaling is poorly characterized. CBP was suppressed in basal-cytokeratin-positive HMECs (CK5/6+, CK14+, CK8–, CK18–, CK19–). Suppression of CBP resulted in loss of reconstituted ECM-mediated growth control and apoptosis and loss of laminin-5 α3-chain expression. Suppression of CBP in normal human mammary epithelial cells (HMECs) resulted in loss of CBP occupancy of the LAMA3A promoter and decreased LAMA3A promoter activity and laminin-5 α3-chain expression. Exogenous expression of CBP in CBP-negative HMECs that have lost reconstituted ECM-mediated growth regulation and apoptosis resulted in (1) CBP occupancy of the LAMA3A promoter, (2) increased LAMA3A activity and laminin-5 α3-chain expression, and (3) enhancement of reconstituted ECM-mediated growth regulation and apoptosis. Similarly, suppression of laminin-5 α3-chain expression in HMECs resulted in loss of reconstituted ECM-mediated growth control and apoptosis. These observations suggest that loss of CBP in basal-cytokeratin-positive HMECs results in loss of reconstituted ECM-mediated growth control and apoptosis through loss of LAMA3A activity and laminin-5 α3-chain expression. Results in these studies may provide insight into early events in basal-type mammary carcinogenesis.

Key words: CBP, Basal cytokeratins, Basal-type breast cancer, Extracellular matrix, Apoptosis, Laminin-5

Introduction
Breast tissue is composed of mammary epithelial cells that rest on extracellular matrix (ECM), and interactions between epithelial cells and ECM regulate normal growth, polarity and apoptosis (Petersen et al., 1992; Strange et al., 1992; Zutter et al., 1995; Ilic et al., 1998; Farrelly et al., 1999; Stupack and Cheresh, 2002). Loss of ECM signaling is thought to be an early event in mammary carcinogenesis and to promote some of the phenotypic changes observed during malignant progression (Petersen et al., 1992; Howlett et al., 1995; Mercurio et al., 2001; Farrelly et al., 1999; Hood and Cheresh, 2002; Stromblad et al., 2002). Carcinogenesis is hypothesized to be a multistep process resulting from the progressive accumulation of genetic damage. Although loss or mutation of specific tumor suppressor genes such as TP53 promotes mammary carcinogenesis (Fabian et al., 1996; Rohan et al., 1998), not all damaged epithelial cells progress to malignancy and many are thought to be eliminated by apoptosis (Thompson, 1995). Mammary gland homeostasis requires a coordinated balance 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; Farrelly et al., 1999; Mercurio et al., 2001; Hood and Cheresh, 2002; Stupack and Cheresh, 2002). Loss of ECM signaling is thought to promote mammary carcinogenesis by preventing the apoptotic elimination of damaged mammary epithelial cells.

The majority of breast cancers are epithelial in origin. The normal mammary gland contains luminal epithelial cells that express luminal-type cytokeratins (CK7, CK8, CK18, CK19) and cells that express stratified epithelial cytokeratins (CK5/6, CK14, CK17) (Wazer et al., 1995; Anbazhagan et al., 1998; Abd El-Rehim et al., 2004). Epithelial breast cancers are broadly divided into those that express luminal keratins (luminal-type) and those that express stratified epithelial cytokeratins (basal-type) (Perou et al., 2000; Foulkes et al., 2003).

We previously used an in vitro model (Howlett et al., 1995; Weaver et al., 1995; Stampfer, 1985) to investigate the potential role of ECM-signaling in promoting apoptosis in basal-cytokeratin-positive human mammary epithelial cell strains (HMECs) that had acutely lost p53 function (Seewald et al.,...
The HMEC strains used in our studies were isolated from reduction mammoplasty tissue by Martha Stampfer (Stampfer, 1985) and express basal-type cytokeratins (CK5/6, CK14+, CK17+) but not luminal-type cytokeratins (CK8−, CK18−, CK19−) (Moll et al., 1982; Taylor-Papadimitriou and Lane, 1987; Wazer et al., 1995; Foulkes et al., 2003). These HMEC strains are distinguished from myoepithelial cells by (1) staining negatively for smooth muscle actin, (2) the presence of cytoplasmic vesicles that stain positively for lipid and exhibit an apical distribution in rECM culture by electron microscopy, and (3) the absence, when viewed by electron microscopy, of plasmalemmal vesicles and tracts of 5-7 nm actin microfilaments (Pitelka, 1983; Seewaldt et al., 1999b; Yee et al., 2003; Seewaldt et al., 2001a). Acute loss of p53 was modeled in these basal-cytokeratin-positive HMEC strains by either retroviral-mediated expression of the human papillomavirus type-16 (HPV-16) E6 protein (Band, 1995; Wazer et al., 1995) or treatment with p53-specific antisense oligonucleotides (AS ODNs). We observed that while p53+/H925-1 HMEC controls grown in reconstituted ECM (rECM) underwent growth arrest on day 7, HMEC-E6 and p53−/H925-1 HMECs underwent apoptosis (Seewaldt et al., 2001a). Although the acute expression of either HPV-16 E6 or suppression of p53 in HMECs promoted enhancement of rECM-mediated apoptosis, HMEC-E6 cells passaged in non-rECM culture rapidly lost both rECM-mediated growth arrest and apoptosis associated with loss of polarized expression of the laminin-5 receptor, α3β1-integrin, and loss of genetic material from chromosome 16 (Seewaldt et al., 2001a). These observations led us to hypothesize that laminin-5−α3β1-integrin growth regulation and polarity signals are critical for targeting the elimination of HMECs that have acutely suppressed levels of p53, and that 16p harbors a gene(s) whose loss and/or rearrangement might promote loss of rECM-mediated growth arrest and apoptosis.

Laminins are ECM glycoproteins that promote mammary gland homeostasis by 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 γ2) 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 and has been implicated in adhesion, migration and invasion. Recent reports indicate that binding of the laminin-5 α3-chain globular LG3 domain to α3β1-integrin mediates cell adhesion and migration (Shang et al., 2001). Dysregulation of laminin-5 expression is observed during carcinogenesis. Whereas benign ductal and lobular epithelial cells demonstrate continuous laminin-5 staining at the epithelial-stromal interface, primary breast cancers and breast cancer cells exhibit loss of laminin-5 α3-chain expression (Martin et al., 1998). Loss of laminin-5 α3-chain expression has also been observed in prostate cancer (Hao et al., 2001), while epigenetic inactivation of all three laminin-5 encoding genes has been observed in lung cancer cells (Sathyanarayana et al., 2003). There is also evidence that increased laminin-5 γ2-chain expression and cleavage may be associated with tissue remodeling and tumor invasion. Matrix metalloproteinase (MMP)-dependent mammary gland involution coincides with binding of the laminin-5 γ2-chain MMP-cleavage fragment, DIII, to the epidermal growth factor receptor (Schenk et al., 2003). Mammary glands from β1,4-galactosyltransferase 1-null mice exhibit excess mammary gland branching associated with (1) decreased laminin-5 α3-chain expression, (2) increased expression and cleavage of laminin-5 γ2-chain, and (3) increased MMP expression (Steffgen et al., 2002). Increased expression of laminin-5 γ2-chain is observed at the invasive front of tumors associated with poor prognosis (Pyke et al., 1994; Yamamoto et al., 2001; Niki et al., 2002) and cooperative interactions between laminin-5 γ2-chain and MMPs are associated with an aggressive phenotype in melanoma (Seftor et al., 2001). These observations suggest that dysregulation of laminin-5 signaling are important for cancer progression.

Our prior studies indicated that laminin-5−α3β1-integrin-growth regulation signals may be critical for targeting the elimination of HMECs (Seewaldt et al., 2001a). Little is known about the regulation of laminin-5 gene transcription in normal mammary epithelial tissue or about the molecular mechanism underlying the loss of laminin-5 expression observed in early breast carcinogenesis. The human LAMA3A promoter is known to contain three binding sites of the dimeric transcription factor activating protein 1 (AP-1) (Virolle et al., 1998; 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 bp is critical for baseline transcription of laminin-5 α3-chain (Miller et al., 2001). CREB-binding protein (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.

Detailed cytogenetic analysis performed for this report indicated that chromosome 16p13 is the critical area whose loss and/or rearrangement promoted loss of rECM-mediated growth arrest and apoptosis (Seewaldt et al., 2001a). CBP is a nuclear protein located at chromosome band 16p13.3 that regulates proliferation, differentiation and apoptosis (Giles et al., 1997; Yao et al., 1998). 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 the majority of benign and malignant papillary neoplasms of the breast and loss or amplification of 16p is frequently observed in premalignant breast lesions (Linninger et al., 1998; Tsuda et al., 1998; Aubele et al., 2000). Taken together, these observations suggest that loss of CBP expression promotes mammary carcinogenesis.

This report describes a role for CBP in mediating laminin-5 α3-chain expression in basal-cytokeratin-positive HMEC strains and subsequent enhancement of rECM-mediated growth control and apoptosis. Observations in this model system may predict a critical role for CBP in regulating basal-cytokeratin-positive mammary epithelial cell homeostasis through modulation of laminin-5 α3-signaling.

**Materials and Methods**

**Cell culture and media**

Normal human mammary epithelial cell (HMEC) strains AG11132 and AG11134 (M. Stampfer, 1972/RA7 and #48R, respectively) were purchased from the National Institute of Aging, Cell Culture Repository (Coriell Institute, National Institute of Aging, Cumden, NJ) (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) supplement with 4 μg/ml bovine pituitary extract (Clonetics #CC4009), 5 μg/ml insulin (Sigma, St Louis, MO), 10 ng/ml epidermal growth factor (UBL, Lake Placid, NY), 0.5 μg/ml hydrocortisone (Sigma), 10⁻³ M isoproterenol (Sigma), and 10 nM HEPES buffer (Standard Media). Cells were cultured at 37°C in a humidified incubator with 5% CO₂/95% air.

Large-scale rECM culture

Large-scale rECM culture was used to prepare total RNA or protein lysate for analysis using techniques previously developed by the laboratory of Mina Bissell (Roskelley 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% (vivo) rECM.

Cell growth and proliferation in rECM culture

Proliferation was assessed by Ki67 staining index, whereby 5 μm 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. Growth was measured by counting the number of DAPI-stained nuclei per cell cluster in cryosections of cells grown in rECM by previously published methods (Weaver et al., 1997).

Detection of apoptosis

TUNEL staining in HMECs grown in rECM for 5-11 days was carried out as previously described (Seewaldt et al., 2001a). 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. Electron microscopy was as previously described (Seewaldt et al., 1997a). Fifty colonies were scored for the number of TUNEL-positive cells as a percentage of the total number of cells scored. The apoptotic index was determined and cells were prepared for electron microscopy and immunostaining.

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 scrA3342V (22 mer, nucleotide position 3342-3363) was chosen to be the scrambled sequence of the antisense ODN to ensure identical nucleotide content and minimize differences potentially attributable to nucleic acid content, and 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 potentially attributable to nucleic acid content, and based on lack of suppression of CBP-specific ODNs (0.001 to 0.1 μM final concentration) on ice. rECM cultures were prepared as above and 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 constant supply of ODNs. The diameter of the growing colonies was determined and cells were prepared for electron microscopy and immunostaining.

Retroviral gene expression

Expression of HPV-16 E6 was as previously described (Seewaldt et al., 2001a). Exogenous expression of CBP in HMECs was as for HPV-16 E6 with the following modifications. The retroviral vector harboring the coding sequences for CBP was generated from the pcDNA3 recombinant plasmid by methods previously described (Seewaldt et al., 1995). The pcDNA3 plasmid was digested with BamHI and the released plasmid inserted into the BamHI cloning site of the dephosphorylated pLXSN plasmid. Correct orientation and sequence was verified by direct sequencing. Ten micrograms of purified plCBPSN retroviral construct plasmid was transfected via Cellfectin™ (Invitrogen, Carlsbad, CA) into the PE 501 murine ecotropic retrovirus packaging cell line (Seewaldt et al., 1995). Expression of the exogenous construct was confirmed by PCR and protein expression was confirmed by western analysis (Seewaldt et al., 2001a; Seewaldt et al., 2001b).

Cytogenetic analysis of early and late passage HMECs

Spectral karyotypic 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., 2001a; Seewaldt et al., 2001b).

Western blotting

Preparation of cellular lysates and immunoblotting were performed as previously described (Seewaldt et al., 1997b; Seewaldt et al., 1999a). For CBP expression, the blocked membrane was incubated with 1:200 dilution of the CBP C20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For laminin-5 expression, the membrane was incubated with a 1:100 dilution of the C19 antibody to the laminin-5 α3-chain (Santa Cruz Biotechnology). Loading control was provided by 1:200 dilution of the 119 antibody to β-actin (Santa Cruz Biotechnology). All CBP and laminin bands were normalized to actin. The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Immunostaining

Immunostaining was carried out as previously described (Seewaldt et

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### Table 1. CBP-specific antisense ODN sequences

| Target gene | Sequences* | Size | Status |
|-------------|------------|------|--------|
| CBP         | A3342V     | 5'⁻CACCTCGAGTTTCTTTACTCC-3'⁻ | 22 bp | Active |
|             | scrA3342V  | 5'⁻ATTCTCGACATCGTCTCTGTT-3'⁻ | 22 bp | Inactive |

*The first and last three base pairs of each ODN sequence were modified by phosphorothioate.
Differential gene expression studies

Cells were grown in rECM using 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 performed 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 performed 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 performed 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 performed 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.

Semi-quantitative RT-PCR

To confirm microarray data, relative transcript levels were analyzed by semi-quantitative 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 (Virolle et al., 2002), and 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 MgCl2, 50 mM KCl, pH 8.3, 2.5 units Taq polymerase, and 2.0 μl cDNA. Reaction conditions for β-actin were 300 nM forward primer, 300 nM reverse primer, 250 μM of each dNTP, 10 mM Tris-HCl, 1.5 mM MgCl2, 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 Applied Biosciences GeneAmp PCR system 2400 (Applied Biosciences). Preliminary reactions were performed 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 was analyzed by electrophoresis in 1.2-1.5% agarose gels containing ethidium bromide, visualized under UV light, and quantitated. All samples were performed in triplicate and normalized to β-actin as the control.

LAMA3A reporter studies

A 1403 bp region of the laminin-5 α3-chain (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′. The PCR product was cloned into pCRII (Invitrogen),

### Table 2. Laminin and integrin primers

| Gene   | Primer set                  | Cycle conditions          | PCR cycle number |
|--------|-----------------------------|---------------------------|------------------|
| ITGA3  | F: 5′-AAGCCAAGTCTGGAGACT-3′ | 94°C 3 min 94°C 30 sec    | 22               |
|        | R: 5′-GTAGTATTTGGCCAGCTT-3′ | 60°C 1 min 72°C 1 min     |                  |
| ITGB1  | F: 5′-GGAAGGGATTCCTGAAAGT-3′ | 94°C 3 min 94°C 30 sec    | 19               |
|        | R: 5′-GGACACAGGATAGGTTGGA-3′ | 54°C 30 sec 72°C 1 min    |                  |
| LAMA3  | F: 5′-TGTGGATCTTGGGCGAC-3′  | 94°C 3 min 94°C 30 sec    | 20               |
|        | R: 5′-TGCCCATATGTAGCCCTCTG-3′ | 58°C 30 sec 72°C 1 min    |                  |
| LAMB3  | F: 5′-TGAGTTTCAGCAGGTTACTC-3′ | 95°C 3 min 95°C 1 min     | 23               |
|        | R: 5′-TACCTGCTCCATTGGGTCTC-3′ | 55°C 1 min 72°C 1 min     |                  |
| LAMC2  | F: 5′-CTGAATGATGGGCAATGCCAC-3′ | 95°C 3 min 95°C 1 min     | 22               |
|        | R: 5′-GCTCTGGATATCAACCTTCTG-3′ | 55°C 1 min 72°C 1 min     |                  |
| β-actin| F: 5′-GTCCTGCTGCAACAGGCCTC-3′ | 94°C 2 min 94°C 15 sec    | 18               |
|        | R: 5′-CAACATGATCTGCTGCTTCTC-3′ | 55°C 30 sec 72°C 30 sec   |                  |

by semi-quantitative 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 (Virolle et al., 2002), and 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 MgCl2, 50 mM KCl, pH 8.3, 2.5 units Taq polymerase, and 2.0 μl cDNA. Reaction conditions for β-actin were 300 nM forward primer, 300 nM reverse primer, 250 μM of each dNTP, 10 mM Tris-HCl, 1.5 mM MgCl2, 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 Applied Biosciences GeneAmp PCR system 2400 (Applied Biosciences). Preliminary reactions were performed 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 was analyzed by electrophoresis in 1.2-1.5% agarose gels containing ethidium bromide, visualized under UV light, and quantitated. All samples were performed in triplicate and normalized to β-actin as the control.

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digested out with HindIII and BamHI, and cloned into the reporter plasmid pBLCA5 (American Type Culture Collection, Manassas, VA). Cells were transfected with the resultant pBLAMA3aCAT5 reporter plasmid using previously published transfection conditions and controls (Seewaldt et al., 1997a). Transfection control was provided by the pcMV-GH plasmid (Seewaldt et al., 1997a). Transfected cells were plated in Standard Media in T-25 flasks pre-treated with Poly-HEME, treated with 5% (vivo) rECM for 24 hours, and harvested for CAT activity assays as previously described (Seewaldt et al., 1997a). CAT reporter activity was normalized to GH concentration (Nichols Institute, San Juan Capistrano, CA) and total protein as previously described (Seewaldt et al., 1997a).

**LAMA3A chromatin immunoprecipitation**

Occupancy of the AP-1-rich region of LAMA3A promoter from positions −387 to −127 bp was tested by chromatin immunoprecipitation (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% (vivo) 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 crosslink cellular proteins. The formaldehyde was quenched by adding 1.0 ml of 250 mM glycine followed by a 5 minute RT incubation. Cells were then rinsed twice in ice-cold PBS containing protease inhibitor cocktail (4 μg/ml epibastatin hydrochloride, 2 μg/ml calpain inhibitor II, 2 μg/ml pepstatin A, 4 μg/ml mastoparan, 4 μg/ml leupeptin hydrochloride, 4 μg/ml aprotinin, 1 mM TPCK, 1 mM phenylmethylsulfonyl fluoride, and 100 μM TLCK), pelleted, and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1, and 1× protease inhibitor cocktail). Samples were then sonicated 3×15 seconds each with a 1 minute 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, 1× 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°C, followed by the addition of 45 μl of protein A-sepharose and 2 μg sheared salmon sperm DNA, and then incubated an additional 1 hour on a shaker at 4°C. Sepharose beads were then collected and washed sequentially for 10 minutes 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 (0.25% 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 NaHCO₃. The eluate was heated at 65°C overnight to reverse the formaldehyde crosslinking. DNA fragments were recovered by phenol/chloroform extraction followed by ethanol precipitation and then amplified by using PCR primers, sense 5’-AAAGCTATTCCATCGAAATGTTGGGACCCGAGAAGGCTAAAACACATTTCAAG-3 and antisense 5’-TCTAGATCAGGGCCCTCACTGCAGCCGTCCTGCTGACTGC-3’. Approximately 1.1% of the total chromatin was used as the input control and 16% was used for immunoprecipitation. Thirty micro liters of PCR product were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide and visualized under UV light. All samples were tested in triplicate.

**Suppression of LAMA3 by siRNA**

The double-stranded siRNA oligo targeting exon 29 of LAMA3 mRNA was purchased from Ambion (Austin, TX). Control non-silencing siRNA was provided by Qiagen (Alameda, CA). Preliminary studies were conducted on HMEC cells. The total amount of LAMA3 siRNA and time required for optimal suppression was determined to be 2.0 μg for 12 hours. HMEC cells were transfected with 2.0 μg of LAMA3 siRNA using Cellfectin™ (Invitrogen). Twelve hours after transfection, RNA was harvested using the Aurum™ Total RNA kit (Bio-Rad Laboratories, Hercules, CA). Semi-quantitative RT-PCR was performed to confirm suppression of LAMA3 expression. cDNA was prepared for RT-PCR from 50 ng total RNA with Superscript™ II reverse transcriptase (Invitrogen). β-Actin and LAMA3 PCR reaction conditions were performed as described above except products were amplified for 24 and 29 cycles, respectively. Twenty microliters of PCR product were run on 1.5% agarose gels stained with ethidium bromide and visualized with Kodak ID™ Image Analysis Software (Eastman Kodak). For initial LAMA3 suppression studies, early passage HMECs were transfected with 2.0 μg of LAMA3 siRNA, or control siRNA, 12 hours prior to culturing in rECM. For stable suppression of LAMA3 in HMEC-E6 cells with siRNA, the pSilencer 4.1-CMV puro expression vector was purchased from Ambion. The LAMA3 siRNA #2 sequence (target: 5’-AAGGCTAAAAACACATTTCAAG-3’, Ambion; #8284), already shown to suppress LAMA3 mRNA in transient transfection, was used to design two 55-mer DNA oligos to be annealed and inserted into the pSilencer 4.1-CMV puro vector. A hairpin siRNA template was created by annealing the following oligos: top-strand oligo, 5’-GAT CCG CCT AAA ACA CAT TTC AAG AGA CTT GTA GAT GGT TTG AGC CTT GAT-3’; and bottom-strand oligo, 5’-AGC TTA AGG CTA AAA AAC ATT TCA AGT CTC TTG AAC TTG AAG TGT GTT GC CCG-3’ (Qiagen). The annealed oligos were ligated to pSilencer 4.1-CMV through their HindIII and BamHI ends. Transfection of expression plasmid was performed in HMEC-E6 cells by using Cellfectin™ (Invitrogen) and selection with puromycin. Suppression of LAMA3 mRNA was confirmed by RT-PCR as described above.

**Results**

Late passage HMEC-E6 cells exhibit rearrangement of the CBP locus at chromosome 16p13 and a decrease in CBP protein expression

We previously performed SKY-based cytogenetic analysis on 35 unique late passage HMEC-E6 cells to identify rearrangements that might pinpoint the chromosomal location of potential gene(s) whose loss might promote loss of rECM-mediated growth control and apoptosis induction. The most frequent chromosomal losses involved (1) 16p (26/35 cells, 74%), (2) 12p (17/35 cells, 49%), (3) 21p (17/35 cells, 49%), (4) 22p (16/35 cells, 46%), (5) 2q (13/35 cells, 37%), (6) 1q (11/35 cells, 31%), and (7) 14p (10/35 cells, 29%) (Seewaldt et al., 2001a). These studies suggested that chromosome 16p harbored a gene(s) whose loss and/or rearrangement might play a role in loss of rECM-mediated growth control and apoptosis induction.

The predominant types of chromosomal changes involving chromosome 16p (and other chromosomes) were deletions, whole-arm translocations, and dicentric chromosomes with breakpoints in the pericentromeric and/or telomeric regions (Seewaldt et al., 2001a). However, two of 35 cells had specific translocations or deletions involving a more distal region of 16p. One cell exhibited a 16p deletion and retained material proximal to 16p13, and a second cell exhibited an unbalanced translocation der(16)(t(13;16)(q12;p13) that affected band 16p13 (Fig. 1A,B). These observations indicated that the gene
of importance was possibly located in the distal region of 16p, at 16p13.

Chromosomal band 16p13 is the locus of the CBP gene (Giles et al., 1997; Yao et al., 1998). Since CBP is known to play a role in growth control and apoptotic signaling, we hypothesized that loss of CBP protein expression might promote loss of rECM-mediated growth control and apoptosis induction in HMEC-E6 cells. We found that these cells also had decreased levels of CBP by western blotting (Fig. 1C). The relative CBP level in late passage HMEC-E6 cells was 8% (P<0.01) and 5% (P<0.01) that of early passage HMEC-E6 and HMEC-LXSN cells, respectively. There was no significant difference (P>0.05) in the relative amount of CBP in early passage HMEC-E6 and HMEC-LXSN cells.

Laminin-5 expression is decreased in late passage HMEC-E6 cells

We previously observed that enhancement of rECM-mediated growth regulation and apoptosis in early passage HMEC-E6 cells required polarized expression of the laminin-5 receptor, α3/β1-integrin (Seewaldt et al., 2001a). Differential gene expression studies, semi-quantitative RT-PCR, and western analysis were performed to test whether the loss of rECM-mediated growth control and apoptosis and loss of CBP expression 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 (Fig. 2A, Table 3). Semi-quantitative RT-PCR

![Fig. 1. Partial karyotype and CBP expression of late passage HMEC-E6 cells (passage 20). (A) Partial karyotype of a single mitotic cell demonstrating two copies of an unbalanced translocation between chromosomes 13 and 16 involving 16p13 (arrows). Spectral karyotyping in classification colors (red, chromosome 13 material; orange, chromosome 16 material). (B) Partial karyotypes of two cells that demonstrate either loss of a more distal region of 16p or rearrangement at the CBP locus, 16p13. (C) CBP protein expression is decreased in 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.

**Table 3. Differential gene expression data**

| Affymetrix ID | Gene name   | LX+MG (max) | LX+MG (min) | E6E+MG (max) | E6E+MG (min) | P-value  | Fold* |
|---------------|-------------|-------------|-------------|--------------|--------------|----------|-------|
| L34155        | Laminin-α3  | 27320.5     | 24983.1     | 12660.1      | 11419.4      | 3.15E-04 | -2.19 |
| U17760_mal    | Laminin-β3  | 20186.5     | 13978.7     | 6650.9       | 5177.5       | 2.60E-04 | -2.96 |
| U31201_cds1   | Laminin-γ2  | 2113.7      | 1764.8      | 2210.2       | 1892.2       | 7.48E-01 | 1.05  |
| U31201_cds2   | Laminin-γ2  | 12003.5     | 10606.4     | 6279.3       | 5727.3       | 5.49E-03 | -1.91 |
| M59911        | Integrin-α3 | 3999.9      | 3699.8      | 4443.4       | 3507.5       | 7.74E-01 | 1.05  |
| X07979        | Integrin-β1 | 17214.6     | 9457.1      | 16541.4      | 13266.4      | 8.79E-01 | 1.03  |

| Expression   | LXSN+MG vs E6E+MG |
|---------------|--------------------|
| Affymetrix ID | Gene name   | LX+MG (max) | LX+MG (min) | E6L+MG (max) | E6L+MG (min) | P-value  | Fold* |
| L34155        | Laminin-α3  | 27320.5     | 24983.1     | 733.2        | 607.7        | 7.26E-08 | -38.0 |
| U17760_mal    | Laminin-β3  | 20186.5     | 13978.7     | 1310.3       | 811.2        | 2.92E-06 | -15.8 |
| U31201_cds1   | Laminin-γ2  | 2113.7      | 1764.8      | 140.3        | 259          | 1.01E-05 | -18.9 |
| U31201_cds2   | Laminin-γ2  | 12003.5     | 10606.4     | 793.8        | 687.3        | 3.18E-06 | -15.3 |
| M59911        | Integrin-α3 | 3999.9      | 3699.8      | 6047.5       | 4928.4       | 1.06E-01 | +1.37 |
| X07979        | Integrin-β1 | 17214.6     | 9457.1      | 20614.6      | 17432.6      | 1.41E-01 | +1.36 |

*Values highlighted in bold indicate a fold change >2.0 with P<0.01.
confirmed a 98% decrease in laminin-5 α3-chain (P<0.01), a 46% decrease in laminin-5 β3-chain (P<0.01), and a 41% decrease in laminin-5 γ2-chain (P<0.01) mRNA expression relative to early passage HMEC-LXSN controls (Fig. 2B,C). Late passage HMEC-E6 cells also exhibited a 97% decrease in laminin-5 α3-chain (P<0.01), a 28% decrease in laminin-5 β3-chain (P>0.05), and a 37% decrease in laminin-5 γ2-chain (P>0.05) mRNA expression relative to early passage HMEC-E6 controls (Fig. 2B,C). There was no significant change (P>0.05) in the level of α3/β1-integrin mRNA expression (Fig. 2A-C).

Laminin-5 α3-chain protein has been previously shown to exhibit both a processed and unprocessed form (Aumailley et al., 2003). Western analysis demonstrated that late passage HMEC-E6 cells exhibited an 86% (P<0.01) decrease in unprocessed laminin-5 α3-chain protein relative to early passage HMEC-LXSN cells (Fig. 2D).

Loss of CBP expression in late passage HMEC-E6 cells correlates with a loss of CBP binding to the LAMA3A promoter and loss of LAMA3A promoter activity

We observed that late passage HMEC-E6 cells grown in rECM culture exhibit reduced levels of CBP protein expression and loss of laminin-5 α3-chain expression. We tested whether the observed decrease in CBP and laminin-5 α3-chain expression in late passage HMEC-E6 cells correlated with loss of CBP binding to the LAMA3A promoter and decreased LAMA3A promoter activity.

The human LAMA3A promoter contains three AP-1 sites at positions −387, −185 and −127 bp (Virolle et al., 1998; Miller et al., 2001). The AP-1 site at position −185 bp is critical for basal activity in mammary epithelial cells (Virolle et al., 1998; Miller et al., 2001). Chromatin immunoprecipitation (ChIP) was performed in rECM-resistant, ‘CBP-poor’ late passage HMEC-E6 cells and controls to test whether the observed decrease in laminin-5 α3-chain expression and 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 bp) (Virolle et al., 1998; 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. By contrast, rECM-resistant, late passage HMEC-E6 cells, which expressed decreased CBP and low levels of laminin-5 α3-chain, demonstrated markedly
decreased CBP binding (Fig. 3A). 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.

To test for 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 no. AF279435) and grown in rECM culture. Early passage HMEC-E6 cells and early and late passage HMEC-LXSN controls exhibited a similar level of LAMA3A activity (Fig. 3B). By contrast, late passage HMEC-E6 cells exhibited a 91% decrease ($P<0.001$) in LAMA3A promoter activity relative to early passage HMEC-E6 cells (Fig. 3B).

**Fig. 3.** Loss or suppression of CBP expression inhibits CBP occupancy of the LAMA3A promoter and suppresses LAMA3A promoter activity and laminin-5 expression. (A) Late passage HMEC-E6 cells do not exhibit CBP occupancy of the 277 bp AP-1-rich region of the LAMA3A promoter. ChIP was performed in HMEC-LXSN controls (LXSN) (passage 11 and 16), and early passage HMEC-E6 cells (E6) (passage 11) and compared with CBP-poor, late passage HMEC-E6 cells (E6) (passage 18). Input controls test the integrity of the DNA samples. (B) LAMA3A promoter activity was measured in early and late passage HMEC-LXSN controls (LXSN) (passage 11 and 16), and early passage HMEC-E6 cells (E6) (passage 10) and compared with CBP-poor, late passage HMEC-E6 cells (E6) (passage 18). Data represent two independent experiments performed in triplicate. (C) CBP protein expression is suppressed by antisense ODNs. HMEC-LXSN vector controls (LXSN) (passage 12) and early passage HMEC-E6 cells (E6) (passage 10) were treated with 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 added per lane. Actin was used as a loading control. (D) CBP protein expression is suppressed in rECM culture on days 1-11. Early passage HMEC-E6 cells (passage 10) were cultured in rECM and treated daily with active CBP-specific ODN (A3342V) (As) or inactive CBP ODN (scrA3342V) (Scr). Resultant cells were tested for CBP protein expression as described in Materials and Methods. Equal amounts of protein lysate were added per lane and actin was used as a loading control. (E) Early passage HMEC-E6 cells (passage 10) were treated with CBP-specific ODN (A3342A; As) and inactive CBP ODN (scrA3342V) (Scr), grown in contact with rECM, and tested by ChIP to determine whether suppression of CBP expression resulted in a loss of CBP binding to the AP-1-rich site of the LAMA3A promoter. Input controls test the integrity of the DNA samples. (F) Suppression of CBP expression results in decreased LAMA3A promoter activity. Early passage HMEC-LXSN controls (passage 11) and HMEC-E6 cells (passage 11) grown in rECM and treated with CBP-specific antisense ODNs (A3342V) (AS-CBP) exhibit decreased LAMA3A promoter activity relative to cells treated with inactive ODNs (scrA3342V) (scr-CBP). Data represent two independent experiments performed in triplicate. Error bars show standard error. (G) Laminin-5 α3-chain mRNA expression is suppressed by antisense ODNs. Early passage HMEC-E6 cells (passage 12) were cultured in the presence of no treatment (C), inactive CBP ODN (scrA3342V; Scr), and active CBP-specific ODN (A3342V; As). Resultant cells were analyzed for laminin-5 α3-chain mRNA expression by semi-quantitative RT-PCR as described in Materials and Methods. Actin was used as a loading control.
Direct suppression of CBP expression in early passage HMEC-E6 cells results in loss of CBP binding to the LAMA3A promoter, lack of LAMA3A promoter activity and suppression of laminin-5 α3-chain expression

Antisense ODNs were used to suppress CBP expression in HMECs to test whether direct suppression of CBP in early passage HMEC-E6 cells resulted in altered laminin-5 expression. We specifically tested for (1) loss of CBP binding to the AP-1-rich site of the LAMA3A promoter, (2) decrease in LAMA3A activity, and (3) suppression of laminin-5 α3-chain expression. Relative levels of CBP protein expression were tested by western analysis. Early passage HMEC-LXSN controls and early passage HMEC-E6 cells treated with the active, CBP-specific ODN, A3342V, exhibited a 98% (P<0.0001) and 81% (P<0.0001) respective decrease in CBP protein expression relative to untreated controls (Fig. 3C). Cells treated with the inactive CBP ODN, scrA3342V, did not exhibit a statistically significant decrease (P>0.05) in CBP protein expression (Fig. 3C). Early passage HMEC-E6 cells treated daily with CBP-specific ODN, A3342V, and grown in rECM culture exhibited a continued decrease in CBP protein expression at days 1, 7 and 11 (78%, 90% and 94%, respectively) (Fig. 3D).

Early passage HMEC-E6 cells treated with CBP-specific ODNs and grown in rECM demonstrated a loss of CBP occupancy of the AP-1-rich region of the LAMA3A promoter (Fig. 3E), a 94±7% decrease in LAMA3A promoter activity (Fig. 3F), and loss of laminin-5 α3-chain mRNA expression (Fig. 3G). By contrast, early passage HMEC-E6 controls treated with inactive ODNs and grown in rECM demonstrated CBP occupancy of LAMA3A, a statistically non-significant decrease (10%, P>0.05) in LAMA3A activity, and normal levels of laminin-5 α3-chain mRNA expression (Fig. 3E-G). 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 (Virolle et al., 1998; Miller et al., 2001), these observations provide a potential mechanism by which loss of CBP expression might promote loss of LAMA3A promoter activity and laminin-5 α3-chain expression in HMECs.

Exogenous expression of CBP in late passage HMEC-E6 cells promotes CBP binding to the LAMA3A promoter, LAMA3A promoter activity and increased laminin-5 α3-chain expression

Retroviral-mediated gene expression was used to express CBP in late passage HMEC-E6 cells. This allowed us to test directly whether expression of physiologic levels of CBP promoted (1) CBP binding to the AP-1-rich site of the LAMA3A promoter, (2) LAMA3A activity, and (3) increased laminin-5 α3-chain expression. Western analysis and RT-PCR confirmed expression of exogenous expression of CBP (Fig. 4A and data not shown). As previously observed, late passage HMEC-E6 cells exhibited a 90% (P<0.001) decrease in CBP protein levels relative to early passage HMEC-E6 cells. Retroviral-mediated exogenous expression of CBP in late passage HMEC-E6 cells resulted in CBP protein levels that were similar (106%) to that of early passage HMEC-E6 cells. Since CBP protein levels are thought to be tightly regulated, it is important that the
level of CBP protein expression in transduced late passage HMEC-E6 cells was not significantly greater than baseline CBP levels in early passage HMEC-E6 cells, HMEC-LXSN vector controls and parental HMECs (Fig. 4A and data not shown).

Late passage HMEC-E6-CBP* cells expressing exogenous CBP demonstrated CBP occupancy of the AP-1-rich region of the LAMA3A promoter (Fig. 4B). Expression of CBP in late passage HMEC-E6-CBP* cells resulted in LAMA3A promoter activity that was comparable (74%, P<0.01) with early passage HMEC-E6 cells (Fig. 4C). Finally, late passage HMEC-E6-CBP* cells exhibited laminin-5 expression α3-chain protein at levels that were equivalent (122%, P<0.01) to early passage HMEC-E6 cells (Fig. 4D). By contrast, late passage HMEC-E6 CBP* controls transduced with the empty LXSN retroviral vector lacked CBP occupancy of LAMA3A, and exhibited low levels of LAMA3A activity and laminin-5 α3-chain protein expression similarly to late passage HMEC-E6 cells (Fig. 4). These observations further demonstrate that CBP plays an important role in regulating LAMA3A promoter activity and laminin-5 α3-chain expression in HMECs.

Fig. 5. Expression and distribution of α3-integrin and laminin-5 α3-chain in HMECs that express altered levels of CBP. (A) Immunofluorescence characterization of α3-integrin and laminin-5 α3-chain expression in cells sensitive and resistant to rECM-mediated growth arrest and apoptosis. Frozen section of early and late passage HMEC-LXSN controls (LXSN; passage 10 and 16) and HMEC-E6 cells (E6; passage 10 and 18) were grown in rECM for 6 days, cryosectioned, and immunostained for localization of α3-integrin and laminin-5 α3-chain expression. α3-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 (arrowheads). By contrast, CBP-poor late passage HMEC-E6 cells showed dispersed membrane and intracellular staining of α3-integrin (arrow) and qualitatively decreased laminin-5 α3-chain expression.

(B) Immunofluorescent characterization of α3β1-integrin and laminin-5 α3-chain expression in HMECs treated with CBP antisense ODNs. Frozen section of early passage HMEC-LXSN vector controls (LXSN) (passage 11) and HMEC-E6 cells (E6) (passage 11) treated either with CBP antisense ODN (A3342V; CBP-as) or inactive CBP ODN (scrA3342V; CBP-scr). Cells were grown in rECM for 6 days, cryosectioned, and immunostained for α3-integrin or laminin-5 α3-chain. α3-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 (arrowheads). By contrast, HMEC-LXSN and HMEC-E6 cells treated with antisense CBP ODNs demonstrated disorganized membrane and cytosolic staining of α3-integrin (arrows) and markedly reduced laminin-5 α3-chain expression.

(C) Immunofluorescent characterization of α3β1-integrin and laminin-5 α3-chain expression in late passage HMEC-E6 cells expressing exogenous CBP. Frozen sections of immunostained late passage HMEC-E6-CBP* vector controls (CBP(–); passage 17) and late passage HMEC-E6-CBP* cells (CBP(+); passage 17). Cells were grown in rECM for 6 days, cryosectioned, and immunostained for α3-integrin or laminin-5 α3-chain. HMEC-E6-CBP* cells demonstrated disorganized membrane and cytosolic staining of α3-integrin and markedly reduced levels of laminin-5 α3-chain expression. By contrast, HMEC-E6-CBP* cells exhibited a qualitative increase in laminin-5 α3-chain expression. Although there was persistent membrane and cytosolic staining of α3-integrin and laminin-5 α3-chain, there was also increased localization at the basolateral surface (arrowheads).
exhibited reduced levels of CBP protein expression and disorganized expression of both laminin-5 α3-chain and α3-integrin. This observation led us to hypothesize that direct suppression of CBP in HMECs would alter laminin-5 α3-chain and α3-integrin expression and/or distribution in rECM culture. CBP protein expression was suppressed in early passage HMEC-E6 cells and HMEC-LXSN controls by treatment with CBP-specific, antisense ODN (A99424V). HMECs that had suppressed levels of CBP exhibited disorganized plasma membrane and cytosolic distribution of α3-integrin (Fig. 5B) and qualitatively reduced levels of laminin-5 α3-chain expression (Fig. 5B). β1-integrin expression was observed at the basal surface (data not shown). By 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 (Fig. 5B, and data not shown). These observations demonstrate that suppression of CBP protein expression in HMECs alters the distribution of both laminin-5 α3-chain and α3-integrin in rECM culture.

Expression of CBP in HMECs partially restores polarized expression of laminin-5 α3 and integrin-α3 proteins
Late passage HMEC-E6 cells, late passage HMEC-E6-CBP− vector controls, and late passage HMEC-E6-CBP+ cells expressing exogenous CBP were grown in rECM and tested for laminin-5 α3-chain and α3- and β1-integrin expression and distribution by immunocytochemistry. Late passage, CBP-poor HMEC-E6 cells and late passage HMEC-E6-CBP+ control cells grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of α3-integrin and a qualitative decrease in laminin-5 α3-chain expression (Fig. 5C). By contrast, late passage HMEC-E6-CBP+ cells expressing exogenous CBP exhibited partial but not fully polarized expression of laminin-5 α3-chain and α3-integrin on the basal surface (Fig. 5C and data not shown). As predicted by western analysis, there was also a qualitative increase in laminin-5 α3-chain expression in late passage HMEC-E6-CBP+ cells relative to controls (Fig. 5C).

Suppression of CBP expression results in dysregulated proliferation and inhibits apoptosis in rECM culture
Late passage HMEC-E6 cells lost both rECM-mediated growth arrest and apoptosis associated with loss of polarized expression of the laminin-5 receptor, α3β1-integrin (Seewaldt et al., 2001b), and loss of CBP expression. Loss or suppression of CBP in HMECs results in loss of laminin-5 expression. Based on these observations we hypothesized that direct suppression of CBP in early passage HMEC-E6 cells grown in rECM would result in dysregulated proliferation and block apoptosis.

Suppression of CBP expression in early passage HMEC-LXSN controls and HMEC-E6 cells resulted in enhanced proliferation in rECM-culture as measured by Ki-67 staining and DAPI staining of cell nuclei. 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 (Fig. 6A,C). DAPI-stained early passage HMEC-LXSN controls and HMEC-E6 cells treated with either CBP antisense ODN (A3342V; CBP AS) or inactive CBP ODN (scrA3342V; CBP scr). Two hundred cells were surveyed per time point and indices were calculated from an average of three independent experiments. Error bars show standard error.
controls and HMEC-E6 cells treated with inactive ODNs (scrA33243V) did not exhibit a continued increase in Ki-67 staining nor an increase in cell number per cell cluster after day 7 (Fig. 6). These observations show that suppression of CBP protein expression in early passage HMEC-E6 cells and HMEC-LXSN controls results in enhanced proliferation 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 cells treated with CBP-specific antisense ODNs (A33423V) formed large irregular clusters in rECM and did not undergo apoptosis on days 7-11 as assessed by electron microscopy and TUNEL-staining (Fig. 7B,D). By 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 (Fig. 7C,D).

Similarly to early passage HMEC-E6 cells, early passage HMEC-LXSN cells treated with CBP-specific, antisense ODNs (A33423V) formed large irregular clusters in rECM and did not undergo apoptosis (Fig. 7A,E). Early passage HMEC-LXSN controls treated with inactive ODN (scrA33423V) formed morphologically organized structures and did not undergo apoptosis (Fig. 7E and data not shown). These observations demonstrate that suppression of CBP protein expression in 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. Similarly to HMEC strain AG11132 cells, early passage AG11134-E6 cells treated with inactive CBP ODN (scrA33423V) were sensitive to rECM-growth regulation and underwent apoptosis at day 7, early passage AG1134-LXSN controls treated with CBP-specific antisense ODN (A99424V) were resistant to
Expression of CBP in late passage HMEC-E6 cells promotes growth regulation and apoptosis in rECM culture

Exogenous expression of CBP in late passage HMEC-E6 cells resulted in reduced proliferation on days 5-11 relative to late passage HMEC-E6-CBP− controls transduced with the empty LXSNS vector alone (Fig. 8A,B). Proliferation in rECM-culture was measured by Ki-67 staining and DAPI staining as above. Late passage HMEC-E6-CBP− cells expressing exogenous CBP exhibited a decrease in Ki-67 staining and did not exhibit an increase in the cell number per cell cluster after day 5 (Fig. 8A,B). By contrast, late passage HMEC-E6-CBP+ vector control cells demonstrated continued Ki-67 staining and a continued increase in the number of cells per cell cluster on days 7-11 (Fig. 8A,B).

Late passage HMEC-E6-CBP+ cells expressing exogenous CBP and late passage HMEC-E6-CBP− vector controls were tested for the presence of apoptosis by electron microscopy and TUNEL-staining. Late passage HMEC-E6-CBP+ cells underwent apoptosis on day 7 as shown by (1) nuclear condensation (n), (2) cell shrinkage and separation, and (3) margination of chromatin (mr). (E) Percentage of apoptotic cells in late passage HMEC-E6 cells (passage 16) expressing exogenous CBP (CBP(+)) or transduced with the LXSNS control vector (CBP(−)). Apoptosis was measured by TUNEL-staining. Apoptotic index was measured by calculating the percentage of TUNEL-stained cells relative to the total number of cells surveyed. Data represents an average of three independent experiments. Error bars show standard error.
Fig. 9. See next page for legend.
Fig. 9. Suppression of laminin-5 α3-chain in early passage HMEC-E6 cells blocks apoptosis in rECM. (A) Laminin-5 α3-chain mRNA expression is compared by semi-quantitative RT-PCR in three early passage HMEC-E6 cells (passage 10) clones expressing siRNA sequences directed against laminin-5 α3-chain (si#1, si#2, si#3) or control siRNA (si-cont), and in HMEC-E6 control cells (no tx). Actin was used as a loading control. (B) Suppression of laminin-5 α3-chain expression in early passage HMEC-E6 cells results in decreased proliferation in rECM culture. (B) Ki-67 staining indices in early passage HMEC-E6 cells (passage 11) expressing siRNA sequence #2 directed against laminin-5 α3-chain or siRNA control. (C) DAPI staining of cell nuclei demonstrates the number of nuclei per cell cluster formed by early passage HMEC-E6 cells (passage 11) expressing either siRNA sequence #2 directed against laminin-5 α3-chain or siRNA control. Two hundred cells were surveyed per time point and indices were calculated from an average of three independent experiments. Error bars show standard error. (D) Electron micrographs of early passage HMEC-E6 cells (passage 11) expressing siRNA sequence #2 directed against laminin-5 α3-chain (si-laminin-5) or control siRNA (si-control). Early passage HMEC-E6 cells expressing control siRNA underwent apoptosis when grown in rECM for 7 days as shown by (1) nuclear condensation (n), (2) cell shrinkage and separation, and (3) margination of chromatin (mr). By contrast, HMEC-E6 cells expressing siRNA directed against laminin-5 α3-chain did not undergo apoptosis and instead formed large, dense, irregularly shaped multicellular colonies that have no central lumen. (E) Relative levels of TdT-positive cells in early passage HMEC-E6 cells (passage 10) treated either with siRNA sequence #2 directed against laminin-5 α3-chain (laminin-5 si-RNA) or control siRNA (si-control). Apoptosis was measured by TUNEL-staining. Data represents an average of two independent experiments in triplicate. Error bars show standard error. (F) Immunofluorescent characterization of laminin-5 α3-chain expression in early passage HMEC-E6 cells (passage 12) expressing si-RNA directed against laminin-5 α3-chain (si-laminin-5) or control siRNA (si-control). Cells were grown in rECM for 6 days, cryo-sectioned, and immunostained for laminin-5 α3-chain. Laminin-5 α3-chain expression was primarily localized at the basolateral surface in early passage HMEC-E6 cells expressing control siRNA. By contrast, early passage HMEC-E6 cells expressing si-RNA directed against laminin-5 α3-chain exhibited markedly reduced laminin-5 α3-chain expression.

Discussion

Here we demonstrate a mechanism by which loss of CBP expression in basal-cytokeratin-positive HMECs promotes loss of rECM-mediated growth regulation and apoptosis. 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 (Kawasaki et al., 1998; Yao et al., 1998; Shang et al., 2000). In this report, we show that loss or suppression of CBP protein expression in basal-cytokeratin-positive HMECs results in loss of rECM-mediated growth regulation and apoptosis (Figs 6-8). Expression of CBP in CBP-poor late passage HMEC-E6 cells results in restoration of rECM-mediated growth regulation and apoptosis sensitivity (Fig. 8). Consistent with observations in this in vitro system, suppression of CBP protein levels in virgin CBP+/– heterozygote mice results in a 90% incidence of severe mammary gland hyperplasia and hyperlactation (T. P. Yao, personal communication). Taken together, these observations provide evidence that loss of CBP protein expression in mammary epithelial cells promotes loss of rECM-mediated growth regulation and apoptosis.

We observe that loss of CBP expression results in loss of CBP occupancy of the AP-1-rich region of the LAMA3A promoter and inhibits LAMA3A promoter activity and expression of laminin-5 α3-chain (Figs 2, 3, 5). This observation suggests a potential mechanism for CBP regulation of rECM-mediated growth regulation and apoptosis. Both the mouse and human LAMA3A promoter contain an AP-1-rich region (Virolle et al., 1998; Millet 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). We also show that suppression of CBP results in loss of CBP occupancy of the LAMA3A promoter, loss of LAMA3A promoter activity, and decreased laminin-5 α3-chain expression (Fig. 3). Conversely, exogenous expression of CBP in late passage HMEC-E6 cells that express low levels of CBP promotes CBP occupancy of the LAMA3A promoter, an increase in LAMA3A promoter activity, and laminin-5 α3-chain expression (Fig. 4). The decreased production of laminin-5 α3 correlated with loss of CBP occupancy of the AP-1-rich region of the LAMA3A promoter. Direct suppression of laminin-5 α3-chain expression resulted in dysregulated proliferation and loss of apoptosis sensitivity in rECM culture (Fig. 9). Taken together, these observations suggest that CBP occupancy of the LAMA3A promoter promotes laminin-5 α3-chain expression and loss of CBP occupancy inhibits laminin-5 α3-chain expression in early passage HMEC-E6 cells results in loss of rECM-mediated growth control and apoptosis.

Laminin-5 was directly inhibited in early passage HMEC-E6 cells using a siRNA construct targeted against the laminin-5 α3-chain. A 79% (P<0.01) decrease in laminin-5 α3-chain mRNA expression was observed in siRNA-expressing early passage HMEC-E6 cells relative to cells expressing control siRNA or untransduced cells (Fig. 9A). Early passage HMEC-E6 cells expressing siRNA directed against laminin-5 α3-chain grown in rECM exhibited a qualitative decrease in laminin-5 protein expression versus early passage HMEC-E6 cells expressing control siRNA (Fig. 9F). Suppression of laminin-5 expression in early passage HMEC-E6 cells blocked rECM-mediated growth regulation as measured by Ki-67 staining and DAPI staining of cell nuclei. Early passage HMEC-E6 cells treated with siRNA directed against laminin-5 exhibited continued Ki-67 staining and an increase in the cell number per cell cluster after day 5 (Fig. 9B,C). By contrast, early passage HMEC-E6 cells treated with control siRNA demonstrated a decrease in Ki-67 staining and a decrease in the number of cells per cell cluster on days 7-11 (Fig. 9B,C). Early passage HMEC-E6 cells treated with siRNA did not exhibit apoptosis by either electron microscopy or TUNEL-staining (Fig. 9D,E). HMEC-E6 cells treated with control siRNA exhibited apoptosis by both electron microscopy and TUNEL-staining (Fig. 9D,E). Taken together, these observations suggest a role for laminin-5 in regulating proliferation and apoptosis of HMECs in rECM culture.
expression and results in loss of growth regulation and apoptosis in rECM culture.

In contrast to our observation that suppression of CBP inhibits laminin-5 α3-chain expression in HMECs, it has been previously observed that overexpression of the related coactivator 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., 2001b). 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. Although p300 and CBP have many overlapping functions, there is ample evidence that they also have distinct activities. For example, CBP and p300 play distinct roles during retinoic-acid-induced differentiation in F9 cells (Kawasaki et al., 1998; Ugaï 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).

Observations in this model system are consistent with prior studies showing loss of laminin-5 α3-chain expression during early carcinogenesis (Sathyarayana et al., 2003) but do not explain the seemingly paradoxical observation that increased laminin-5 γ2-chain expression and cleavage is associated with tumor invasion (Pyke et al., 1994; Seftor et al., 2001; Yamamoto et al., 2001). However, the rECM-resistant cells used in this model system are not transformed and do not exhibit invasion in in vitro assays and, therefore, do not represent a model of invasive cancer.

Tumorigenesis is thought to be a multistep process; there is increasing evidence that epigenetic changes, including DNA methylation and coactivator/corepressor shifts, may play an important role in modulating gene expression during carcinogenesis. For example, studies have shown that transcription of the E-cadherin gene is downregulated during early carcinogenesis by both promoter hypermethylation and modulation of coactivator/corepressor expression (Thiery, 2003). Similarly to this observation involving E-cadherin regulation, prior studies have demonstrated that laminin-5 α3-chain expression can be inhibited by hypermethylation (Sathyarayana et al., 2003), and here we provide evidence for a second epigenetic mechanism that regulates expression of laminin-5 α3-chain–coactivator loss. In addition, there is evidence that loss of E-cadherin expression may be temporary and that epigenetic control over the expression of E-cadherin would make it possible for E-cadherin to be produced in aggressive primary and metastatic breast tumors (Thiery, 2003). This model of differential regulation of E-cadherin expression may be valuable in reconciling the potentially divergent observations that laminin-5 α3-chain expression is lost during early mammary carcinogenesis and increased laminin-5 γ2-chain expression is associated with tumor invasion and an aggressive phenotype (Pyke et al., 1994; Seftor et al., 2001; Yamamoto et al., 2001; Niki et al., 2002). Further studies will be necessary to determine the role of epigenetic silencing of laminin-5 α3-chain expression in early mammary carcinogenesis.

Observations in this model system may provide insights into the early biology of basal-type epithelial breast cancers. Basal-type epithelial breast cancers occur frequently in young African-American women and are also observed in women who are BRCA1 mutation carriers (Foulkes et al., 2003). Basal-type breast cancers are identified by the following characteristics: ER/PR−, Her2/neu+, p53− and CK5/6+/− (Foulkes et al., 2003). Understanding the early biology of basal-type breast cancers is critical for developing effective prevention strategies. The primary HMEC strain used for these studies is AG11132/172R/AA7, a CK5/6+/−, Her2/neu+, basal-cytokeratin-positive HMEC strain that expresses low levels of ER/PR and was derived from the breast tissue of a young African-American woman (Seewaldt et al., 2001b). Here, we modeled loss of p53 function using expression of HPV-16 E6 to study the role of rECM signaling in mediating apoptosis in this basal-cytokeratin-positive HMEC strain. Importantly, in previous studies, we confirmed that p53-specific antisense ODNs and HPV-16 E6 resulted in the same apoptosis-sensitive phenotype (Seewaldt et al., 2001a). Since basal-type breast cancers typically arise in young African-American women and are ER/PR−, p53− and Her2/neu+, modeling loss of p53 function in ER/PR-poor, Her2/neu+, basal-cytokeratin-positive HMECs derived from a young African-American woman is a potentially relevant model of early basal-type breast carcinogenesis.

Observations in this model system predict that a partial reduction of CBP expression in basal-cytokeratin-positive HMEC strains results in (1) loss of CBP occupancy of the AP-1-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-chain expression blocks rECM-mediated growth control and apoptosis induction 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/laminin-5 α3-chain expression may increase the risk of subsequent invasive basal-type breast cancer.

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