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13. ABSTRACT *(Maximum 200 Words)*

Retinoids are important mediators of normal growth and differentiation of epithelial cells and may play an important role in the chemoprevention of breast cancer. While normal mammary epithelial cells express retinoic acid receptors (RAR) a majority of breast cancers have selectively lost the expression of specific RAR isoforms. This has lead to the hypothesis that loss of retinoic acid receptor function might be an important event in mammary carcinogenesis. In order to investigate the molecular mechanisms by which loss of retinoic acid receptor function might promote the malignant transformation of mammary epithelial cells, we have utilized a dominant-negative approach to inhibit retinoic acid receptor function in normal human mammary epithelial cells (HMECs). We observe in our *in vitro* system that suppression of retinoic acid receptor function in HMECs results in dysregulated growth and inhibits structural differentiation. These observations lead us to hypothesize that retinoids and retinoic acid receptors may be important in regulating mammary epithelial cell growth and differentiation, and therefore, loss of retinoic acid receptor function might promote breast cancer carcinogenesis. The aim of this proposal is to identify and characterize genes activated or suppressed by loss of retinoic acid receptor function in human mammary cells.

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INTRODUCTION:

Retinoids are important for the normal growth and differentiation of epithelial cells. We hypothesize that loss of retinoic acid receptor function might be an important early event in breast cancer carcinogenesis. In Preliminary Data we demonstrated that retinoids are important regulators of proliferation and may promote a more differentiated phenotype in normal mammary epithelial cells. To investigate the hypothesis that loss of normal retinoic acid receptor function might promote carcinogenesis of HMECs, we developed an *in vitro* system in which retinoic acid receptor function is suppressed by expression of a dominant-negative retinoic acid receptor (DNRAR). We observe that HMECs expressing the DNRAR exhibit dysregulated growth and do not exhibit normal structural differentiation *in vitro*. These studies suggest that loss of retinoic acid receptor function in HMECs might result in loss of normal cellular differentiation and thereby may promote mammary carcinogenesis. We used this model system to isolate and characterize retinoid-regulated genes that may be important in the normal growth and differentiation of human mammary epithelial cells. As described in the BODY of this report, we have been successful in identifying a retinoid-regulated gene, the CREB-binding protein, CBP. CBP is a known critical regulator of retinoid-signaling, however, the role of CBP in regulating growth and epithelial cell polarity has been previously unknown. We have been able to provide evidence that CBP functions in a positive feed-back loop with retinoids and the retinoic acid receptor-beta 2. These findings provide important insight into how retinoid and retinoic acid receptors may act to regulate normal mammary epithelial cell growth and polarity.

BODY:

Objective I: Isolation and characterization of retinoid-responsive genes activated or suppressed by retinoic acid receptors in normal human mammary epithelial cells. Formation of a polarized epithelial ductal structure is a characteristic of normal mammary cells and serves to distinguish normal from malignant mammary epithelial cells. We observe that inhibition of retinoic acid receptors in normal human mammary epithelial cells (HMECs) by a dominant-negative retinoic acid receptor (DNRAR) results in dysregulated growth and an inability of cells to form a normal polarized ductal epithelial structure when cultured in 3-dimensional extracellular matrix culture.

A) Isolation of retinoid-regulated genes: We utilized the techniques of "differential display" and differential gene expression studies (gene "chip") to isolate retinoid-regulated genes regulated in normal human mammary epithelial cells (HMECs) that may be critical for mammary epithelial cell polarity and growth regulation. Several candidate genes were identified. Differential gene expression studies were correlated with detailed cytogenetic results in extracellular matrix-resistant and retinoid-resistant mammary epithelial cells (Seewaldt et al. Journal of Cell Biology, 2001, see appendix).

Cytogenetic analysis of extracellular matxix-resistant and retinoid-resistant HMECs demonstrated frequent chromosomal loss of 16p (74%) or rearrangement at 16p13 (16%) (Seewaldt et al. Journal of Cell Biology, 2001, see appendix). **The high frequency of 16p loss in rECM-resistant HMECs suggested that 16p harbors a gene whose loss may be important in resistance to both retinoid and extracellular matrix.** Chromosomal loss at 16p13 is observed in a majority of benign and malignant papillary neoplasms of the breast and

loss of 16p is frequently observed in premalignant breast lesions such as atypical hyperplasia. Chromosome 16p13.3 is the location of the human CREB binding protein (CBP), a nuclear protein important for retinoid-signaling, growth regulation, and apoptosis.

Consistent with our cytogenetic data, we observe that **resistance to extracellular matrix-induced apoptosis is associated with a 75% *decrease* in expression of CBP** (Dietze et al. Microscopy Research and Techniques, 2002; Troch et al. Cancer Research, submitted 2002, see appendix). CBP is a tightly regulated transcription factor that plays a critical regulatory role as an integrator of diverse signaling pathways including those mediated by retinoids, estrogen, and calcium. Given the important role that CBP plays in apoptosis, growth regulation, and retinoid-signaling, we hypothesize that loss of CBP expression may promote loss of extracellular matrix-regulated growth regulation and polarity.

B) Characterization: *Confirming the importance of CBP in regulating polarity and growth regulation in HMECs:* The function of our candidate gene, CBP, in HMECswas examined using sense and anti-sense sequences cloned into either retroviral or inducible expression vectors. HMECs with suppressed CBP were grown in prepared extracellular matrix and analyzed for growth characteristics and the ability to form a normal epithelial ductal structure in prepared extracellular matrix. We observed that suppression of CBP resulted in loss of epithelial cell polarity and growth regulation. Loss of growth regulation was associated with loss of laminin-5 expression and alpha3/beta1 integrin signaling (Dietze et al. Journal of Cell Biology, re-submitted 2003, see appendix).

Demonstration that CBP is regulated by retinoids and retinoic acid receptors. While it is well established that CBP is a co-activators of retinoid-signaling, the transcriptional regulation of CBP is uncharacterized. We tested whether all-*trans*-retinoic acid and the retinoic acid receptor-beta 2 induced the expression of CBP mRNA and protein in mammary epithelial cells (Dietze et al. Microscopy Research and Techniques, 2002, Troch et al. Cancer Research, submitted, 2002, see appendix). We identified that retinoids and the retinoic acid receptor-beta 2 regulate the expression of CBP protein. We then analyzed the promoter of CBP and identified putative retinoic acid receptor response elements in the promoter of CBP. This is an important finding because this suggests that retinoids, retinoic acid receptor-beta 2, and CBP can function in a positive-feed back loop that promotes retinoid-sensitivity in HMECs.

Future Statement of Work (SOW) tasks that will be completed over the next funded period: We will further characterize how CBP regulates mammary epithelial cell polarity through regulation of laminin-5 exprsesion. We will also further analyze our differential gene expression data to identify further down-stream targets of CBP that may play a critical role in retinoid-regulation of mammary epithelial cell growth and polarity.

KEY RESEARCH ACCOMPLISHMENTS:

A) **Development of HMECs resistant to retinoid and extracellular matrix-growth regulation, polarity, and apoptosis.**

B) **Identification of the CREB-binding protein, CBP, as a potential regulator of retinoids and extracellular-matrix signaling.**

C) **Demonstration that suppression of CBP protein expression by antisense oligonucleotides blocks the induction of extracellular matrix-growth regulation and -polarity in HMECs.**

D) **Demonstration that retinoids and the retinoic acid receptor-beta 2 mediate transcription of CBP.**

Reportable Outcomes:

Publications:

1) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., and Caldwell, L.E. Suppression of p53 in human mammary epithelial cells increases sensitivity to extracellular matrix-induced apoptosis. J. Cell Biol., 155: 471-486, 2001.
2) Dietze, E.C. Mrózek, K., Caldwell, L.E., Yokoyama, K., Srinivasan, K., Hobbs, K.B., and **Seewaldt, V.L.** CBP modulates epithelial polarity and resistance to extracellular matrix-induced apoptosis in p53(-) human mammary epithelial cells. Reubmitted to J. Cell Biol, 2003.
3) Dietze, E.C., Caldwell, L.E., Marcom, K., Collins, S.J., Swisshelm, K., Hobbs, K.B., and **Seewaldt, V.L.** Retinoids and retinoic acid receptors regulate growth arrest and apoptosis in human mammary epithelial cells and modulate expression of CBP/p300. Microscopy Res. and Tech., 59:23-40, 2002.
4) Dietze, E.C., Srinivasan, K., Hobbs, K.B., Rieffler, M.B., and **Seewaldt, V.L.** Regulation of CBP/p300 expression by all-*trans*-retinoic acid and RARβ2. Submitted, Cancer Res, 2002.

Abstracts and Presentations:

1) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., Dietze, E., and Caldwell, L.E. Suppression of p53 in Human Mammary Epithelial Cells increases sensitivity to extracellular matrix-induced apoptosis. Loss of p53 function promotes sensitivity to extracellular matrix-induced apoptosis in human mammary epithelial cells. Keystone Symposia, Banff, Alberta, Canada, Feb 10, 2002.

2) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., Dietze, E., Hobbs, K.B., and Caldwell, L.E. Suppression of p53 in Human Mammary Epithelial Cells increases sensitivity to extracellular matrix-induced apoptosis. CBP modulates resistance to extracellular matrix-induced apoptosis in human mammary epithelial cells. AACR, San Francisco, CA. April 10, 2002.

3) Dietze, E.C., Mrozek, K., Caldwell, L.E., Hobbs, K.B., Yokoyama, K., and **Seewaldt, V.L.** CBP modulates resistance to extracellular matrix-induced apoptosis in human mammary epithelial cells. Gordon Conference Mammary Carcinogenesis, Tuscany, Italy, April 2, 2002. Invited oral presentation.

4) Dietze, E.C., Mrozek, K., Caldwell, L.E., Hobbs, K.B., Yokoyama, K., and **Seewaldt, V.L.** CBP/p300 and extracellular matrix-induced apoptosis in human mammary epithelial cells. 18th IUC International Cancer Congress, June 30, 2002, Oslo, Norway.

5) Troch, M., Bowie, M., Dietze, E.C., and **Seewaldt, V.L.** CBP/p300 expression is regulated by a retinoic acid response element. AACR Workshop in Prevention, October 16, 2002, Boston, MA.

6) Troch, M., Bowie, M., Dietze, E.C., and **Seewaldt, V.L.** Retinoids and retinoic acid receptors regulate CBP/p300 expression through a retinoic acid response element. San Antonio Breast Conference, December 13, 2002, San Antonio, TX.

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1) Duke University Assistant Professorship 1/00 (Seewaldt)
2) Promoted to Associate Professor 11/01 (Seewaldt)

Training Supported by this Award:

Svetlana Grupin, Duke Undergraduate Student
Katherine Hobbs, Duke Undergraduate Student

Conclusions:

We have determined that 1) CBP are critical mediators of extracellular matrix regulated-growth and polarity and that 2) retinoids and the retinoic acid receptor-beta 2 regulate CBP transcription. **These observations have important implications:** Currently, we are rapidly developing tools to identify women at high-risk for developing breast cancer. While this new technology holds a great deal of promise, our ability to label women as "high-risk" is rapidly outstripping our ability to develop prevention strategies. In preclinical models we identified a critical role for CBP, in maintaining normal mammary homeostasis. CBP is important for retinoid-, estrogen-, calcium-, and COX-2 signaling. **The development of *in vitro* models of CBP loss and mammary hyperplasia will be extremely valuable for the testing of a wide variety of prevention strategies for breast cancer. Information gained from this proposal can be immediately**

translated to 1) predict breast cancer risk and 2) develop and assess response to prevention agents.

References:

None.

Appendices

1) **Seewaldt, V.L.**, Hockenbery, D., Mrózek, K., and Caldwell, L.E. Suppression of p53 in human mammary epithelial cells increases sensitivity to extracellular matrix-induced apoptosis. J. Cell Biol., 155: 471-486, 2001.
2) Dietze, E.C. Mrózek, K., Caldwell, L.E., Yokoyama, K., Srinivasan, K., Hobbs, K.B., and **Seewaldt, V.L.** CBP modulates epithelial polarity and resistance to extracellular matrix-induced apoptosis in p53(-) human mammary epithelial cells. Submitted to J. Cell Biol.
3) Dietze, E.C., Caldwell, L.E., Marcom, K., Collins, S.J., Swisshelm, K., Hobbs, K.B., and **Seewaldt, V.L.** Retinoids and retinoic acid receptors regulate growth arrest and apoptosis in human mammary epithelial cells and modulate expression of CBP/p300. Microscopy Res. and Tech., 59:23-40, 2002.
4) Dietze, E.C., Srinivasan, K., Hobbs, K.B., Rieffler, M.B., and **Seewaldt, V.L.** Regulation of CBP/p300 expression by all-*trans*-retinoic acid and RARβ2. Submitted, Cancer Res, 2002.

Article

Suppression of p53 function in normal human mammary epithelial cells increases sensitivity to extracellular matrix–induced apoptosis

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Little is known about the fate of normal human mammary epithelial cells (HMECs) that lose p53 function in the context of extracellular matrix (ECM)–derived growth and polarity signals. Retrovirally mediated expression of human papillomavirus type 16 (HPV-16) E6 and antisense oligodeoxynucleotides (ODNs) were used to suppress p53 function in HMECs as a model of early breast cancer. $p53^+$ HMEC vector controls grew exponentially in reconstituted ECM (rECM) until day 6 and then underwent growth arrest on day 7. Ultrastructural examination of day 7 vector controls revealed acinus-like structures characteristic of normal mammary epithelium. In contrast, early passage $p53^-$ HMEC cells proliferated in rECM until day 6 but then underwent apoptosis on day 7. $p53^-$ HMEC-E6 passaged in non-rECM culture rapidly (8–10 passages), lost sensitivity to both rECM-induced growth arrest and polarity, and also developed resistance to rECM-induced apoptosis. Resistance was associated with altered expression of α3-integrin. Treatment of early passage $p53^-$ HMEC-E6 cells with either α3- or β1-integrin function-blocking antibodies inhibited rECM-mediated growth arrest and induction of apoptosis. Our results indicate that suppression of p53 expression in HMECs by HPV-16 E6 and ODNs may sensitize cells to rECM-induced apoptosis and suggest a role for the α3/β1-heterodimer in mediating apoptosis in HMECs grown in contact with rECM.

Introduction

Interactions between extracellular matrix (ECM)* and mammary epithelial cells are critical for the regulation of cellular proliferation, polarity, and apoptosis (Petersen et al., 1992; Strange et al., 1992). These normal interactions may be disrupted during breast carcinogenesis (Petersen et al., 1992; Zutter et al., 1995). It has been observed that although normal mammary epithelial cells undergo growth arrest in culture in the presence of reconstituted ECM (rECM), breast cancer cells and established breast cancer cell lines fail to exhibit similar growth arrest (Petersen et al., 1992). Thus, it is hypothesized that interaction with ECM may serve to distinguish the growth patterns of normal and malignant mammary epithelial cells.

The mechanism by which ECM-mediated signal transduction events might result in changes in gene expression is a subject of current investigation. It has been shown that rECM regulates both biomechanical and biochemical signaling events and conversely that alterations in cell morphology can alter the response of cells to rECM (Folkman and Moscona, 1978; Roskelley et al., 1994). It is hypothesized that because malignant cells have an altered response to rECM, ECM signaling pathways may utilize tumor suppressor checkpoints critical for cellular organization and polarity (Petersen et al., 1992; Howlett et al., 1994; Weaver et al., 1997).

Integrins are heterodimeric cell surface receptors that link ECM to structural and functional components within the cell (Hynes, 1992). There is increasing evidence that integrins are important regulators of normal mammary mor-

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*Abbreviations used in this paper: AS, antisense; ECM, extracellular matrix; HMEC, human mammary epithelial cells; HPV-16, human papillomavirus type 16; rECM, reconstituted ECM; RT, room temperature; SKY, spectral karyotyping; ODN, oligodeoxynucleotide; scrAS, scrambled AS; TdT, terminal deoxynucleotidyl transferase.

Key words: extracellular matrix; mammary epithelial cells; apoptosis; p53; α3/β1-integrin

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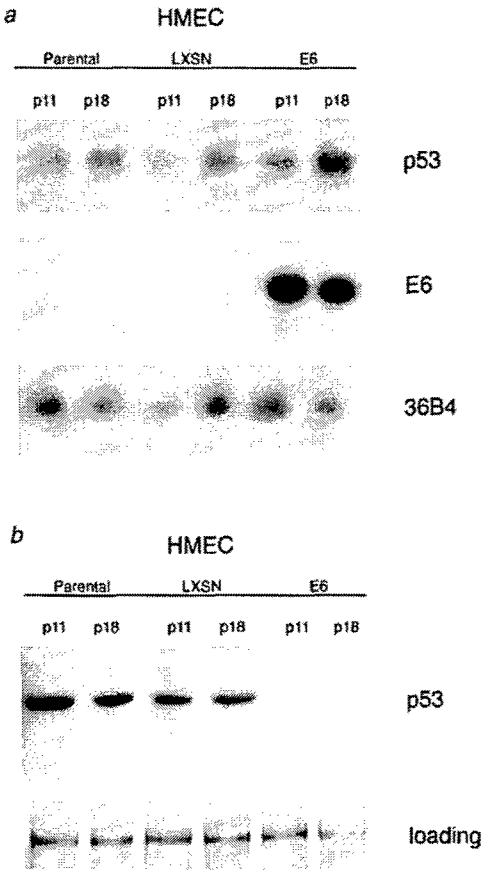

Figure 1. **Expression of p53 and HPV-16 E6 mRNA and p53 proteins in HMECs.** (a) Expression of endogenous p53 and exogenous HPV-16 E6 mRNA in HMECs. Passage 10 and 18 $p53^+$ HMEC-P parental cells (Parental), $p53^+$ HMEC-LXSN controls (LXSN), and $p53^-$ HMEC-E6 cells (E6) were analyzed for p53 and HPV-16 E6 mRNA expression. 10 μg of RNA were loaded per lane. 36B4 served as a loading control. (b) Expression of p53 protein is suppressed in $p53^-$ HMEC-E6 cells. Passage 10 and 18 $p53^+$ HMEC-P parental cells (Parental), $p53^+$ HMEC-LXSN controls (LXSN), and $p53^-$ 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. The protein gel was stained with Coomassie blue, and an unknown 45-kD protein band was used as a loading control.

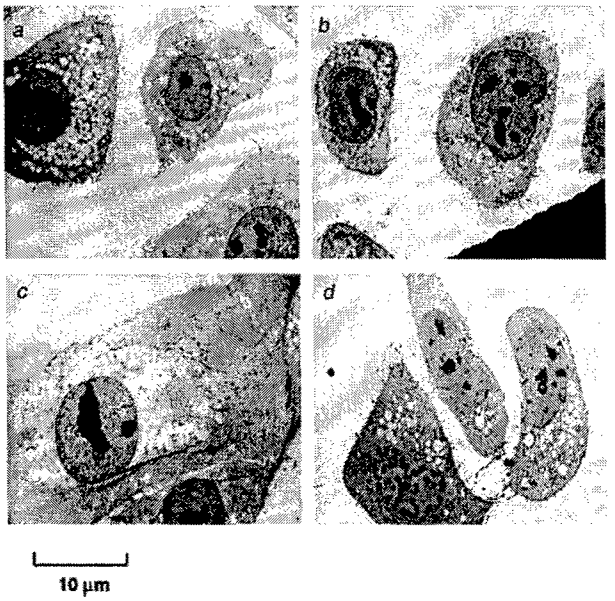

Figure 2. **Morphologic appearance of early and late passage HMECs expressing HPV-16 E6.** Electron micrographs of early passage (passage 10) $p53^+$ HMEC-LXSN controls (a) and $p53^-$ HMEC-E6 cells (b) grown in tissue culture for 4 d were similar in appearance. (c) Late passage (passage 21) $p53^+$ HMEC-LXSN vector control cells were approaching in vitro senescence, were significantly larger than early passage cells, and contained increased numbers of vesicles. (d) Passage 18 $p53^-$ HMEC-E6 cells were similar in appearance (but not identical) to passage 10 cells.

phology, since mammary carcinoma cells frequently demonstrate atypical patterns of integrin expression including loss, downregulation, or improper localization (Koukoulis et al., 1991; Glukhova et al., 1995; Lichtner et al., 1998). In the normal mammary gland, the α3/β1 integrin is expressed at the basal surface of luminal epithelial cells (Koukoulis et al., 1991; Glukhova et al., 1995). In contrast, invasive breast carcinomas demonstrate weak staining and redistribution of the α3/β1 integrin. Recently, the α3/β1 integrin has attracted considerable interest, since its function appears to be versatile. For example, the integrin α3/β1 functions as a cell adhesion receptor for laminin-5 (epiligrin), a major ECM protein present in basement membrane (Xia et al., 1996). The α3/β1-integrin is recruited to focal adhesion contacts in cultured cells and thereby plays an important role in linking ECM to components of the actin cytoskeleton (Carter et al., 1990b; Grenz et al., 1993; DiPersio et al., 1995). Integrin α3/β1 is a critical mediator of intracellular adhesion (Kawano et al., 2001). Studies in keratinocytes suggest that α3/β1 plays a critical role in cell spreading and migration and promotes gap junctional communication (Carter et al., 1990b; Xia et al., 1996; DiPersio et al., 1997). Recently, α3/β1-integrin has been shown to be involved in the initiation apoptosis (Sato et al., 1999). Taken together, these studies illustrate the multifaceted role of α3/β1-integrin in mediating interactions between ECM and epithelial cells and perhaps in initiating apoptosis.

Tissue homeostasis is maintained by a dynamic equilibrium between cellular proliferation and cell death (Evan and Littlewood, 1998). Apoptosis is considered to be the predominant mechanism of cell death and plays a central role in controlling cell number and eliminating cells sustaining DNA damage (Ashkenazi and Dixit, 1998). The role of the tumor suppressor p53 in ECM-induced growth arrest, polarity, and apoptosis is unknown. *TP53* is a cell cycle "checkpoint" gene critical for cell cycle regulation, and it is functionally inactivated in human cancer at a high frequency (Hansen and Oren, 1997). Mutations of the *TP53* gene are detected commonly in breast cancers and are associated with an increased risk of malignancy (Ashkenazi and Dixit, 1998; Levesque et al., 1998). Aberrant expression of p53 in mammary epithelial cells may be a biomarker predicting risk for subsequent breast carcinogenesis. Accumulation of p53 protein in mammary epithelial cells is detected frequently in women at high risk for the development of breast cancer (Fabian et al., 1996) and associated with an increased risk of

progression to breast cancer in women with benign breast disease (Rohan et al., 1998).

Retrovirally mediated introduction of human papillomavirus type 16 (HPV-16) E6 protein into cells provides a model for the isolated loss of p53 function. The E6 protein of the cancer-associated HPV-16 binds to p53 and targets it for degradation through the ubiquitin pathway (Demers et al., 1996). We employed this approach and antisense (AS) oligondeoxyucleotides (ODNs) to acutely suppress p53 function in normal human mammary epithelial cells (HMECs) in order to model p53 loss in the context of ECM signaling.

Our results showed that control HMECs expressing p53 underwent rECM-mediated growth arrest and formed a polarized epithelium. In contrast, HMECs with HPV-16 E6– and ODN-suppressed p53 expression underwent rECM-induced growth arrest followed by apoptosis. $p53^-$ HMEC-E6 cells passaged in non-rECM culture rapidly acquired resistance to rECM-mediated growth arrest, polarity, and apoptosis after 8 –10 passages in culture. Treatment of early passage $p53^-$ HMEC-E6 cells with either α3- or β1-integrin function-altering antibodies (Abs) blocked rECM-mediated growth arrest and induction of apoptosis. Observations in our model system suggest that rECM may play an important role in the induction of apoptosis in early passage $p53^-$ HMECs via an α3/β1 signaling pathway.

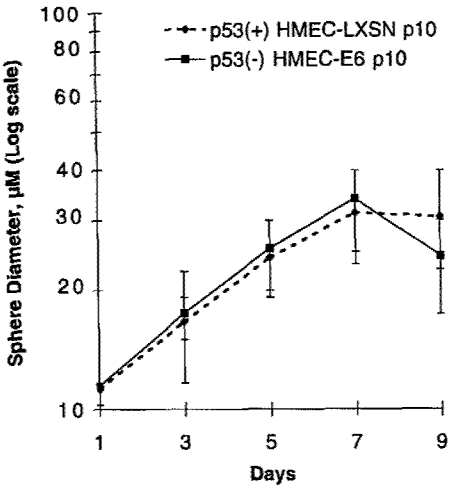

Figure 3. **rECM-induced growth arrest of early passage $p53^-$ HMEC-E6 cells and $p53^-$ HMEC-LXSN controls.** The mean diameter of spheres formed by $p53^+$ HMEC-LXSN controls (passage 10) and $p53^-$ HMEC-E6 cells (passage 10) were plotted as a function of days in culture. Cells were plated in rECM on day 0 and the diameter of growing spherical cell colonies measured with an eye piece equipped with micrometer spindle. For both $p53^+$ HMEC-LXSN controls and $p53^-$ HMEC-E6 cells, the 20 largest colonies were measured at each time point. These data are representative of three separate experiments.

Results

p53 protein suppression in HMECs

Retrovirally mediated expression of the HPV-16 E6 protein was used to suppress normal intracellular p53 protein levels in HMECs. The pLXSN16E6 retroviral vector containing the coding sequence for HPV-16 E6 viral protein has been described previously (Demers et al., 1994). Actively dividing passage 9 AG11132 cells were infected with the retroviral vector LXSN16E6 or the control vector LXSN, and the infected cells were selected as described in Materials and methods. All experiments described subsequently were performed on mass cultures. HMEC-P parental cells, $p53^+$ HMEC-LXSN controls, and $p53^-$ HMEC-E6 cells were passaged serially in culture on plastic tissue culture plates. As previously observed (Seewaldt et al., 1999a,b), $p53^+$ HMEC-LXSN controls and HMEC-P parental cells exhibited a progressive increase in doubling time with continued serial passaging and entered "phase b" growth plateau at about passage 23 (Romanov et al., 2001). In contrast, $p53^-$ HMEC-E6 cells exhibited an extended lifespan in culture and entered a growth plateau at passage 40.

Northern blot analysis was performed on $p53^+$ HMEC-P parental cells, $p53^-$ HMEC-E6 cells, and $p53^+$ HMEC-LXSN controls (passage 11 and 18) to test for HPV-16 E6 mRNA expression. Expression of the LTR-initiated E6 mRNA transcript was observed in $p53^-$ HMEC-E6 cells at passage 11 and 18 but not in parental or vector controls (Fig. 1 a).

Western blots were performed on $p53^+$ HMEC-P parental cells, $p53^-$ HMEC-E6 cells, and $p53^+$ HMEC-LXSN controls (passage 11 and 18) to determine the relative levels of p53 protein expression. Expression of p53 protein was observed in $p53^+$ HMEC-P parental cells and $p53^+$ HMEC-LXSN vector controls but was not detectable by Western analysis in $p53^-$ HMEC-E6 cells at either passage 11 or 18 (Fig. 1 b).

Cytogenetic analysis of early and late passage $p53^+$ and $p53^-$ HMECs

Cytogenetic analysis of $p53^+$ HMEC-P parental cells, $p53^+$ HMEC-LXSN controls, and early passage $p53^-$ HMEC-E6 cells was performed using both spectral karyotyping (SKY) and DAPI staining to (a) test whether parental and vector control cells exhibited a normal karyotype and (b) verify that early passage $p53^-$ HMEC-E6 cells did not exhibit specific karyotypic abnormalities.

38 early passage $p53^+$ HMEC-P parental cells (passage 8) were karyotyped. 35 (92%) metaphase cells had a normal diploid karyotype. Three cells (8%) exhibited random chromosome loss.

Similarly, 22 early passage $p53^+$ HMEC-LXSN control cells (passage 10) were karyotyped. 20 (91%) $p53^+$ HMEC-LXSN metaphase cells had a normal diploid karyotype. Two cells (9%) exhibited random chromosome loss.

22 late passage $p53^+$ HMEC-LXSN control cells (passage 17) were karyotyped. 18 (82%) $p53^+$ HMEC-LXSN metaphase cells had a normal diploid karyotype. Three cells (13%) exhibited random chromosome loss. One cell (5%) was tetraploid (92 chromosomes). These results are consistent with karyotypes reported for late "phase a" (pregrowth plateau) HMECs (Romanov et al., 2001).

A total of 21 early passage $p53^-$ HMEC-E6 metaphase cells (passage 10) were karyotyped. Two cells were analyzed by SKY, and 19 cells were analyzed using inverted and contrast-enhanced DAPI staining. The majority of cells (12

Table I. **Karyotype of late passage p53⁻ HMEC-E6 cells**

Cell number	Karyotype
S14	32<2n>,XX,−1,−2,−5,−8,−11,−12,−14,−16,−16,−17,−18,−20,−21, der(10;21)(q10;q10),−22,+mar
S21	35,XX,−2,−3,−5,−6,−7,−8,−11,dic(12;20)(p1?1;p13),−16,del(16)(p11),−18,−19
S24	36,−X,t(X;14)(q2?8;q24),r(5),der(6)del(6)(p22)del(6)(q11),−9,−10,−10,−13, dic(14;21)(q32;q22),−15,−15,der(17;21)(q10;q10),−22
S32	36,XX,−1,−3,−5,−6,del(8)(q13q24),−9,−11,−13,−17,−18,−19
S17	39,X,−X,−1,del(3)(p11),der(6;21)(q10;q10),del(8)(q24),del(12)(p13),−15,del(16)(p11),−18,−19,−21
S19	38,XX,−1,−2,−5,−7,dic(9;20)(p11;p13),−15,dic(16;21)(p11;p13),−18
S6	40,XX,del(1)(q3?1),−3,der(6)t(3;6)(?;p11),−10,der(12;16)(q10;q10),−15,−17,−10, der(?21)t(20;?21)(p11;q11)
S30	41,X,dic(X;12)(q28;p11),del(8)(?p11),der(16;21)(q10;q10),der(15;21)(q10;q10), dic(17;18)(p11;p11.3),−22
S33	41,XX,dic(5;21)(p1?5;p1?3),dic(12;21)(p1?2;q22),dic(15;17)(p13;q25),dic(16;22)(p11;p1?3), dic(18;20)(p11.3;q13.?1)
S13	41,XX,−1,−4,−6,−10,dic(12;17)(p13;p1?3),add(16)(p11),−21
S34	42,XX,del(2)(q11),del(9)(q11),del(12)(p1?2),dic(12;16)(p11;q24),−15,−21,−21
S7	44,XX,dic(6;8)(p25;p23),−13,del(17)(p11),der(21;22)(q10;q10)
S9	44,X,dic(X;21)(p22;p1?1),del(2)(q11),+der(2),+der(2)t(2;16)(?;?q1?3),−16,−17,−19
S2	44,XX,del(7)(q11),trc(7;21;20)(q36;p11q21−22;p13),del(16)(?p11)
S20	44,XX,der(12;16)(q10;q10),dic(20;21)(p13;p1?1)
S26	44,XX,dic(10;17)(q26;p1?1),−12
S28	44,X,dic(X;15)(q11;p11),−16
S29	44,XX,−5,del(16)(p11)
S27	45,XX,−10,der(16)t(10;16)(q11;p11),t(20;20)(q13.1;q13.3)
S16	45,XX,+15,dic(15;15)(q26;p1?3),der(12;16)(q10;q10),der(20)t(18;20)(?q12;p13)
S5	45,XX,dic(14;21)(q3?2;p11),−15,del(16)(p11),del(17)(p11),+21,der(21)t(14;21)(?;?p11)
S18	46,XX,csb(2)(p11),del(9)(q12),del(12)(p13),der(16;21)(q10;q10),dic(20;21)(p13;p13)
S10	49,XX,+2,dic(3;6)(p26;p25)×2,+dic(3;22)(p11;p13),+5,+5,t(5;9)(q35;q34), dic(6;20)(p25;q13.?1),−7,+8,+10,+dic(12;6;19)(p13;?;p13.?3),−13,+14,−15,+16, der(16)t(13;16)(q1?2;p13)x2,+der(17)t(17;19)(p11;?p12),+18,dic(20;20)(p13;q13.3),−21
S15	55,X,t(X8)(q2?8p21),+der(X)t(X2)(p22?),−1,del(2)(q11),+del(2)(?p11),+3,+4,+5,+5,+7,+7,−9,+11, +11,dic(12;21)(p13;q22)?dup(12)(p11p13),+13,+14,−16,del(16)(p1?2),−19,+20,+22
S35	61<3n>,−X,−X,−X,−1,−2,−3,−3,−4,+5,−7,−7,+8,+9,−12, der(12)t(X;12)(q21;p13),+14,−15,−16,−20
S1	63<3n>,XXX,−2,−3,der(3)del(3)(p21)del(3)(q2?3),+4,der(6)inv(6)(p25q23)dic(66)(p25q27),+7,+10, +11,+12,dic(12;22)(p13;p13)×2,−14,−15,−16,−16,dic(20;20)(p13;q13.3),−21,der(21;21)(q10;q10)
S25	84,XXX,del(X)(q13),−3,−5,del(6)(p11),−8,−8,dic(14;16)(p11;p11)×2,dic(17;21)(p13;q22)×2
D1	46,XX,del(16)(p1?1)
D3	43,XX,−15,−19,−20
D10	41,X,−X,dic(7;21)(p22;q22),−11,dic(12;?)(p13;?),−15,dic(16;?)(p1?1;?),−20,−21,−22,+2mar
D6	44,XX,−6,der(12;16)(q10;q10)
D9	44,XX,der(16;20)(q10;q10),der(17;21)(q10;q10)
D12	41,XX,−12,add(15)(p11−13),−16,add(17)(p1?1),−20,−21,−21
D14	44,XX,+9,dic(?9;12)(q34;p13),der(16;17)(q10;q10),dic(21;21)(q22;q22)
D2	45,XX,der(12;14)(q10;q10)

cells, 57%) had a normal diploid chromosome content, three cells had random chromosome loss (14%), and the remaining cells were aneuploid. In three cells (14%), multiple losses of whole chromosomes occurred, resulting in the chromosomes number of 30, 32, and 36, respectively. The other three cells (14%) were either tetraploid (92 chromosomes) or hypotetraploid (90 and 80 chromosomes). Only two cells, both near diploid and both studied using DAPI staining, displayed structural chromosome changes: inv(20)(p11q13.1) in one cell, and del(X)(p21), dic(14; 19)(q32;q13.4), and a marker chromosome in another.

In contrast to early passage cells, late passage p53⁻ HMEC-E6 (passage 18) were markedly abnormal with numerical and structural chromosome aberrations. A total of 35 metaphase cells were analyzed: 27 using SKY and 8 using inverted and contrast-enhanced DAPI staining (Table I). These results have been published previously (Seewaldt et al., 2001). No cell had a normal diploid karyotype. The predominant types of structural changes were deletions, whole arm translocations, and dicentric chromosomes with breakpoints in the pericentromeric and/or telomeric regions. Although a majority of the 35 cells contained complex chromosomal rearrangements, each resistant cell analyzed was unique. This suggests that chromosome aberrations observed in the late passage p53⁻ HMEC-E6 cell population resulted from a generalized event causing karyotypic instability that is inconsistent with the outgrowth of a mutant clone.

Morphologic appearance of early and late passage HMECs with HPV-16 E6–suppressed p53 expression

Early passage p53⁺ HMEC-LXSN controls (passage 10) and p53⁻ HMEC-E6 cells (passage 10) were similar in appear-

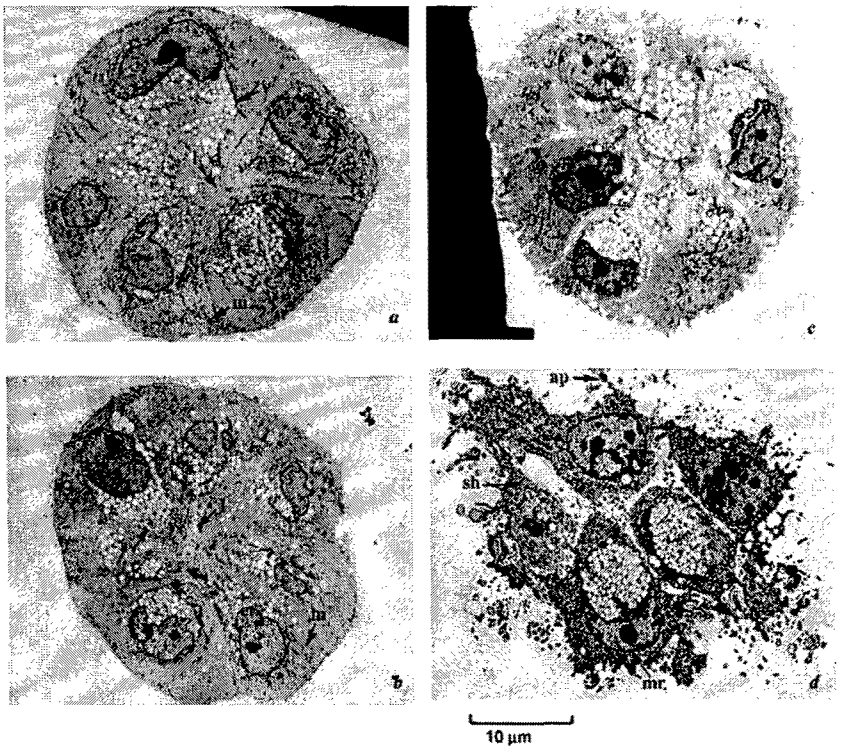

Figure 4. **Early passage p53⁻ HMEC-E6 cells undergo apoptosis when cultured in contact with rECM starting at 7 d.** (a and b) Electron micrographs of p53⁺ HMEC-LXSN vector control cells (passage 10) grown in rECM for 7 (a) and 14 (b) d. p53⁺ HMEC-LXSN controls formed acini-like structures, which demonstrated a central lumen (l) consistent with nonlactating mammary glandular epithelium: (1) nuclei were aligned predominantly with the basal surface, (2) secretory vesicles (v) were present on the luminal surface and not on the basal surface, and (3) mitochondria (m) were located at the basolateral surface. (c and d) p53⁻ HMEC-E6 cells grown in rECM for 6 (c) and 7 (d) d. At 6 d (c), p53⁻ HMEC-E6 cells are organized around a central lumen, there is cell separation (s), nuclei are primarily oriented at the basal surface, and vesicles (v) are present typically at the luminal surface. At 7 d (d), p53⁻ HMEC-E6 cells demonstrated evidence of apoptosis including (1) nuclear condensation, (2) cell shrinkage (sh) and separation, (3) margination of chromatin (mr), and (4) the presence of apoptotic bodies (ap) containing cytoplasmic elements.

ance (Fig. 2, a and b). Late passage p53⁺ HMEC-LXSN cells (passage 21) exhibited morphologic changes characteristic of senescence including increased cell size and increased cytoplasmic vesicles (Fig. 2 c). In contrast, late passage p53⁻ HMEC-E6 cells (passage 18) were morphologically similar (but not identical) to passage 10 cells (Fig. 2 d).

rECM-induced growth arrest of HMECs is not altered by the acute expression of HPV-16 E6

Early passage p53⁺ HMEC-LXSN controls (passage 10) and p53⁻ HMEC-E6 cells (passage 10) were grown in prepared rECM as a single cell suspension. Both p53⁺ HMEC-LXSN and p53⁻ HMEC-E6 cells grew exponentially in rECM until day 6 and then growth arrested on day 6–7 (Fig. 3). They formed a uniform population of spherical colonies. The mean diameter of p53⁺ HMEC-LXSN and p53⁻ HMEC colonies on day 7 was 30.5 ± 7.4 and 33 ±5.5 μm, respectively (Fig. 3). There was a decrease in the mean sphere diameter of p53⁻ HMEC-E6 cells relative to controls on day 9, but this was not statistically significant. These data suggest that the acute suppression of p53 function in HMECs by HPV-16 E6 alters neither proliferation of cells in contact with rECM nor rECM-induced growth inhibition as measured by colony diameter in this in vitro system.

Acute expression of HPV-16 E6 induces apoptosis in rECM-growth arrested HMECs in the absence of p53 expression

EM was performed on early passage rECM growth-arrested p53⁺ HMEC-LXSN controls and p53⁻ HMEC-E6 cells to test whether suppression of p53 expression by HPV-16 E6 altered the response of HMECs to rECM. p53⁺ HMEC-LXSN control cells (passage 10) proliferated in contact with rECM until day 6 and then underwent rECM-induced growth arrest on day 6–7.

As observed previously (Seewaldt et al., 1997b), day 7 p53⁺ HMEC-LXSN cells grown in rECM exhibited an acinus-like structure consistent with normal nonlactating mammary glandular epithelium (Fig. 4 a). There was no evidence of apoptosis. Day 14 p53⁺ HMEC-LXSN cells likewise did not exhibit evidence of apoptosis (Fig. 4 b).

In contrast, early passage p53⁻ HMEC-E6 cells (passage 10) proliferated in rECM until day 6 and then underwent

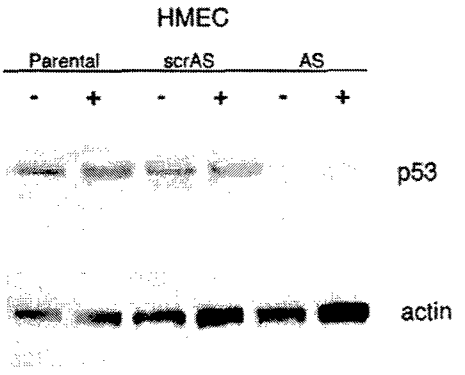

Figure 5. **Expression of p53 protein is suppressed in p53⁻ HMEC-AS cells.** Passage 10 p53⁺ HMEC-P parental cells (Parental), p53⁺ HMEC-scrAS controls (scrAS), and p53⁻ HMEC-AS cells (AS) were grown with (+) or without rECM (−) and analyzed for p53 protein expression as described in Materials and methods. Equal amounts of protein lysate were loaded per lane. Hybridization with Abs to actin serves as the loading control.

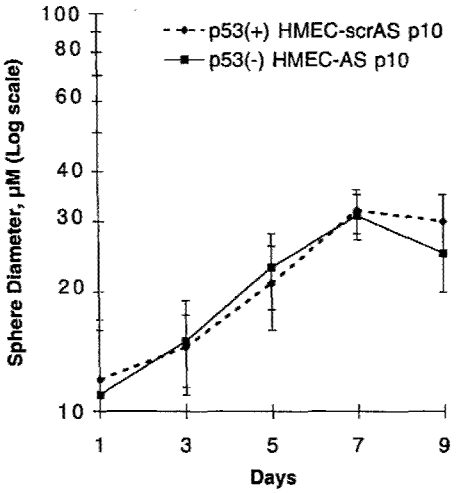

Figure 6. **rECM-induced growth arrest of early passage p53⁻ HMEC-AS cells and p53⁺ HMEC-scrAS controls.** The mean diameter of spheres formed by p53⁺ HMEC-scrAS controls (passage 10) and p53⁻ HMEC-AS cells (passage 10) are plotted as a function of days in culture. Cells were plated in rECM on day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with micrometer spindle. The 20 largest colonies were measured at each time point. These data are representative of three separate experiments.

apoptosis on day 7. There was no morphologic evidence of apoptosis in day 6 rECM cultures (Fig. 4 c). On day 7, 100 early passage p53⁻ HMEC-E6 cell clusters were surveyed for the presence or absence of apoptosis. 98 clusters (98%) exhibited morphologic evidence of apoptosis by the following criteria: (a) nuclear condensation, (b) cell shrinkage and separation, (c) margination of chromatin, (d) the presence of apoptotic bodies, and (e) mitochondrial condensation (Fig. 4 d).

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, (a) AG1134-LXSN controls underwent growth arrest and formed an acinus-like structure in contact with rECM at day 7 (unpublished data), and (b) early passage AG11134-E6 cells exhibited morphologic evidence of apoptosis at day 7 (unpublished data).

The terminal deoxynucleotidyl transferase (TdT) method was also used to detect the presence or absence of apoptotic strand breaks in day 7 rECM culture. The 3′-hydroxyl termini of apoptotic-induced strand breaks were labeled with biotin-dUTP by exogenous TdT and were detected in situ by HRP-conjugated streptavidin. Day 7 rECM growth-arrested early passage p53⁺ HMEC-LXSN controls (passage 10) did not demonstrate evidence of apoptosis (unpublished data). In contrast, early passage day 7 p53⁻ HMEC-E6–transduced cells (passage 10) grown in contact with rECM exhibited apoptotic strand breaks (unpublished data).

These observations indicate that whereas early passage p53⁺ HMEC-LXSN controls undergo rECM-induced growth arrest, early passage p53⁻ HMEC-E6 cells undergo rECM-mediated growth arrest on day 6 followed by induction of apoptosis on day 7. Results from this in vitro model of rECM-induced apoptosis in HMECs suggest an association between HPV-16 E6–induced suppression of p53 function and the induction of rECM-mediated apoptosis.

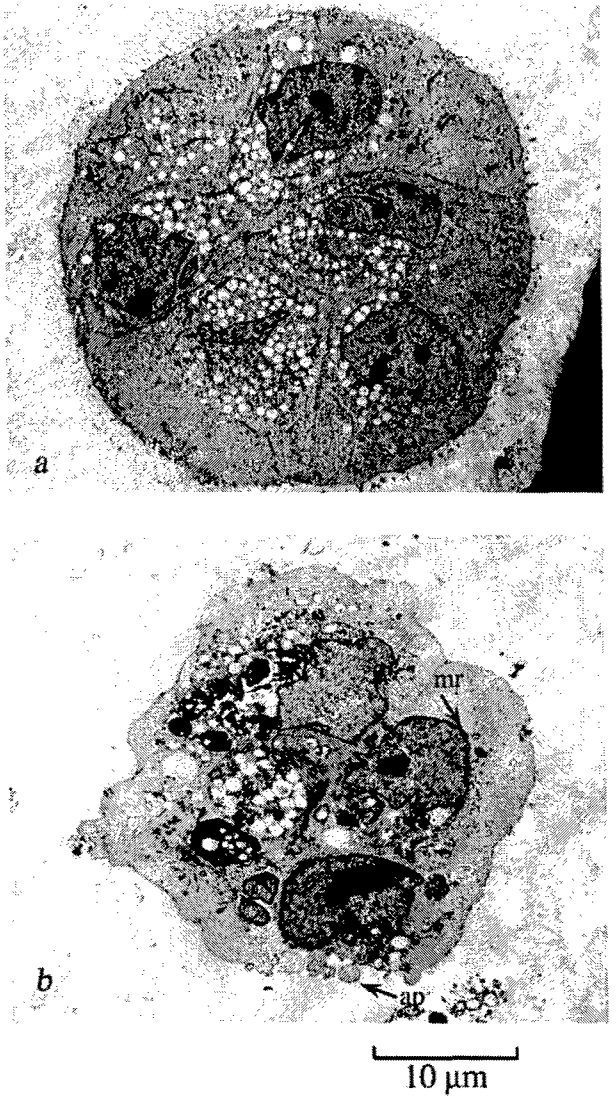

Figure 7. **Early passage p53⁻ HMEC-AS cells undergo apoptosis when cultured in contact with rECM starting at 7 d.** (a) Electron micrographs of p53⁺ HMEC-scrAS control cells (passage 10) grown in rECM for 14 d. p53⁺ HMEC-LXSN controls formed acini-like structures. (b) p53⁻ HMEC-AS cells grown in rECM for 7 d demonstrated evidence of apoptosis including (1) nuclear condensation, (2) cell shrinkage and separation, (3) margination of chromatin (mr), and (4) the presence of apoptotic bodies (ap) containing cytoplasmic elements.

Acute suppression of p53 by an AS approach in HMECs promotes sensitivity to rECM-mediated apoptosis

Since HPV-16 E6 may have effects other than the suppression of p53, an AS approach was used to test whether the acute suppression of p53 function promotes sensitivity to rECM-mediated apoptosis. p53 protein expression was suppressed using a p53 AS ODN in HMECs. Western blot analysis demonstrated almost complete suppression of p53 protein expression in HMECs treated with the p53 AS ODN (p53⁻ HMEC-AS) and no suppression of p53 protein expression in HMECs treated with a scrambled sequence of the p53 AS ODN (p53⁺ HMEC–scrambled AS [scrAS]) (Fig. 5).

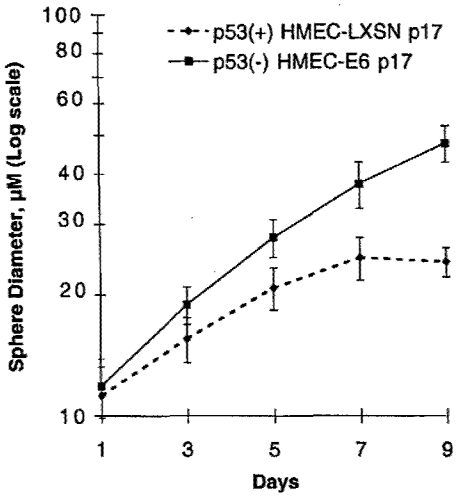

Figure 8. **Late passage p53⁻ HMEC-E6 cells are resistant to rECM-induced growth arrest.** The mean diameter of spheres formed by late passage p53⁺ HMEC-LXSN controls (passage 17) and p53⁻ HMEC-E6 cells (passage 18) are plotted as a function of days in culture as described in Materials and methods. These data are representative of three separate experiments.

Consistent with results obtained in early passage p53⁻ HMEC-E6 cells, the acute suppression of p53 function by an AS approach did not alter the ability of p53⁻ HMEC-AS or p53⁺ HMEC-scrAS controls to proliferate in rECM or to undergo rECM-mediated growth arrest (Fig. 6). Mean diameter of p53⁺ HMEC-scrAS and p53⁻ HMEC-AS colonies on day 7 was 32 ± 4.0 and 31 ± 5.0 μm, respectively (Fig. 6). These data suggest that the acute suppression of p53 function in HMECs by an AS approach neither alters proliferation of cells in contact with rECM nor does it alter rECM-induced growth inhibition as measured by colony diameter.

We tested whether inhibition of p53 protein expression by an AS approach promoted rECM-mediated apoptosis. On day 7, 100 early passage p53⁻ HMEC-AS cell clusters were surveyed for the presence or absence of apoptosis. Similar to early passage p53⁻ HMEC-E6 cells, p53⁻ HMEC-AS cells underwent apoptosis on day 7 (Fig. 7 b). 90 clusters (90%) exhibited morphologic evidence of apoptosis by the following criteria: (a) nuclear condensation, (b) cell shrinkage and separation, (c) margination of chromatin, (d) the presence of apoptotic bodies, and (e) mitochondrial condensation (Fig. 7 b). In contrast, early passage p53⁺ HMEC-scrAS cells did not exhibit morphologic evidence of apoptosis on day 7 or 14 (Fig. 7 a; unpublished data). These observations support the hypothesis that the acute suppression of p53 function promotes rECM-mediated apoptosis.

Late passage p53⁻ HMEC-E6 cells acquire resistance to rECM-induced growth arrest

Starting at passage 16–18, p53⁻ HMEC-E6 cells acquired resistance to rECM-induced growth arrest as evidenced by continued increase in sphere diameter from day 5 to 9 (Fig. 8). The mean sphere diameter was 37 ± 3 μm at day 7 and 48 ± 5 μm at day 9. In contrast, passage 17 p53⁺ HMEC-LXSN cells exhibited sensitivity to rECM-induced growth arrest (Fig. 8).

Late passage HMECs with HPV-16 E6-suppressed p53 function exhibit dysregulated growth and are resistant to rECM-induced apoptosis

Late passage p53⁺ HMEC-LXSN controls (passage 17) and p53⁻ HMEC-E6 cells (passage 18) were cultured in rECM. In contrast to early passage p53⁻ HMEC-E6 cells, late passage cells demonstrated disorganized growth and no evidence of apoptosis at day 7 or 14 (Fig. 9, a and b). Similar to early passage p53⁺ HMEC-LXSN vector controls, electron micrographs of late passage controls demonstrated a normal acinus-like structure without evidence of apoptosis at day 7 (unpublished data) and 14 (Fig. 9 c). Passage 18 p53⁻ HMEC-E6 cells grown in rECM formed disorganized multilayered irregularly shaped colonies (Fig. 9, a and b). There was no morphologic evidence of apoptosis in passage 18 p53⁻ HMEC-E6 cells grown in rECM. A second HMEC strain AG11134 was tested to confirm that these results were not strain specific. Late passage AG11134-E6 cells were grown in rECM. Similar to late passage p53⁻ HMEC-E6 cells, AG11134-E6 cells formed disorganized aggregates of cells and did not demonstrate evidence of apoptosis (unpublished data). These results suggest that p53⁻ HMEC-E6 cells passaged in vitro acquire resistance to rECM-induced apoptosis.

Late passage p53⁻ HMEC-E6 cells grown in contact with rECM lack polarized expression of E-cadherin

Early and late passage p53⁺ HMEC-LXSN and early passage p53⁻ HMEC-E6 cells grown in contact with rECM demonstrated basolateral expression of E-cadherin, including lateral staining at cell–cell junctions consistent with a polarized epithelium (Fig. 10, a–c). 20 cell clusters were surveyed per data point. These results are similar to what is observed in normal breast sections and consistent with results obtained by other investigators when mammary epithelial cells are grown in contact with rECM (Weaver et al., 1997; Spancake et al., 1999). In contrast, late passage p53⁻ HMEC-E6 cells exhibited punctate dispersed and intracellular staining consistent with a loss of epithelial polarity (Fig. 10 d). These results are similar to what is observed in tumorigenic mammary epithelial cells that lack epithelial polarity grown in rECM (Weaver et al., 1997).

Laminin is critical for the induction of apoptosis in early passage p53⁻ HMEC-E6 cells

To identify the component of rECM that promotes apoptosis, early passage p53⁻ HMEC-E6 cells and p53⁺ HMEC-LXSN controls were cultured in growth factor–depleted Matrigel™, collagen I/IV gels, and collagen I/IV supplemented with laminin. Early passage p53⁻ HMEC-E6 cells underwent apoptosis when cultured for 7 d in growth factor–depleted Matrigel™ (Fig. 11 a), suggesting that depletion of growth factors present in the Matrigel™ are not critical for the induction of apoptosis. Early passage p53⁺ HMEC-LXSN controls did not undergo apoptosis when cultured in growth factor–depleted Matrigel™ (Fig. 11 b). When early passage p53⁻ HMEC-E6 cells were cultured in the presence of collagen I/IV, there was no evidence of apoptosis after 7 and 14 d (Fig. 11 c; unpublished data). Likewise, early passage p53⁺ HMEC-LXSN controls did not exhibit evidence of apoptosis when grown in the presence of

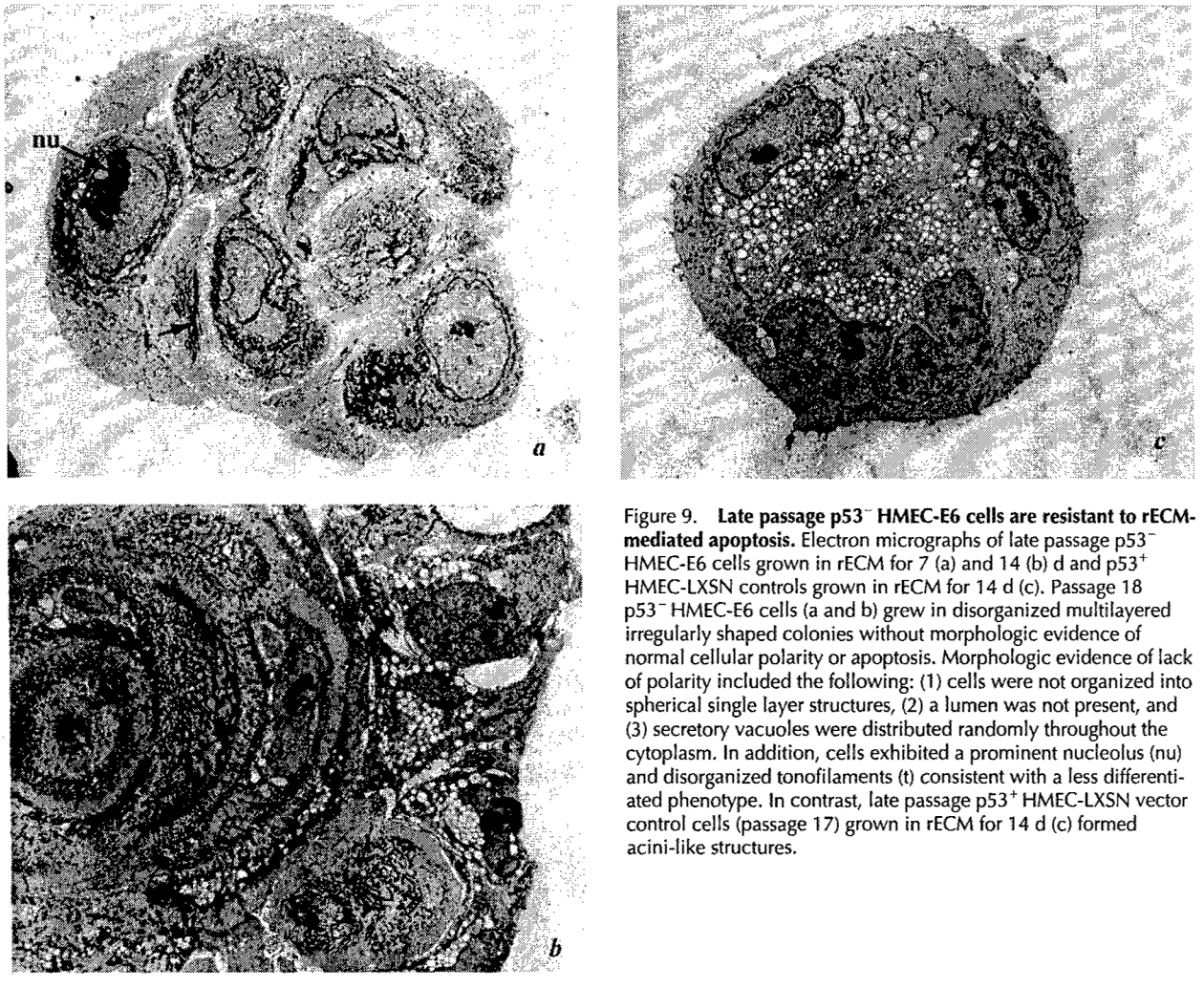

Figure 9. **Late passage p53⁻ HMEC-E6 cells are resistant to rECM-mediated apoptosis.** Electron micrographs of late passage p53⁻ HMEC-E6 cells grown in rECM for 7 (a) and 14 (b) d and p53⁺ HMEC-LXSN controls grown in rECM for 14 d (c). Passage 18 p53⁻ HMEC-E6 cells (a and b) grew in disorganized multilayered irregularly shaped colonies without morphologic evidence of normal cellular polarity or apoptosis. Morphologic evidence of lack of polarity included the following: (1) cells were not organized into spherical single layer structures, (2) a lumen was not present, and (3) secretory vacuoles were distributed randomly throughout the cytoplasm. In addition, cells exhibited a prominent nucleolus (nu) and disorganized tonofilaments (t) consistent with a less differentiated phenotype. In contrast, late passage p53⁺ HMEC-LXSN vector control cells (passage 17) grown in rECM for 14 d (c) formed acini-like structures.

collagen I/IV (Fig. 11 d). Interestingly, neither early passage p53⁺ HMEC-LXSN controls nor p53⁻ HMEC-E6 cells formed acinar structures when grown in collage I/IV (Fig. 11, c and d). When human placental laminin was added to the collagen I/IV gel, both early passage p53⁻ HMEC-E6 cells and p53⁺ HMEC-LXSN vector controls developed branching tubular structures that terminated in spherical aggregates (Fig. 11, e and g). Early passage p53⁻ HMEC-E6 cells present in both branched and aggregate structures exhibited evidence of apoptosis at 7–10 d (Fig. 11 g). By day 14, no cells were visualized by EM (unpublished data). Likewise, early passage p53⁻ HMEC-E6 cells cultured on a laminin-coated substratum in the presence of a 1:1 dilution of standard medium to laminin formed similar three-dimensional branching aggregates that also underwent apoptosis on days 7–10 as determined by EM (unpublished data). In contrast, early passage p53⁺ HMEC-LXSN controls grown in collagen I/IV supplemented with laminin did not exhibit evidence of apoptosis at 14 d (Fig. 11 h). These observations suggest a critical role for laminin in the induction of apoptosis in p53⁻ HMEC-E6 cells.

α3-integrin expression is altered in rECM-resistant late passage p53⁻ HMEC-E6 cells

Early and late passage p53⁺ HMEC-LXSN controls and p53⁻ HMEC-E6 cells were tested for α2-, α3-, α6-, β1-, and β4-integrin expression by immunohistochemistry. All early and late passage p53⁺ HMEC-LXSN controls and early passage p53⁻ HMEC-E6 cells stained positively for α2-, α3-, and β1-integrins and very weakly for α6- and β4-integrins (Fig. 12; unpublished data). Late passage p53⁻ HMEC-E6 cells grown in non-rECM culture exhibited a qualitative decrease in expression of α3-integrin (Fig. 12 d).

Early and late passage p53⁺ HMEC-LXSN controls and p53⁻ HMEC-E6 cells were grown in rECM and tested for α3- and β1-integrin expression by immunohistochemistry (clones P1F2 and P4C10, respectively). Early and late passage p53⁺ HMEC-LXSN controls and early passage p53⁻ HMEC-E6 cells exhibited polarized basal expression of α3- and β1-integrins (Fig. 13). In contrast, late passage p53⁻ HMEC-E6 cells grown in rECM demonstrated disorganized plasma membrane and cytosolic expression of α3-integrins (Fig. 13 d). Redistribution of α3-integrins has been seen

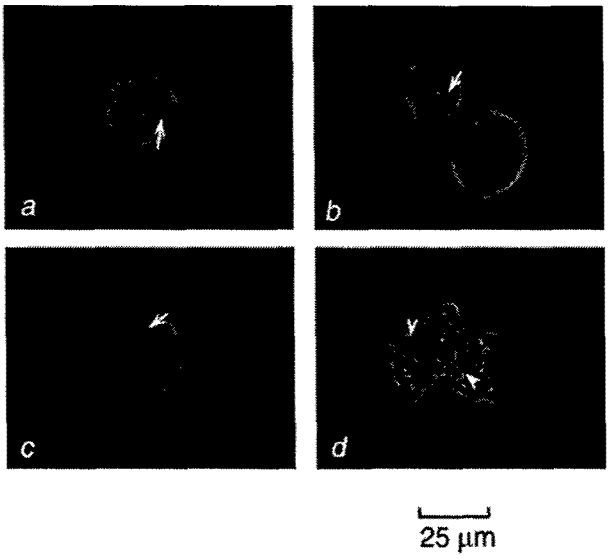

Figure 10. **Localization of E-cadherin in p53$^+$ HMEC-LXSN and p53$^-$ HMEC-E6 cells using immunofluorescence microscopy.** Frozen section of early passage p53$^+$ HMEC-LXSN controls (passage 11) (a), early passage p53$^-$ HMEC-E6 cells (passage 11) (b), late passage p53$^+$ HMEC-LXSN controls (passage 16) (c), and late passage p53$^-$ HMEC-E6 cells (passage 21) (d) grown in rECM for 6 d, cryosectioned, and stained with a mAb to E-cadherin as described in Materials and methods. E-cadherin was localized at the basolateral surface and at points of cell–cell contact in early and late passage p53$^+$ HMEC-LXSN cells and in early passage p53$^-$ HMEC-E6 cells (a–c, arrows). In contrast, late passage p53$^-$ HMEC-E6 cells showed punctate dispersed membrane and intracellular staining (d, arrowheads).

previously by other investigators in association with loss of mammary epithelial cell polarity in rECM culture and is consistent with these findings (Weaver et al., 1997). Late passage p53$^-$ 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 (Fig. 13 h).

α3- and β1-integrin function-altering Abs inhibit ECM-induced growth arrest of early passage p53$^+$ and p53$^-$ HMEC-E6 cells

Early passage p53$^-$ HMEC-E6 cells (passage 10) were treated with either α3- or β1-integrin function-altering Abs or control mouse nonimmune IgG and grown in prepared rECM as a single cell suspension. As expected, p53$^+$ HMEC-LXSN controls and p53$^-$ HMEC-E6 cells treated with nonimmune mouse IgG or β1-integrin–stimulating Ab (clone B3B11) grew exponentially in rECM until day 6 and then growth arrested on day 6–7 (Fig. 14 a; unpublished data), forming a uniform population of spherical colonies. In contrast, p53$^+$ HMEC-LXSN controls and p53$^-$ HMEC-E6 cells treated with α3- or β1-integrin blocking Abs (clones P1B5 and JB1A, respectively) did not undergo growth arrest on day 6–7 and continued to proliferate (Fig. 14, b and c). Mean diameter of control p53$^-$ HMEC-E6 colonies treated with nonspecific mouse IgG on day 7 and 9 was 30 ± 3 and 27 ± 4 μm, respectively (Fig. 14 a). Mean diameter of p53$^-$ HMEC-E6 cells treated with α3- or β1-integrin blocking Abs on day 9 was 51 ± 5 and 52 ± 4 μm, respectively (Fig. 14 c). p53$^+$ HMEC-LXSN controls

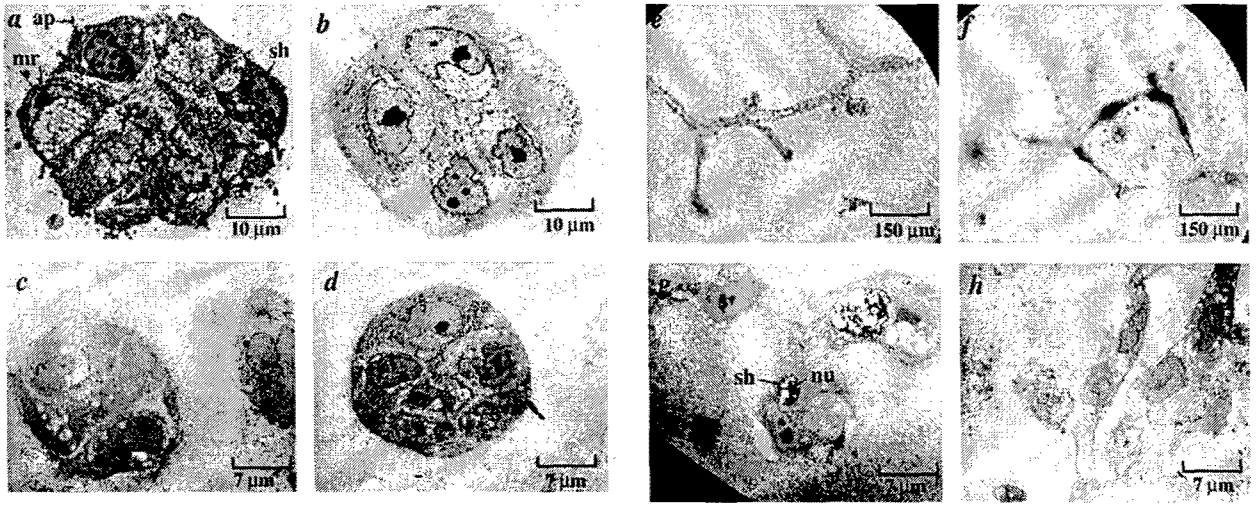

Figure 11. **Early passage p53$^-$ HMEC-E6 cells undergo apoptosis when cultured in contact with growth factor–depleted rECM and laminin as evidenced by EM.** At 7 d (a), passage 11 p53$^-$ HMEC-E6 cells grown in contract with growth factor–depleted rECM demonstrated evidence of apoptosis including the following: (1) nuclear condensation, (2) cell shrinkage (sh) and separation, (3) margination of chromatin (mr), and (4) the presence of apoptotic bodies (ap) containing cytoplasmic elements. In contrast, passage 11 p53$^+$ HMEC-LXSN controls (b) grown in contact with growth factor–depleted rECM did not demonstrate evidence of apoptosis at 14 d. Passage 11 p53$^-$ HMEC-E6 cells (c) and passage 11 p53$^+$ HMEC-LXSN controls (d) grown in collagen I/IV for 7 d did not demonstrate evidence of apoptosis by morphologic criteria. Light micrographs of passage 11 p53$^-$ HMEC-E6 cells grown in collagen I/IV supplemented with laminin (e) exhibited branching structures that terminated in spherical cell clusters. Electron micrographs of passage 11 p53$^-$ HMEC-E6 cells grown in collagen I/IV supplemented with laminin (g) demonstrated morphologic evidence of apoptosis including cell shrinkage (sh) and nuclear condensation (nu) at 7 d. Light micrographs of passage 11 p53$^+$ HMEC-LXSN vector controls grown in collagen supplemented with laminin demonstrated branching structures similar to those exhibited by early passage p53$^-$ HMEC-E6 cells (f) but did not demonstrate evidence of apoptosis by EM (h).

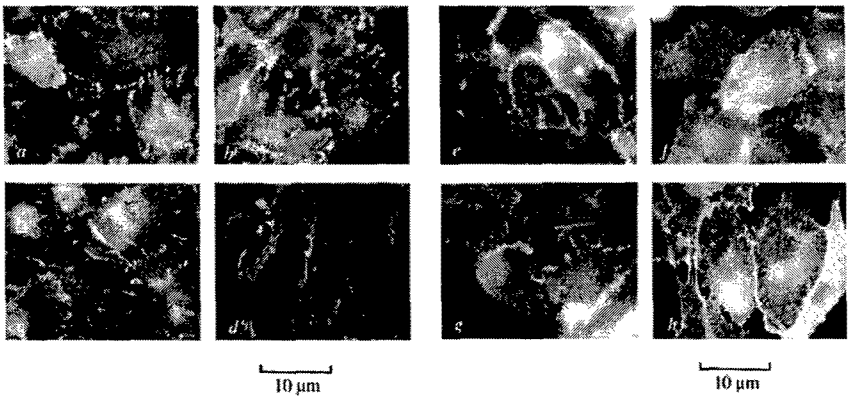

Figure 12. **Late passage p53⁻ HMEC-E6 cells exhibit decreased expression of integrin α3.** Early passage p53⁺ HMEC-LXSN controls (passage 10) (a and e), early passage p53⁻ HMEC-E6 cells (passage 10) (b and f), late passage p53⁺ HMEC-LXSN controls (passage 18) (c and g) and late passage p53⁻ HMEC-E6 cells (passage 21) (d and h) were grown on glass coverslips for 48 h and then stained by indirect Immunofluorescence with mAb P1F2 against integrin α3 (a–d) or with P4C10 against β1-integrin (e–h).

treated with α3- or β1-integrin blocking Abs demonstrated similar results (Fig. 14 b). These data show that interruption of α3/β1-integrin signaling by a blocking Ab inhibits rECM-mediated growth arrest in both p53⁺ HMEC-LXSN controls and early passage p53⁻ HMEC-E6.

α3- and β1-integrin function-altering Abs inhibit rECM-mediated apoptosis of early passage p53⁻ HMEC-E6 cells

Early passage p53⁻ HMEC-E6 cells and early passage p53⁺ HMEC-LXSN controls were treated with either α3- or β1-integrin function-altering Abs or control mouse nonimmune IgG and grown in rECM. Electron micrographs of early passage p53⁻ HMEC-E6 cells and early passage p53⁺ HMEC-LXSN controls treated with either α3- or β1-integrin blocking Abs (clones P1B5 and JB1A, respectively) demonstrated disorganized cell aggregates without evidence of apoptosis at day 7 or 14 (Fig. 15; unpublished data). Early passage p53⁻ HMEC-E6 cells treated with β1-integrin blocking Abs also exhibited increased cell–cell separation (Fig. 15 b). Control p53⁻ HMEC-E6 cells treated with nonspecific mouse IgG or β1-integrin stimulatory Ab (clone B3B11) underwent apoptosis on day 7 (unpublished data). Control p53⁺ HMEC-LXSN controls treated with nonspecific mouse IgG did not exhibit apoptosis at day 7 (unpublished data). These results suggest that α3/β1-integrin-mediated signal transduction may be critical for the induction of apoptosis in early passage p53⁻ HMEC-E6 cells grown in contact with rECM.

Discussion

p53 is a critical regulator of cell cycle control, and the high frequency with which p53 is functionally inactivated in human malignancy attests to its key role in preventing the clonal expansion of mutated cells (Hansen and Oren, 1997; Goetz et al., 2001). Although there is extensive experimental evidence demonstrating the importance of p53 as a tumor suppressor and in promoting differentiation, little is known about the fate of normal human cells that acutely lose p53 function in the context of rECM regulatory signals.

Abnormal p53 expression in benign breast tissue is associated with the subsequent development of breast cancer and may represent a very early event in breast carcinogenesis (Fabian et al., 1996; Levesque et al., 1998; Rohan et al., 1998). Interactions between mammary epithelial cells and ECM play a critical role in maintaining normal tissue homeostasis and are likely to be disrupted during breast carcinogenesis. In this report, we describe an in vitro system for investigating interactions between HMECs with suppressed p53 protein expression and rECM as a potential model of early mammary carcinogenesis.

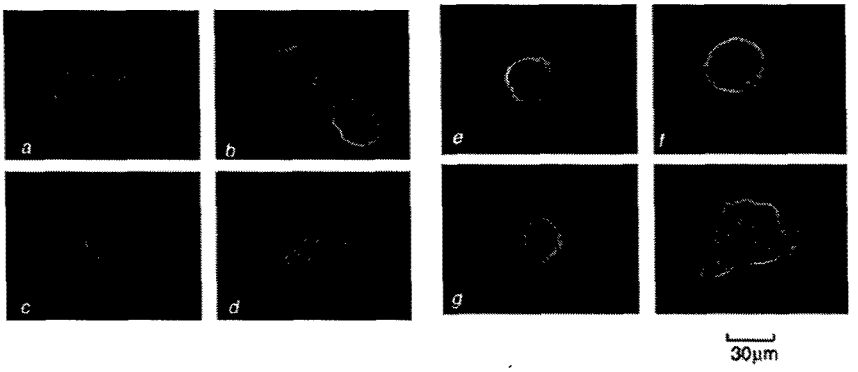

Figure 13. **Localization of α3- and β1-integrin expression in p53⁺ HMEC-LXSN and p53⁻ HMEC-E6 cells using immunofluorescence microscopy.** Frozen section of early passage p53⁺ HMEC-LXSN controls (passage 11) (a and e), early passage p53⁻ HMEC-E6 cells (passage 11) (b and f), late passage p53⁺ HMEC-LXSN controls (passage 16) (c and g), and late passage p53⁻ HMEC-E6 cells (passage 21) (d and h) grown in rECM for 6 d, cryosectioned, and stained with monoclonal Abs to α3- (a–d) and β1- (e–h) integrins as described in Materials and methods. Integrins α3 and β1 were localized to the cell-rECM junction in early and late passage p53⁺ HMEC-LXSN cells and in early passage p53⁻ HMEC-E6 cells (a–c and e–g). In contrast, late passage p53⁻ HMEC-E6 cells demonstrated disorganized membrane and cytosolic staining (d).

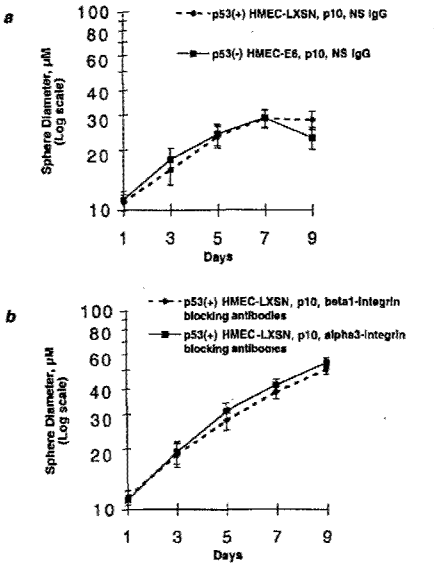

Figure 14. **Treatment with α3- and β1-integrin function-altering Abs blocks rECM-mediated growth arrest in p53$^+$ and p53$^-$ HMECs.** The mean diameter of spheres formed by p53$^+$ HMEC-LXSN controls, passage 10 (a and b) and p53$^-$ HMEC-E6 cells, passage 10 (a and c) treated with α3- or β1-integrin function-altering Abs (b and c) or nonimmune mouse IgG (a) is 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 micrometer spindle. The 20 largest colonies were measured at each time point. Both early passage (passage 10) p53$^+$ HMEC-LXSN controls and p53$^-$ HMEC-E6 transduced cells treated with nonimmune mouse IgG control antiserum proliferated when grown in contact with rECM until day 7 and then underwent growth arrest. In contrast, early passage (passage 10) p53$^+$ HMEC-LXSN and p53$^-$ HMEC-E6 cells treated with α3- and β1-integrin function-altering Abs were resistant to rECM-induced growth arrest and continued to proliferate after day 7. These data are representative of three separate experiments.

Retrovirally mediated expression of HPV-16 E6 and AS ODNs were used to acutely inhibit p53 protein expression in HMECs (Figs. 1 and 5). The combination of these approaches allowed us to utilize both viral and nonviral methods to suppress p53. We observed that early passage p53$^+$ HMEC control cells underwent rECM-mediated growth arrest on day 6–7 and formed acinus-like structure (Figs. 3, 4, and 10). In contrast, early passage p53$^-$ HMEC-E6 cells and early passage p53$^-$ HMEC-AS cells proliferated until day 6 (Figs. 3, 4, and 6) and then underwent apoptosis on day 7 as evidenced by EM and by in situ TdT staining (Figs. 4 and 7; unpublished data). These observations suggest that the acute suppression of p53 function in HMECs by HPV-16 E6 and by AS ODNs may promote sensitivity to rECM-induced apoptosis.

ECM has been shown to provide essential signals for mammary epithelial cell survival and in their absence cells undergo apoptosis (Streuli et al., 1991; Strange et al., 1992; Pullan et al., 1996). The critical relationship between ECM signaling and p53 expression is highlighted by a recent report that ECM survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis (Ilic et al., 1998). However, there is significant evidence that growth arrest, differentiation, and survival signals may also promote apop-

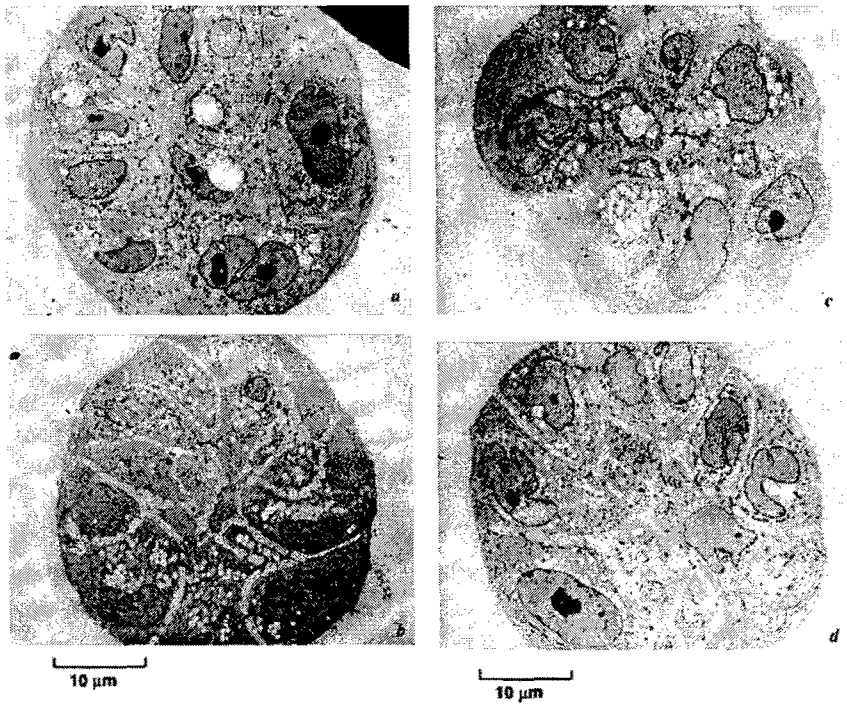

Figure 15. **Inhibition of α3- and β1-integrin signaling blocks induction of rECM-mediated apoptosis.** Electron micrographs of passage 10 p53$^-$ HMEC-E6 cells (a and b) and passage 10 p53$^+$ HMEC-LXSN controls (c and d) treated with 10 μg/ml α3- (a and c) or 20 μg/ml β1- (b and d) integrin blocking Abs and then grown in rECM for 7 d. Cells grew in multilayered colonies and did not growth arrest at day 6–7. There was no morphologic evidence of apoptosis. Evidence of lack of polarity included the following: (1) cells are not organized into spherical single layer structures, (2) a lumen was not present, and (3) vacuoles and nuclei were randomly distributed throughout the cytoplasm. Cells treated with β1-integrin blocking Abs exhibited cell–cell separation. There was no evidence of apoptosis.

tosis in genetically damaged cells (Seewaldt et al., 1995; Hong and Sporn, 1997; Mancini et al., 1997; Seewaldt et al., 1997b).

Our observation that the acute suppression of p53 in HMECs promotes sensitivity to apoptosis is consistent with several reports in human and mouse primary fibroblasts that the acute suppression of p53 results in apoptosis sensitivity (Hawkins et al., 1996; Wahl et al., 1996; Lanni et al., 1997). The mechanism by which the acute loss of p53 function might promote apoptosis is unknown. However, we observe that immediately after suppression of p53 by HPV-16 E6, HMECs exhibited a high percentage of tetraploid cells (14%) and an increased sensitivity to apoptosis. The tumor suppressor p53 is a critical component of the spindle checkpoint that ensures the maintenance of diploidy, and an increase in tetraploidy has been observed in cultured fibroblasts from p53-deficient mouse embryo fibroblasts (Cross et al., 1995). We speculate that it is possible that a loss in the spindle checkpoint might promote the induction of apoptosis when early passage $p53^-$ HMEC cells are exposed to either survival or growth arrest signals. Alternatively, p53 also plays an important role in differentiation, and therefore lack of p53 might result in cellular disregulation that promotes cell death. We observe that early passage $p53^-$ HMECs are sensitive to the induction of apoptosis by other agents that only promote growth arrest in $p53^+$ HMECs at equimolar concentrations. For example, we observed that although tamoxifen promotes G1/S-phase arrest in early passage $p53^+$ HMEC controls, tamoxifen promoted apoptosis in early passage $p53^-$ HMECs (Dietze et al., 2001; Seewaldt et al., 2001). Taken together, these observations provide evidence that the acute suppression of p53 might promote the induction of apoptosis when cells are exposed to growth arrest or survival signals.

Although early passage p53 HMEC-E6 cells underwent apoptosis when cultured in rECM, late passage cells were resistant to rECM-induced growth arrest, did not exhibit epithelial polarity, and failed to undergo apoptosis (Fig. 9). Loss of epithelial polarity is based on morphologic appearance by EM and lack of polarized expression of E-cadherin (Figs. 9 and 10). The development of apoptosis resistance correlated with the appearance of complex karyotypic abnormalities. Unlike early passage cells, late passage $p53^-$ HMEC-E6 cells continued to proliferate in rECM, formed multilayered aggregates of cells, and subsequently did not undergo apoptosis (Figs. 8 and 9). Based upon observations in our in vitro system, we propose that resistance to rECM-mediated growth arrest and polarity may promote resistance to apoptosis.

We investigated which component of rECM may be proapoptotic. Early passage $p53^-$ HMEC-E6 cells grown in collagen did not form acinar structures and did not undergo apoptosis (Fig. 11). Based on these observations, we speculate that the presence of collagen I/IV in a 1:1 ratio in our model system is not sufficient to induce apoptosis nor promote an acinar structure. When laminin was added to collagen I/IV gels, early passage $p53^-$ HMEC-E6 cells formed organized branched tubular structures that terminated in spherical cell clusters and underwent apoptosis (Fig. 11). Early passage $p53^-$ HMEC-E6 cells cultured in laminin suspension, in the absence of a plastic substratum for adhesion, formed similar three dimensional structures and likewise underwent apoptosis. This suggests that contact with laminin is critical for the induction of apoptosis in early passage $p53^-$ HMEC-E6 cells.

Integrin $\alpha3\beta1$ is a critical mediator of intracellular adhesion and an important receptor for laminin-5 (Xia et al., 1996; Kawano et al., 2001). Recently, $\alpha3/\beta1$-integrin has been shown to play a potential role in the initiation apoptosis in T cells (Sato et al., 1999). Resistance to rECM-mediated apoptosis in late passage $p53^-$ HMEC-E6 cells was associated with altered expression of $\alpha3$-integrin (Figs. 12 and 13). Redistribution of $\alpha3$-integrin has been seen previously by other investigators in association with loss of mammary epithelial cell polarity in rECM culture and is consistent with our findings (Weaver et al., 1997). Treatment of early passage $p53^-$ HMEC-E6 cells with $\alpha3$- and $\beta1$-integrin function-altering Abs blocked rECM-mediated growth arrest and inhibited the induction of apoptosis (Figs. 14 and 15). Taken together, these observations suggest an important role for $\alpha3/\beta1$ signaling in rECM-mediated growth regulation and apoptosis.

Previous investigators have tested the ability of $\alpha3$- and $\beta1$-integrin blocking Abs to mediate growth of breast cell lines in rECM and in collagen and fibrin gels (Howlett et al., 1995; Alford et al., 1998). HMT-3522, a nontumorigeneic breast cell line, demonstrated decreased proliferation in rECM culture when treated with either the inhibitory anti–$\alpha3$-integrin Ab P1B5 or anti–$\beta1$-integrin Ab AIIB2 (Howlett et al., 1995). In contrast, we observed dysregulated proliferation when we treated HMECs with either the inhibitory anti–$\alpha3$-integrin Ab P1B5 or anti–$\beta1$-integrin Ab JB1A. The induction of stimulatory or inhibitory functions by Abs directed to defined integrin subunits has been observed previously for both anti–$\beta1$- and anti–$\alpha3$-integrin Abs (Lenter et al., 1993; Driessens et al., 1995; Lichtner et al., 1998) and is felt to be highly cell type specific. We hypothesize that utilization of nonimmortalized cells with low levels of $\alpha6\beta4$-integrin expression may account for differences between our results and those obtained by Howlett et al. (1995).

In conclusion, we have shown that whereas $p53^+$ HMEC-LXSN cells undergo growth arrest and form polarized epithelium when grown in contact with rECM, $p53^-$ HMEC-E6 and $p53^-$ HMEC-AS cells that have acutely lost p53 function undergo apoptosis. Resistance to rECM-mediated growth arrest and polarity results in resistance to rECM-mediated apoptosis and is associated with altered expression of $\alpha3$-integrin. Treatment of apoptosis-sensitive early passage $p53^-$ HMEC-E6 cells with either $\alpha3$- or $\beta1$-integrin function-altering Abs results in loss of rECM-mediated growth arrest and resistance to rECM-mediated apoptosis. We suggest that sensitivity and resistance to rECM-mediated apoptosis in $p53^-$ HMECs is dependent on the ability to form a polarized epithelium and may require $\alpha3/\beta1$-integrin signaling.

Materials and methods

Cell culture and media

HMEC strains AG11132 and AG11134 (M. Stampfer, Lawrence Berkeley National Laboratory, Berkeley, CA) were purchased from the National In-

stitute of Aging, Cell Culture Repository (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 ~20–25 passages. AG11132 and AG11134 cells exhibit a low level of estrogen receptor staining characteristic of normal mammary cells. AG11132 and AG11134 were at passage 8 at the time of receipt. HMECs were grown in standard medium: mammary epithelial cell basal medium (Clonetics) supplemented with 4 mg/ml bovine pituitary extract (CC4009; Clonetics), 5 mg/ml insulin (Upstate Biotechnology), 10 ng/ml EGF (Upstate Biotechnology), 0.5 mg/ml hydrocortisone (Sigma-Aldrich), 10^{-5} M isoproterenol (Sigma-Aldrich), and 10 mM Hepes buffer (Sigma-Aldrich). G418 (GIBCO BRL) containing standard medium was prepared by the addition of 300 mg/ml of G418 to standard medium. Cells were cultured at 37°C in a humidified incubator with 5% CO2/95% air. Mycoplasma testing was performed as reported previously (Seewaldt et al., 1997a).

Retroviral transduction

The LXSN16E6 retroviral vector containing the HPV-16 E6 coding sequence (provided by D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) has been described previously (Halbert et al., 1991; Demers et al., 1994). HMECs (passage 9) were plated in four T-75 tissue culture flasks (Corning) 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 of 1:1 in the presence of 4 μg/ml polybrene (Sigma-Aldrich) to log-phase cells grown in T-75 flasks. The two remaining T-75 flasks were not infected with virus. After 48 h, the two flasks containing transduced cells and one flask with untransduced cells were passaged 1:3 (passage 10) and selected with standard medium containing 300 μg/ml G418. Cells were grown in G418 containing standard medium for 1 wk until 100% of control untransduced cells were dead. The transduction efficiency was high during selection, cells were passaged 1:3 at the completion of selection (passage 11), and cells 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 cells expressing the HPV-16E6 construct were designated $p53^-$ HMEC-E6, and vector control clones were designated $p53^+$ HMEC-LXSN. All cells were maintained in standard medium after transfection in the absence of G418 selection to ensure that any chromosomal abnormalities or apoptosis resistance observed was not due to continued exposure to G418. All experiments were performed on mass cultures.

p53 oligonucleotides

The p53 AS oligonucleotide is an 18-mer targeting the region of the initiation codon (six base pairs immediately before the first and the first four coding codons): 5′-CGGCTCCTCCATGGCAGT-3′. This AS ODN has been used previously by several investigators to suppress p53 function (Bi et al., 1994; Capoulade et al., 2001). The p53 control ODN (5′-CGGCTCCTCATGGCAGT-3′) was chosen to be a scrambled sequence of the AS ODN to ensure identical nucleotide content and minimize differences potentially attributable to nucleic acid content (Capoulade et al., 2001). In all ODNs, the first and the last three nucleotides were phosphorothiolate modified to increase stability in vitro.

Early passage $p53^+$ HMEC-P parental cells were plated in T-75 plates in standard medium. After allowing 24 h for attachment, cell cultures were treated for 72 h with either 0.1 μM active or scrAS p53 ODNs. The culture medium was replaced by new standard medium containing fresh ODNs every 24 h. Western blot analysis was performed to confirm suppression of p53 expression as described below. The resulting film images were digitized and quantitated using Eastman Kodak, Co. 1D image analysis software.

rECM culture was as follows: cells were trypsinized, and ~10^4 cells were resuspended in 100 μl rECM containing 0.1 μM of either active or p53 scrAS ODN on ice. rECM cultures were prepared as described below. rECM cultures were overlayed with standard medium containing 0.1 μM of either active or scrambled p53 ODNs. Overlay medium was changed every 24 h to ensure a fresh supply of ODNs. The diameter of the growing colonies was determined, and cell colonies were prepared for EM as described below. To measure p53 protein expression in cells grown in rECM culture, colonies were released from the matrix by 60-min incubation at 37°C with dispase (5,000 U/ml caseinolytic activity; Collaborative Research). Released cells were washed once using ice-cold PBS with 5 mM EDTA and twice with PBS alone. The resulting pellet was tested for p53 protein expression by Western blot analysis as described below.

Western blotting

Preparation of cellular lysates and immunoblotting were performed as previously described (Seewaldt et al., 1995, 1997b). p53 expression was detected using a 1:100 dilution of mouse anti-human p53 Ab-2 (Oncogene Research Products) and detected by ECL Western blotting detection reagents (Amersham Pharmacia Biotech) as described by the manufacturer.

Northern blot analysis

RNA was extracted with guanidium isothiocyanate and subjected to Northern blotting in formaldehyde denaturing gels as described previously (Seewaldt et al., 1995). 10 mg of RNA were loaded per lane. Molecular probes used in the Northern blot analysis were as follows: the human p53 probe was a 1.9-kb BamH1 fragment (Seewaldt et al., 1997b), and the 36B4 probe was a 700-bp PstI fragment that was used as a loading and transfer control probe (Seewaldt et al., 1995).

HMEC culture in reconstituted ECM

Mammary epithelial cells were grown in rECM by methods developed by Bissell and others (Folkman and Moscona, 1978; Howlett et al., 1994; Roskelley et al., 1994; Seewaldt et al., 1997b). 100 μl of rECM (Matrigel™; Collaborative Research) or growth factor–depleted rECM (growth factor–reduced Matrigel™; Collaborative Research) were added per well to a 48-well plate and allowed to gel at 37°C for 20 min. $p53^-$ HMEC-E6–transduced cells and $p53^+$ HMEC-LXSN vector controls were trypsinized, counted, and pelleted in a sterile microcentrifuge tube. Approximately 10^4 cells were resuspended in 100 μl rECM on ice, gently overlaid on the initial undercoating of ECM, and allowed to gel at 37°C for 20 min. Standard medium was then added, and wells were inspected to ensure an equal distribution of cells in each well. Cells were grown for 5–14 d in culture. Medium was changed daily.

For integrin-blocking experiments, ~10^4 $p53^+$ HMEC-LXSN vector control cells (passage 11) or $p53^-$ HMEC-E6 cells (passage 10) were pelleted and resuspended in 100 μl standard medium containing either Abs to α3- and β1-integrins (Chemicon International) or control nonimmune mouse IgG for 15 min at room temperature (RT). Final concentration of α3-integrin blocking Ab (CDW496, clone P1B5) was 10 μg/ml, β1-integrin blocking Ab (CD29, clone JB1A) was 20 μg/ml, and β1-integrin stimulatory Ab (CD29, clone B3B11) was 10 μg/ml. 100 μl rECM was added to the cell suspension, gently mixed, and overlaid as described above. 1 ml standard medium containing the above respective concentration of blocking Ab was added to each well and changed every other day. Cells were grown for 5–9 d in rECM culture.

Collagen/laminin morphogenesis assays

Collagen and collagen/laminin gels were prepared by a modification of methods developed by Alford et al. (1998). Collagen type I (Sigma-Aldrich) and human placental collagen type IV (Sigma-Aldrich) were solubilized in 0.018 N acetic acid for a final concentration of 3 mg/ml each. Three parts collagen type I were mixed with one part collagen type IV. The collagen I/IV solution was neutralized by mixing 8 vol of collagen solution with 1 vol of sterile PBS and 1 vol of sterile 0.1 M NaOH for a final pH of 7.4. 100 μl of neutralized collagen I/IV solution were added per well to a 48-well plate and allowed to gel at 37°C for 20 min. Approximately 10^4 early passage $p53^-$ HMEC-E6 cells and $p53^+$ HMEC-LXSN controls were resuspended in 100 μl neutralized collagen I/IV solution on ice, gently overlaid on the initial undercoating of collagen, and allowed to gel at 37°C for 20 min. Standard medium was then added, and wells were inspected to ensure an equal distribution of single cells suspended in each well. Cells were grown for 5–9 d in culture and then prepared for EM as described previously (Seewaldt et al., 1997b). For collagen/laminin gels, nine parts collagen I/IV were added to one part human placental laminin (Sigma-Aldrich), and gels were prepared as above. Laminin cultures were prepared as follows: 48-well plates were coated with 100 μl neutralized collagen I/IV solution and baked at 65°C for 24 h. This was repeated three times. 100 μl of human placental laminin were added per well and baked at 50°C until the laminin solution hardened. This was repeated three times. Approximately 10^4 early passage $p53^-$ HMEC-E6 cells were suspended in a 1:1 mixture of standard medium and human placental laminin. Cells were grown for 7 d and prepared for EM as previously described (Seewaldt et al., 1997b).

Transmission EM

$p53^-$ HMEC-E6 cells and $p53^+$ HMEC-LXSN vector control cells were grown in contact with rECM as described above, and EM was preformed as described previously (Seewaldt et al., 1997b).

Cell growth determination in rECM culture

Cell growth was determined by the following criteria: the size of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. For both $p53^+$ HMEC-LXSN vector controls and $p53^-$ HMEC-E6–transduced cells, the 20 largest colonies were measured.

Detection of apoptosis by in situ TUNEL

$p53^-$ HMEC-E6 cells and $p53^+$ HMEC-LXSN vector control cells were grown in contact with rECM as described above for 5–9 d. Cells were then fixed in PBS with 10% formalin and embedded in paraffin. Sections were deparaffinized and quenched in methanol containing 2.1% hydrogen peroxide. Antigen retrieval was achieved by placing slides in 10 mM citric acid at 95°C for 10 min. Nuclear proteins were stripped with 20 μg/ml proteinase K, and slides were washed in deionized water. Positive controls were immersed in DN buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 4 mM magnesium chloride, 0.1 mM DTT) for 5 min at RT and then incubated with DNAseI (Roche) in DN buffer for 10 min at RT. Negative controls were treated with 5% FBS. All samples were immersed in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1.0 mM cobalt chloride) for 5 min at RT. Sections were covered with TdT/Bio-14-dATP solution (800 μl TdT buffer containing 120 U terminal transferase and 50 nM Bio-14-dATP [GIBCO BRL]), incubated for 1 h at RT, and then the reaction was terminated with PBS. Sections were blocked with 2% BSA for 10 min at RT and treated with ABC solution (Elite). Sections were allowed to complex on ice for 30 min, incubated for 30 min at 37°C, and were washed in PBS. Sections were stained with DAB (2% nickel chloride, 0.1% hydrogen peroxide) for 3 min at RT and counterstained.

E-cadherin immunostaining

Early and late passage $p53^-$ HMEC-E6 cells and $p53^+$ HMEC-LXSN vector control cells were grown in rECM as described for 6 d and embedded in OCT (Miles). Cells were snap frozen, and 5-μm sections were obtained. Sections were fixed in for 30 min at RT with 3.7% formaldehyde in PBS and were blocked with 0.5% heat-denatured BSA (HD-BSA) in PBS for 1 h at RT. Cells were then incubated for 30 min with mouse anti–human E-cadherin Ab (BD Signal Transduction Laboratories), diluted in PBS with 0.5% HD-BSA for 30 min at RT, and then washed six times with PBS at RT. For Immunofluorescence, cells were incubated with FITC-conjugated goat anti–mouse Ab at a 1:200 Ab dilution (Santa Cruz Biotechnology, Inc.) 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.

Immunodetection of integrin expression

Early and late passage $p53^-$ HMEC-E6 cells and $p53^+$ HMEC-LXSN vector control cells were grown on glass coverslips for 48 h in standard medium. Cells were fixed in 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 h at RT. Cells were then incubated with a primary Ab diluted in PBS with 0.5% HD-BSA for 1 h at RT and washed six times with PBS at RT. Abs against integrin subunits α2 (P1H5), α3 (P1F2, P1B5), and β1 (P4C10) were a gift from William Carter (Fred Hutchinson Cancer Research Center) and have been described previously (Wayner and Carter, 1987; Wayner et al., 1988; Carter et al., 1990a,b). Abs against integrin α6 (GoH3) and β4 (3E1) were obtained from Chemicon International. For Immunofluorescence, cells were incubated with either FITC-conjugated goat anti–mouse Ab at a 1:1,500 dilution or goat anti–rat Ab at a 1:4,000 dilution (Dako) in PBS with 0.5% HD-BSA for 1 h at RT and washed. Sections were mounted in a solution containing 25 mg/ml of 1,4-diazobicyclo-(2,2,2)octane in glycerol and visualized for immunofluorescence using a ZEISS LSM 410 fluorescence microscope.

For rECM culture, early and late passage $p53^-$ HMEC-E6 cells and $p53^+$ HMEC-LXSN vector control cells were grown in contact with rECM for 6 d in standard medium. Cells were embedded in OCT, snap frozen, and sectioned as described above. Sections were fixed in for 20 min at RT with 2% formaldehyde in 0.1 M sodium cacodylate, 0.1 M sucrose, pH 7.2, and blocked with 0.5% HD-BSA in PBS for 1 h at RT. Sections were stained with Abs against integrin subunits α3 (P1F2) and β1 (P4C10) and visualized as described above.

Cytogenetic analysis of early passage transduced and parental HMECs

Cultures of $p53^+$ HMEC-P parental cells (passage 10), $p53^+$ HMEC-LXSN vector controls (passage 10), $p53^-$ HMEC-E6 cells (passage 10), and $p53^-$ HMEC-E6 cells (passage 18) were checked for sufficient numbers of dividing cells and exposed to colcemid (GIBCO BRL) at a final concentration of 0.01–0.02 μg/ml for 2–3 h. Subsequently, the cells were released from flasks by trypsinization, exposed to hypotonic solution, and fixed as described previously (Mrózek et al., 1993). Chromosome preparations were made, and after appropriate aging slides were subjected to SKY, a method that enables simultaneous display of all human chromosomes in different colors (Schröck et al., 1996). Additional slides were also stained with DAPI (Vector Laboratories) alone. For SKY, the slides were hybridized with the SKY probe mixture containing combinatorially labeled painting probes for each of the autosomes and sex chromosomes (Applied Spectral Imaging) for 42–45 h at 37°C. The hybridization and detection procedures were performed according to the manufacturer's protocol (Applied Spectral Imaging), and chromosomes were counterstained with DAPI in antifade solution. The multicolor hybridizations were visualized with the SpectraCube SD 200 system (Applied Spectral Imaging) mounted on the ZEISS Axioplan 2 epifluorescence microscope using a custom-designed optical filter (SKY-1; Chroma Technology). The DAPI images of all metaphase cells were acquired using a DAPI-specific optical filter. Spectral analysis and classification were performed using SkyView 1.2r visualization and analysis software (Applied Spectral Imaging). The assignment of breakpoints in structural abnormalities was made on comparison of images of SKY classified chromosomes with the images of the same chromosomes stained with DAPI that were inverted electronically and contrast enhanced by SkyView 1.2r software. Karyotypic abnormalities were classified according to the recommendations of the International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

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Retinoids and Retinoic Acid Receptors Regulate Growth Arrest and Apoptosis in Human Mammary Epithelial Cells and Modulate Expression of CBP/p300

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KEY WORDS mammary epithelial cells; retinoic acid receptor; extracellular matrix; CBP; p300

ABSTRACT Retinoids and retinoic acid receptors (RARs) are important mediators of normal epithelial cell homeostasis. To assess the role of retinoids and RARs in regulating growth arrest and apoptosis in benign and malignant mammary epithelial cells, two model systems were developed: 1) RAR function was suppressed in retinoid-sensitive normal human mammary epithelial cells (HMECs) by the dominant-negative retinoic acid receptor, RARα403 (DNRAR), and 2) retinoid-resistant MCF-7 breast cancer cells were transduced with a functional RARβ2. Inhibition of RAR function by the DNRAR in HMECs resulted in retinoid-resistance, increased proliferation, and dysregulated growth when cells were cultured in reconstituted extracellular matrix (rECM). Expression of RARβ2 in MCF-7 cells resulted in sensitivity to retinoid-induced growth arrest and apoptosis. The CREB-binding protein (CBP) and the homologous protein p300 are tightly regulated, rate-limiting integrators of diverse signaling pathways and are recruited during retinoid-mediated transcriptional activation. The relationship between retinoid receptor expression, growth regulation, and transcriptional regulation of CBP/p300 is poorly understood. Inhibition of RAR function in HMECs by DNRAR suppressed expression of CBP/p300 and expression of RARβ2 in MCF-7 cells promoted induction of CBP/p300 when cells were treated with 1.0 μM all-*trans*-retinoic acid (ATRA). These results suggest that ATRA and RARs regulate growth arrest of HMECs and modulate CBP/p300 protein expression. Since CBP and p300 are normally present in limiting amounts, their regulation by ATRA and RARs may be an important element in the control of transcriptional activation of genes regulating growth arrest and apoptosis. *Microsc. Res. Tech. 59: 23–40, 2002.*

INTRODUCTION

Vitamin A (retinol) and its derivatives (retinoids) are important for normal cellular growth and differentiation (Hong et al., 1997). The actions of retinoids are ultimately thought to be mediated through specific nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) belonging to the steroid/thyroid superfamily of transcription factors (Mangelsdorf et al., 1994). Multiple retinoic acid receptors have been identified; among these are RARα, β, and γ. RARα is expressed ubiquitously in adult tissue and RARγ is primarily expressed in skin. RARβ is unique because it is primarily expressed in epithelial cells and exhibits induced expression in response to retinoic acid (ATRA) that is mediated by an enhancer element, the retinoic acid response element (RARE), within its promoter (DeThe et al., 1990; Hoffmann et al., 1990). While normal cultured human mammary epithelial cells (HMECs) express RARβ mRNA, most breast cancer cell lines fail to express this gene (Swisshelm et al., 1994; Seewaldt et al., 1995, 1997a). Furthermore, RARβ mRNA expression is upregulated in senescent HMECs, suggesting that this gene may be involved in regulating terminal differentiation in mammary epithelial tissue (Swisshelm et al., 1994).

Retinoids have also been found to be important for the prevention of certain cancers. Retinoids can halt the progression of disease in premalignant lesions of the oral cavity, cervix, and skin and are effective in preventing the development of second primary tumors of the aerodigestive tract and lung (Lippmann et al., 1993; Volkes et al., 1993). There is also evidence that retinoids may be important for the prevention of breast cancer. The risk of breast cancer is increased for

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women with a lower dietary intake of vitamin A and beta carotene but not for women with dietary deficiencies of vitamin C or E (Hunter et al., 1993).

The potential association between vitamin A deficiency and the development of cancer suggests that retinoid-dependent signaling pathways have a role in the suppression of carcinogenesis. Selective loss of expression of RARβ has been observed in ductal carcinoma in situ and in a majority of breast cancers, suggesting that loss of RARβ function may be an important early event in breast cancer development (Widschwendter et al., 1997; Xu et al., 1997). Furthermore, loss of heterozygosity at the chromosomal region 3p22-25 containing the coding sequence for the RARβ is the most frequent chromosomal aberration observed in morphologically normal lobules adjacent to breast cancer (Deng et al., 1996). This suggests that in a subset of sporadic breast cancer a tumor suppressor gene at 3p22-25 may be important in initiation of early progression of mammary tumorigenesis (Deng et al., 1996). Recently, an alternative mechanism for the development of retinoid-resistance has been observed in breast cancer cells (Sommer et al., 1999). RARβ4 is an alternatively spliced product of the RARβ P2 promoter that may function as a naturally occurring dominant-negative repressor of RAR-mediated growth suppression (Nagpal et al., 1992) and promote loss of mammary epithelial polarity (Berard et al., 1994). Five-fold increased expression of RARβ4 has been detected in breast cancer cell lines relative to HMECs (Sommer et al., 1999). These observations suggest that there may be multiple mechanisms to account for the development of retinoid-resistance in mammary epithelial cells.

One group of transcriptional cofactors that may be recruited by RARs is the CREB-binding protein (CBP)/p300 family which plays a critical role in integrating multiple signal transduction pathways by the induction of selective gene transcription (Yao et al., 1998; Robyr et al., 2000). CBP and p300 have been shown to be required for growth arrest and apoptosis (Vo et al., 2001). Retinoids are important mediators of growth arrest in epithelial cells and CBP and p300 are transcriptional coactivators of the RAR family of nuclear receptors (Kawasaki et al., 1998; Yao et al., 1998). This raises the possibility that the regulation of gene expression by RARs could be in part mediated by the availability of CBP and p300. In the work presented here, we examine the role of retinoids and retinoic acid receptors in mammary epithelial cell growth regulation, apoptosis, and the regulation of CBP/p300 expression.

Abbreviations

ATRA	all-*trans*-retinoic acid
RAR	retinoic acid receptor
RARβ2	retinoic acid receptor-beta isoform-2
RARE	retinoic acid response element
HMEC	normal human mammary epithelial cell
DNRAR	dominant-negative retinoic acid receptor-α403
ECM	extracellular matrix
rECM	reconstituted extracellular matrix
CBP	CREB-binding protein
Cdk	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
TdT	terminal deoxynucleotidyl transferase
pRB	retinoblastoma protein
CAT	chloramphenicol acetyl transferase

ALL-*TRANS*-RETINOIC ACID AND RETINOIC ACID RECEPTORS MEDIATE GROWTH ARREST IN NORMAL AND MALIGNANT MAMMARY EPITHELIAL CELLS

Retinoids play a critical role in epithelial cell homeostasis and the loss of retinoid receptor function is felt to be an important event in mammary carcinogenesis. We developed two experimental systems to test the hypothesis that retinoids and RARs are critical mediators of growth arrest and apoptosis in mammary epithelial cells. In the first experimental system, a dominant-negative approach was utilized to suppress retinoic acid receptor function in HMECs. In the second system, a functional RAR-β2 was expressed in breast cancer cell lines that fail to express RAR-β2 and are resistant to the growth inhibitory effects of all-*trans*-retinoic acid (ATRA).

ATRA Mediates Growth Arrest in HMECs

HMECs express RAR-α, RAR-β2, and RAR-γ and are sensitive to the growth inhibitory effects of ATRA (Swisshelm et al., 1994; Seewaldt et al., 1995, 1997a). Growth inhibition is independent of p53 expression (Seewaldt et al., 1999b). We and others previously identified that ATRA regulates cyclin D1 levels by post-translational proteolysis (Langenfield et al., 1997; Seewaldt et al., 1997b) and that retroviral-mediated overexpression of cyclin D1 inhibits ATRA-mediated growth arrest and differentiation in HMECs (Seewaldt et al., 1999a). The cyclin D1 promoter does not contain either a retinoic acid response element (RARE) or an AP-1 site. It is felt that the primary target of ATRA during the induction of growth arrest is the regulation of ubiquitin-mediated proteolysis of cyclin D1 through recognition of the cyclin D1 C-terminal PEST sequence (Langenfield et al., 1997; Seewaldt et al., 1999a). Cyclin D1/cdk4 complexes are thought to play a major role in phosphorylating the retinoblastoma protein (pRB) during G_1-phase (Kato et al., 1993). Recent studies suggest that pRB is the downstream target of cyclin D1, as suppression of hyperphosphorylated pRB (ppRB) inhibits ATRA-mediated growth arrest (Dietze et al., 2000). These observations suggest that ATRA mediates growth inhibition in HMECs by a cyclin D1/pRB-mediated pathway.

Suppression of RAR Function in HMECs

A truncated RAR exhibiting dominant-negative activity was utilized to inhibit RAR function in HMECs to test whether inhibition of RAR function results in loss of ATRA-mediated growth inhibition. Dominant-negative retinoic acid receptors have been used to unravel the physiologic function of retinoids in several cell types (Tsai et al., 1992, 1993, 1994; Anderson et al., 1995; Imakado et al., 1995; Saitou et al., 1995). For this study we utilized a dominant-negative mutant of RARα (RARα403) driven by a retroviral LTR (DNRAR) (Tsai et al., 1993). This RARα derivative is a negative transcriptional regulator that can simultaneously block all wild-type RAR isoforms (−α, −β, and −γ) (Damm et al., 1993). This truncated mutant RAR retains the abil-

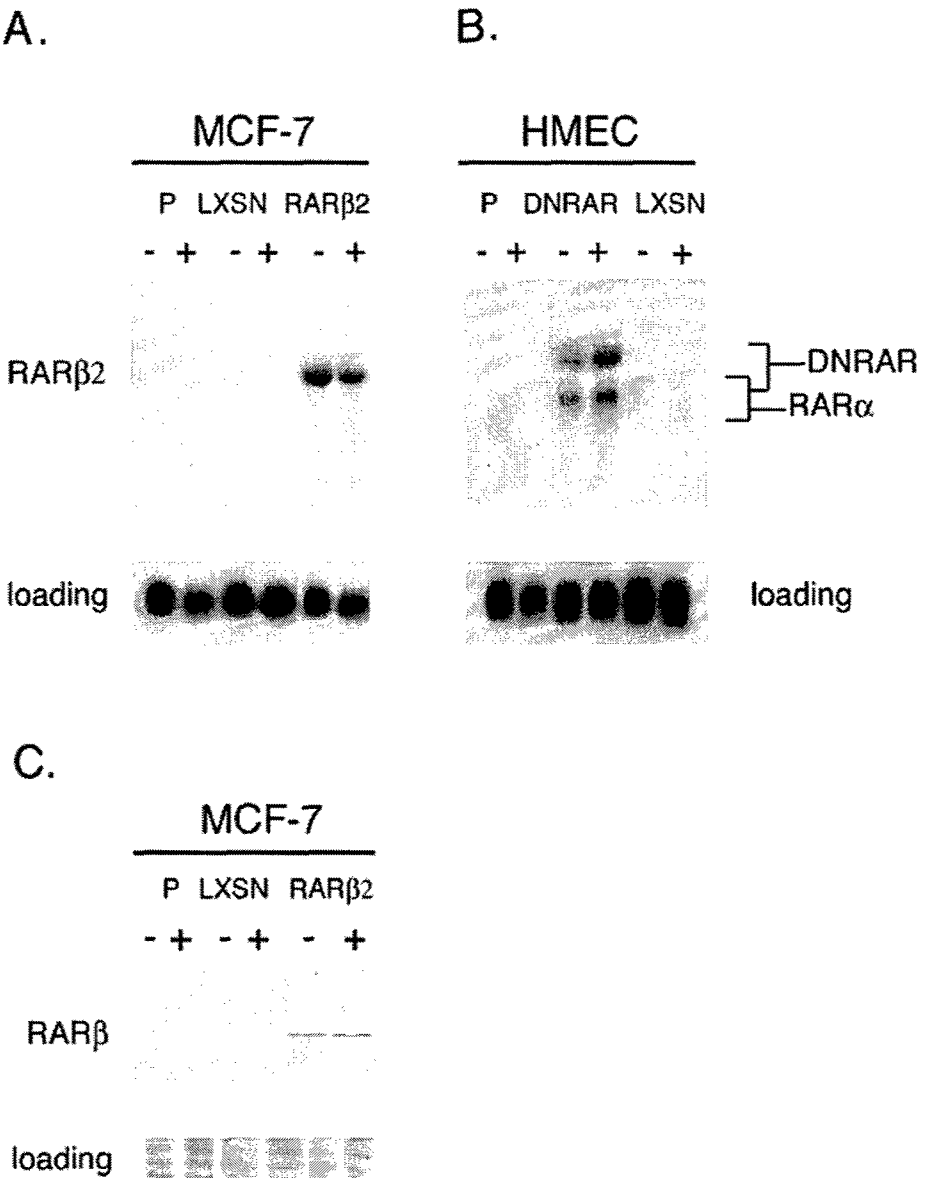
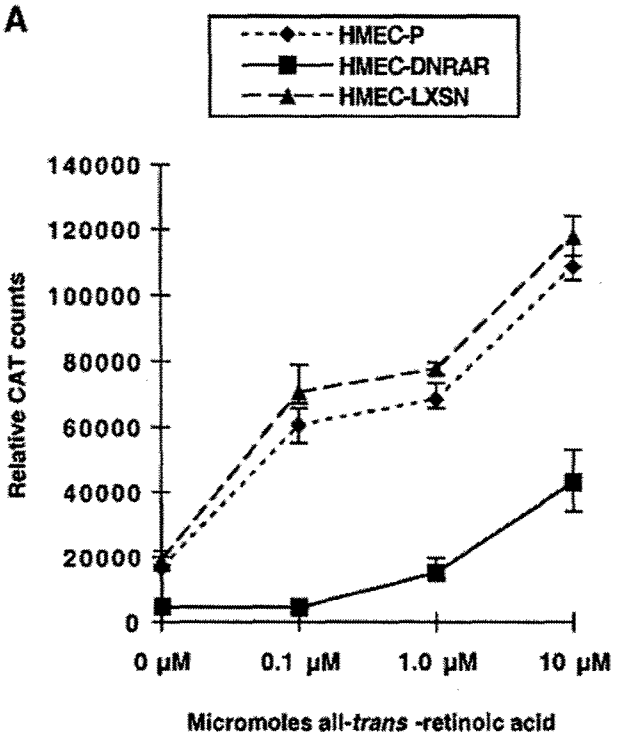
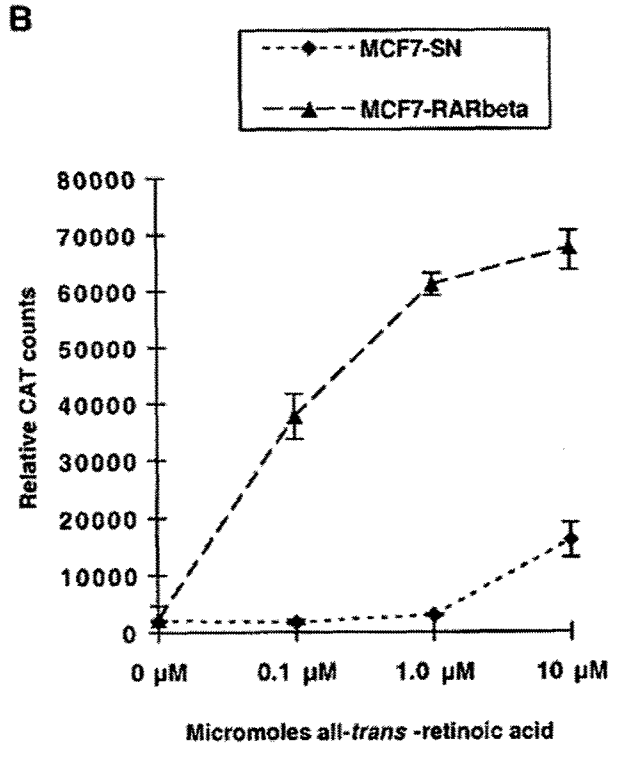


Fig. 1. Expression of transduced constructs in normal and malignant mammary epithelial cells. **A:** Expression of exogenous RARβ2 mRNA in MCF-7 breast cancer cells. Northern analysis of MCF7-P parental cells (P), MCF7-RARβ2 transduced breast cancer cell lines (RARβ2), and vector controls (LXSN) treated for 48 hours without (−) or with (+) 1.0 μM ATRA. Ten μg of total RNA were loaded per lane. 36B4 served as a loading control. **B:** Expression of the RARα403 DNRAR construct in HMECs. Northern analysis was performed on RNA extracted from HMEC-P parental cells (P), from HMEC-DNRAR cells infected with LRARα403SN retroviral vector (DNRAR), and from HMEC-LXSN vector control cells (LXSN). Cells were treated without (−) or with (+) 1.0 μM ATRA for 48 hours. The blot was probed with a 1.3 kb (SmaI) RARα cDNA fragment. Ten μg of total RNA were loaded per lane. 36B4 serves as a loading control. **C:** RARβ protein expression in transduced MCF-7 breast cancer cells. Western blot of MCF7-RARβ2 cells (RARβ2), MCF7-P parental cells (P), and MCF7-LXSN vector controls (LXSN) treated without (−) or with (+) 1.0 μM ATRA for 48 hours. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. One hundred μg of protein lysate were loaded per lane. The blot was probed with an RARβ-specific polyclonal antiserum (Seewaldt et al., 1995). Unidentified 45 kd and 65 kd protein bands were used as a loading control.

ity to dimerize with RXR and bind retinoic acid response elements, suggesting that the DNRAR acts by forming transcriptionally inactive heterodimers that compete for DNA binding with the natural RAR-RXR heterodimers (Damm et al., 1993). Control cells were infected by the control LXSN retroviral vector (without insert).

Expression of the transduced RARα403 DNRAR construct was confirmed by Northern analysis of total cellular RNA extracted from both HMEC-LXSN vector controls and HMEC-DNRAR cell lines utilizing an RARα probe. As expected, HMEC-LXSN demonstrated expression of endogenous RARα mRNA (Fig. 1B). Expression of the transduced DNRAR insert was observed





in HMEC-DNRAR cells at >10-fold higher than the endogenous RARα (Fig. 1B). As previously observed, both a 4.8- and a 3.0-kb DNRAR transcript were observed (Tsai et al., 1993).

Expression of RARβ2 in Breast Cancer Cells

To test the role of RARβ2 in mediating breast cancer cell growth arrest we constructed an LXSN-based retroviral vector harboring the coding sequence of the human RARβ2 gene. This vector includes a 94 bp sequence of 5′ untranslated adenosine deaminase sequence to place the cDNA in an optimal translational context (Seewaldt et al., 1995), as well as the neomycin phosphotransferase selectable marker. This RARβ retroviral construct is designated LaRARβ2SN. The breast cancer cell line MCF-7 does not express RARβ2 mRNA and is relatively resistant to the growth inhibitory effects of ATRA at concentrations less than 5 μM (Swisshelm et al., 1994). MCF-7 cells were infected with the LaRARβ2SN retroviral vector and G418 resistant cells were isolated. These transductants were designated MCF7-RARβ2. Vector control cells were obtained by infecting MCF-7 with the retroviral vector LXSN without insert and were designated MCF7-LXSN. Experiments depicted in the following figures were performed on MCF7-RARβ2-clone-9 and MCF7-LXSN-clone-2 and are representative of experiments performed in 5 RARβ2- and 3 LXSN-transduced MCF7 clones. Northern and Western blots were performed on viral-transduced MCF-7 cells to determine the levels of RARβ2 mRNA and protein expression, respectively. The LTR-initiated 4.8-kb RARβ2 mRNA was observed in MCF7-RARβ2 cells but not in MCF7-P parental cells nor in MCF7-LXSN controls (Fig. 1A). Endogenous RARβ2 mRNA was not expressed by MCF7-LXSN or MCF7-P parental cells (Fig. 1A), nor was it reactivated by the expression of the exogenous RARβ2. RARβ protein expression was determined by Western blotting using an RARβ-specific polyclonal antiserum. The expected single 53-kd band was detected in cellular lysate from MCF7-RARβ2 transduced clones but not in the lysate of MCF7-LXSN controls or MCF7-P cells (Fig. 1C).

Modulation of RAR Function in Benign and Malignant Mammary Epithelial Cells Alters ATRA-Induced RARE Activity

DNRAR, RARα403, blocks ATRA-stimulated transcription both by binding ATRA and competing directly

Fig. 2. Modulation of RAR function and expression alters ATRA-induced RARE activity. **A:** DNRAR construct LRARα403SN inhibited ATRA-mediated *trans*-activation of βRARE in HMEC-DNRAR cells (passage 12–14) relative to HMEC-P parental cells (passage 12–14) and HMEC-LXSN vector controls (passage 12–14). **B:** Expression of RARβ2 in retinoid-resistant MCF-7 cells increased ATRA-mediated *trans*-activation relative to MCF7-P parental cells and HMEC-LXSN vector controls. Cells were transfected with pRRE4-tkCAT plasmid, treated with 0, 0.1, 1.0, or 10 μM ATRA for 24 hours and cell lysates were assayed for CAT reporter activity. pCMV-GH was used as a transfection efficiency control. CAT counts were corrected for growth hormone activity and total protein (Seewaldt et al., 1997a). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. Data represent an average of three independent transfections performed in duplicate.

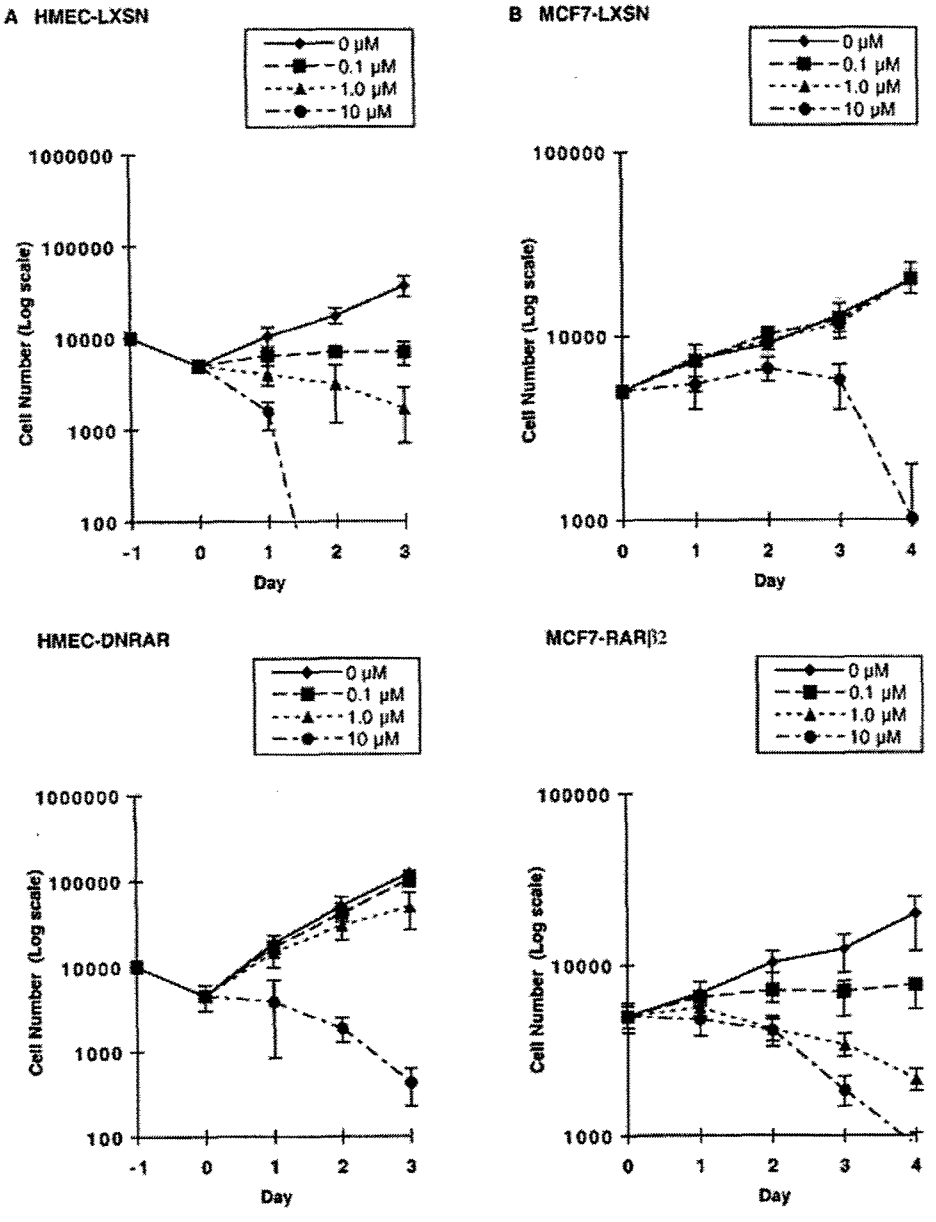


Fig. 3. ATRA and RARs regulate growth arrest in normal and malignant mammary epithelial cells. **A:** Suppression of RAR function by a DNRAR blocked ATRA-mediated growth inhibition of HMECs. Growth curves of HMEC-LXSN vector controls (passage 12) and HMEC-DNRAR cells (passage 12). **B:** Expression of RARβ2 in retinoid-resistant MCF-7 breast cancer cells resulted in sensitivity to ATRA. Growth curves of MCF7-LXSN vector controls and MCF7-RARβ2 transduced cells. Cells were plated on Day −1 in the appropriate standard media, in triplicate, at 1×10^4 cells per well in 12-well plates. Cells were re-fed on Day 0 with standard media containing 0, 0.1, 1.0, or 10 μM ATRA. Cells were trypsinized and counted in triplicate. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. These data are representative of three separate experiments.

TABLE 1. Percentage distribution of cells in G_1- and S-phase in ATRA-treated cells

Cell Type	ATRA (M)	Day 0	Day 1	Day 2	Day 3	Day 4
HMEC-LXSN	10^{-7}	60/20	60/21	63/15	65/12	67/11
	10^{-6}	60/20	60/19	65/11	73/03	79/02
HMEC-DNRAR	10^{-7}	55/30	53/31	54/30	57/30	58/29
	10^{-6}	55/30	56/30	57/29	63/25	69/22
MCF7-LXSN	10^{-6}	61/20	62/19	61/21	60/18	60/21
MCF7-RARβ2	10^{-6}	63/16	68/10	68/10	70/03	73/01

Adherent HMEC-LXSN vector controls, HMEC-DNRAR transduced cells, MCF7-LXSN vector controls, and MCF7-RARβ2 transduced cells were treated for 0 to 4 days with 0.1 μM or 1.0 μM ATRA. The percentage of cells in G_1- and S-phase of the cell cycle were determined by flow cytometry and recorded in tabular form (%G_1-/%S-phase). ATRA was dissolved in ethanol and Day 0 controls received an equivalent volume of solvent. These data are representative of three separate experiments.

for endogenous RARs to form inactive heterodimers that compete with active dimers for the RARE. DNRAR- and LXSN-transduced HMECs were not subcloned and all experiments were performed on mass culture. To confirm dominant-negative activity of the DNRAR construct in HMECs, loss of sensitivity to ATRA was tested by transient expression of chloramphenicol acetyl transferase (CAT) driven by a RARE

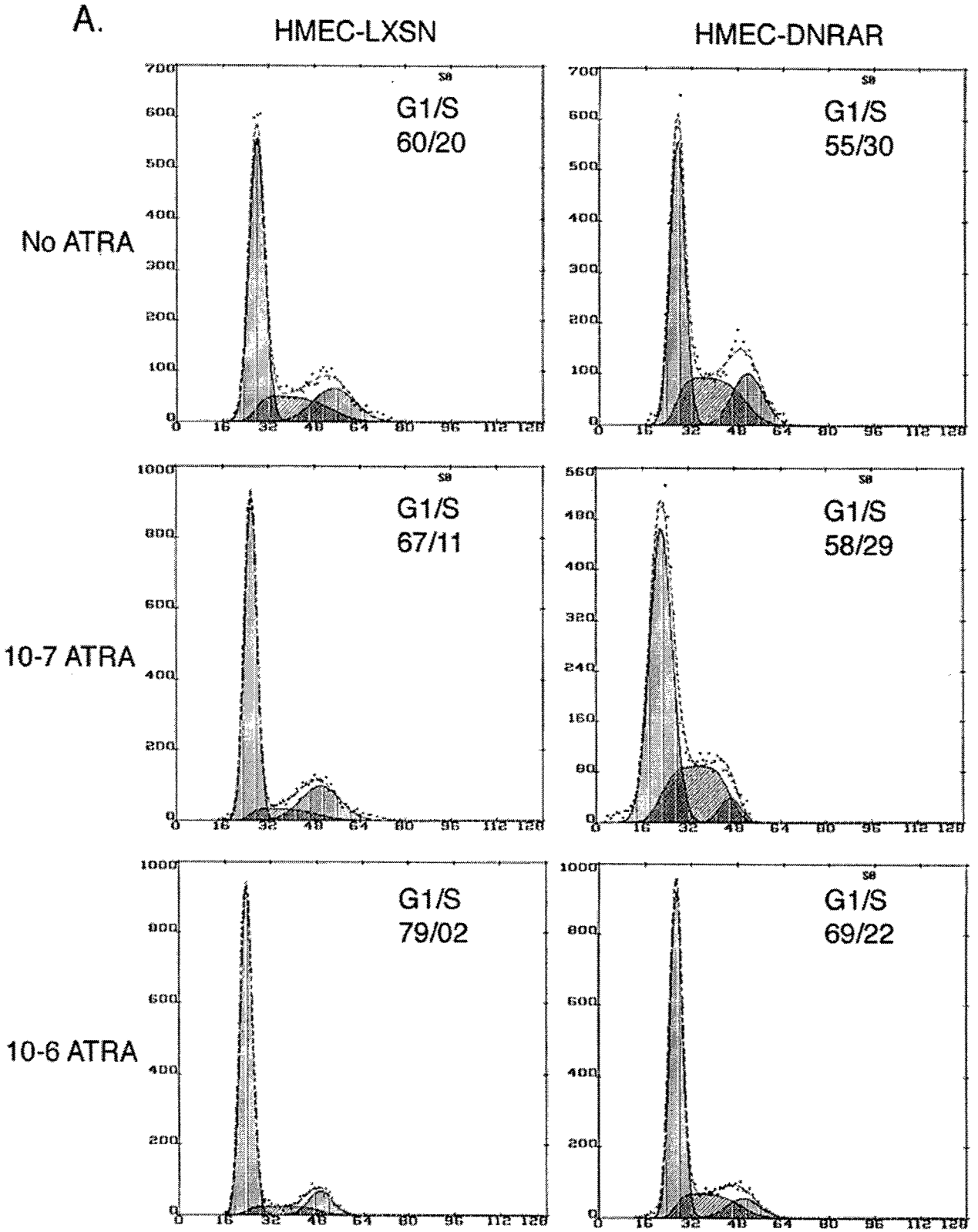


Fig. 4. Modulation of RAR function and expression alters the cell cycle distribution of normal and malignant mammary epithelial cells. **A:** Cell cycle analysis of HMEC-LXSN vector controls (passage 12) and HMEC-DNRAR cells (passage 12) treated with 0, 0.1, or 1.0 μM ATRA and (**B**) MCF7-LXSN vector controls and MCF7-RARβ2 cells treated with 0 or 1.0 μM ATRA for 4 days. Cells were trypsinized and harvested for cell cycle analysis on Day 4. The percentage of cells in G_1 and S-phase of the cell cycle was determined by flow cytometry. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. These data are representative of three separate experiments.

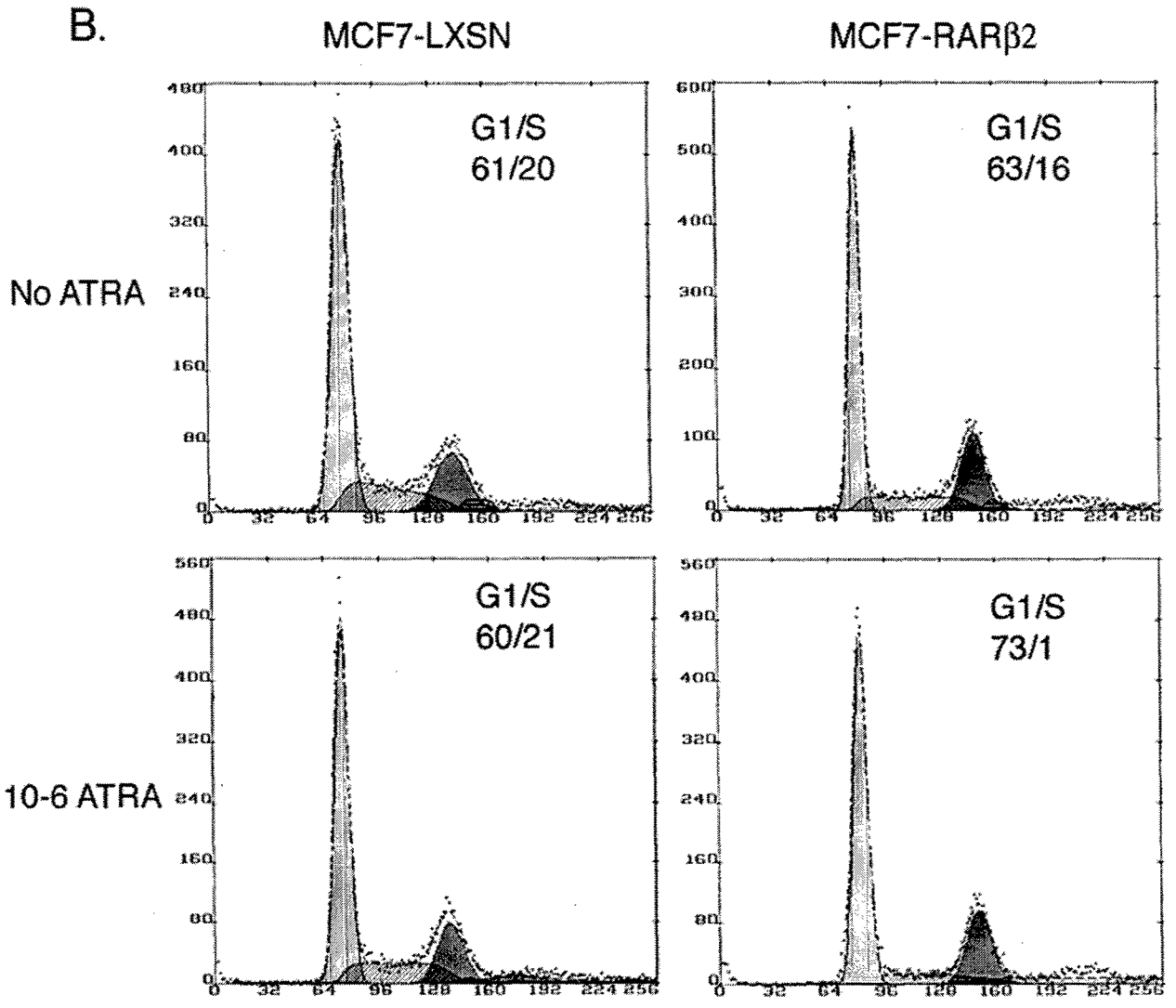


Figure 4. (Continued.)

sequence (Seewaldt et al., 1997b). HMEC-LXSN and HMEC-P parent cells treated with 0.1–10 μM ATRA exhibited a 3–6-fold increase in RARE-CAT activity relative to untreated cells (Fig. 2A). In contrast, HMEC-DNRAR cells were resistant to ATRA-mediated *trans*-activation of the RARE at concentrations of 0.1–1.0 μM ATRA and only showed partial induction at concentrations of 10 μM ATRA (Fig. 2A). This observed inhibition of ATRA-mediated *trans*-activation in the DNRAR expressing HMECs relative to controls demonstrates the dominant-negative activity of our construct in HMECs and is consistent with previous observations utilizing this construct (Tsai et al., 1993). At 10 μM ATRA there was only partial suppression of ATRA-mediated *trans*-activation, indicating that the dominant-negative activity of this construct could be overcome by increasing concentrations of ATRA. These data demonstrate the ability of the DNRAR to inhibit normal RAR function in HMECs.

The ability of ATRA to stimulate RARE-mediated transcription was tested in retinoid-resistant MCF7-LXSN vector controls and MCF7-RARβ2 transduced cells. A 15–30-fold increase in RARE activity was detected in MCF7-RARβ2 cells treated with 0.1–10 μM ATRA (Fig. 2B). In contrast, increased RARE activity was not detected in MCF7-LXSN controls treated with 0.1 and 1.0 μM ATRA, but an 8-fold increase was detected after treatment with 10 μM ATRA (Fig. 2B). These observations suggest that expression of RARβ2 in retinoid-resistant MCF-7 breast cancer cell lines increases the ability of ATRA to induce RARE-mediated transcription.

ATRA and RARs Regulate Proliferation of Mammary Epithelial Cells

Surprisingly, untreated HMEC-DNRAR cells (passage 10–15) exhibited a significantly decreased doubling time relative to untreated HMEC-LXSN vector

controls and HMEC-P parental cells at the same passage number (Fig. 3A). In cultures passaged in parallel, we observed that HMEC-DNRAR cells had a 12–18-hour doubling time while HMEC-LXSN vector controls (passage 12) and HMEC-P parental cells exhibited a 24-hour doubling time (data not shown). These observations demonstrate that suppression of RAR function by a dominant-negative approach results in dysregulated proliferation in HMECs (Seewaldt et al., 1997a). The precise molecular mechanism by which inhibition of RAR function results in increased proliferation in untreated HMECs is currently an area of investigation in our laboratory.

Expression of the DNRAR resulted in resistance to the growth-inhibitory effects of ATRA. Increased growth inhibition of HMEC-LXSN controls was observed with increasing concentrations of ATRA and increasing time of exposure (Fig. 3A, Table 1). HMEC-DNRAR cells were resistant to the growth-inhibitory effects of ATRA relative to controls. Growth of HMEC-DNRAR cells was inhibited at 10 μM ATRA, suggesting that either the block could be overcome at higher concentrations of ATRA or that cells experienced a direct cytotoxic effect (Fig. 3A). This shifting of the dose–response curve to ATRA is evidence that the DNRAR has dominant-negative activity in HMECs. These data also demonstrate that ATRA inhibits the proliferation of HMECs in culture and that inhibition of RAR function blocks this growth inhibition.

MCF7-LXSN vector control cells were relatively resistant to the growth-inhibitory effects of 1.0 μM ATRA (Fig. 3B). Retinoid-resistance was overcome at 10 μM ATRA. In contrast, MCF7-RARβ2 transduced cells exhibited growth inhibition when treated with 0.1–1.0 μM ATRA. Increased growth inhibition was observed with increasing concentrations of ATRA (Fig. 3B, Table 1). These data provide evidence that expression of a functional RARβ2 in retinoid-resistant MCF-7 breast cancer cells results in increased sensitivity to the antiproliferative effects of ATRA. The importance of RARβ2 in regulating growth inhibition is supported by observations made by other investigators (Liu et al., 1996; Sun et al., 2000)

Modulation of RAR Function in Benign and Malignant Mammary Epithelial Cells Results in Altered Cell Cycle Distribution

To further investigate the ability of RARs to regulate growth arrest of HMECs, flow cytometric (FACS) analysis was performed on HMEC-DNRAR cells (passage 13–14) and HMEC-LXSN vector controls (passage 13–14) (Fig. 4A, Table 1). Analysis of isolated nuclei stained with propidium iodide demonstrated that untreated HMEC-DNRAR cells had a 50 ± 5% increase in the percentage of cells in S-phase relative to untreated HMEC-LXSN vector control cells (Fig. 4A). This is consistent with the observation that HMEC-DNRAR cells have an increased rate of proliferation relative to HMEC-LXSN controls.

HMECs whose normal RAR function was inhibited by the DNRAR were relatively resistant to the ATRA-mediated G_1 block observed in vector control cells: HMEC-LXSN treated with 0, 0.1, and 1.0 μM ATRA for 4 days had 20%, 11%, and 2% of cells in S-phase, respectively (Fig. 4A, Table 1). In contrast, 30%, 29%, and 22% of HMEC-DNRAR cells treated with 0, 0.1, and 1.0 μM ATRA, respectively, for 4 days were in S-phase (Fig. 4A, Table 1). These results suggest that RARs are important mediators of ATRA-mediated growth inhibition and that suppression of RAR function in HMECs results in a significant increased percentage of cells in S-phase relative to controls.

The ability of RARβ2 to suppress proliferation in the breast cancer cell line MCF-7 was then tested. FACS analysis was performed on MCF7-RARβ2 transduced cells and MCF7-LXSN controls treated with 0 and 1.0 μM ATRA to further investigate the effect of RARβ2 expression on MCF-7 cell cycle progression. MCF7-RARβ2 cells treated with 1.0 μM ATRA for 4 days exhibited a 16 ± 3% increase in the percentage of cells in G_1 and a 94 ± 5% decrease in the percentage of cells in S-phase (Fig. 4B, Table 1). In contrast, MCF7-LXSN vector control cells did not exhibit a decrease in the percentage of cells in S-phase when treated with 1.0 μM ATRA for 4 days (Fig. 4B, Table 1). This cell cycle analysis suggests a critical role for ATRA and RARβ2 in mediating the G_1/S-phase distribution of MCF-7 cells.

RETINOIDS AND RETINOIC ACID RECEPTORS MEDIATE APOPTOSIS IN MALIGNANT BUT NOT IN NORMAL MAMMARY EPITHELIAL CELLS

There is evidence that retinoid receptors may be important mediators of apoptosis. Apoptosis plays an important role in embryogenesis, in normal tissue involution, and in regulating the death of terminally differentiated cells (Evans et al., 1998). There is evidence that retinoid-mediated truncation defects of the embryonic limb are the result of RAR-β2-mediated apoptosis (Soprano et al., 1994). Tumor response to therapy may depend on the ability of individual malignant cells to undergo apoptosis (Evans et al., 1998).

In addition to the above-noted growth arrest, MCF7-RARβ2 transduced cells underwent morphologic changes consistent with apoptosis beginning 4 days after treatment with 1.0 μM ATRA. These changes included nuclear condensation, loss of adherence, and cell shrinkage (Fig. 5). ATRA-treated MCF7-LXSN cells and untreated MCF7-RARβ2 transduced cells did not exhibit evidence of apoptosis (Fig. 5). Untreated MCF7-RARβ2 transduced cells exhibited modest cytoplasmic shrinkage relative to vector controls (Fig. 5), likely reflecting the presence of endogenous retinoids present in the fetal calf serum used in the cell culture medium.

The terminal deoxynucleotidyl transferase (TdT) method was also used to detect apoptotic strand breaks. In this assay, 3′ hydroxyl termini of apoptotic-induced strand breaks are labeled with biotin-dUTP by exogenous TdT and can be detected in situ or by flow cytometry after staining with avidin-conjugated fluorescein isothiocyanate (Seewaldt et al., 1997a). Incorporation of labeled dUTP was seen in MCF7-RARβ2 transduced cells treated with 1.0 μM ATRA for 6 days. This was not observed in untreated MCF7-LXSN and

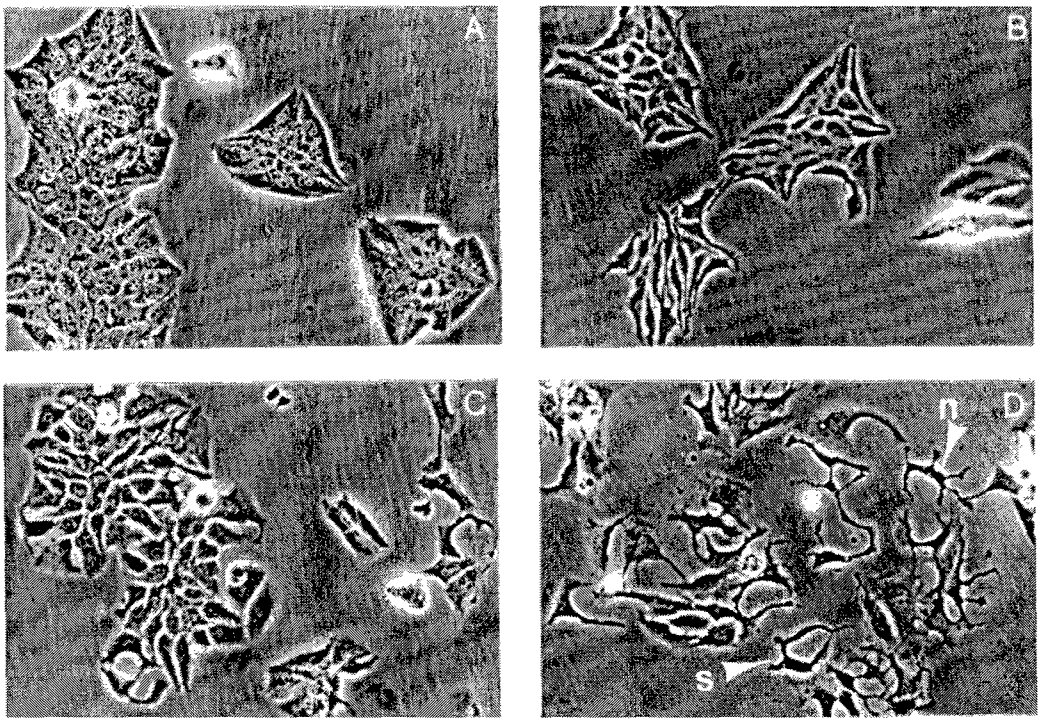


Fig. 5. Morphologic effects of retinoic acid on RARβ2 transduced MCF-7 cells and vector controls. Representative MCF7-LXSN vector controls (**A,C**) and MCF7-RARβ2 transduced cells (**B,D**) were incubated with (**C,D**) and without (**A,B**) 1.0 μM ATRA for 6 days. MCF7-RARβ2 transduced cells treated with ATRA exhibited nuclear condensation (n) and cell shrinkage (s). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent.

MCF7-RARβ2 controls or ATRA-treated MCF7-LXSN vector cells (Fig. 6). Similar results have been subsequently observed by other investigators (Liu et al., 1996). Taken together, these observations indicated that ATRA and RARβ2 mediate apoptosis in the breast cancer cell line MCF-7.

In order to determine whether ATRA mediates apoptosis in HMECs, we treated HMECs with ATRA and tested for apoptosis by morphologic criteria and by biochemical parameters. HMECs treated with 1.0 μM or 10 μM ATRA and examined by electron microscopy did not exhibit morphologic changes characteristic of apoptosis (data not shown). Internucleosomal DNA fragmentation is characteristic of apoptosis and distinguishes it from other modes of cell death such as necrosis (Holtz et al., 1992; Tepper et al., 1992). Similarly, we did not observe increased fragmented cytoplasmic DNA by the diphenylamine assay 2, 4, or 6 days after treatment of HMECs with 1.0 μM ATRA or 10 μM (data not shown). Moreover, ethidium bromide-stained DNA extracted from ATRA-treated HMECs did not demonstrate DNA laddering following 2, 4, or 6 days treatment with 1.0 μM ATRA (data not shown). Finally, apoptotic strand breaks were not detected by TdT staining in HMECs after 5 days treatment with 1.0 μM ATRA (data not shown). These data together suggest that while ATRA induces growth arrest in HMECs, ATRA does not induce apoptosis in HMECs.

INHIBITION OF RETINOID RECEPTOR FUNCTION IN NORMAL MAMMARY EPITHELIAL CELLS BLOCKS GROWTH REGULATION AND POLARITY BY RECONSTITUTED EXTRACELLULAR MATRIX (rECM)

Interaction between basement membrane and mammary epithelial cells is thought to play an important role in regulating growth arrest and epithelial polarity (Folkman et al., 1978; Petersen et al., 1992; Alford et al., 1998). There is evidence that these normal interactions are disrupted early during mammary carcinogenesis. While HMECs undergo growth arrest in culture in the presence of rECM, breast cancer cells and established breast cancer cell lines fail to exhibit similar growth arrest (Petersen et al., 1992). Thus, it is hypothesized that interaction with basement membrane may serve to distinguish the growth patterns of normal and malignant mammary epithelial cells.

To test the role of retinoid receptors in ECM-induced growth arrest and polarity, HMEC-LXSN vector controls and HMEC-DNRAR transduced cells were grown in rECM as a single cell suspension. HMEC-LXSN vector controls grew exponentially until Day 6 and then growth arrested on Day 7 (Fig. 7) forming a uniform population of spherical colonies. Mean diameter of these colonies at Day 9 was 23.5 μ (±10 μ) (Fig. 7). In contrast, HMEC-DNRAR transduced cells continued to proliferate within rECM beyond the time point when

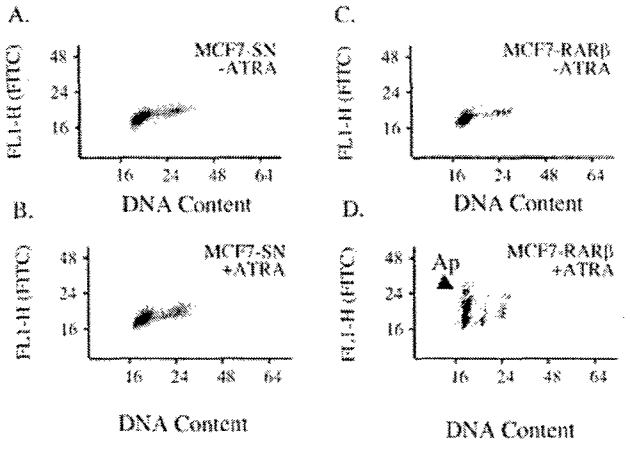


Fig. 6. Fluorescence labeling of apoptotic strand breaks by the TdT method in ATRA-treated MCF-7 cells. Representative MCF7-RARβ2 transduced cells (**C,D**) and MCF7-LXSN vector controls (**A,B**) treated with (**B,D**) or without (**A,C**) 1.0 μM ATRA for 6 days. Cells were incubated with TdT in the presence of biotin-labeled dUTP (Boehringer Mannheim). Detection was with avidin-conjugated fluorescein isothiocyanate (Seewaldt et al., 1995). MCF7-RARβ2 transduced cells treated with ATRA demonstrated apoptotic strand breaks (ap). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent.

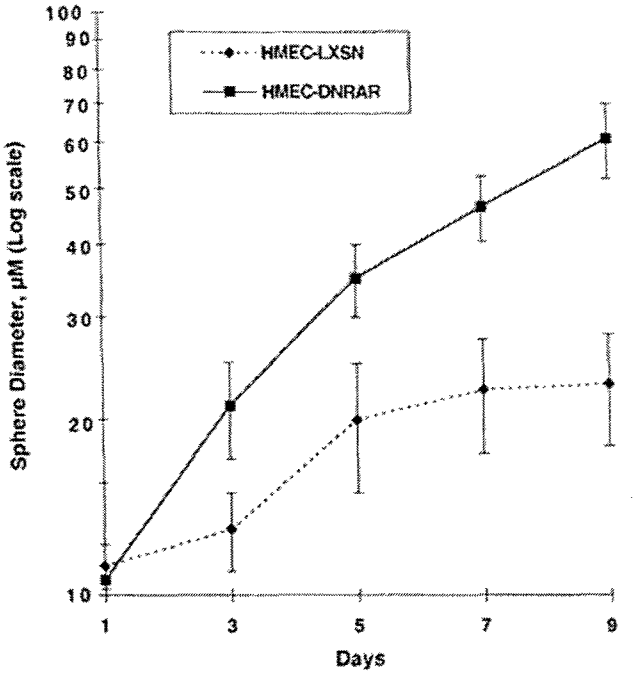


Fig. 7. Inhibiting RAR receptor activity by a DNRAR enhances the proliferation of HMECs cultured in rECM. The mean diameter of spheres formed by HMEC-LXSN vector controls (passage 10) and HMEC-DNRAR cells (passage 10) whose retinoid receptor function is inhibited by a DNRAR is plotted as a function of days in culture. Cells were plated in rECM on Day 0 and the diameter of growing spherical cell colonies was measured with an eyepiece equipped with micrometer spindle. For both HMEC-LXSN vector controls and HMEC-DNRAR transduced cells, the 20 largest colonies were measured at each time point.

vector control cells underwent growth arrest (Fig. 7). Moreover, in contrast with control cells, HMEC-DNRAR transduced cells formed large, dense, irregular colonies. The mean diameter of these colonies was 62 μ (±10 μ) at Day 9, which was significantly larger than that of HMEC-LXSN vector control cells (Fig. 7). These data suggest that inhibition of RAR function in HMECs may result in loss of growth inhibition by rECM.

We next tested whether loss of retinoid receptor function could inhibit the formation of a normal epithelial ductal structure in vitro. Light micrographs of toluidine blue-stained, Epon-embedded, thick sections of HMEC-LXSN vector control cells cultured for 14 days in rECM demonstrated regular, spherical colonies (Fig. 8A,B). Electron micrographs show that HMEC-LXSN control cells grew in organized, acinus-like structures consisting of a single layer of epithelial cells, connected by numerous desmosomes, organized around a central lumen (Fig. 9A,B).

A fundamental property of normal epithelial cells is their ability to organize into polarized structures manifested by the characteristic location of intracellular organelles. When HMEC-LXSN control cells were cultured in rECM and examined by electron microscopy, the resulting acinus-like structure exhibited cellular organization typical of normal polarized epithelium: 1) microvilli were primarily distributed on the luminal and lateral surfaces but not on the basal surface, 2) secretory vacuoles were present on the luminal surface and not on the basal surface, and 3) mitochondria were primarily located at the basal surface (Fig. 9A,B).

In contrast, light micrographs of HMEC-DNRAR transduced cells grown in rECM for 14 days exhibited large, dense, irregularly shaped multilayered clusters of cells (Fig. 8C,D). Unlike normal control cells, the surface of these cell clusters projected irregularly into the surrounding rECM. Electron micrographs demonstrated disorganized clusters of cells with an absence of normal epithelial polarity (Fig. 9C,D). Evidence of lack of polarity included: 1) cells were not organized into spherical, single layered structures; 2) a lumen was not present; 3) microvilli were present on all cell surfaces; and 4) secretory vacuoles and mitochondria were randomly distributed throughout the cytoplasm. These data suggest that suppression of RAR function results in increased proliferation and inhibits the ability of HMECs to form a polarized ductal epithelial structure in rECM. Our results suggest that ATRA and retinoic acid receptors may play an important role in rECM-signal transduction critical for maintaining a normal mammary epithelial phenotype and loss of RAR function may thereby promote mammary carcinogenesis.

EXPRESSION OF RARβ2 IN BREAST CANCER CELLS PROMOTES GROWTH REGULATION BY RECM AND INDUCTION OF APOPTOSIS

We tested whether expression of RARβ2 in MCF-7 breast cancer cells might inhibit proliferation in rECM. Both MCF7-RARβ2 and MCF7-LXSN cells were cul-

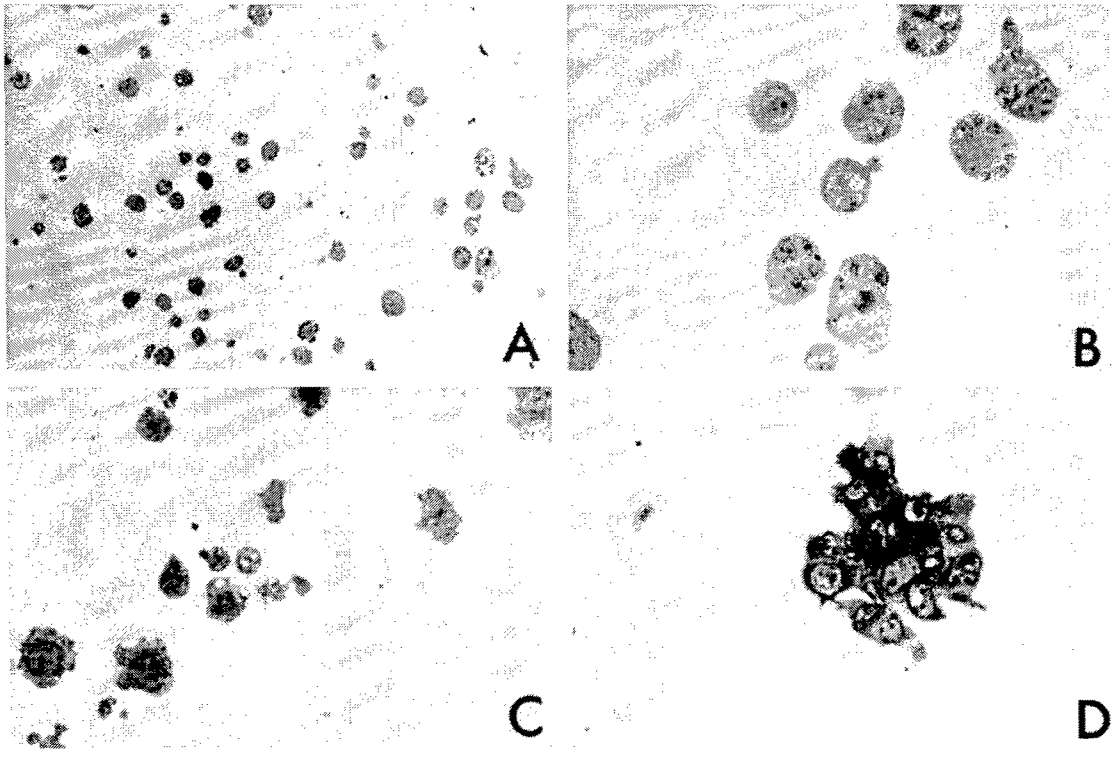


Fig. 8. Morphologic appearance of HMECs with DNRAR mediated suppression of RAR function grown in rECM. **A,B:** HMEC-LXSN vector control cells grown in rECM for 14 days. Cells form acinus-like spherical colonies that underwent growth arrest after 7 to 8 days in culture. Vector control cells demonstrate regular, spherical colonies surrounding a central lumen with sharply delineated boundaries, consistent with differentiated mammary glandular epithelium. **C,D:** HMEC-DNRAR transduced cells whose retinoid receptor was inhibited by a DNRAR grown in rECM for 14 days. Cells formed large, dense irregularly shaped multicellular colonies with no central lumen and did not undergo growth arrest after 14 days in culture. Magnification of **A,C** = ×10 objective; magnification of **B,D** = ×40 objective. Epon-embedded 1 μ sections were stained with toluidine blue.

tured in prepared rECM as a single cell suspension. MCF7-LXSN vector control cells grew exponentially in rECM and formed large, dense, irregular colonies. Mean diameter of these colonies at Day 9 was 39 μ (±5.0 μ) (Fig. 10). In contrast, MCF7-RARβ2 transduced cells grew exponentially until Day 5 and then exhibited a progressively reduced growth rate (Fig. 10). The mean diameter of these colonies on Day 9 was 22 μ (±4.0 μ), which was significantly lower than that of MCF7-LXSN vector control cells (Fig. 10). These data suggest that expression of RARβ2 function in retinoid-resistant MCF-7 breast cancer cells may promote growth inhibition by rECM.

To investigate the potential mechanism for the observed reduction in proliferation in rECM, 20 MCF7-RARβ2 cell clusters were surveyed by electron microscopy on Day 5. Nineteen clusters (95%) exhibited morphologic evidence of apoptosis by the following criteria: 1) cell shrinkage, 2) margination of chromatin, and 3) the presence of apoptotic bodies (Fig. 11). In contrast, MCF7-LXSN vector control cells grown in rECM for 5–9 days did not exhibit morphologic evidence of apoptosis (Fig. 11 and data not shown). These data suggest that expression of RARβ2 in breast cancer cells grown in contact with rECM may promote the induction of apoptosis.

RETINOIDS AND RETINOIC ACID RECEPTORS REGULATE EXPRESSION OF CBP/P300 IN NORMAL AND MALIGNANT MAMMARY EPITHELIAL CELLS

CBP and p300 are thought to be present in the cell in limiting quantities and it is hypothesized that various transcription factors compete for their binding (Tanaka et al., 1997). Although CBP and p300 are highly homologous and appear to have many overlapping functions, some of their functions are clearly distinct. In F9 cells treated with retinoic acid, both CBP and p300 are required for G_1 arrest and apoptosis but only p300 seems to be required for differentiation (Kawasaki et al., 1998). Furthermore, in regulating growth arrest CBP and p300 appear to target different genes. Finally, mice that are heterozygous nulls for either CBP or p300 have different phenotypes (Tanaka et al., 1997). Transcriptional regulation of CBP or p300 levels has not been demonstrated. However, it was recently shown that HSC-3 oral carcinoma cells treated with 9-*cis*-retinoic acid exhibit increased levels of CBP and p300 protein on Western blotting (Hayashi et al., 2000, 2001). It has been clearly established that the levels of CBP and p300 expression are very important in regulating gene expression and that alterations in CBP levels have important implications in human disease

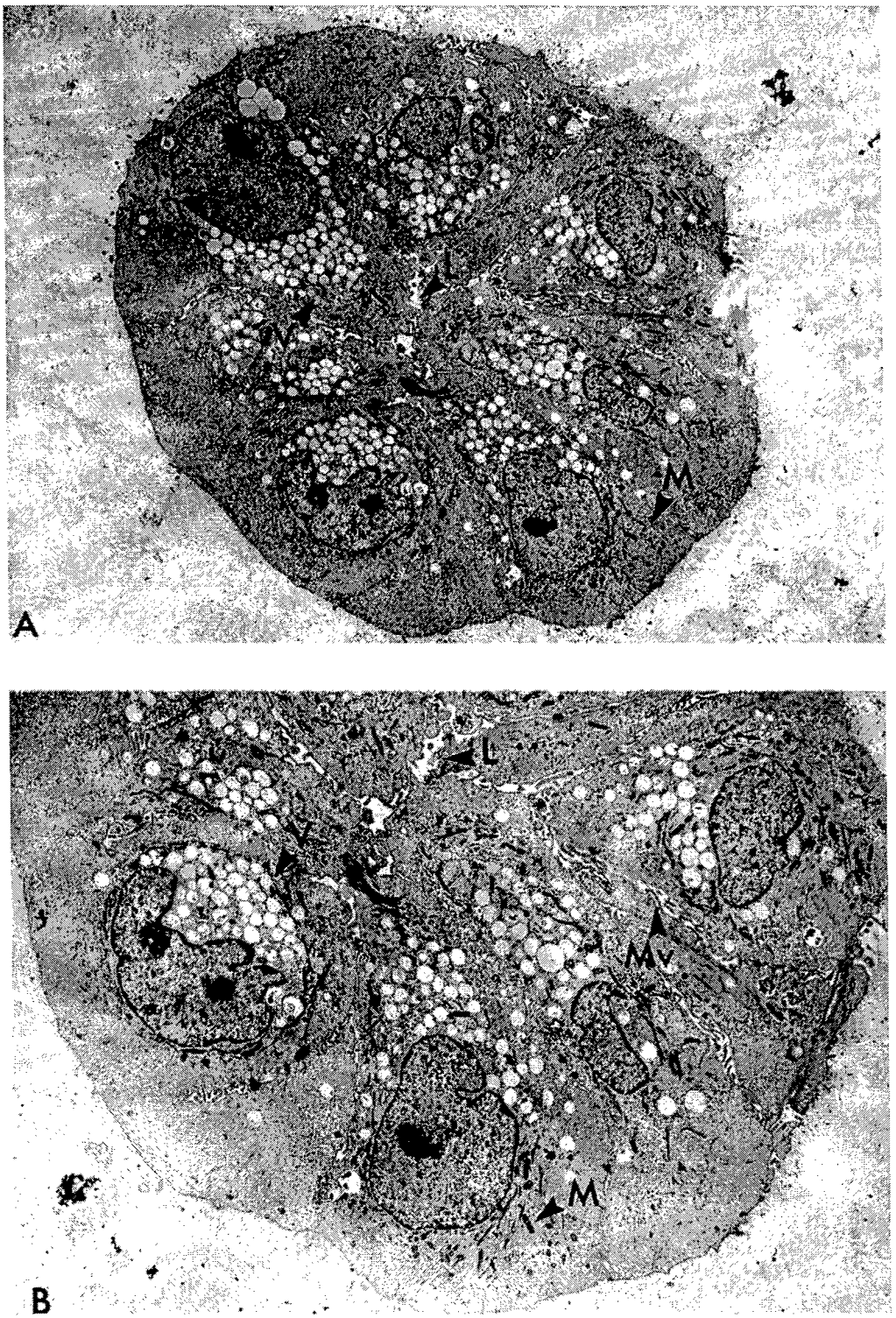


Fig. 9. Phenotypic changes observed by electron microscopy in HMECs expressing a DNRAR suggest that RARs play an important role in the formation of a polarized ductal epithelium. **A,B:** Electron micrographs of HMEC-LXSN vector control cells grown in rECM for 14 days (magnification of **A** and **B** are ×1,500 and ×2,500, respectively). HMEC-LXSN control cells formed acini-like structures that demonstrated a central lumen (L) surrounded by correctly polarized luminal cells consistent with differentiated mammary glandular epithelium. Morphologic evidence of polarity included: 1) microvilli (Mv) were primarily distributed on the luminal and lateral surfaces, 2) secretory vacuoles (V) were present on the luminal surface and not the basal surface, and 3) mitochondria (M) were primarily located at the basal surface. **C,D:** HMEC-DNRAR transduced cells whose RAR function was inhibited by a DNRAR grown in rECM (magnification **C** and **D** are ×1,000 and ×2,500, respectively). Cells grew in large, disorganized multilayered, irregularly shaped colonies, with no lumen formation and a loss of normal cellular polarity. Evidence of lack of polarity included: 1) cells were not organized into spherical, single layer structures, 2) a lumen was not present, 3) mitochondria (M) were present throughout the cell, and 4) secretory vacuoles (V) were randomly distributed throughout the cytoplasm. These morphologic changes observed in HMEC-DNRAR cells suggest that loss of RAR function in HMECs inhibits the formation of a polarized ductal epithelium in vitro.

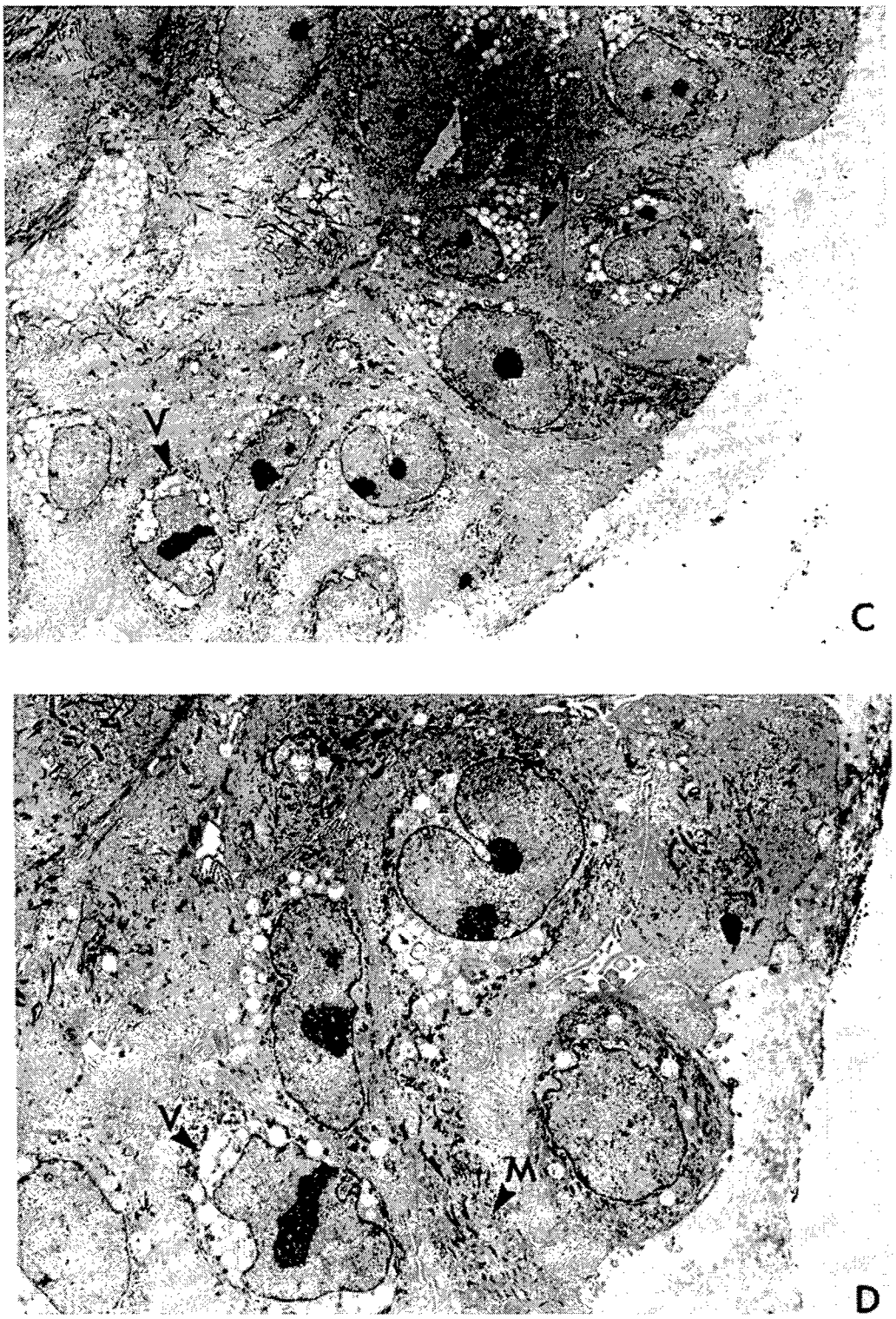


Figure 9. (Continued.)

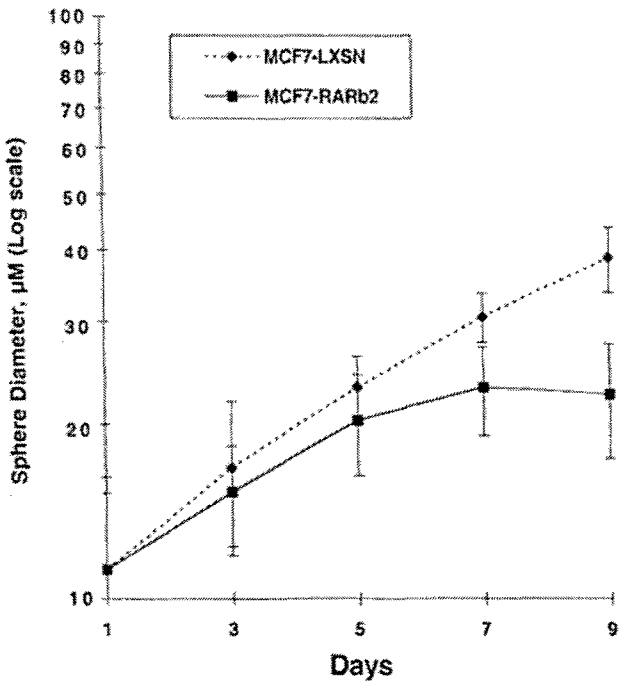


Fig. 10. Expression of RARβ2 suppresses proliferation of MCF-7 cells in rECM. The mean diameter of spheres formed by MCF7-LXSN vector controls and MCF7-RARβ2 cells is plotted as a function of days in culture. Cells were plated in rECM on Day 0 and the diameter of growing spherical cell colonies was measured with an eyepiece equipped with micrometer spindle. For both MCF7-LXSN vector controls and MCF7-RARβ2 transduced cells, the 20 largest colonies were measured at each time point.

(Giles et al., 1998). Furthermore, chromosomal loss at 16p and 16p13, the CBP locus, is observed in many early breast lesions (Linninger et al., 1998; Lu et al., 1998; Aubele et al., 2000).

CBP and p300 Protein Expression Is Modulated by ATRA and RAR Function in HMECs

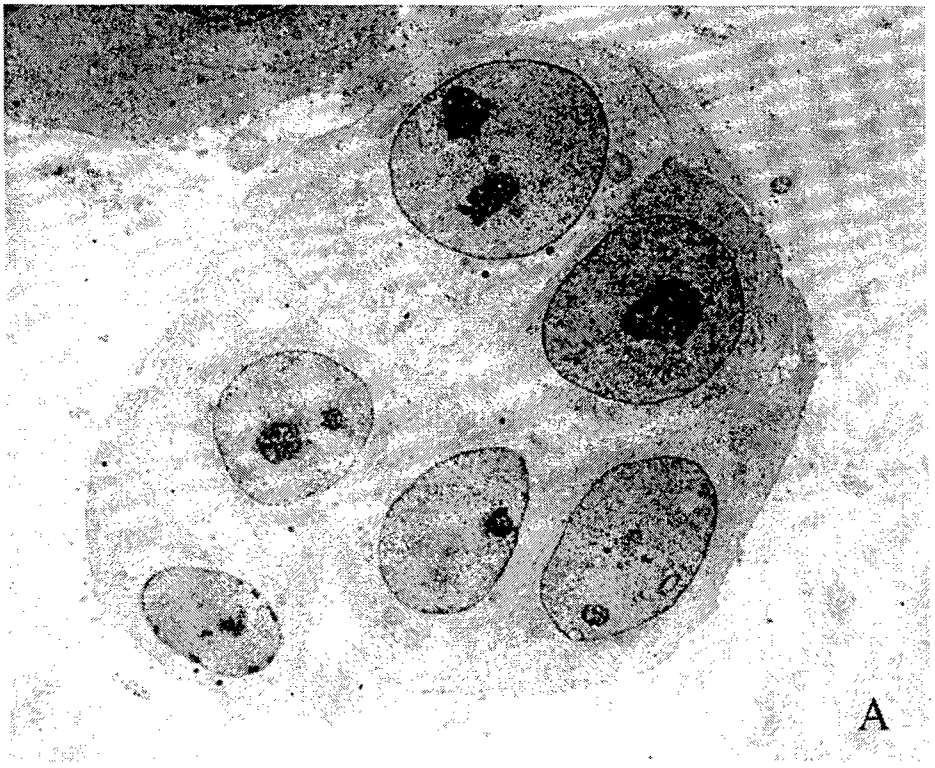
Both CBP and p300 protein levels were decreased in untreated HMEC-DNRAR cells relative to HMEC-LXSN controls (Fig. 12A, Table 2). There was a 50 ± 11% ($P < 0.05$) decrease in the level of CBP and a 76 ± 12% ($P < 0.01$) decrease in the level of p300. When treated for 24 hours with 1.0 μM ATRA, HMEC-LXSN cells showed a 60 ± 9.8% ($P < 0.01$) and a 14 ± 8.6% (n.s.) increase in the amount of CBP and p300, respectively. However, consistent with suppression of RARE-driven CAT expression, HMEC-DNRAR cells treated for 24 hours with 1.0 μM ATRA showed no change in the level of CBP. In contrast, p300 levels increased by 54 ± 16% ($P < 0.05$) (Table 2). Expression of a functional RARβ2 in MCF-7 cells was associated with increased ATRA-mediated induction of CBP and p300 protein expression (Fig. 12B, Table 2). There was a 130 ± 18% ($P < 0.01$) increase in the level of CBP in MCF7-RARβ2 cells treated with 1.0 μM ATRA relative to a 22 ± 13% ($P < 0.05$) induction in similarly treated MCF7-LXSN vector controls. p300 protein levels increased by 160 ± 11% ($P < 0.01$) in MCF7-RARβ2 cells treated with 1.0 μM ATRA and did not increase in ATRA-treated MCF7-LXSN vector controls. These observations suggest a potential association between RARs, ATRA, and CBP/p300 protein expression. Since RARs recruit CBP and p300 during transcription and the pool of CBP is limited, the ATRA associated increase of CBP and p300 pools in the cell may allow RARs to effectively recruit them.

CONCLUSIONS

The ability of retinoic acid receptors to modulate growth arrest and apoptosis in benign and malignant mammary epithelial cells was tested by 1) suppression of RAR function in retinoid-sensitive HMECs and 2) expression of a functional RARβ2 in retinoid-resistant MCF-7 breast cancer cells.

A dominant-negative approach was utilized to study the role of RAR function in mediating proliferation of HMECs. The DNRAR, RARα403, has the advantage of being able to simultaneously interfere with all RAR isoforms with a single construct in a specific human cell type. Previous studies utilizing transgenic mouse lines resulting from targeted disruption of specific RARs by homologous recombination have been complicated because the loss of a specific isoform has in some cases resulted in embryonic lethality (RXRα) (Kastner et al., 1994) or in other cases, no phenotypic alteration (RARβ2) (Mendelson et al., 1994). In the latter case, it is postulated that other members of the RAR family or isoform can compensate for loss of a specific RAR isoform, and therefore no phenotypic alteration is observed. This suggests that multiple receptors must be knocked out to completely disable the RAR signaling pathway. We observed that inhibition of retinoic acid function by a dominant-negative approach in HMECs results in 1) dysregulated growth associated with an increased percentage of cells in S-phase, 2) resistance to the induction of G_1 arrest by ATRA, and 3) loss of rECM growth regulation and polarity. Taken together, these results indicate that ATRA and RARs play an important role in regulating growth arrest in HMECs. Alternatively, it is possible that the DNRAR may heterodimerize with a member of the steroid–thyroid superfamily of nuclear receptors other than RAR, such as the retinoid-X receptor (RXR). For this reason it is possible that the observed effects may be due to direct or indirect interference with either RAR, RXR, or non-retinoid nuclear hormone receptor signaling (Saitou et al., 1995; Li et al., 2001).

Loss of retinoid receptor function is observed early during breast carcinogenesis (Widschwendter et al., 1997; Xu et al., 1997). The mechanism by which loss of RARβ expression might promote breast carcinogenesis is not fully understood, but it is hypothesized that RARβ2 may play a critical role in mediating the growth-inhibitory effects of ATRA in mammary epithelial cells (Seewaldt et al., 1995; Liu et al., 1996). This hypothesis is strengthened by the recent observation that suppression of RARβ2 in H157 cells by an antisense approach inhibited ATRA-mediated growth inhibition and RARE binding activity and transactivation (Sun et al., 2000). We observed that MCF7-RARβ2 transduced cells exhibited increased sensitivity to ATRA- and rECM-mediated growth arrest and apoptosis. Other investigators have confirmed that ATRA and RARβ2 are critical mediators of apoptosis in MCF-7 cells (Liu et al., 1996). Interestingly, while MCF7-RARβ2 transduced cells were sensitive to apoptosis, HMECs did not undergo apoptosis when treated



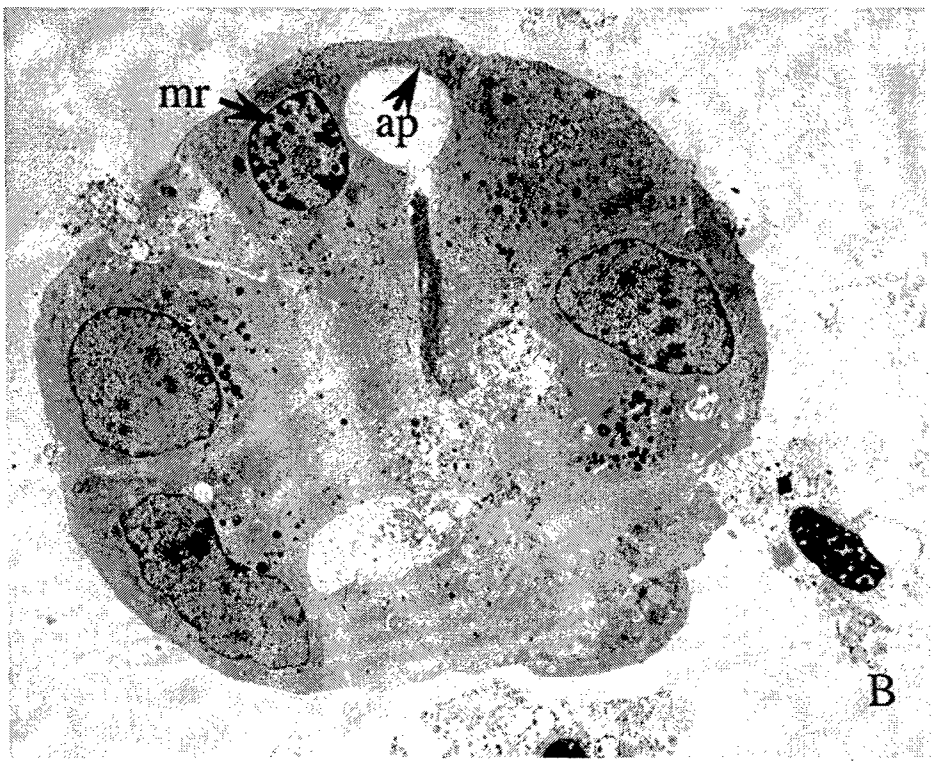


Fig. 11. Phenotypic changes observed by electron microscopy in RARβ2-transduced MCF-7 breast cancer cells suggest that RARs play a role in rECM-mediated apoptosis. **A:** Electron micrographs of MCF7-LXSN vector control cells grown in rECM for 5 days. MCF7-LXSN control cells formed disorganized aggregates of cells. **B:** In contrast, MCF7-RARβ2 transduced cells grown in rECM exhibited morphologic evidence of apoptosis. Evidence of apoptosis included: 1) cell shrinkage and separation, 2) margination of chromatin (mr), and 3) the presence of apoptotic bodies (ap) containing cytoplasmic elements. Magnification = ×1,500.

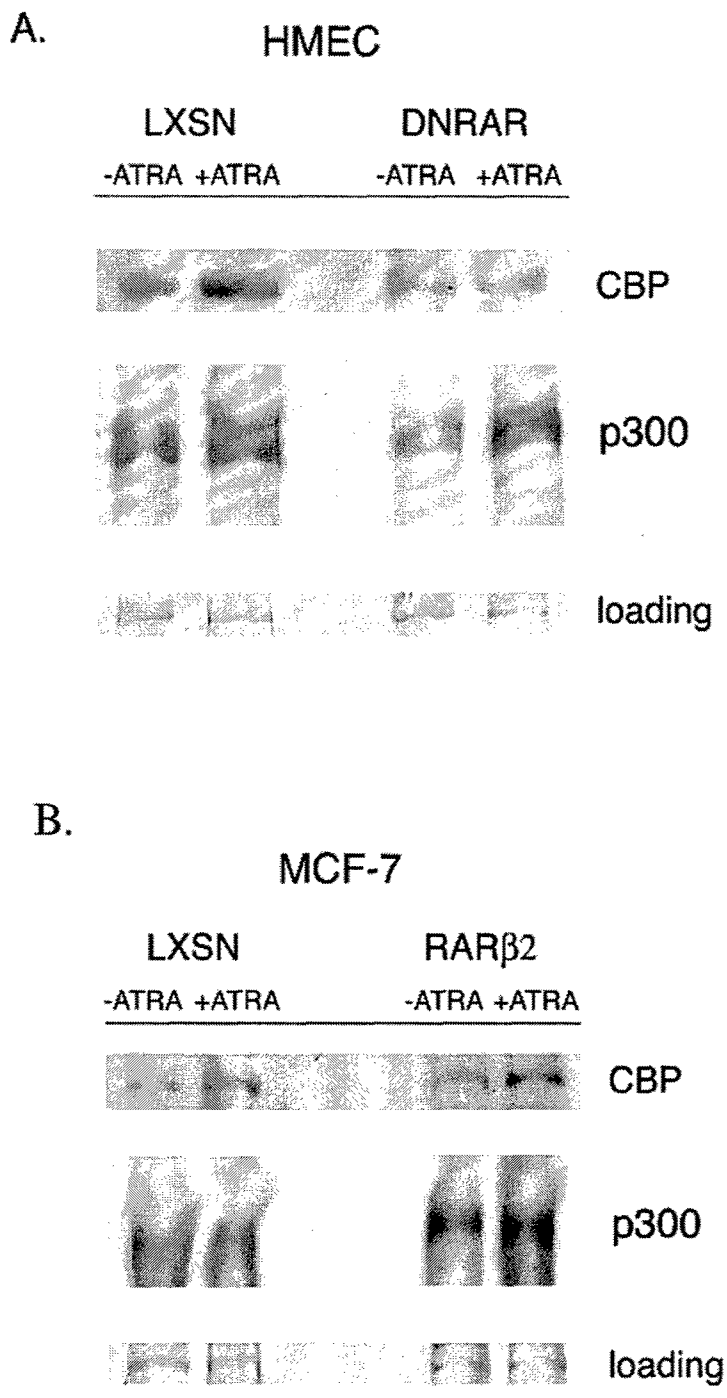


Fig. 12. ATRA-treatment and RAR expression correlate with CBP/p300 expression in normal and malignant mammary epithelial cells. **A:** Western analysis of CBP/p300 protein expression in HMEC-DNRAR cells (DNRAR) and in HMEC-LXSN vector control cells (LXSN). **B:** Expression of CBP/p300 protein in MCF7-RARβ2 transduced breast cancer cell lines (RARβ2) and vector controls (LXSN). Cells were treated with or without 1.0 μM ATRA for 48 hours. The blot was probed with a CBP- and p300-specific polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. An unidentified 90 kd protein band was used as a loading control.

with ATRA or grown in contact with rECM. This is consistent with other reports of differentiation agents mediating growth arrest in normal cells and apoptosis in cancer cells.

Tissue homeostasis is maintained by a dynamic equilibrium between cellular proliferation and cell death (Evans et al., 1998). Apoptosis is felt to be the predominant mechanism of cell death and plays a central role in controlling cell number and in eliminating cells sustaining DNA damage (Thompson et al., 1995). Cancer cells typically have an immature phenotype representing a block in the normal differentiation pathway. Recently, agents such as retinoids that are capable of inducing differentiation have been utilized in vitro and in vivo in an attempt to restore the normal differentiation program in malignant cells. Restoration of normal growth arrest and differentiation signals in cancer cells appears to activate apoptosis. For example, retinoids induce growth arrest and differentiation of acute promyelocytic leuke-

TABLE 2. CBP/p300 expression in ATRA-treated transduced normal and malignant mammary epithelial cells

	HMEC-LXSN		HMEC-DNRAR	
	− ATRA	+ ATRA	− ATRA	+ ATRA
CBP	0.63 ± 0.10	1.0 ± 0.01	0.31 ± 0.07	0.28 ± 0.07
p300	0.88 ± 0.09	1.0 ± 0.01	0.22 ± 0.04	1.16 ± 0.01
	MCF7-LXSN		MCF7-RARβ2	
	− ATRA	+ ATRA	− ATRA	+ ATRA
CBP	0.45 ± 0.06	0.55 ± 0.09	0.44 ± 0.08	1.0 ± 0.01
p300	0.24 ± 0.04	0.24 ± 0.03	0.38 ± 0.04	1.0 ± 0.02

Adherent HMEC-LXSN vector controls, HMEC-DNRAR transduced cells, MCF7-LXSN vector controls, and MCF7-RARβ2 transduced cells were treated for 48 hours with 1.0 μM ATRA. The relative levels of CBP/p300 protein levels were determined by Western analysis and quantitated using Kodak 1D Image Analysis Software. ATRA was dissolved in ethanol and untreated controls received an equivalent volume of solvent. These data are representative of three separate experiments.

mic cells followed by apoptosis (Oshahi et al., 1992; Park et al., 1994) and treatment of human colon adenocarcinoma cells with Herbimycin A results in growth arrest and differentiation followed by apoptosis (Mancini et al., 1997). We observe that ATRA and RARs induce growth arrest followed by apoptosis in RARβ-transduced breast cancer cells, but ATRA induces growth arrest without apoptosis in normal mammary cells. These observations suggest that RAR-initiated normal growth and differentiation signals may promote apoptosis in abnormal cells, perhaps as part of the normal cellular regulatory pathways that target the elimination of damaged cells. We hypothesize that loss of these RAR-derived signals could promote the development of malignancy by permitting the clonal expansion of mutated cells.

CBP and p300 are highly homologous nuclear proteins important for growth regulation and apoptosis. CBP/p300 are known to modulate a diverse range of signaling pathways, including those regulated by estrogens, retinoids, interferon, and p53 (Goldman et al., 1997; Snowden et al., 1998; Vo et al., 2001). Transcriptional regulation of CBP or p300, however, has not been well studied to date and the promoter sequences of CBP and p300 are unknown. There is evidence that RARβ might be critical for CBP/p300 expression (Hayashi et al., 2001). In this study, we observed that 1.0 μM ATRA induced CBP/p300 protein expression in HMECs and in MCF7-RARβ2 cells. Retinoid-resistant MCF7-LXSN cells, lacking RARβ2 expression, treated with 1.0 μM ATRA exhibited no induction of p300 protein expression and only slight increase in CBP protein expression. Suppression of RAR function in HMECs correlated with suppression of CBP/p300 expression. The dominant-negative activity of the DNRAR, RARα403, is sufficient to block induction of CBP but not p300 by 1.0 μM ATRA. Taken together, these results suggest a potential role for ATRA and RARs in mediating the expression of CBP/p300; however, the molecular mechanism for this relationship has not been defined. Since CBP and p300 pools are limited, any alteration in their levels may impact the transcription of genes regulated in part by CBP/p300. It is also important to consider that since the expression of the DNRAR in HMECs increased levels of p300, it is possible that the increased proliferation observed in HMEC-DNRAR cells may reflect increased levels of p300.

Our observations suggest the following model: loss of RARβ2 function in mammary epithelial cells may result in altered CBP/p300 expression, thereby resulting in loss of growth regulation and promotion of mammary carcinogenesis. Since CBP/p300 are integrators of retinoid signaling and we observe that CBP/p300 protein levels are regulated by RARs and ATRA, it is possible that this loss of growth regulation involves a positive feedback loop. Alternatively, increased expression of a DNRAR such as RARβ4 may result in dysregulated proliferation and promote the clonal expansion of mammary epithelial cells harboring reduced CBP/p300 levels. CBP/p300 are critical regulators of growth arrest, differentiation, and apoptosis. Therefore, loss of RAR function might disrupt this normal cycle of breast epithelial cell homeostasis, leading to an expanded abnormal epithelial cell population. Further mutations of cellular oncogenes or tumor suppressor genes in this expanded population of cells might then lead to the development of overt breast cancer. This model predicts that the loss of RARβ and subsequent downregulation of CBP/p300 might be a relatively early event in the pathogenesis of breast cancer.

NOTE ADDED IN PROOF

Fan et al. (Fan, S, et al. 2002. p300 modulates the BRCA1 inhibition of estrogen receptor activity. Cancer Research 62; 141–151.) have recently shown that p300, but not CBP, transcription and protein levels are downregulated in breast and prostate cancer cell lines by BRCA1. Downregulation of p300 results in the inhibition of estrogen receptor-alpha transcriptional activity.

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CBP/p300 Modulates Resistance to Reconstituted Extracellular Matrix-Induced 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 growth regulation, polarity, and apoptosis. Loss of ECM-sensitivity is thought to be a critical early event in mammary carcinogenesis. We previously observed that resistance to apoptosis in late passage p53(-) HMEC-E6 cells grown in prepared ECM (rECM) was associated with a loss of sensitivity to rECM-mediated growth arrest and polarity. rECM-resistance was also associated with loss of chromosome arm 16p (74%) or an unbalanced translocation involving 16p13, the CREBP binding protein (CBP) locus and 2) decreased CBP protein expression. To test the role of CBP and rECM-signaling in promoting apoptosis, rECM function was suppressed in HMECs using a dominant-negative retinoid receptor (DNRAR), RARα403. Expression of the DNRAR in HMECs inhibited rECM-mediated polarity, growth regulation and apoptosis and inhibited expression of CBP, and the related protein, p300. To directly test the role of CBP/p300 in rECM-mediated apoptosis, CBP or p300 expression was suppressed by an antisense approach. Inhibition of CBP or p300 function resulted in loss of rECM-growth regulation and apoptosis. These observations suggest a critical role for CBP/p300 in rECM-mediated growth regulation and apoptosis in HMECs.

INTRODUCTION

Normal breast tissue is composed of mammary epithelial cells that rest on extracellular matrix (ECM)[1]. The interaction between epithelial cells and ECM is important for regulation of normal growth, polarity, and differentiation (Petersen, et al. 1992; Strange, et al. 1992; Zutter, et al. 1995). Loss of ECM-signaling is thought to be an early event in mammary carcinogenesis and it is hypothesized that interactions with ECM may distinguish the growth patterns of normal and malignant mammary epithelial cells (Petersen, et al. 1992).

Mutations of the *TP53* gene are frequently detected in breast cancers and are associated with an increased risk of malignancy (Ashkenazi and Dixit 1998). Accumulation of p53 protein in mammary epithelial cells is frequently detected in women at high risk for the development of breast cancer (Fabian, et al. 1996) and is associated with an increased risk of breast cancer in women with benign hyperplastic breast tissue (Rohan, et al. 1998). While the role of p53 as a tumor suppressor is well understood, the role of ECM-signal transduction in eliminating normal human mammary epithelial cells (HMECs) that have acutely lost p53 function is unknown.

We previously developed an *in vitro* model to investigate p53 loss in HMECs in the context of ECM-derived growth regulation signals (Seewaldt, et al. 2001*a*). p53 function was first suppressed in HMECs by either 1) retroviral-mediated expression of the Human Papillomavirus Type-16 (HPV-16) E6 protein or 2) an antisense approach; the resultant p53(-) HMECs and p53(+) HMEC controls were then cultured in reconsituted ECM

(rECM) (Seewaldt, et al. 2001*a*). While p53(+) HMEC vector controls and parental cells grown in rECM underwent growth arrest on Day 7, p53(-) HMECs proliferated in rECM until Day 6 and then underwent apoptosis on Day 7. While the acute suppression of p53 in HMECs promoted sensitivity to rECM-mediated apoptosis, p53(-) HMECs expressing E6 (p53(-) HMEC-E6 cells) passaged in non-rECM culture rapidly (8 to 10 passages) acquired resistance to both rECM-mediated growth arrest and apoptosis (Seewaldt, et al. 2001*a*). These observations suggest a critical role for rECM-signaling in promoting apoptosis in HMECs that have acutely sustained p53 loss.

In this report we investigated the role of 1) rECM-growth arrest and polarity in mediating apoptosis and 2) the CREBP binding protein, CBP, and the related protein, p300 in regulating rECM-mediated growth arrest, polarity, and apoptosis *in vitro*.

Retinoid receptors play an important role in regulating mammary epithelial cell growth and apoptosis (Seewaldt, et al. 1995, Seewaldt, et al. 1997*a*). We previously observed that suppression of retinoid receptor function in HMECs by a dominant-negative retinoid receptor (DNRAR), RARα403, resulted in loss of rECM-induced growth arrest and polarity (Seewaldt, et al. 1997*a*). Here, we investigated whether loss of rECM-induced growth regulation and polarity by expression of the DNRAR, blocked rECM-apoptosis in early passage p53(-) HMEC-E6 cells.

We also observed the relationship between expression of the CREBP binding protein, CBP and apoptosis-sensitivity. CBP expression was of interest because late passage,

rECM-resistant p53(-) HMEC-E6 cells similarly exhibited decreased expression of CBP protein and loss of chromosome arm 16p (74%). In addition, one cell exhibited chromosomal loss distal to part of 16p13 and a second cell demonstrated a unbalanced translocation involving 16p13. CBP is a nuclear protein important for regulating growth, differentiation, and apoptosis that is located at chromosome band 16p13.3 (Yao, et al. 1998; Giles, et al. 1997). 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 observed in premalignant breast lesions (Lininger, et al. 1998; Tsuda, et al. 1998; Aubele, et al. 2000). CBP and the related protein, p300, are key integrators of diverse signaling pathways including the p53-, estrogen-, and retinoid-signaling pathways (Kawasaki, et al. 1998; Robyr, et al. 2000). Based on these observations we hypothesized that CBP protein expression might be critical for rECM growth regulation and apoptosis in p53(-) HMEC cells, and suppression of CBP expression could promote apoptosis-resistance.

We report that suppression of CBP or the related protein, p300, by an antisense approach inhibited rECM-mediated growth arrest, polarity and apoptosis in early passage p53(-) HMEC-E6 cells. Observations in our model system have important clinical implications as they predict a critical role for CBP/p300 in rECM-signaling.

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, has a limited life span in culture, and fails to divide after approximately 20 to 25 passages. HMECs exhibit a low level of estrogen receptor staining characteristic of normal mammary 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 (UBI, Lake Placid, NY), 10 ng/ml epidermal growth factor (UBI), 0.5 μg/ml hydrocortisone (Sigma), 10^{-5}M isoproterenol (Sigma, St. Louis, MO), and 10 mM HEPES buffer (Sigma) [Standard Media]. G418 (Gibco, Grand Island, NY) containing Standard Media was prepared by the addition of 300 μg/ml of G418 to 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 9) 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. 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 10) and selected with Standard Media containing 300 μg/ml G418. Cells were grown in G418 containing Standard Media for four to seven days, until 100% of control untransduced cells were dead. The transduction efficiency was high during selection, cells were passaged 1:3 at the completion of selection (passage 11), and cells 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 p53(+) HMEC-P. Transduced AG11132 cells expressing the HPV-16E6 construct were designated p53(-) HMEC-E6 and vector control clones were designated p53(+) 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.

Double retroviral transductions

See Figure 1 for schema. Construction of the LRAR α403SN dominant-negative retroviral vector has been previously described (Tsai, et al. 1992). Transducing virions from either the PA317-LRAR α403SN or the control PA317-LXSN (without insert) retroviral

producer line were added at a multiplicity of infection of 1:1 in the presence of 4 μg/ml Polybrene (Sigma) to log-phase HMECs. One T-75 flask was not infected with virus. After 48 hours, the flasks containing transduced cells and one flask with untransduced cells were selected with Standard Media containing 300 μg/ml G418. Cells were cultured in G418-containing media for four to seven days, until 100% of control untransduced cells were dead. Cells were then placed in Standard Media without G418.

The second phase of the double transductions was accomplished as follows: 1) A T-75 flask of cells tranduced with LRAR α403SN (DNRAR) was infected with either virions from PA317-LXSHE6 or PA317-LXSH, at a multiplicity of infection of 1:1 and selected with 300 μg/ml hygromycin for 4 to 7 days. 2) One T-75 flask of HMECs transduced with LXSN was infected with either virions from PA317-LXSHE6 or PA317-LXSH, at a multiplicity of infection of 1:1 and selected with hygromycin for four to seven days. All cells were then passaged serially in culture in the absence of selection. Double transduced HMECs expressing the 1) LXSN/LXSH constructs were designated p53(+) HMEC-LX/LX, 2) LXSN/HPV-16E6 constructs were designated p53(-) HMEC-LX/E6, 3) LRAR α403SN/LXSH constructs were designated p53(+) HMEC-DN/LX, and 4) LRAR α403SN/HPV-16E6 constructs were designated p53(-) HMEC-DN/E6.

Western Blotting

Preparation of cellular lysates and immunoblotting were performed as previously described (Seewaldt, et al. 1997*b*). For p53 expression, the membrane was blocked with 20% bovine serum albumin (Sigma) in PBS overnight at RT and then incubated with a

1:100 dilution of mouse anti-human p53 (Oncogene Science Ab-2). For CBP/p300 expression, the blocked membrane was incubated with either 1/200 CBP C1 or 1/300 p300 N15 antibody (Santa Cruz Biotechnology) in TBS with 0.05% Tween-20 (Sigma). The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software.

HMEC Culture in rECM

HMECs were grown in rECM by methods developed by Bissell and others (Folkman and Moscona, 1978; Howlett et al., 1995). 100 µl of rECM (Growth Factor Depleted Matrigel^{tm}, 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 9 days in culture.

Cell Growth Determination in rECM Culture

Cell growth was determined by the following criteria: The size of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. For all transduced HMECs, the 20 largest colonies were measured.

E-cadherin Immunostaining

Transduced HMECs were grown in rECM as described above for 6 days and embedded in O.C.T. (Miles). Cells were snap frozen and 5 micron sections were obtained. Sections were fixed in for 30 min at RT with 3.7% formaldehyde in PBS and were blocked with 0.5% heat-denatured bovine serum albumin (HD-BSA) in PBS for 1 hr at RT. Cells 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 immunoflurorescence, cells were incubated with FITC-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).

Transmission Electron Microscopy

Transduced HMECs were grown in contact with rECM as described above. Electron microscopy was as previously described (Seewaldt, et al. 1999; Seewaldt, et al, 2001*a*).

Cytogenetic analysis of early and late passage HMECs

Cytogenetic analyses of p53(+) HMEC-LXSN (passage 10), p53(-) HMEC-E6 (passage 10), and p53(-) HMEC-E6 (passage 20) were performed as previously described (Mrózek, et al. 1993; Schröck, et al. 1996; Seewaldt, et al. 2001*a*).

Suppression of CBP/p300 expression

Nine antisense oligonucleotides (ODNs) to human CBP and p300 were generated by the PAS program (Ugai et al., 1999). The CBP antisense A3342V ODN (24-mer, nucleotide

position 3342-3363) and the p300 antisense ODNs A3851T (20-mer, nucleotide position 3851-3870) and A5765T (20-mer, nucleotide position 5765-5784) were initially chosen on the basis of selective inhibition of CBP or p300 protein expression, respectively, in MCF-7 cells (data not shown); suppression was confirmed in HMECs. Inactive CBP ODN A2172Z (26 mer, nucleotide position 2172-2197) and inactive p300 ODN A2811W (23 mer, nucleotide position 2811-2833) were selected based on lack of suppression of CBP and p300 respectively in MCF-7 and HMECs. Scrambled CBP ODN A3342V (24-mer) and scrambled ODN A3851T (20-mer) were chosen to be scrambled sequences of the antisense ODNs to ensure identical nucleotide content and minimize differences potentially attributable to nucleic acid content. These ODNs were also selected based on lack of suppression of CBP and p300 respectively in mammary epithelial cells. (See Table 1 for a list of ODNs tested.) In all ODNs, the first and last three nucleotides were phosphorothiolate modified to increase their stability *in vitro*. Early passage p53(+) HMEC-LXSN controls and p53(-) 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 0.1 μM active or inactive ODNs. Every 24 hr the culture media was replaced by new Standard Media containing fresh ODNs. Western analysis was performed to confirm suppression of either CBP or p300 expression as described above. The resulting film images were digitized and quantitated using Kodak 1D Image Analysis Software. For rECM culture, early passage p53(+) HMEC-LXSN controls and p53(-) HMEC-E6 cells were trypsinized and approximately 1 x 10^4 cells were then resuspended in 100 μl rECM containing either CBP- or p300-ODN (0.01 to 0.1 μM final concentration) on ice. rECM cultures were prepared as above. rECM cultures were overlayed with Standard Media

containing either CBP- or p300-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 as described above.

Results

Suppression of rECM-mediated growth regulation and polarity in early passage p53(-) HMEC-E6 cells and controls.

We previously investigated p53 loss in the context of rECM-signaling. p53 function was suppressed in HMECs by either 1) retroviral-mediated expression of HPV-16 E6 (p53(-) HMEC-E6) or 2) an antisense approach (p53(-) HMEC-AS) (Seewaldt, et al. 2001*a*). Controls were provided by 1) expression of the empty LXSN retroviral vector (p53(+) HMEC-LXSN) or 2) inactive oligonucleotides (p53(+) HMEC-scrAS), respectively. We observed that while early passage p53(-) HMECs underwent apoptosis when cultured in rECM, p53(-) HMEC-E6 cells passaged in non-rECM culture rapidly developed resistance to rECM-mediated growth regulation and apoptosis (Seewaldt, et al. 2001*a*). Based on these observations, we hypothesized that inhibition of rECM-signaling might block apoptosis in early passage p53(-) HMEC-E6 cells. To test this hypothesis, rECM-growth regulation and -polarity were suppressed by expression of the DNRAR, RARα403 (Seewaldt, et al. 1997*a*) prior to expression of HPV-16 E6.

Retroviral-mediated gene transfer was utilized to express the DNRAR in HMECs. After selection, HPV-16 E6 was expressed in HMEC-DN cells and p53(+) HMEC-LX controls. See Figure 1 for double transduction and selection schema. Northern analysis was performed on LRARα403SN-infected cells and vector controls to test for DNRAR (RARα403) mRNA expression utilizing an LRARα probe. The LTR-initiated 4.8- and 3.0-kb DNRAR mRNA transcripts were observed in p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells, but they were not present in p53(+) HMEC-LX/LX vector controls or early passage p53(-) HMEC-LX/E6 cells (Figure 2). The endogenous 3.6 kb RARα mRNA transcript was also detected in all HMECs at low levels (Figure 2).

Expression of HPV-16 mRNA was detected in p53(-) HMEC-LX/E6 and p53(-) HMEC-DN/E6 cells but not in p53(+) HMEC-LX/LX or p53(+) HMEC-DN/LX cells (Figure 2). p53 protein expression was observed in p53(+) HMEC-LX/LX vector controls and p53(+) HMEC-DN/LX cells, but p53 was not detectable by Western analysis in early passage p53(-) HMEC-LX/E6 cells or in p53(-) HMEC-DN/E6 cells (Figure 3).

The functional activity of the DNRAR was tested. Increased growth inhibition of p53(+) HMEC-LX/LX controls and early passage p53(-) HMEC-LX/E6 was observed with increasing concentrations of ATRA and increasing time of exposure (Figure 4*a, c*). In contrast, p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells were relatively resistant to the growth inhibitory effects of ATRA (Figure 4*b, d*). As previously observed, growth inhibition of DNRAR-transduced HMECs could be overcome at 10 μM ATRA (Figure 4*b, d*) suggesting that the block could be overcome by higher concentrations of ATRA or

that cells experienced a direct cytotoxic effect (Seewaldt, et al. 1997*a*). This shifting of the dose-response curve to ATRA is evidence that the DNRAR has functional activity in p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells.

We previously observed that p53(+) HMEC-controls (p53(+) HMEC-LXSN and p53(+) HMEC-scrAS) and early passage p53(-) HMECs (p53(-) HMEC-E6 and p53(-) HMEC-AS cells) were sensitive to rECM-mediated growth arrest (Seewaldt, et al. 2001*a*). Similarly, early passage p53(+) HMEC-LX/LX vector controls and early passage p53(-) HMEC-LX/E6 cells grew exponentially in rECM until Day 6 and then growth arrested on Day 6 to 7 (Figure 5) forming a uniform population of spherical colonies. Mean diameter of p53(+) HMEC-LX/LX and early passage p53(-) HMEC-LX/E6 colonies on Day 7 were 28 microns (+/- 9.0 microns) and 30 microns (+/- 10 microns), respectively (Figure 5). Similar to late passage p53(-) HMEC-E6 cells, p53(+) HMEC-DN/LX cells and p53(-) HMEC-DN/E6 cells were resistant to rECM-induced growth arrest as evidenced by a lack of rECM-growth inhibition at Day 7 and continued increase in sphere diameter until at least Day 9 (Figure 5). Mean diameter of p53(+) HMEC-DN/LX cells and p53(-) HMEC-DN/E6 cells were 41 microns (+/- 10.0 microns) and 42 microns (+/- 9.0 microns), respectively (Figure 5). These data suggest that expression of DNRAR, RARα403 in early passage p53(-) HMEC-E6 cells and in early passage p53(+) HMEC-LXSN controls results in increased proliferation in rECM.

We previously observed that p53(+) HMEC controls and early passage p53(-) HMECs exhibited polarized distribution of E-cadherin (Seewaldt, et al. 2001*a*). Similarly, early

passage p53(+) HMEC-LX/LX controls and early passage p53(-) HMEC-LX/E6 cells grown in contact with rECM demonstrated basolateral expression of E-cadherin, including lateral staining at cell-cell junctions, consistent with a polarized epithelium (Figure 6). Twenty cell clusters were surveyed per data point. These results are consistent with what is observed in normal breast sections and are consistent with results obtained by other investigators when mammary epithelial cells are grown in contact with rECM (Weaver et al., 1997; Spancake et al., 1999). Similar to late passage p53(-) HMEC-E6 cells, p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells exhibited punctate, dispersed and intracellular staining consistent with a loss of epithelial polarity (Figure 6) (Seewaldt, et al. 2001*a*). These results are consistent with prior observations in tumorigenic mammary epithelial cells grown in rECM (Weaver, et al. 1997).

Suppression of rECM-growth arrest and -polarity in early passage p53(-) HMEC-E6 cells blocks rECM-induced apoptosis.

We tested whether suppression of rECM-mediated growth arrest by a DNRAR inhibited apoptosis. Similar to late passage p53(-) HMEC-E6 cells, early passage p53(-) HMEC-DN/E6 cells were resistant to rECM-growth regulation and polarity and did not undergo apoptosis when cultured in rECM for 7-9 days (Figure 7) (Seewaldt, et al. 2001*a*). In contrast, early passage p53(-) HMEC-LX/E6 were sensitive to rECM-growth regulation and underwent apoptosis when cultured in rECM (Figure 7). This is consistent with our prior observations in early passage p53(-) HMECs. These results suggest that suppression of rECM growth arrest by a DNRAR inhibits rECM-mediated apoptosis in early passage p53(-) HMEC-E6 cells.

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) AG1134-LX/LX controls underwent growth arrest and formed an acinus-like structure in contact with rECM at Day 7 (data not shown), 2) early passage AG11134-LX/E6 cells were sensitive to rECM-growth regulation and exhibited morphologic evidence of apoptosis at Day 7 (data not shown), and 3) AG1134-DN/LX and AG1134-DN/E6 cells were resistant to r-ECM growth arrest and did not undergo apoptosis at Day 7-9 (data not shown).

Late passage p53(-) HMEC-E6 cells acquire resistance to rECM-induced apoptosis associated with chromosome abnormalities leading to preferential loss of chromosome arm 16p.

Retinoid-signaling and the actions of the DNRAR in HMECs are complex. To better define potential mediators of apoptosis-resistance, cytogenetic analysis was performed on p53(-) HMEC-E6 cells and controls. Our goal was to determine whether rECM-resistance correlated with the acquisition of specific chromosome aberrations and correlate this with the expression of known mediators of retinoid-signaling and/or apoptosis.

As previously described, no clonal abnormalities were found in early or late passage p53(+) HMEC-LXSN vector control cells (passages 10 and 17) or in parental HMECs (Seewaldt, et al. 2001*a*, Seewaldt, et al. 2001*b*). These results are consistent with karyotypes reported for "phase a" (pre-growth plateau) HMECs (Romanov et al., 2001).

Twelve of 21 cells, (57%) of early passage p53(-) HMEC-E6 metaphase cells (passage 10) were cytogenetically normal, including 3 cells with random chromosome loss. The remaining cells were aneuploid (Seewaldt, et al. 2001*a*, Seewaldt, et al. 2001*b*). In contrast to early passage cells, all 35 late passage p53(-) HMEC-E6 cells (passage 20) studied cytogenetically (27 using SKY and 8 using DAPI staining) were markedly abnormal and each cell exhibited multiple chromosomal losses and gene rearrangements (Seewaldt, et al. 2001*a*, Seewaldt, et al. 2001*b*). The most frequent losses involved the following chromosomal arms: 16p (26 cells, 74%), 12p (17 cells, 49%), 21p (17 cells, 49%), and 17p (14 cells, 40%). The high frequency of loss of 16p in late passage p53(-) HMEC-E6 cells suggests that 16p harbors a gene(s) whose loss and/or rearrangement may be important in the development of resistance to rECM-mediated growth control and to rECM-induced apoptosis. On further analysis, we hypothesized that the gene of importance may be located in the distal region of 16p, at 16p13, because one cell with 16p deletion retained material proximal to 16p12, while in another cell, an unbalanced translocation der(16)t(13;16)(q1?2;p13) affected band 16p13 (Figure 8). This band is a locus of the CBP gene encoding the CBP protein. CBP is both an important mediator of apoptosis, growth regulation, and retinoid-signaling (Yao, et al. 1998; Giles, et al. 1997).

Sensitivity and resistance to rECM-mediated growth regulation and apoptosis correlates with altered-regulation of CBP/p300 protein expression.

Expression of CBP and the related protein, p300, was tested in apoptosis-sensitive and –resistant p53(-) HMEC-E6 cells and controls. rECM-resistance in late passage p53(-)

HMEC-E6 cells correlated with a 41% ($p \geq 0.01$) and 87% ($p \geq 0.01$) decrease in CBP and p300 expression, respectively, relative to p53(+) HMEC LXSN controls (Figure 9). Increased sensitivity to rECM-mediated apoptosis in early passage p53(-) HMEC-E6 cells correlated with a 143% ($p \geq 0.01$) and 104% ($p \geq 0.01$) respective increase in CBP and p300 expression (Figure 9). These observations suggest a potential role for CBP/p300 in mediating sensitivity to rECM-mediated growth regulation and apoptosis.

CBP/p300 protein expression in rECM-resistant HMEC expressing the DNRAR.

Early passage p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells were resistant to rECM-mediated growth arrest and polarity. Early passage p53(-) HMEC-DN/E6 failed to undergo apoptosis when cultured in rECM. Both CBP and p300 protein levels were decreased in early passage p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells relative to early passage p53(+) HMEC-LX/LX controls (Figure 10). There was an 87% and 93% respective decrease in the level of CBP and a 93% and 96% respective decrease in the level of p300.

Suppression of CBP/p300 by an antisense approach in p53(+) and p53(-) HMECs.

Antisense ODNs were utilized to directly test the role of CBP and p300 expression in rECM-mediated growth regulation and apoptosis in early passage p53(+) HMEC-LXSN controls and early passage p53(-) HMEC-E6 cells. Suppression of CBP or p300 in HMECs was confirmed by Western analysis (Figure 11). Early passage p53(+) HMEC-LXSN vector controls and p53(-) HMEC-E6 cells treated with the active CBP-specific A3342V ODN exhibited a 95% and 92% respective decrease in CBP protein expression.

Values were obtained relative to untreated controls. No decrease in CBP was observed when early passage p53(+) HMEC-LXSN cells or p53(-) HMEC-E6 cells were treated with the scrambled CBP ODN, scrA3342V ODN (Figure 11). Early passage p53(+) HMEC-LXSN controls and p53(-) HMEC-E6 cells treated with the p300-specific active A3851T ODN exhibited a respective 85% and 90% decrease in p300 protein expression. There was a 23% decrease in early passage p53(+) HMEC-LXSN cells treated with the scrambled p300 ODN, scrA3851T (Figure 11). Early passage p53(-) HMEC-E6 cells treated with scrA3851T did not exhibit a decrease in p300 protein expression.

Suppression of CBP or p300 in early passage HMECs by an antisense approach enhances proliferation in rECM.

Treatment of early passage p53(+) HMEC-LXSN cells and early passage p53(-) HMEC-E6 cells with CBP- or p300-specific ODNs resulted in resistance to rECM-induced growth arrest. This was evidenced by a lack of rECM-growth inhibition at Day 7 to 9 (Figure 12). Treatment of early passage p53(-) HMEC-E6 cells or p53(+) HMEC-LXSN vector controls with scrambled ODNs did not alter rECM-mediated growth inhibition (Figure 12). These data suggest that suppression of CBP or p300 in p53(+) HMEC-LXSN controls or early passage p53(-) HMEC-E6 cells by an antisense approach promotes increased proliferation of cells in contact with rECM.

HMECs with suppressed CBP or p300 protein expression grown in contact with rECM lack polarized expression of E-cadherin

Early passage p53(-) HMEC-E6 cells and p53(+) HMEC-LXSN controls treated with either active CBP or p300 ODNs exhibited punctate, dispersed and intracellular staining consistent with a loss of epithelial polarity (Figure 13). In contrast, early passage p53(+) HMEC-LXSN controls and early passage p53(-) HMEC-E6 cells treated with either scrambled CBP or p300 ODNs demonstrated basolateral expression of E-cadherin, including lateral staining at cell-cell junctions, consistent with a polarized epithelium (Figure 13). Twenty cell clusters were surveyed per data point. These results indicate that suppression of either CBP or p300 in p53(+) HMEC-LXSN controls and early passage p53(-) HMEC-E6 cells by ODNs inhibits the formation of a polarized epithelium in rECM culture.

Suppression of CBP or p300 in early passage p53(-) HMEC-E6 cells by an antisense approach inhibits rECM-mediated apoptosis.

We tested whether suppression of CBP or p300 protein expression by ONDs blocked rECM-mediated apoptosis in early passage p53(-) HMEC-E6 cells. Early passage p53(-) HMEC-E6 treated with either CBP- or p300-specific antisense ODNs formed large irregular clusters in rECM and did not undergo apoptosis (Figure 14). There was no evidence of apoptosis in early passage p53(-) HMEC-LXSN controls treated with either CBP or p300 ODNs and cultured in rECM. Early passage p53(-) HMEC-E6 cells treated with either scrambled CBP or p300 ODNs underwent apoptosis (Figure 14). Early passage p53(+) HMEC-LXSN controls treated with either scrambled CBP or p300 ODNs underwent growth arrest (Figure 12) but not apoptosis (data not shown). These observations demonstrate that suppression of CBP or p300 protein expression in early

passage p53(-) HMEC-E6 cells by antisense ODNs inhibits rECM-mediated apoptosis.

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 AG1134-E6 cells and early passage AG11134-LXSN controls treated with CBP- or p300-specific antisense ODNs were resistant to rECM-mediate growth regulation and did not undergo apoptosis (data not shown), 2) early passage AG11134-E6 cells treated with scrambled CBP or p300 ODNs were sensitive to rECM-growth regulation and underwent apoptosis at Day 7 (data not shown), and 3) early passage AG1134-LXSN controls treated with scrambled CBP or p300 ONDs were resistant to r-ECM growth arrest and did not undergo apoptosis at Day 7-9 (data not shown).

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). However, there is significant evidence that growth arrest, differentiation, and survival signals may also promote apoptosis in genetically damaged cells (Seewaldt, et al., 1995; Hong and Sporn, 1997; Mancini, et al. 1997; Seewaldt, et al. 1997*b*; Seewaldt, et al. 2001*a*).

We previously observed that unlike early passage p53(-) HMECs, late passage p53(-) HMEC-E6 cells were resistant to rECM-induced growth arrest, polarity, and apoptosis (Seewaldt, et al. 2001*a*). In this report, we tested whether suppression of rECM-mediated growth arrest and polarity signaling in early passage p53(-) HMEC-E6 cells might block apoptosis. We previously observed that suppression of retinoid receptor function in p53(+) HMECs by DNRAR, RARα403, lead to loss of rECM-induced growth arrest and polarity (Seewaldt, et al. 1997*a*). Observations in this *in vitro* system are morphologically similar to what is observed in mammary hyperplasia without atypia .

Loss of retinoid-sensitivity is hypothesized to be an early event in mammary carcinogenesis (Widschwendter, et al. 1997; Xu, et al. 1997). Furthermore, loss of heterozygosity at the chromosomal region 3p22-25 containing the coding sequence for the RAR-β is the most frequent chromosomal aberration observed in morphologically benign lobules adjacent to breast cancer (Deng, et al. 1996). This suggests that in a subset of

sporadic breast cancers, a tumor suppressor gene at 3p22-25 may be important in the initiation of early progression of mammary tumorigenesis (Deng, et al. 1996). Recently, an alternative mechanism for the development of retinoid-resistance has been observed in breast cancer cells (Sommer, et al. 1999). RARβ4 is an alternatively spliced product of the RARβ P2 promoter and functions as a naturally occurring dominant-negative repressor of RAR-mediated growth suppression (Nagpal, et al. 1992). Expression of RARβ4 in mammary epithelial cells promotes the loss of growth regulation and epithelial polarity, similar to what is observed for the DNRAR (Berard, et al. 1994). Based on these observations, it appears that 1) the normal function of retinoid receptors may involve regulating the proliferation of normal mammary cells and 2) loss of receptor function might disrupt normal ECM-mediated tissue homeostasis and thus promote mammary hyperplasia.

When HPV-16 E6 was acutely expressed in DNRAR-transduced HMECs, the resulting early passage p53(-) HMEC-DN/E6 cells did not undergo apoptosis when grown in rECM and instead formed large, disorganized aggregates. These observations suggest that inhibition of rECM-induced growth arrest and polarity, by expression of a DNRAR, blocks induction of apoptosis in p53(-) HMEC-E6 cells.

The mechanism by which HMEC-ECM derived signals might regulate gene expression is poorly understood. It has been recently shown that rECM regulates both biomechanical and biochemical signaling events and, conversely, alterations in cell morphology can alter the response of cells to rECM (Howlet, et al. 1995; Roskelley, et al. 1994; Dhawan, et al.

1994). It is hypothesized that because malignant cells have an altered response to rECM, rECM-signaling pathways may utilize tumor suppressor checkpoints critical for cellular organization and polarity (Roskelley, et al. 1994; Dhawan, et al. 1994).

A preferential loss of chromosome arm 16p was observed in a majority (74%) of rECM-resistant, late passage p53(-) HMEC-E6 cells. One resistant cell exhibited loss distal to part of 16p13 and a second cell exhibited an unbalanced translocation involving 16p13. Loss of chromosome 16p correlated with the development of resistance to rECM-mediated growth arrest, polarity, and apoptosis (Seewaldt, et al. 2001*a*). The high frequency of 16p loss suggested that loss of a particular gene or genes located in this chromosome arm, likely in its distal region at 16p13, may be critical for the development of resistance to rECM.

Chromosome 16p13 is the location of the human CBP, a nuclear protein important for p53-function, growth regulation, and apoptosis (Yao, et al. 1998; Giles, et al. 1997). CBP is a tightly regulated transcription factor that plays a critical regulatory role as an integrator of diverse signaling pathways including those mediated by estrogen and retinoids (Kawasaki, et al. 1998; Robyr, et al. 2000). Given the important role CBP plays in both apoptosis and maintenance of a normal mammary phenotype, we hypothesize that modulation of CBP expression could either promote or suppress resistance to rECM-signaling pathways that might target the apoptotic elimination of p53(-) HMECs.

We compared protein expression of CBP, and the related protein, p300, in apoptosis-sensitive and -resistant HMECs. CBP and p300 protein expression was suppressed in rECM-resistant, late passage p53(-) HMEC-E6 cells and early passage p53(-) HMEC-DN/E6 cells. Current models suggest that CBP and p300 are present in limiting amounts and transcriptional regulation may be, in part, achieved through competition for these cofactors (Kawasaki, et al. 1998). Full suppression of CBP/p300 in mouse "knock out" models is embryonic-lethal and only partial suppression of CBP/p300 is required for a phenotype. Inhibition of either CBP or p300 protein expression in apoptosis-sensitive, early passage p53(-) HMEC-E6 cells by antisense ODNs resulted in loss of rECM-mediated growth arrest polarity and apoptosis. Taken together these observations suggest a critical role for CBP and p300 expression in regulating apoptosis in p53(-) HMEC-E6 cells grown in rECM.

We observe that both CBP and p300 play a role in rECM-mediated growth arrest and apoptosis. While other investigators have observed that both p300 and CBP play an important role in retinoic acid-induced apoptosis, these co-activators appear to play a distinct role during retinoic acid-induced differentiation in F9 cells (Kawasaki, et al. 1998; Ugai, et al. 1999). It is possible that the difference between these previous studies and our results can be accounted for by differences in cell type or perhaps due to the presence or absence of rECM.

Our model system predicts that 1) ECM-mediated growth arrest signals may play an important role in eliminating HMECs that acutely develop loss of p53 function and 2)

inhibition of these critical regulatory signals, including CBP/p300 expression, might promote the clonal expansion of damaged HMECs. These observations have important clinical implications by suggesting that the 1) development of mammary hyperplasia, 2) loss of chromosome 16p, or 3) perhaps, loss of retinoic acid receptor function might significantly increase the risk of breast cancer in women who are at high risk for p53 mutations.

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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; **RAR**, retinoic acid receptor; **DNRAR**, dominant-negative retinoic acid receptor; **CBP**, CREBP binding protein.

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Table 1: CBP- and p300-specific antisense ODN sequences

Target gene	Sequences	Size	Status
CBP			
A3342V	5'-CACTTCAGGTTTCTTTTCATCC -3'	22 bp	Active
A2172Z Inactive	5'-CTTCTAGTTCTTTTTGTATCTTGTAG-3'	26 bp	
scrA3342V Inactive	5'- ATTCTCATCATCGTCTTCGTTC-3'	22 bp	
p300			
A3851T	5'- TTGTTGGTGGTGTAGGTGTC-3'	20 bp	Active
A5765T	5'-GTTTTCTCTCTTCTTCCTCC-3'	20 bp	Active
A2811W Inactive	5'-CTCATCATTGGGTTTTGAGAATT-3'	23 bp	
scrA3851T Inactive	5'-TTTAGTGCGTTGGTGGTGTG-3'	20 bp	

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

Figure 1: Schema for inhibition of RAR function and suppression of p53 expression in HMECs. RAR function was first suppressed by infection with LRARα403SN **(DNRAR)**, control cells were transduced with the LXSN retroviral vector (without insert) **(LXSN)**, and cells were selected with G418. p53 function was suppressed by infection with the LXSHE6 retroviral vector **(E6)**, control cells were transduced with the LXSH retroviral vector **(LXSH)**, and cells were selected with hygromycin.

Figure 2: Expression of dominant-negative construct LRARα403SN in HMECs. Northern analysis was performed on tRNA extracted from p53(-) HMEC-LX/LX vector controls **(LX/LX)** (passage 12), early passage p53(-) HMEC-E6 cells **(LX/E6)** (passage 12), late passage p53(-) HMEC-E6 cells **(E6(L))** (passage 24), p53(+) HMEC-DN/LX cells **(DN/LX)** (passage 12), and p53(-) HMEC-DN/E6 cells **(DNRAR/E6)** (passage 13). The blot was probed with a 1.3-kb (SmaI) RAREα cDNA fragment. Ten micrograms of total RNA were loaded in each lane. 36B4 served as a loading control.

Figure 3: p53 protein expression is suppressed in HMECs transduced with HPV-16 E6. p53(+) HMEC-LX/LX vector controls **(LX/LX)** (passage 12), p53(+) HMEC-DN/LX cells **(DN/LX)** (passage 13), early passage p53(-) HMEC-LX/E6 cells **(LX/E6)** (passage 12), and p53(-) HMEC-DNRAR/E6 cells **(DNRAR/E6)** (passage 12) were analyzed for p53 protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. A Coomassie blue-stained unknown 45 kD protein band served as a loading control.

Figure 4: Inhibition of RAR function by a DNRAR results in resistance to ATRA-mediated growth inhibition in p53(+) and p53(-) HMECs. Growth curves of ATRA-treated HMEC-LX/LX vector controls (passage 12) *(a)*, p53(+) HMEC-DN/LX cells (passage 11) *(b)*, early passage p53(-) HMEC-LX/E6 transduced cells (passage 12) *(c)*, and p53(-) HMEC-DN/E6 cells (passage 12) *(d)*. Cells were plated on Day –1 in duplicate and treated on Day 0 with Standard Media containing 0, 0.1, 1.0, and 10 μM ATRA. Each well was counted in triplicate.

Figure 5: Inhibition of RAR function by a DNRAR results in loss of rECM-induced growth arrest in both p53(+) and p53(-) HMECs. The mean diameter of spheres formed by p53(+) HMEC-LX/LX vector controls (passage 12) *(a)*, p53(+) HMEC-DN/LX cells (passage 11) *(a)*, early passage p53(-) HMEC-LX/E6 cells (passage 13) *(b)*, and p53(-) HMEC-DN/E6 cells (passage 12) *(b)* were plotted as a function of days in culture. Cells were plated in prepared basement membrane on Day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. The 20 largest colonies were measured at each time point.

Figure 6: Localization of E-cadherin in HMECs expressing a DNRAR using immunofluorescence microscopy. Frozen section of early passage p53(+) HMEC-LX/LX vector controls (passage 11) *(a)*, p53(-) HMEC-LX/E6 cells (passage 11) *(b)*, p53(+) HMEC-DN/LX cells *(c)*, and p53(-) HMEC-DN/E6 cells (passage 12) *(d)* grown in rECM for 6 days, cryosectioned, and stained with a monoclonal antibody to E-cadherin as described in Materials and Methods. E-cadherin was localized at the basolateral surface and at points of cell-cell contact in p53(+) HMEC-LX/LX cells and in p53(-) HMEC-LX/E6 cells *(a, b,* arrows*)*. In contrast, p53(+) HMEC-DN/LX and p53(-) HMEC-DN/E6 cells showed punctate, dispersed membrane and intracellular staining *(c, d,* arrowheads*)*.

Figure 7: Inhibition of RAR function in early passage p53(-) HMECs cells blocks rECM-mediated apoptosis. *(a)* Electron micrographs of p53(+) HMEC-LX/LX vector control cells (passage 10) grown in rECM for 9 days. p53(+) HMEC-LX/LX control cells formed acini-like structures that demonstrated a central lumen surrounded by correctly polarized luminal cells consistent with differentiated mammary glandular epithelium: 1) nuclei were predominantly aligned with the luminal surface **(n)**, 2) secretory vesicles **(v)** were present on the luminal surface and not on the basal surface, and 3) mitochondria were primarily located at the basal surface. *(b)* Early passage p53(-) HMEC-LX/E6 cells (passage 10) grown in rECM for 9 days demonstrated evidence of apoptosis: 1) nuclear condensation, 2) cell shrinkage and separation, 3) margination of chromatin **(mr)**, and 4) apoptotic bodies **(ap)** containing cytoplasmic elements. *(c)* Day 9 culture of p53(+) HMEC-DN/LX cells (passage 12) whose retinoid receptor was inhibited by a DNRAR formed large, dense, irregularly shaped multicellular colonies with no central lumen and did not undergo apoptosis. *(d)* Day 9 culture of p53(-) HMEC-DN/E6 also demonstrated large, multilayered collections of cells and did not undergo apoptosis.

Figure 8: Partial karyotypes of a representative late p53(-) 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 9: CBP/p300 protein expression is suppressed in apoptosis-resistant late passage p53(-) HMEC-E6 cells and p53(+) HMEC-DN/E6 cells. p53(+) HMEC-LX/LX vector controls **(LX/LX)** (passage 12), early passage p53(-) HMEC-LX/E6 cells **(LX/E6)** (passage 12), p53(+) HMEC-DN/LX cells **(DN/LX)** (passage 11), late passsge p53(-) HMEC-E6 cells **(E6(L))** (passage 20), and p53(-) HMEC-DN/E6 cells **(DN/E6)** (passage 11) were analyzed for CBP/p300 protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin was used as a loading control.

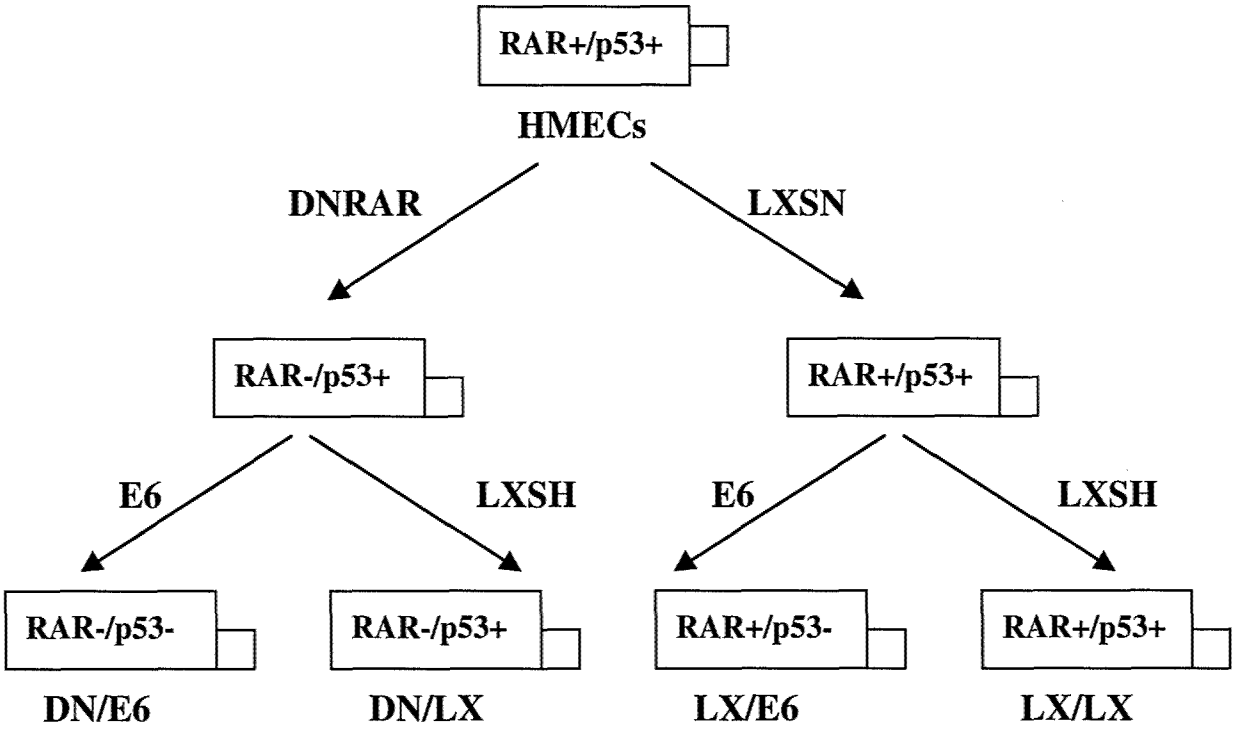
Figure 10: CBP/p300 protein expression is suppressed in HMECs transduced with the DNRAR RARα403. p53(+) HMEC-LX/LX vector controls **(LX/LX)** (passage 12), early passage p53(-) HMEC-LX/E6 cells **(LX/E6)** (passage 12), p53(+) HMEC-DN/LX cells **(DN/LX)** (passage 13), and early passage p53(-) HMEC-DNRAR/E6 cells **(DNRAR/E6)** (passage 12) were analyzed for CBP and p300 protein expression as described in Materials and Methods. Equal amounts of protein lysate were loaded per lane. Actin served as a loading control.

Figure 11: CBP/p300 protein expression is suppressed by antisense ODNs in p53(+) HMEC-LXSN vector control cells and early passage p53(-) HMEC-E6 cells. p53(+) HMEC-LXSN vector controls **(LXSN)** (passage 12) and early passage p53(-) HMEC-E6 cells **(E6)** (passage 12) were cultured in the presence of **(1)** no treatment, **(2)** scrambled p300 ODN (scrA3851T), **(3)** scrambled CBP ODN (A3342V), **(4)** p300 antisense ODN (asA3851T), and **(5)** CBP antisense ODN (asA3342V). Resultant cells were analyzed for CBP/p300 protein expression as described in Materials and Methods. One hundred micrograms of protein lysate were loaded per lane. Actin was used as a loading control.

Figure 12: Inhibition of CBP or p300 expression by an antisense approach results in loss of rECM-induced growth arrest in both p53(+) and p53(-) HMECs. The mean diameter of spheres formed by HMEC-LXSN vector controls (passage 10) *(a, b)* and p53(-) HMEC-E6 cells (passage 11) *(c, d)* treated with either 1) CBP antisense ODN (A3342V) **(CBP-as)** or scrambled CBP ODN (A3342) **(CBP-scr)** *(a, c)* or 2) p300 antisense ODN (A3851T) **(p300-as)** or scrambled p300 ODN (A2811W) **(p300-scr)** *(b, c)* were plotted as a function of days in culture. Cells were plated in prepared basement membrane on Day 0, and the diameter of growing spherical cell colonies was measured with an eye piece equipped with a micrometer spindle. The 20 largest colonies were measured at each time point.

Figure 13: Localization of E-cadherin in HMECs treated with CBP and p300 antisense ODNs using immunofluorescence microscopy. Frozen section of early passage p53(+) HMEC-LXSN vector controls (passage 11) *(a, c, e, g)*, p53(-) HMEC-E6 cells (passage 11) *(b, d, f, h)*, treated with either CBP antisense ODN (A3342V) *(a, b)*, scrambled CBP ODN (A3342) *(c, d)*, p300 antisense ODN (A3851T) *(e, f)*, or scrambled p300 ODN (A2811W) *(g, h)* grown in rECM for 6 days, cryosectioned, and stained with a monoclonal antibody to E-cadherin as described in Materials and Methods. E-cadherin was localized at the basolateral surface and at points of cell-cell contact in p53(+) HMEC-LXSN and p53(-) HMEC-E6 cells treated with either scrambled CPB or scrambled p300 ODNs *(c, d, g, h* arrows*)*. In contrast, p53(+) HMEC-LXSN and p53(-) HMEC-E6 cells treated with either antisense CPB or antisense p300 ODNs showed punctate, dispersed membrane and intracellular staining *(a, b, e, f* arrowheads*)*.

Figure 14: Inhibition of CBP or p300 by an antisense approach in early passage p53(-) HMECs blocks rECM-mediated apoptosis. *(a, c)* Electron micrographs of p53(+) HMEC-LXSN vector control cells (passage 10) with suppressed CBP *(a)* or p300 *(c)* function grown in prepared rECM for 9 days. p53(+) HMEC-LXSN control cells treated with either CBP antisense (A3342V) ODN *(a)* or p300 antisense (A3851T) ODN *(c)*. Cells formed large, dense, irregularly shaped multicellular colonies with no central lumen and did not undergo growth arrest in culture. *(b, d)* Similarly, early passage p53(-) HMEC-E6 cells (passage 10) treated with either CBP antisense (A3342V) *(b)* or p300 antisense (A3851T) ODN *(d)* grown in prepared extracellular matrix for 9 days formed irregular multilayered colonies. In contrast, early passage p53(-) HMEC-E6 cells (passage 10) treated with either scrambled CBP ODN (scrA3342V) *(e)* or scrambled p300 ODN (scrA3851T) *(f)* underwent apoptosis when grown in rECM for 9 days as evidenced by 1) nuclear condensation **(n)**, 2) cell shrinkage and separation, and 3) margination of chromatin **(mr)**.


RAR+/p53+
HMECs
DNRAR
LXSN
RAR-/p53+
RAR+/p53+
E6
LXSH
E6
LXSH
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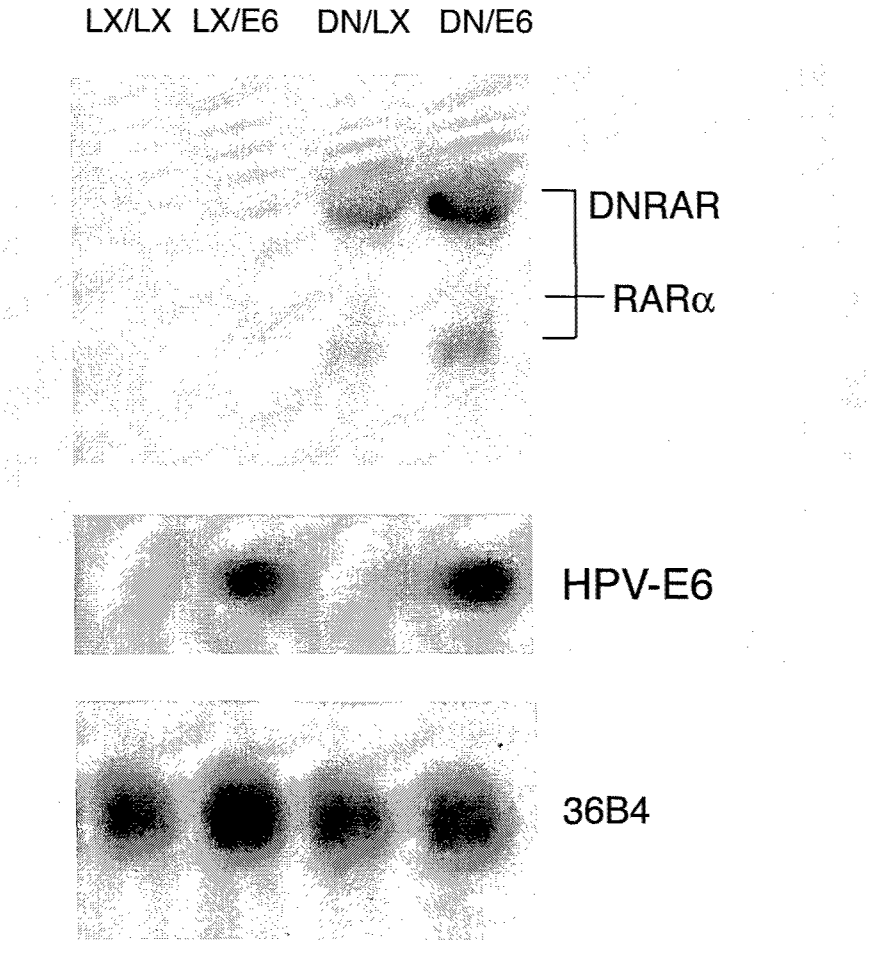
LX/LX

HMEC



HMEC

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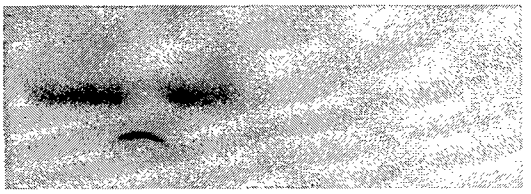
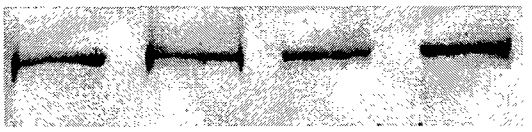


p53



loading

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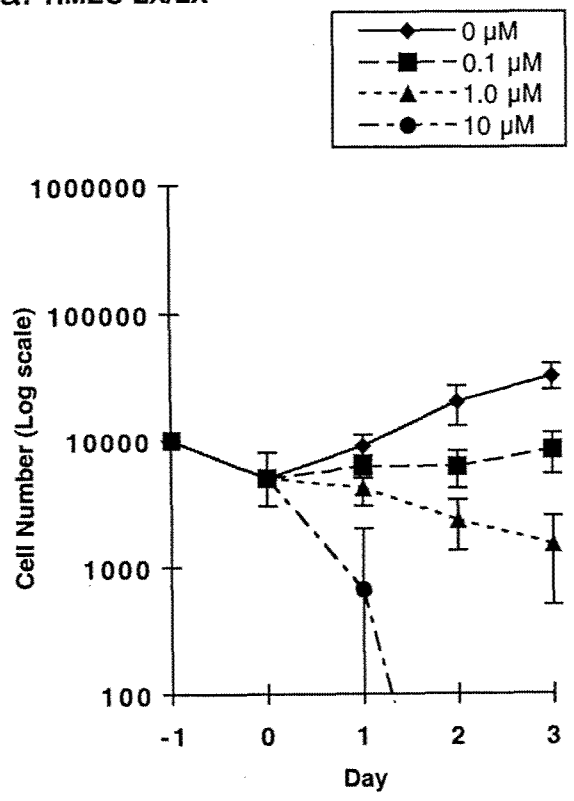

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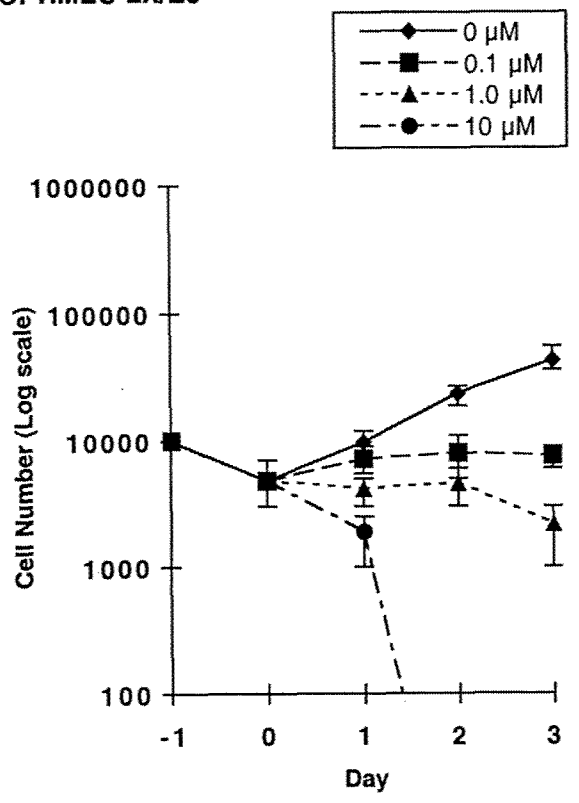

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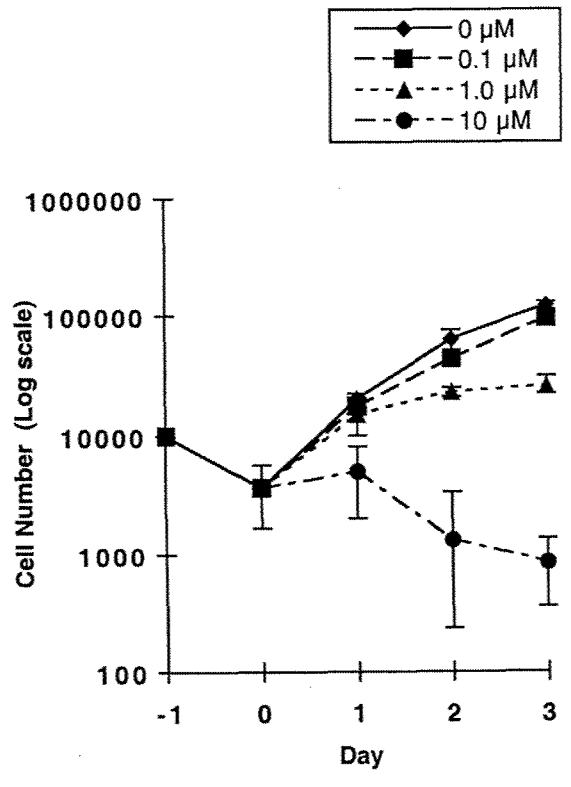

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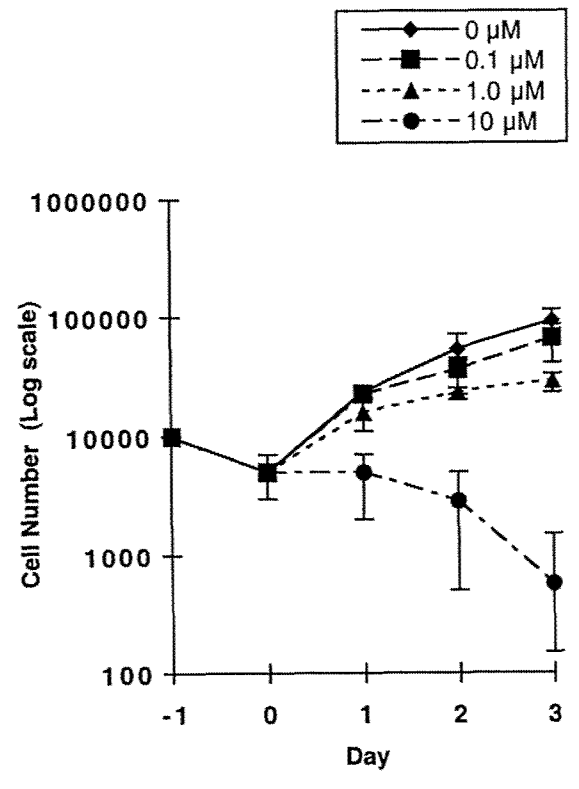

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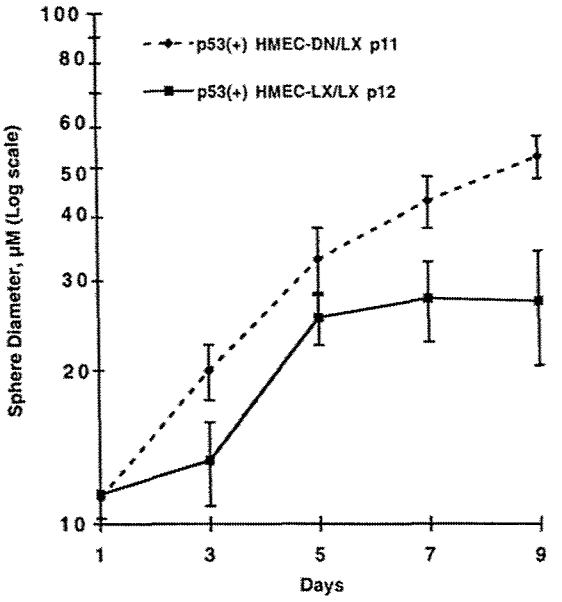
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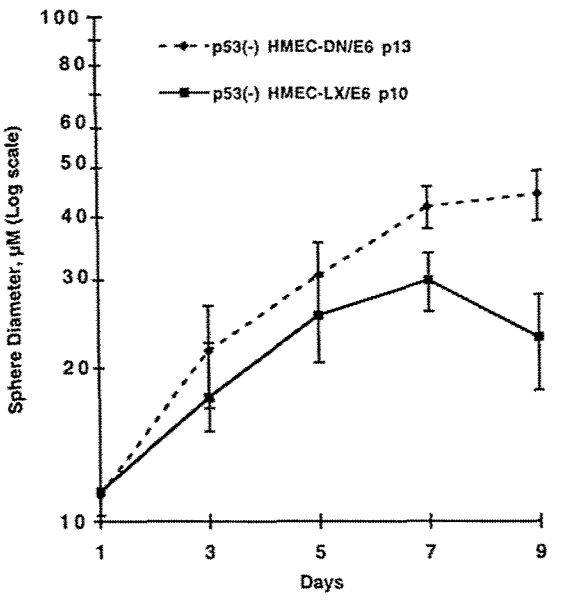
p53(+) HMEC-LX/LX p12
Sphere Diameter, µM (Log scale)
100
80
60
50
40
30
20
10
1
3
5
7
9
Days

b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p53(-) HMEC-LX/E6 p10
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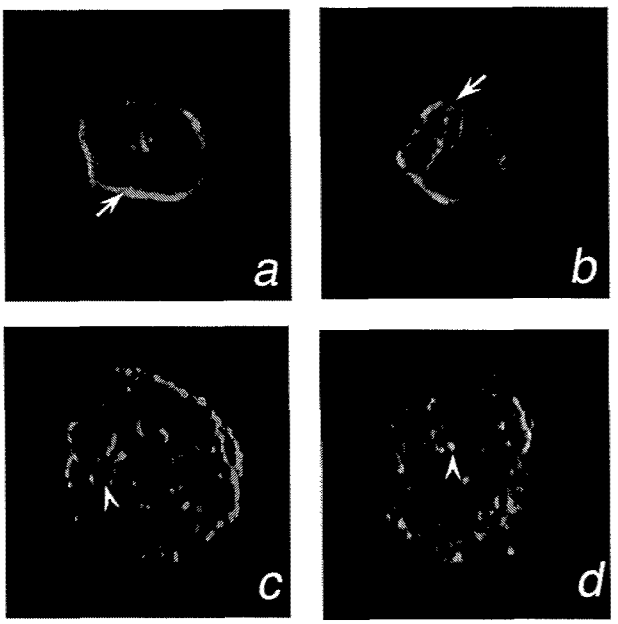
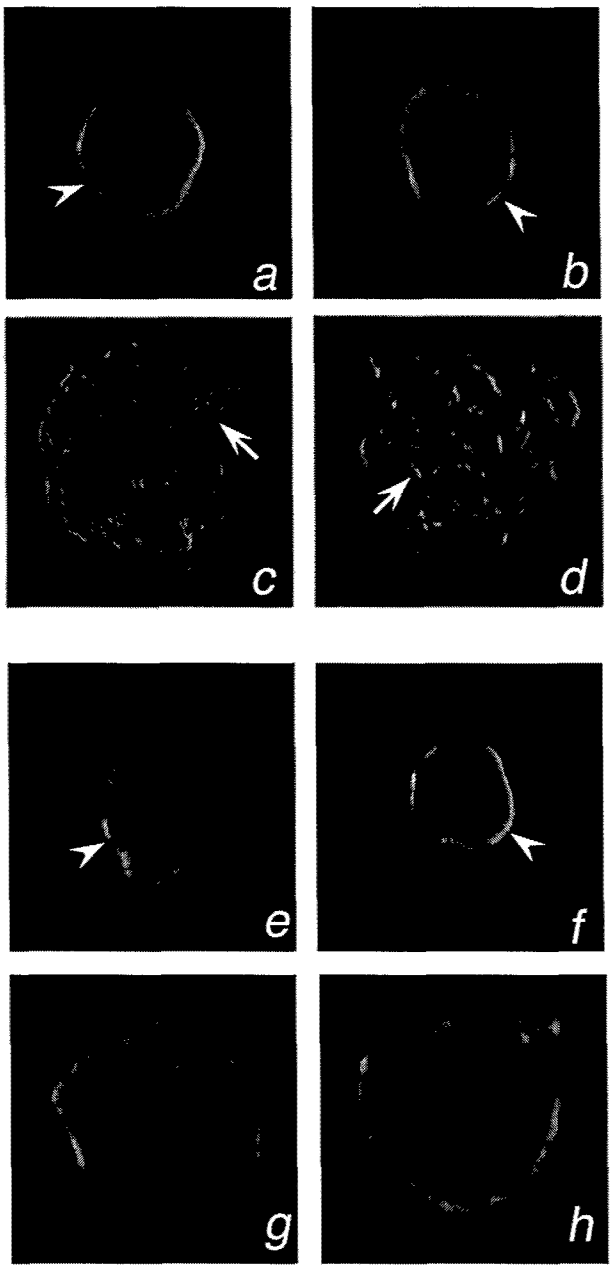
30
20
10
1
3
5
7
9
Days


a
b
c
d
25 μm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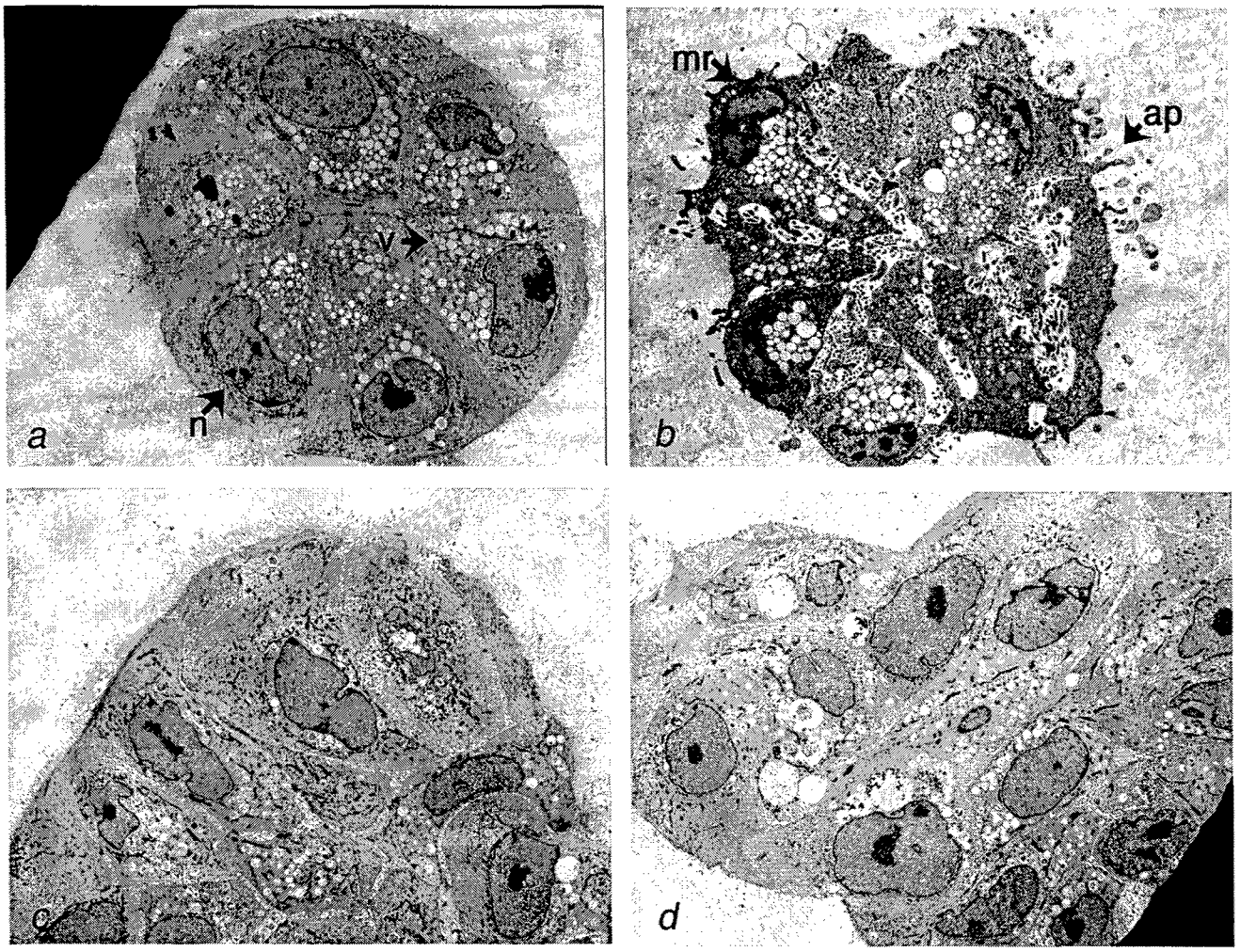
b
c
d
e
f
g
h
25 μm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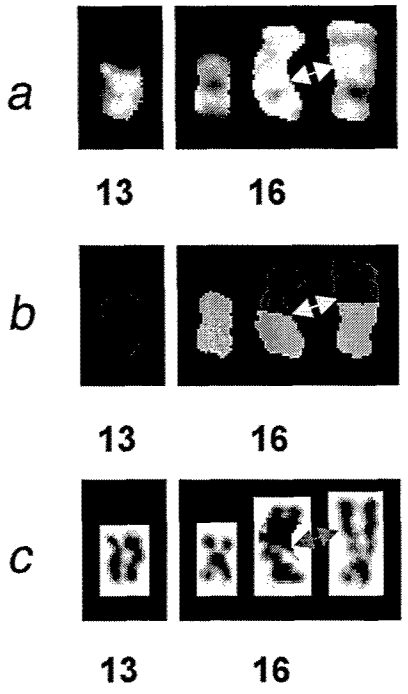
v
n
b
mr
ap
c
d
10 µm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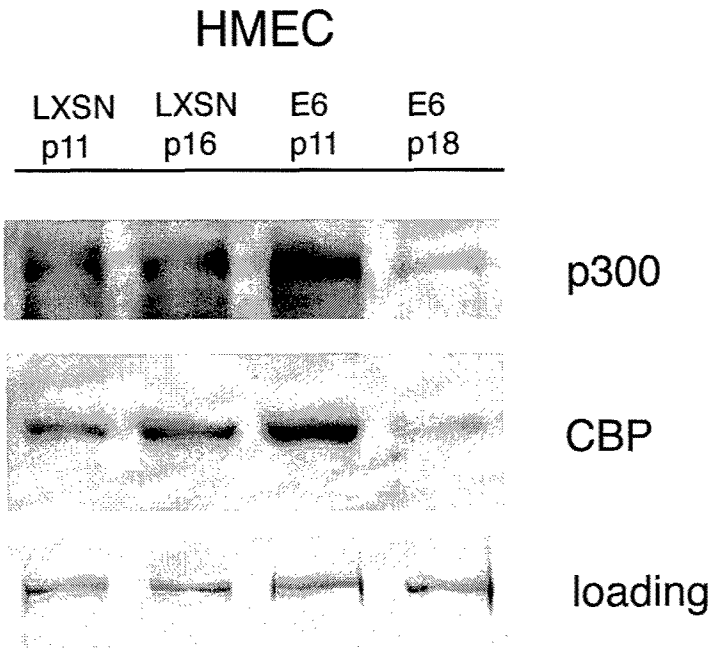
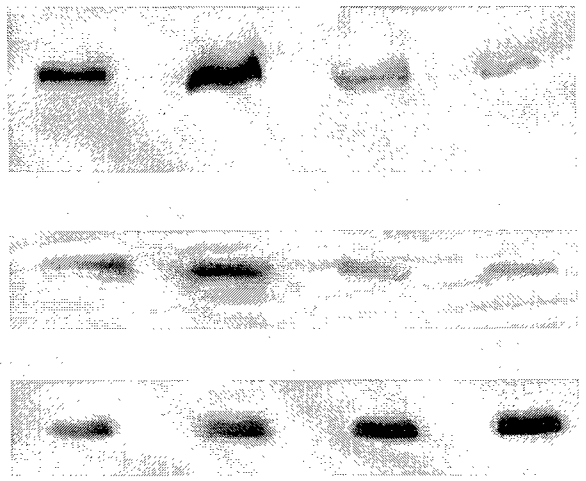
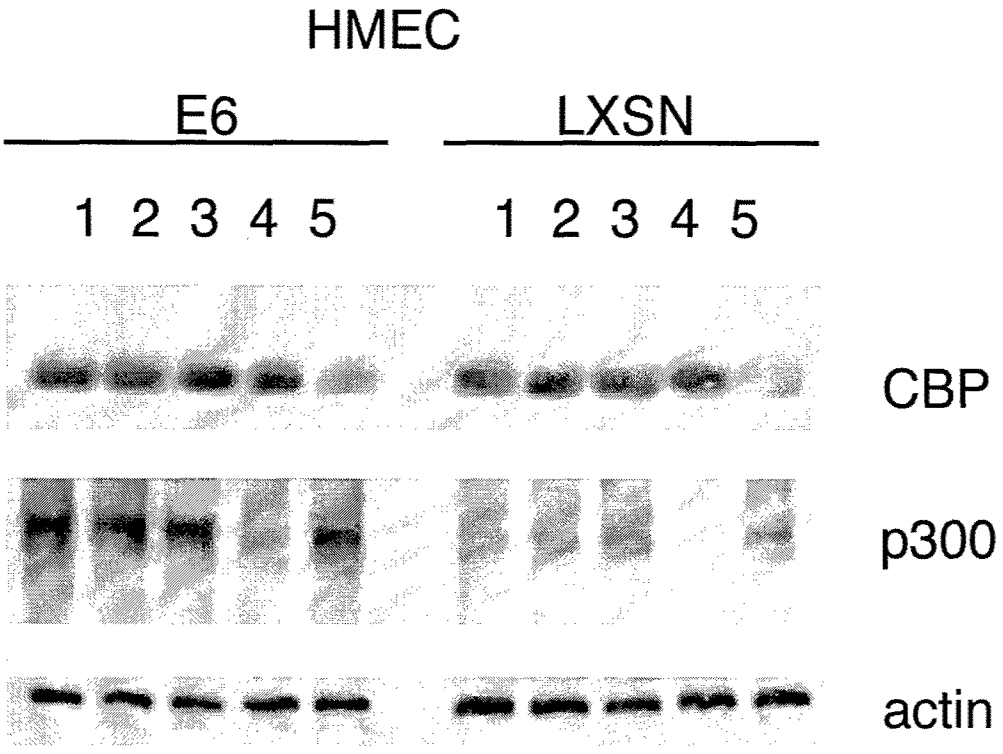
13
16
b
13
16
c
13
16

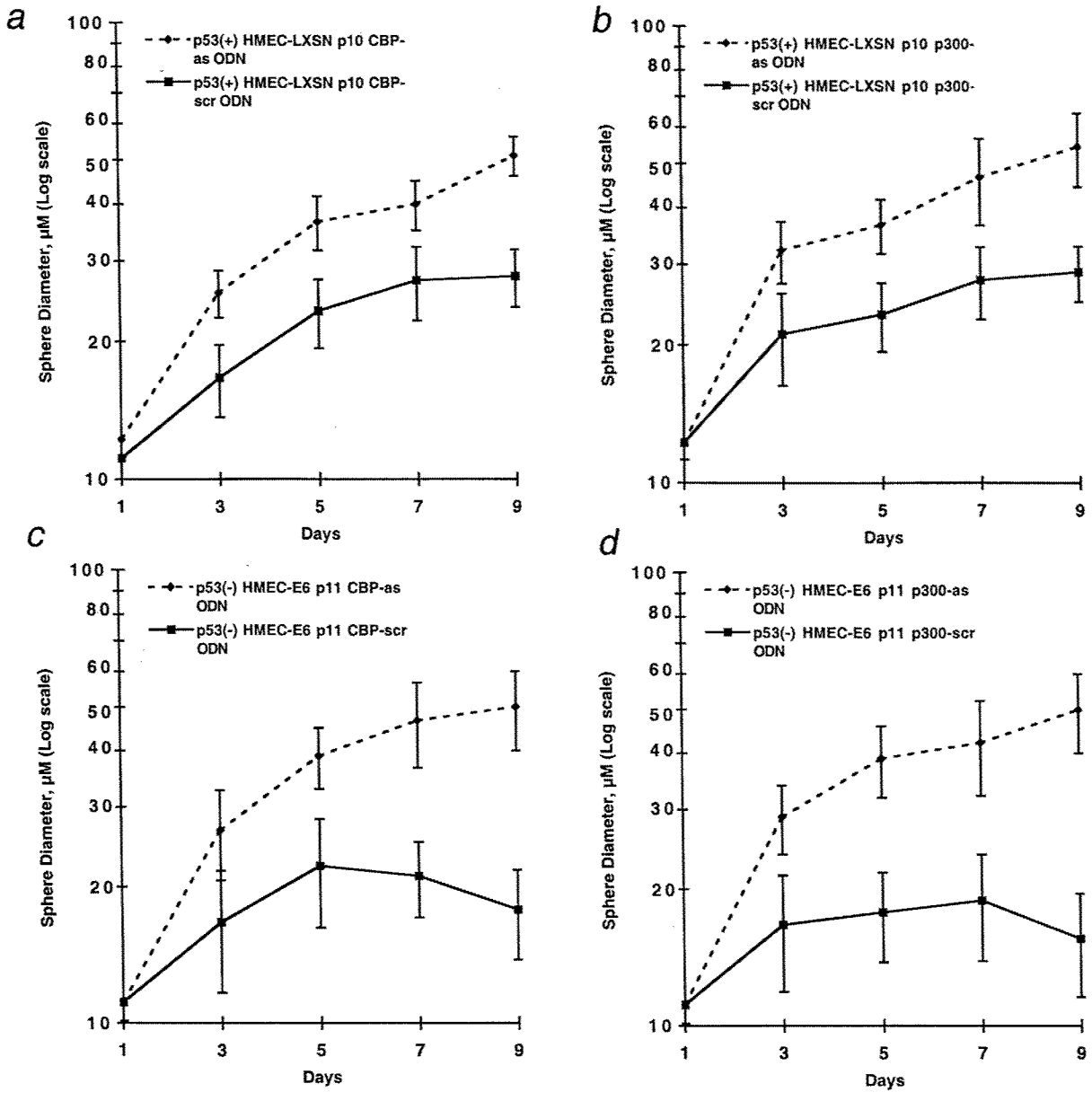

HMEC
LXSN p11
LXSN p16
E6 p11
E6 p18
p300
CBP
loading

HMEC

LX/LX LX/E6 DN/LX DN/E6




HMEC
E6
LXSN
1 2 3 4 5
1 2 3 4 5
CBP
p300
actin


a
p53(+) HMEC-LXSN p10 CBP-as ODN
p53(+) HMEC-LXSN p10 CBP-scr ODN
Sphere Diameter, µM (Log scale)
Days
b
p53(+) HMEC-LXSN p10 p300-as ODN
p53(+) HMEC-LXSN p10 p300-scr ODN
Sphere Diameter, µM (Log scale)
Days
c
p53(-) HMEC-E6 p11 CBP-as ODN
p53(-) HMEC-E6 p11 CBP-scr ODN
Sphere Diameter, µM (Log scale)
Days
d
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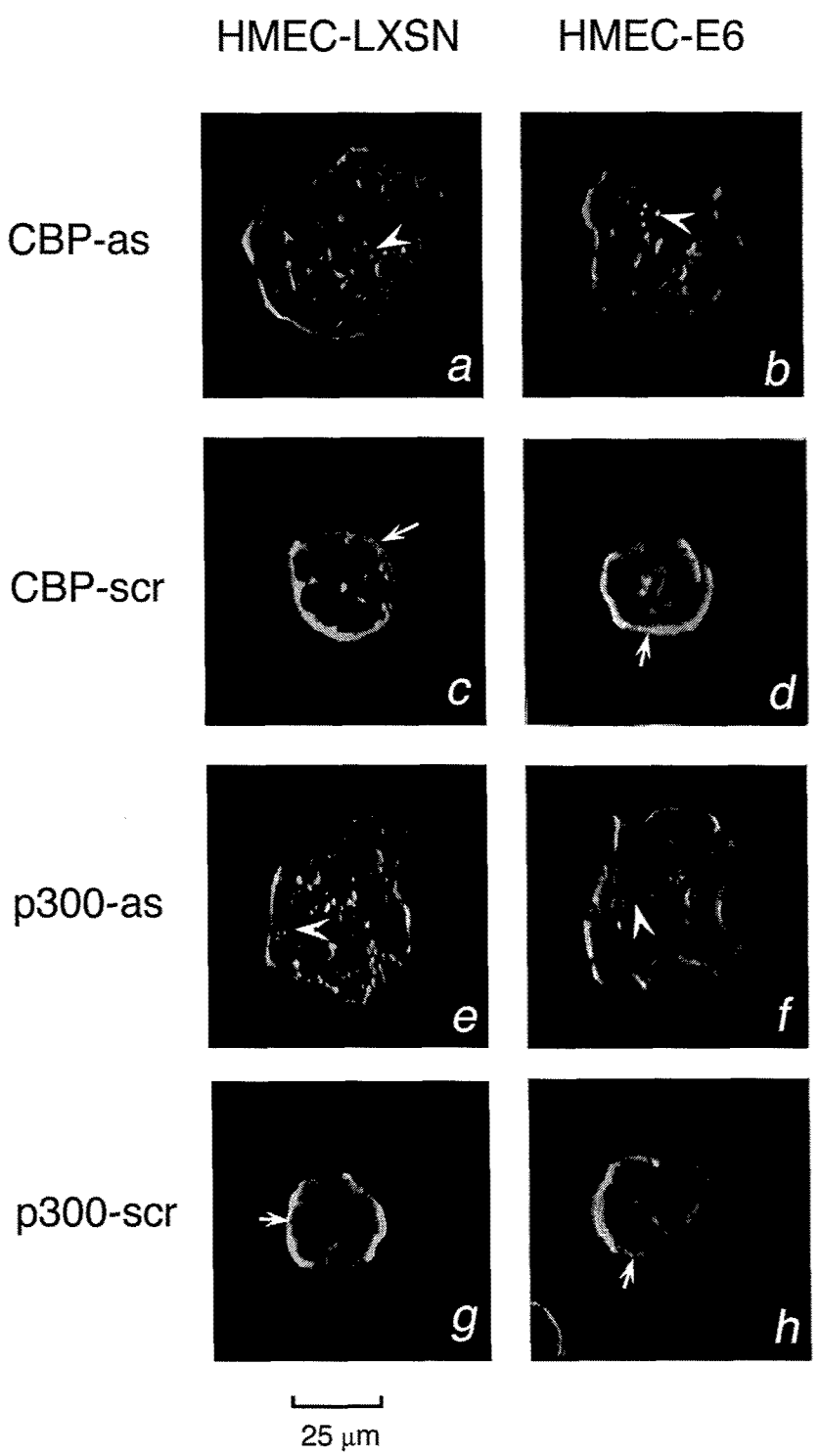
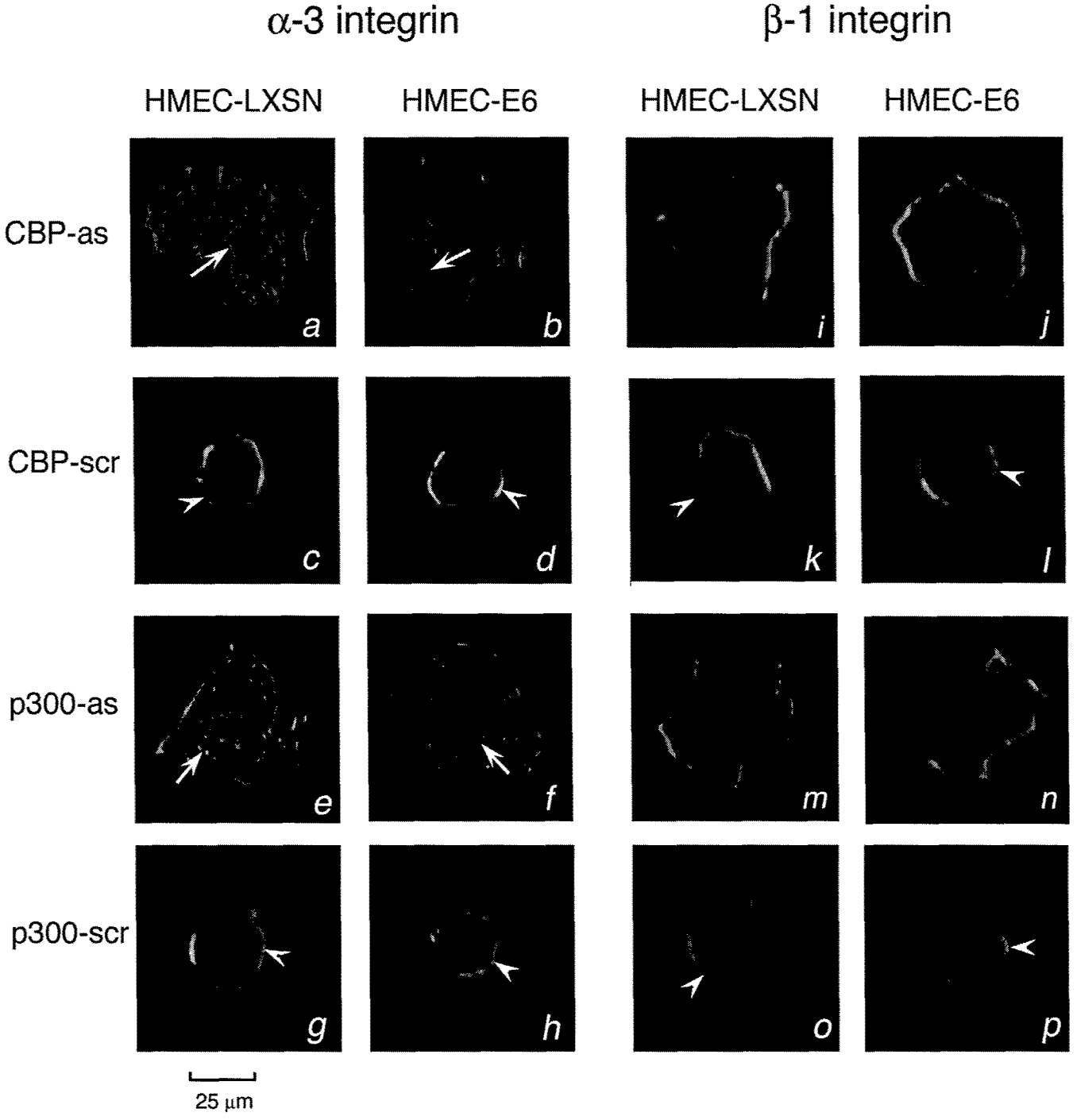
Sphere Diameter, µM (Log scale)
Days


HMEC-LXSN
HMEC-E6
CBP-as
CBP-scr
p300-as
p300-scr
a
b
c
d
e
f
g
h
25 μm


α-3 integrin
β-1 integrin
HMEC-LXSN
HMEC-E6
HMEC-LXSN
HMEC-E6
CBP-as
CBP-scr
p300-as
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a
b
i
j
c
d
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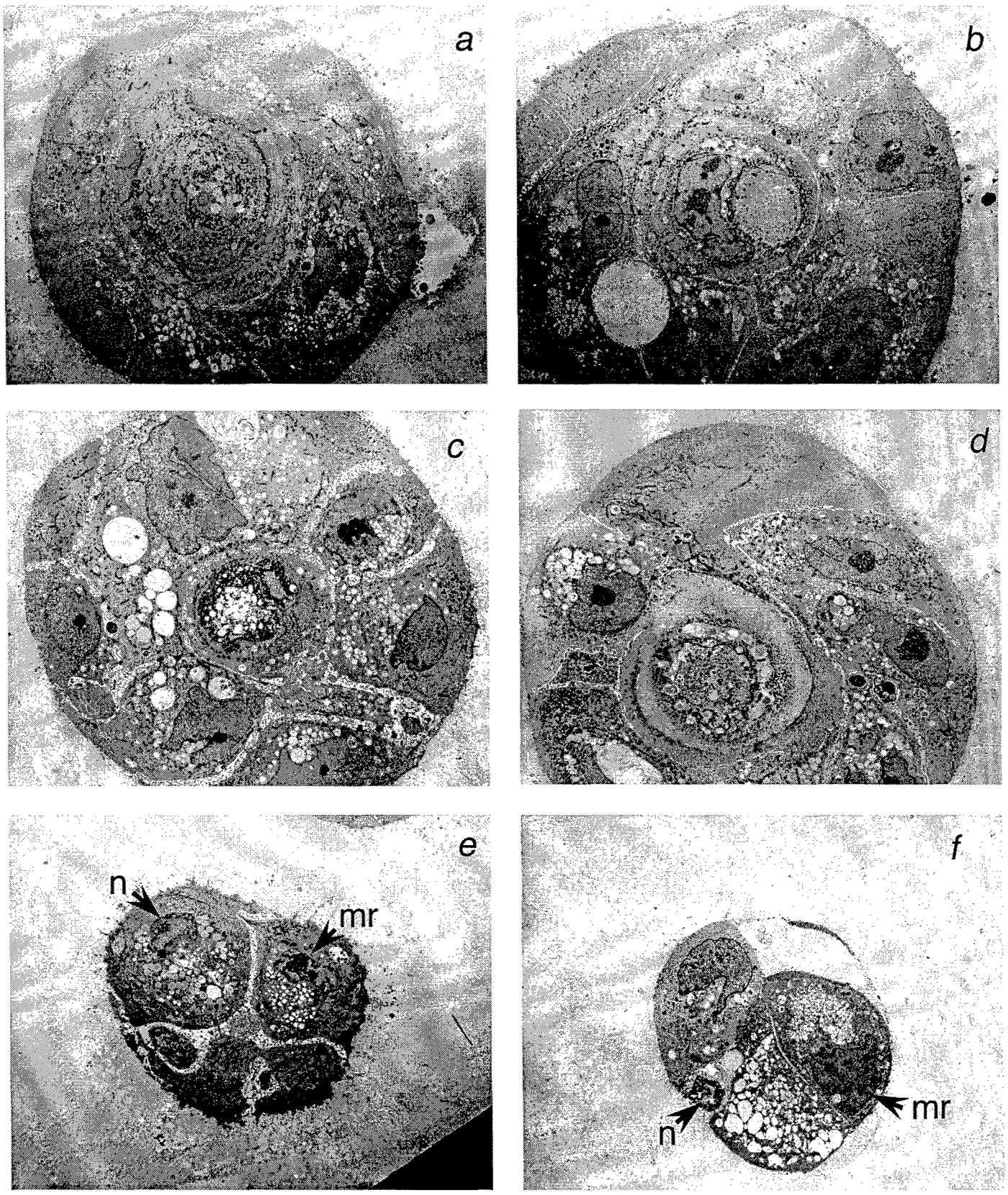
e
f
m
n
g
h
o
p
25 μm


a
b
c
d
e
n
mr
f
n
mr
10 μm

A Functional Retinoic Acid Response Element in the Promoter Regions of CBP and p300: A Rational for Retinoid-Based Chemoprevention [1]

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Running Title: Transcriptional Regulation of CBP and p300

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Key words: retinoic acid response element, all-*trans*-retinoic acid, retinoic acid receptor β2, CBP, and p300.

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[3] The abbreviations used are: CBP, CREB binding protein; RAR, retinoic acid receptor; RXR, retinoid X receptor; HPV, human papillomavirus; RTS, Rubenstein-Taybi Syndrome; HMEC, human mammary epithelial cell; DNRAR, dominant negative RARα403; RARE, retinoic acid response element; ATRA, all-*trans*-retinoic acid; TTBS, 0.1% Tween 20-Tris buffered saline; AS-, antisense; ODN-, oligodeoxynucleotide; Scr-, scrambled; rECM, prepared extracellular matrix; TSA, trichostatin A.

Abstract

CBP and p300 are known co-activators of retinoid-signaling, however, the transcriptional regulation of CBP/p300 is poorly characterized. Two systems were used to test the role of retinoids and retinoic acid receptors (RARs) in regulating CBP/p300 transcription: 1) retinoid-resistant MCF-7 cells were transduced with RARβ2 and 2) retinoid sensitivity was blocked in normal human mammary epithelial cells (HMECs) using the dominant negative RAR, RARα403. Expression of RARβ2 in MCF-7 cells promoted induction of CBP/p300 by ATRA. Inhibition of RAR function in HMECs blocked ATRA-mediated induction of CBP/p300. Retinoic acid response elements were identified in the promoters of CBP and p300; ATRA-inducible, RARβ2-mediated *trans*-activation was demonstrated. These observations indicate that retinoids and RARs regulate CBP/p300 expression through a RARE. Since CBP and p300 are 1) normally present in limiting amounts and 2) recruited during ATRA-mediated transcriptional, their regulation by ATRA and RARs may be important for transcriptional control of ATRA-regulated gene expression and the efficacy of retinoid-based chemoprevention.

Introduction

CBP and p300 are: 1) important coregulators of retinoid-, p53-, and nuclear hormone receptor-signaling, 2) required for BRCA1-mediated repair of double-stranded DNA breaks, 3) and hypothesized to be tumor suppressor genes (1-5). The ability of various signaling pathways to regulate transcription depends on the particular pathway's ability to compete for CBP and/or p300 (1, 2). The activity of CBP and p300 can be altered by phosphorylation and binding of proteins such as high risk HPV E6 protein, SV40 large T antigen, and adenovirus E1A protein (1, 2, 6). CBP or p300 loss has been observed in AML, treatment related CML and MDS, gastric cancer, and colorectal cancer as well as cell lines derived from lung, breast, and pancreatic cancer cells (3, 5, 7). CBP levels are decreased in Rubinstein-Taybi syndrome in which a loss of activity in one CBP allele is observed and is associated with developmental defects, an increased risk of cancer, and premature breast development (3, 8).

Retinoids are important mediators of growth and differentiation in epithelial cells and have been successfully utilized as chemoprevention agents (9-15). The majority of retinoid actions are mediated through specific nuclear retinoid receptors, RARs and RXRs, which act as transcription regulators and exhibit cross talk with estrogen- and prostaglandin-signaling pathway (9, 10). RARβ2 is unique because it is primarily expressed in epithelial cells and is positively regulated by retinoids and the RARβ2 P2 promoter retinoic acid response element (RARE) (9). RARβ2 is tumor suppressor in

breast and lung cancer and progressive loss of RARβ2 expression is observed during epithelial carcinogenesis (9,10). Importantly, while retinoids and RARβ2 mediate growth arrest and differentiation in HMECs; restoration of RARβ2 function in breast cancer cells promotes apoptosis (11, 15, 16).

Loss of RARβ2 expression has been observed in oral dysplasia as well as breast, lung, prostate, cervical, esophageal, and gastric carcinomas (9, 10) and is hypothesized to be the result of both genetic and epigenetic events. Two mechanisms have been proposed: 1) loss of heterozygosity (LOH) and 2) promoter methylation (17, 18). Loss of heterozygosity at the *RARβ2* loci (3p24) is frequently observed in breast cancers and is thought to be a late mechanism for retinoid-resistance (17). In contrast, RARβ2 P2 promoter methylation has been observed in atypical and dysplastic mammary epithelial cells and is thought to be an important early mechanism for loss of RARβ2 expression (18).

Recently, p300, but not CBP, was shown to be transcriptionally regulated by BRCA1 in breast and prostate cancer cell lines (19). In these cell lines, BRCA1 downregulated p300 mRNA. T he d ecrease in p300 resulted in inhibition of ERα activity, which could be rescued by overexpression of either p300 or CBP in prostate cells (19). Furthermore, the 9-*cis*-retinoic acid treated oral carcinoma cell line HSC-3 showed increased levels of CBP and p300 protein expression (20). These data suggest that, like p300 and BRCA1, CBP and p300 levels may be directly regulated by retinoids and that transcriptional

regulation of CBP and p300 levels may be important in determining global transcriptional coregulation by CBP and p300 activity.

In this study we observe that CBP and p300 have functional RAREs in their respective promoter regions. We hypothesize that CBP/p300 and RARβ2 might act in a concerted manner to suppress tumor growth. These observations have important implications for developing future retinoid-based prevention strategies for epithelial carcinogenesis.

Materials and Methods

Materials and Cells. All chemicals and biochemicals were obtained from Sigma, cell culture reagents and primers from GIBCOBRL, and cell culture plasticware from Corning unless otherwise noted. A 1.0 mM all-*trans*-retinoic acid (ATRA) stock solution was prepared in ethanol and stored in opaque tubes at –70°C. ATRA stocks were prepared and ATRA was used under reduced yellow lighting. Control cultures received equivalent volumes of ethanol (0.1%).

MCF-7 breast tumor cells were a gift of the late Ruth Sager. This MCF-7 variant is retinoid-resistant and does not express RARβ2 (11). Expression of a functional RARβ2 in these retinoid-resistant MCF-7 cells (MCF7-RARβ2) resulted in increased sensitivity to ATRA-mediated 1) growth inhibition and 2) *trans*-activation of a RARE as previously described (11). Controls were provided by MCF-7 cells transduced with the LXSN

retrovirus without insert (MCF7-LXSN) (11). Standard media used for MCF-7 cell culture was supplemented MEM-α medium as previously described (11). Transduced MCF-7 cells were routinely maintained in standard tumor cell media plus 1.0 mg/ml G418 except for the last two passages before experimental use. Growth media was not processed to remove endogenous retinoids.

Normal human mammary epithelial cells (HMECs) were a gift of Martha Stampfer and were derived from reduction mammoplasty (15). Expression of the dominant-negative RAR, RARα403 in HMECs (HMEC-DNRAR) has been previously described (16). RARα403 is a truncated RARα with dominant-negative activity against all RAR isoforms (-α, -β, -γ) (16). Expression of RARα403 in HMECs blocked sensitivity to ATRA-mediated 1) growth inhibition and 2) *trans*-activation of a RARE (16). Controls were provided by HMECs transduced with the LXSN retrovirus without insert (HMEC-LXSN) (16). Culture conditions and selection are as previously described (15, 16). Growth media was not processed to remove endogenous retinoids.

COS-7L cells (GIBCO) were grown in serum free media, VP-SFM, supplemented with 4 mM glutamine. All cells were cultured at 37^0C in a humidified incubator with 5% CO_2/95% air.

Reporter Plasmid Construction. RARE Reporter Constructs. Complimentary oligos were designed from each of the potential retinoic acid response element (RARE) sites within 4000 bp of the transcription start sites of CBP and P300. A HindIII site was

placed at the 5' end and a BamHI site was placed at the 3' end of each oligo (Qiagen Operon). Complimentary oligos were annealed at 95°C for 5 minutes and then allowed to slowly cool to room temperature. The annealed CBP and P300 oligos were double digested with HindIII and BamHI, and then inserted into the reporter plasmid pBLCAT5, which was purchased from ATCC. Potential consensus RARE sequences and forward and reverse primer sequences are listed in Table 1.

SDS-PAGE and Western Analysis. Three T-75 flasks of each cell type were grown to ~50% confluency, treated for 48 hr with 1.0 μM ATRA, harvested as previously described (15), and stored in 35 μl aliquots at -70^0C for electrophoresis. The lysate was separated by 8% SDS-PAGE, transferred to a PVDF as described (15). The blocked membrane was incubated with either a 1) 1/200 dilution of a monoclonal antibody directed against CBP or 2) a 1/300 dilution of a monoclonal antibody directed against p300 (C1 and N15, respectively, Santa Cruz Biotechnology) in TBS with 0.05% Tween-20 (Sigma). The film image was digitized and quantitated using Kodak1D™ software (Eastman Kodak).

Semiquantitative RT-PCR. First-strand cDNA was obtained from 3 μg (MCF-7) of total RNA using the Superscript First-Strand Synthesis System for RT-PCR, in a final volume of 20 μl (Invitrogen, Life Technologies). Amplification of the 272 bp CBP fragment was performed with following primers, forward 5'-TCAGTCAACATCTCCT TCGC-3' and reverse 5'-TGTTGAACATGAGCCAGACG-3'. The forward and reverse primer sequences, respectively, for p300 were 5'-AATGATGAGTGAAAATGCCAGT

GTGCCC-3', and 5'-GCAGTCGCTTGGGCTTGGGTAT-3'. All PCR reactions were performed in a GeneAmp PCR System 2400 (PerkinElmer). Preliminary PCR was carried out to determine a cycle number that was within linear range. The conditions used to amplify CBP from MCF-7 cDNA are as follows. Each PCR reaction contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 1 mM dNTPs, 200 nM primers, 1.25 units of recombinant Taq polymerase (Roche), and 2.0 µl cDNA in a final volume of 50 µl. Following an initial denaturation at 94°C for 4 min, 28 cycles of 94°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec was carried out with a final extension at 72°C for 5 min. Amplification of p300 from MCF-7 cDNA was performed using the following conditions. Each PCR reaction contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 1.0 mM dNTPs, 100 nM primers, and 2.0 µl cDNA in a final volume of 50 µl. After an initial denaturation at 94°C for 4 min, 2.0 units of recombinant Taq polymerase (Roche) were added to each reaction. Amplification was continued for 26 cycles at 94°C for 15 sec, 53°C for 30 sec, and 72°C for 3 min. The final extension was 72°C for 5 min. β-actin was amplified as a positive control using forward (5'-GCTCGTCGTCGACAAC GGCTC-3') and reverse (5'-CAAACATGATCTGGGTCATCTTCTC-3') primer sequences. Amplification of β-actin from MCF-7 cDNA was carried out for 20 cycles and following the reaction and thermal cycling conditions used for CBP.

The CBP PCR products were visualized on 1.2% agarose gels stained with ethidium bromide and the p300 PCR products were run on 1% agarose/ethidium bromide gels. Gel images were obtained using a Kodak Digital Science™ Image Station 440 CF (Eastman

Kodak). The products were quantified using Kodak Digital Science™ 1D software (Eastman Kodak). All PCR reactions were done in triplicate.

Transient Expression Assay. COS7-L cells were transfected with the reporter constructs described above and with and without the RARβ2 expression plasmid, phRAR-β. pRARE4TKCAT provided a positive control for RARE activity. Transfected cells were treated with 1.0 μM ATRA for 24 hrs. Transfection conditions and controls and CAT activity assays are as previously described (16).

Results and Discussion

ATRA and RARβ2 Regulate CBP and p300 mRNA and Protein Expression in MCF-7 C ells. Expression of RARβ2 in retinoid resistant-MCF-7 cells 1) increased baseline CBP and p300 levels and 2) promoted ATRA-mediated induction of CBP and p300 (Fig. 1A, B, Table 2). Baseline CBP and p300 1) mRNA and 2) protein levels were increased in retinoid-sensitive MCF7-RARβ2 cells relative to retinoid-resistant MCF7-LXSN controls (Fig. 1A, B, Table 2). There was a XX-fold ($p < 0.0X$) increase in baseline CBP mRNA and a XX-fold increase ($p < 0.00X$) in baseline CBP protein expression in MCF7-RARβ2 cells relative to MCF7-LXSN controls. Similarly, there was a XX-fold ($p < 0.00X$) increase in baseline p300 mRNA expression and a XX-fold ($p < 0.00X$) increase in baseline p300 protein expression.

Treatment of MCF7-RARβ2 cells with 1.0 μM ATRA for 48 hr resulted in a 3.2-fold

increase ($p < 0.005$) in CBP mRNA and 2.3-fold increase ($p < 0.01$) in CBP protein expression. In contrast, similarly treated retinoid-resistant MCF7-LXSN vector controls exhibited only a slight decrease in CBP mRNA and an insignificant increase in CBP protein expression ($p > 0.005$). MCF7-RARβ2 cells treated with 1.0 μM ATRA for 48 hr exhibited a 2-fold increase ($p < 0.005$) in p300 mRNA and a 2-fold increase ($p < 0.005$) in p300 protein expression. There was a slight decrease in p300 mRNA expression in ATRA-treated MCF7-LXSN controls and p300 protein levels did not increase in ATRA-treated MCF7-LXSN controls. (Fig. 1A, B, Table 2).

Expression of the dominant-negative RAR (DNRAR), RARα403, in retinoid-sensitive, RARβ2(+) HMECs 1) suppressed baseline CBP and p300 expression and 2) blocked ATRA-mediated induction of CBP and p300. Baseline CBP and p300 1) mRNA and 2) protein levels were decreased in retinoid-resistant HMEC-DNRAR cells relative to retinoid-sensitive HMEC-LXSN (Fig. 1C, D, Table 2). There was a 2.2-fold ($p < 0.01$) decrease in baseline CBP mRNA and a 2.0-fold decrease ($p < 0.005$) in baseline CBP protein expression in HMEC-DNRAR cells relative to HMEC-LXSN controls. Similarly, there was a 10-fold ($p < 0.005$) decrease in baseline p300 mRNA expression and a 2.3-fold ($p < 0.005$) decrease in baseline p300 protein expression.

ATRA-mediated induction of CBP and p300 was blocked by expression of a DNRAR. Retinoid-sensitive HMEC-LXSN cells were treated for 48 hr with 1.0 μM ATRA, showed a 12- ($p < 0.005$) and 28-fold ($p < 0.005$) increase in the amount of CBP and p300 mRNAs expression respectively. HMEC-DNRAR cells treated for 48 hr with 1.0

μM ATRA showed an insignificant induction in the level of CBP or p300 mRNA ($p > 0.005$). When treated for 48 hr with 1.0 μM ATRA, HMEC-LXSN cells showed a 1.6- ($p < 0.005$) and 1.5-fold ($p < 0.005$) increase in the amount of CBP and p300 protein respectively. HMEC-DNRAR cells treated for 48 hr with 1.0 μM ATRA showed no significant change ($p > 0.005$) in CBP or p300 protein expression (Fig. 1C, D, Table 2).

These data 1) indicate that both CBP and p300 levels are, in part, regulated by RARs and ATRA and 2) are consistent with the observation made by Hayashi *et al* that expression of CBP and p300 in HSC-3 cells are increased by treatment with 9-cRA (20). Since CBP and p300 recruitment during transcriptional regulation is crucial to ATRA signaling, and the pools of CBP and p300 are regulated by ATRA, the observed ATRA associated increase of CBP and p300 may play an important role in the cellular response to retinoid exposure. In addition, retinoid treatment would be expected to alter global modulation of CBP and p300 dependent transcriptional control.

Transactivation of CAT Expression by ATRA and the CBP or p300 Promoter. While ATRA treatment of retinoid-sensitive MCF7-RARβ2 and HMEC-LXSN cells results in increased CBP and p300 mRNA expression, a functional RARE in either the CBP or p300 promoter region has not been demonstrated.

Baseline RARE-CAT activity was tested in COS7-L cells utilizing a CAT reporter plasmid coupled to 4 copies of the RARβ2 RARE (pRARE4TKCAT). Baseline RARE activity is low and not induced by 0.01 – 10.0 μM ATRA (Fig. 2). Co-transfection of

COS7-L cells with pRARE4TKCAT and the RARβ2 expression plasmid (phRAR-β) leads to increased baseline RARE-activity and a X-fold induction of RARE activity after treatment with 1.0 μM ATRA for 24 hr.

Potential consensus RAREs sequences were identified in the promoters of both CBP and p300 (Table 1A). Four CBP (CBP 1-4) and 3 p300 (p300 1-3) potential RARE sequences regions were 1) cloned into the pBLCAT5 CAT reporter plasmid and 2) tested for ATRA-mediated *trans*-activation in COS7-L cells (Fig. 2). CBP-3 showed 4-fold induction of CAT activity after treatment with 1.0 μM ATRA for 24 hr in the presence of RARβ2 (Fig. 2). RARβ2 expression was required for CBP-3 activity (Fig. 2). CBP-2 did not demonstrate "classic" RARE-induction (Fig. 2). CBP-1 and 4- were not induced by ATRA (Fig. 2 and data not shown). p300-3 demonstrated a X-fold induction of CAT activity after treatment with 1.0 μM ATRA for 24 hr (Fig. 2). p300-1 and p300-3 were not induced by ATRA (Fig. 2). These data indicate that the promoter regions of both CBP and p300 have at 1) least one active RARE and that 2) transcription is induced by ATRA and RARβ2.

As demonstrated, both CBP and p300 have a functional RARE(s) in their promoter regions. Our observations indicate that RARβ2 expression plays a direct role in the physiologic regulation of CBP and p300. Since CBP and p300 are known co-activators of retinoid signaling and have been observed to induce RARβ2 expression via a RARE we hypothesize that a positive feedback loop may exist between 1) RARβ2 and 2) CBP and p300. Our observations have important clinical implications because they suggest

that either loss of RARβ2 or CBP/p300 due to genetic or epigenetic causes would have a profound effect on the efficacy of retinoid-based chemoprevention.

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Table 1. Consensus RAREs within 4000 bp of the transcription start sites of CBP and p300.

A.

GENE	Potential RARE (bp upstream from start sequence)	Type
CBP-1	**(3348)**-TGTCAGGGAATGGCTCA ACAGTCCCTTACCGAGT-**(3332)**	ER5
CBP-2	**(2669)**-AGGTGACATTTGAACT TCCACTGTAACTTGA-**(2654)**	PAL4 DR1, DR3
CBP-3	**(2083)**-TGAACTCCTGACCTCAGGTGA ACTTGAGGACTGGAGTCCACT-**(2063)**	2 x ER1 2 x DR2
CBP-4	**(1543)**-AGGTGAGGGTGTCTGCCCT TCCACTCCCACAGACGGGA-**(1525)**	PAL7 DR6
p300-1	**(3376)**-CTGACCTCGTGATCC GACTGGAGCACTAGG-**(3362)**	DR2
p300-2	**(3077)**-CTGACCTCCTGATCC GACTGGAGGACTAGG-**(3063)**	ER1 DR2
p300-3	**(2541)**-TGACCTCAGGTGATCC ACTGGAGTCCACTAGG-**(2529)**	ER1 DR4

B.

Oligo Name	Sequence 5' – 3'
CBP 1	AAAAAAGCTTTCCACTCCCACAGACGGGAGGATCCCCCC
CBP 1-R	GGGGGGATCCTCCCGTCTGTGGGAGTGGAAAGCTTTTTT
CBP 2	AAAAAAGCTTACTTGAGGACTGGAGTCCACTGGATCCCCCC
CBP 2-R	GGGGGGATCCAGTGGACTCCAGTCCTCAAGTAAGCTTTTTT
CBP 3	AAAAAAGCTTTCCACTGTAAACTTGAGGATCCCCCC
CBP3-R	GGGGGGATCCTCAAGTTTACAGTGGAAAGCTTTTTT
CBP 4	AAAAAAGCTTACAGTCCCTTACCGAGTGGATCCCCCC
CBP 4-R	GGGGGGATCCACTCGGTAAGGGACTGTAAGCTTTTTT
P300-1	AAAAAAGCTTCTGACCTCGTGATCCGGATCCCCCC
P300-1-R	GGGGGGATCCGGATCACGAGGTCAGAAGCTTTTTT
P300-2	AAAAAAGCTTTGACCTCCTGATCCGGATCCCCCC
P300-2-R	GGGGGGATCCGGATCAGGAGGTCAAAGCTTTTTT
P300-3	AAAAAAGCTTCTGACCTCAGGTGATCCGGATCCCCCC
P300-3-R	GGGGGGATCCGGATCACCTGAGGTCAGAAGCTTTTTT

(A) RARE types are indicated as orientation followed by spacing (e.g.: ER4 is an everted repeat with 4 bp between the ends). The designation for RARE types is: ER, everted repeat; DR, direct repeat; and PAL, palindrome. **(B)** CBP and p300 forward and reverse **(R)** primer sequences corresponding to potential RARE sites within the promoter regions of CBP and p300.

Table 2. Relative CBP and p300 expression in ATRA-treated transduced MCF-7 cells (A) and HMECs (B).

(A)	MCF7-LXSN		MCF7-RARβ2	
	-ATRA	+ATRA	-ATRA	+ATRA
CBP mRNA	0.36 ± 0.2 *	0.20 ± 0.05 *	0.31 ± 0.1 *^	1.0 ± 0.0
protein	0.45 ± 0.06 *	0.55 ± 0.09 *	0.44 ± 0.08 *^	1.0 ± 0.0
p300 mRNA	0.20 ± 0.05 *	0.072 ± 0.03 *	0.51 ± 0.041 *^	1.0 ± 0.0
protein	0.24 ± 0.04 *	0.24 ± 0.03 *	0.38 ± 0.04 *^	1.0 ± 0.0
(B)	**HMEC-LXSN**		**HMEC-DNRAR**	
	-ATRA	+ATRA	-ATRA	+ATRA
CBP mRNA	0.082 ± 0.07 *^	1.0 ± 0.0	0.041 ± 0.06 *	0.082 ± 0.03 *
protein	0.63 ± 0.10 *^	1.0 ± 0.0	0.31 ± 0.07 *	0.28 ± 0.07 *
p300 mRNA	0.036 ± 0.05 *^	1.0 ± 0.0	0.016 ± 0.03 *	0.053 ± 0.02 *
protein	0.66 ± 0.03 *^	1.0 ± 0.0	0.064 ± 0.06 *	0.37 ± 0.1 *

HMEC-LXSN vector controls, HMEC-DNRAR transduced cells, MCF7-LXSN vector controls, or MCF7-RARβ2 transduced cells were treated for 24 hr with 1.0 μM ATRA. T he relative levels of CBP/p300 protein levels were determined by Western analysis, the mRNA levels by

semiquantitative PCR, and both were quantitated using Kodak 1D Image Analysis Software. These data are the means of three separate experiments (*/^ = protein or mRNA level of CBP/p300 significantly different for $p < 0.005$ when: 1) compared to the treatment/cell type giving a relative value of 1(*) or 2) in the same cell type when no treatment is compared to ATRA treatment (^)).

Fig. 1: **(A)** RT-PCR analysis of CBP and p300 mRNA expression in MCF7-RARβ2 transduced cells **(RARβ2)** and MCF7-LXSN vector controls **(LXSN)**. **(B)** Expression of CBP and p300 protein in MCF7-RARβ2 cells **(RARβ2)** and MCF7-LXSN controls **(LXSN)**. **(C)** RT-PCR analysis of CBP and p300 mRNA expression in HMEC-DNRAR cells **(DNRAR)** and in HMEC-LXSN vector control cells **(LXSN)**. **(D)** Western analysis of CBP and p300 protein expression in HMEC-DNRAR cells **(DNRAR)** and in HMEC-LXSN controls **(LXSN)**. Cells were treated with **(+RA)** or without **(-RA)** 1.0 μM ATRA for 48 hr. Actin serves as a loading control.