CCAAT/Enhancer Binding Protein δ (C/EBPδ) Regulation and Expression in Human Mammary Epithelial Cells: I. "Loss of Function" Alterations in the C/EBPδ Growth Inhibitory Pathway in Breast Cancer Cell Lines

G.S. Sivko[1] and J.W. DeWille[2]*

[1]Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio
[2]Department of Veterinary Biosciences and Division of Molecular Biology and Cancer Genetics, Ohio State Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio

Abstract   "Loss of function" alterations in growth inhibitory signal transduction pathways are common in cancer cells. In this study, we show that growth arrest (GA) treatments—serum and growth factor withdrawal and growth inhibitory IL-6 family cytokines (Interleukin-6 and Oncostatin M (OSM))—increase STAT3 phosphorylation (pSTAT3), increase CCAAT enhancer binding protein δ (C/EBPδ) gene expression and induce GA of primary, finite-lifespan human mammary epithelial cells (HMECs), and immortalized breast cell lines (MCF-10A and MCF-12A). In contrast, serum and growth factor withdrawal from human breast cancer cell lines (MCF-7, SK-BR-3, T-47D, and MDA-MB-231) for up to 48 h induced a relatively modest increase in pSTAT3 levels and C/EBPδ gene expression and resulted in varying levels of GA. In most breast cancer cell lines, IL-6 family cytokine treatment increased pSTAT3 levels and C/EBPδ gene expression, however, growth inhibition was cell line dependent. In addition to "loss of function" alterations in growth inhibitory pathways, breast cancer cell lines also exhibit "gain of function" alterations in growth signaling pathways. The Akt growth/survival pathway is constitutively activated in T-47D and MCF-7 breast cancer cells. The Akt inhibitor LY 294,002 significantly enhanced T-47D growth inhibition by serum and growth factor withdrawal or IL-6 family cytokine treatment. Finally, we show that activation of the pSTAT3/C/EBPδ growth control pathway is independent of estrogen receptor status. These results demonstrate that "loss of function" alterations in the pSTAT3/C/EBPδ growth inhibitory signal transduction pathway are relatively common in human breast cancer cell lines. Defective activation of the pSTAT3/ C/EBPδ growth inhibitory signal transduction pathway, in conjunction with constitutive activation of the Akt growth stimulatory pathway, may play a synergistic role in the etiology or progression of breast cancer. J. Cell. Biochem. 93: 830–843, 2004. © 2004 Wiley-Liss, Inc.

Key words: CCAAT/enhancer binding protein δ (C/EBPδ); cytokine; growth arrest; mammary epithelial cells; signal transducer and activator of transcription 3 (STAT3)

Breast cancer is the second leading cause of cancer-related deaths in US women. Approximately 180,000 new cases of breast cancer are diagnosed each year in the US, and despite improved diagnostic and treatment modalities, annual breast cancer deaths exceed 40,000 [Greenlee et al., 2000]. Breast cancer development is a multi-step process, beginning with ductal or lobular atypical hyperplasia with progression to ductal or lobular carcinoma in situ (DCIS/LCIS), and eventually to invasive carcinoma and metastasis [Beckmann et al., 1997; Russo and Russo, 2001]. At the molecular level, breast cancer is characterized by the progressive accumulation of genetic errors in growth control genes and DNA repair genes, resulting in the characteristics associated with cancer cells—loss of contact inhibition, uncontrolled growth, immortalization, and invasion [Beckmann et al., 1997].

Maintenance of appropriate cell-cycle progression is controlled by a series of checkpoints and interdependent growth stimulatory and growth inhibitory signaling pathways. One of the major goals in breast cancer research is to identify mammary epithelial cell growth-control pathways that may play a role in the development or progression of breast cancer. We previously reported that C/EBPδ, a member of the CCAAT/enhancer binding protein family of nuclear proteins, plays a key role in mouse mammary epithelial cell growth control in vitro and in vivo [O'Rourke et al., 1997; Gigliotti and DeWille, 1998; Gigliotti and DeWille, 1999; O'Rourke et al., 1999a,b; Hutt et al., 2000; Hutt and DeWille, 2002]. We found that serum and growth factor withdrawal or cytokine treatment of HC11 mouse mammary epithelial cells initiates a signal transduction cascade that includes activation of latent cytoplasmic signal transducer and activator of transcription 3 (STAT3), translocation of activated STAT3 dimers to the nucleus, STAT3 binding to consensus acute phase response elements (APREs) in the mouse C/EBPδ gene promoter, transactivation of the *C/EBPδ* gene, and growth arrest (GA) [Hutt et al., 2000; Hutt and DeWille, 2002]. In addition, we showed that phosphorylated/activated STAT3 (pSTAT3) and C/EBPδ levels increase during mammary gland involution, and that virgin female C/EBPδ knockout mice exhibit aberrant mammary epithelial cell proliferation and mammary gland ductal hyperplasia [Gigliotti et al., 2003]. These results indicate that the STAT3/C/EBPδ growth-control signal transduction pathway plays a functionally significant role in normal mouse mammary epithelial cell growth regulation.

C/EBPδ is a member of the C/EBP family of nuclear proteins. Six C/EBP family members have been identified, including C/EBPα, C/EBPβ (also known as CRP2, NF-IL6, LAP, AGP/EBP, IL-6BP, and NF-M), C/EBPγ, C/EBPδ (CRP3, NF-IL6β CELF), C/EBPε, and C/EBPζ (CHOP10, GADD153) [Lekstrom-Himes and Xanthopoulos, 1998]. C/EBP family members are expressed in a tissue-specific manner and function in a wide range of cellular activities, including the regulation of cell growth and differentiation. C/EBPs function in the control of cell growth and differentiation by transcriptional activation of downstream target genes, such as GADD45γ, and protein–protein interactions with cell-cycle regulatory proteins such as Rb, p21, cdk2, and cdk4 [Cao et al., 1991; Hurst, 1995; Jung et al., 2000; Harris et al., 2001].

Recent reports indicate that alterations in the structure and expression of specific C/EBPs influence tumorigenesis. For example, C/EBPα plays a key role in granulocyte differentiation, and mutations in the C/EBPα gene are common in a subset of acute myeloid leukemia (AML) patients [Tenen, 2001; Gombart et al., 2002]. Alterations in C/EBPα structure and expression have also been reported in human lung cancer [Halmos et al., 2002]. A second C/EBP family member, C/EBPβ, has been linked to carcinogen-induced skin tumorigenesis [Zhu et al., 2002]. Polyak et al. recently utilized serial analysis of gene expression (SAGE) to investigate the molecular alterations associated with breast cancer progression. Their analysis of approximately 50,000 unique transcripts identified C/EBPδ as one of a small subset of genes that were consistently down-regulated with progression from normal human mammary epithelium to breast carcinoma [Porter et al., 2001].

Breast cancer cell lines derived from primary tumors and metastatic tumors from pleural effusions fairly accurately reflect the characteristics of cancer cells in vivo [Lacroix and Leclercq, 2004]. Many of these cell lines, such as the MCF-7, T-47D, SK-BR-3, and MDA-MB-231 are relatively well-characterized genotypically and phenotypically in terms of chromosome number, hormone receptor status, gene expression profile, and relative invasiveness [Lacroix and Leclercq, 2004]. The functional significance of the pSTAT3/C/EBPδ growth-control signal transduction pathway, however, has not been systematically investigated in human mammary epithelial cells (HMECs) or human breast cancer cell lines. Therefore, the goals of the present study were to: (1) determine the functional role of the pSTAT3/C/EBPδ growth-control signal transduction pathway in non-transformed HMECs (primary and immortalized), (2) investigate potential "loss of function" alterations in the pSTAT3/C/EBPδ growth-control signal transduction pathway in human breast cancer cell lines, and (3) investigate potential "gain of function" alterations which could override growth inhibitory signaling resulting from activation of the pSTAT3/C/EBPδ pathway.

The results indicate that primary, finite-lifespan HMECs, and immortalized, non-trans-

formed HMEC lines exhibit growth inhibition in response to standard GA conditions; i.e., serum and growth factor withdrawal and cytokine treatment. This growth inhibition is characterized by the activation of the pSTAT3/C/EBPδ growth-control signal transduction pathway and down-regulation of the Akt growth-stimulatory pathway. Growth inhibition of MCF-12A cells was also demonstrated by direct over-expression of C/EBPδ using the clonogenic colony growth assay. Human breast cancer cell lines respond poorly to growth inhibition by standard GA conditions. Human breast cancer cell lines exhibit defects in STAT3 activation, C/EBPδ gene expression and/or C/EBPδ down-stream gene function in response to GA conditions. Some human breast cancer cell lines (T-47D and MCF-7) exhibit constitutive activation of the Akt pathway, which contributes to an ineffective GA response. Finally, we show that the pSTAT3/C/EBPδ growth control signal transduction pathway is independent of estrogen receptor status. These results demonstrate that "loss of function" alterations in the STAT3/C/EBPδ growth-arrest signal transduction pathway are common in human breast cancer cell lines and may play a role in the development or progression of clinical breast cancer.

MATERIALS AND METHODS

Cell Culture

Cell culture media and components were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA) and Sigma-Aldrich Inc. (St. Louis, MO). Cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in ATCC recommended phenol red free media with the addition of 100 U/ml penicillin:100 μg/ml streptomycin and 500 ng/ml Fungizone (Invitrogen) unless otherwise specified. MCF-10A and MCF-12A cell lines were growth arrested in 1:1 DMEM/F-12 with 0.1% horse serum. T-47D cell line was growth arrested in RPMI 1640 (Sigma) with 0.1% FBS. MCF-7 cell line was cultured in DMEM without phenol red (Sigma) with 10 μg/ml bovine insulin, 4 mM L-glutamine, 10 ng/ml human EGF, 100 U/ml penicillin:100 μg/ml streptomycin, 500 ng/ml Fungizone, and 10% FBS. GA media (GAM) contained only 0.1% FBS. MDA-MB-231 cell line was purchased from ATCC and cultured in Minimum Essential Eagle's Media (with Earle's salts and non-essential amino acids, without L-glutamine, phenol red and sodium bicarbonate) (Sigma), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U/ml penicillin:100 μg/ml streptomycin, 500 ng/ml Fungizone, and 10% heat-inactivated FBS. GAM contained only 0.1% heat-inactivated FBS. SK-BR-3 cell line was growth arrested in 0.1% FBS. HMECs were purchased from Clonetics (Cambnex Inc., East Rutherford, NJ) and cultured in mammary epithelial basal medium (MEBM) with the addition of 13 mg/ml BPE, 10 μg/ml hEGF, 5 mg/ml Insulin, 0.5 mg/ml Hydrocortisone, 50 mg/ml Gentamicin, and 50 μg/ml Amphotericin-B, all supplied by Clonetics. GAM contained only the Gentamicin and Amphotericin-B.

GA and Cytokine Addition Studies

Near confluent (70%) cells were rinsed and then cultured in serum and growth factor deprived media (specified above for each cell line) for designated time periods. Human IL-6 and Oncostatin M (OSM) were obtained from Peprotech (Rocky Hill, NJ), reconstituted in sterile water and were added at a dose of 50 ng/ml. Cytokine addition studies were performed similar to the GA studies by incubating near confluent cells in either GA or growth media for the designated time periods with and without the addition of IL-6/OSM.

[3H]Thymidine/Cell-Cycle Experiments

Cell-cycle experiments were conducted on cells cultured in 12-well plates. Cells were plated at ∼100,000 cells/well and allowed to reach 70% confluence before initiating GA. [3H]Thymidine was added 2 h prior to collection. Media containing [3H]thymidine was removed and cells were washed in the wells with 10% trichloroacetic acid three times at 4°C (1 × 10 min, 2 × 5 min). Cells were dissolved in 250 μl 0.3N NaOH/1% SDS and the solution transferred to a vial containing 5 ml scintillation fluid. Samples were dissolved and then counted using a liquid scintillation counter.

Northern Blot Analysis

Total RNA was isolated using RNAzol B (Tel-Test, Inc., Friendswood, TX), run on 1.2% agarose gels and transferred to Duralon UV membrane followed by UV crosslinking. Filters were hybridized for a minimum of 2 h. DNA probes were labeled with [alpha-32P]dCTP using a Random Primers Labeling Kit (Invitrogen),

added to the hybridization mixture and allowed to hybridize a minimum of 2 h. C/EBPδ probe was a 700 bp fragment isolated from Research Genetics Clone 82850. TTK (Threonine Tyrosine Kinase-ATCC 80028) was used as an S-phase/growth marker. Human GA specific protein 1 (GAS1) probe was isolated from ATCC Clone 959480. Cyclophylin (CP) was used as a loading control.

Western Blot Analysis

Whole cell protein was isolated using a whole cell lysis buffer containing 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% IGEPAL, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, 1× complete protease (Boehringer, Mannheim), 1 mM NaF, 1 mM $NaVO_3$, 1 mM Na_2MoO_4, 100 nM okadaic acid. Proteins were run on precast Bio-Rad 10–12% Tris-HCl gels and were transferred to PVDF membrane. Rabbit polyclonal C/EBPδ, C/EBPβ, and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal STAT3, phospho-STAT3(Tyr705), Akt and phospho-Akt(Ser473) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Westerns were blocked in 10% milk with primary and secondary antibody addition in 5% milk. Signal was developed using ECL and ECL Plus detection systems (Amersham Biosciences).

Clonogenic Assay

Transfections were performed using Lipofectamine Plus (Invitrogen). MCF-12A cells were transfected with 1 μg of pcDNA3 or pcDNA3-full length C/EBPδ constructs. Transfected cells were selected for with media containing 400 μg/ml G418 (Geneticin). Colonies were stained with 0.5% crystal violet in 20% methanol.

Estrogen and Tamoxifen Assays

MCF-7 cells plated at 70% confluence were growth arrested for 72 h in DMEM without phenol red with the addition of 0.1% charcoal-stripped fetal calf serum (Life Technologies). 17β-Estradiol (Sigma) was added at 100 pM to growth arrested cells with or without the addition of 100 nM 4-hydroxytamoxifen (4-OHT, Sigma).

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) and Bonferroni posttest analysis.

RESULTS

Serum and Growth Factor Withdrawal and OSM Addition Activate STAT3, Induce C/EBPδ mRNA and Protein and Result in GA of Finite-Lifespan HMECs

To determine whether C/EBPδ gene expression was induced during G_0 GA of HMECs, finite-lifespan HMECs were growth arrested by standard methods-serum and growth factor withdrawal and growth-inhibitory cytokine (OSM) addition. The initial experiment assessed the influence of serum and growth factor withdrawal (GAM) and OSM treatment in both complete growth media (CGM) and GAM on HMEC C/EBPδ mRNA levels. Compared to growing (untreated) cultures, each GA treatment significantly induced (twofold to fourfold) C/EBPδ mRNA levels (Fig. 1a, lane 1 vs. lanes 2–7).

We next investigated the influence of serum and growth factor withdrawal and OSM treatment on STAT3 phosphorylation, C/EBPδ gene expression and primary HMEC GA. STAT3 protein levels were constitutive in all HMEC samples regardless of treatment (Fig. 1b). Compared to growing (subconfluent, growing) cultures, serum, and growth factor withdrawal GA treatment induced a relatively modest level of phosphorylated STAT3 (pSTAT3) (Fig. 1b, lane 1 vs. lane 2). OSM treatment induced the highest levels of pSTAT3, particularly when the OSM treatment was combined with serum and growth factor withdrawal (GAM) (Fig. 1, lanes 2–7). C/EBPδ protein was detected in HMEC cultures that exhibited STAT3 phosphorylation, even if the level of pSTAT3 was relatively modest (Fig. 1b, lanes 2–7). This may result from efficient transcriptional activation from relatively modest amounts of pSTAT3, or autoregulation of the C/EBPδ gene promoter [O'Rourke et al., 1999a]. The relative induction of C/EBPδ protein in response to the individual growth-arrest treatments paralleled the relative induction of C/EBPδ mRNA levels (Fig. 1a, lanes 2–7).

The influence of growing and growth-arrest treatments was assessed by [3H]thymidine incorporation (Fig. 1c). [3H]Thymidine incorporation increased in growing (GR) HMECs cultured for 24 and 48 h in CGM (Fig. 1c). Addition of OSM to HMECs in CGM (GR-OSM) significantly reduced [3H]thymidine incorporation at both 24 and 48 h time points
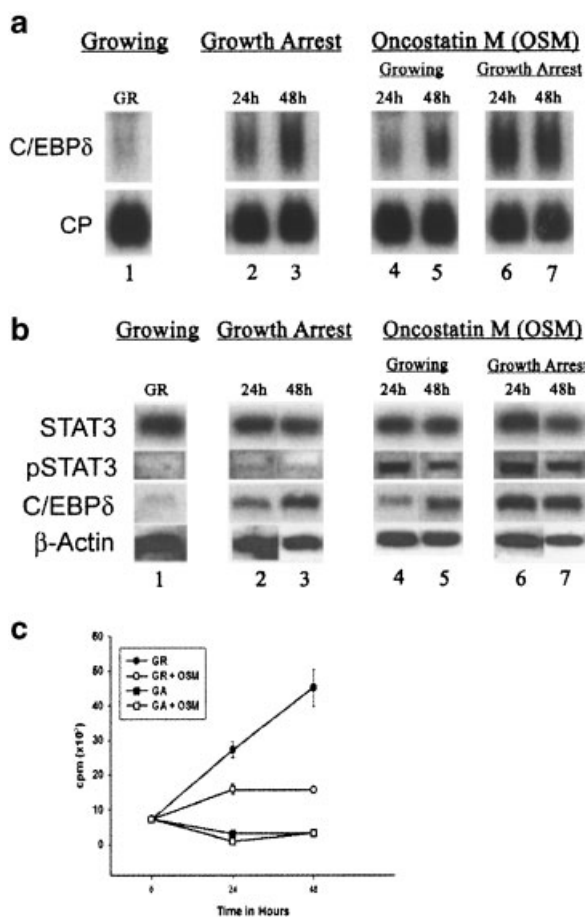
Fig. 1. Induction of C/EBPδ mRNA and protein and decrease in [³H]thymidine incorporation in primary HMECs following two methods of growth arrest-serum and growth factor withdrawal (growth arrest media, GA) and Oncostatin M (OSM) treatment. **a**: RNA was isolated from HMECs under the following conditions and probed for C/EBPδ and CP (cyclophylin): (**lane 1**) growing (50% confluent), (**lanes 2–3**) near confluent plates 24 and 48 h following serum and growth factor withdrawal, (**lanes 4–5**) near confluent plates in growth media with 50 ng/ml OSM for 24 and 48 h, and (**lanes 6–7**) near confluent plates in growth arrest media containing 50 ng/ml OSM for 24 and 48 h. **b**: Whole cell protein was isolated from HMECs under the conditions described above in (a). Western blots were performed and probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ, and β-actin (loading control). **c**: [³H]Thymidine incorporation of HMECs initially plated at 35% confluence under the following conditions: (**lane 1**) growth media (GR), (**lane 2**) growth media containing 50 ng/ml OSM (GR-OSM), (**lane 3**) growth arrest media (GA), and (**lane 4**) growth arrest media containing 50 ng/ml OSM (GA-OSM).

($P < 0.0001$) compared to untreated HMECs in CGM (GR). Serum and growth factor withdrawal alone (GA) and in combination with OSM treatment (GA-OSM) significantly reduced HMEC [³H]thymidine incorporation compared to growing cultures ($P < 0.0001$). These

results indicate that serum and growth factor withdrawal and OSM treatment induce cell-cycle exit and growth inhibition of primary, finite-lifespan HMECs.

Serum and Growth Factor Withdrawal and IL-6 Family Cytokine Addition Increases pSTAT3 Levels, Increases C/EBPδ mRNA, and Protein Levels, and Initiates GA in Immortalized Non-Tumorigenic HMEC Lines MCF-10A and MCF-12A

Previous reports by Stampfer et al. have correlated "loss of function" alterations in specific growth control genes with HMEC immortalization [Stampfer et al., 1997; Romanov et al., 2001; Yaswen and Stampfer, 2001; Yaswen and Stampfer, 2002]. These results suggest that specific "loss of function" alterations may facilitate the progressive accumulation of growth regulatory defects that characterize immortalization and transformation. To further investigate the association between STAT3 activation, C/EBPδ induction and the initiation of G_0 GA, we utilized two immortalized, but non-tumorigenic HMEC lines, MCF-10A and MCF-12A. MCF-10A and MCF-12A cell lines exhibit near diploid karyotypes and have undergone relatively few chromosome rearrangements [Soule et al., 1990; Paine et al., 1992].

Serum and growth factor withdrawal alone (growth arrest), and serum and growth factor withdrawal plus cytokine treatment (GA + OSM, GA + IL-6) from MCF-10A and MCF-12A cells induced changes in gene expression consistent with cell-cycle exit and G_0 GA. TTK mRNA levels (S phase marker) declined from "Growing" (Fig. 2a,b, lane 1) to 48 h G_0 GA/cytokine treatment (Fig. 2a,b, lane 1 vs. lanes 2–10). GAS1 mRNA levels (G_0 GA marker) are virtually undetectable in "Growing" MCF-10A and MCF-12A cells (Fig. 2a,b, lane 1). GAS1 mRNA levels increase significantly in both MCF-10A and MCF-12A cells in response to G_0 GA/cytokine treatments (Fig. 2a,b, lane 1 vs. lanes 2–10). GAS1 mRNA levels are maximal in 48 h GA + OSM treated MCF-10A and MCF-12A cells (Fig. 2a,b, lane 7).

Consistent with the results from primary HMECS (Fig. 1), STAT3 was constitutively expressed in both MCF-10A and MCF-12A cells cell lines under all conditions. pSTAT3 was minimally induced with serum and growth factor withdrawal-induced GA treatment (Fig. 2c,d, lanes 2–4). However, the combination of serum
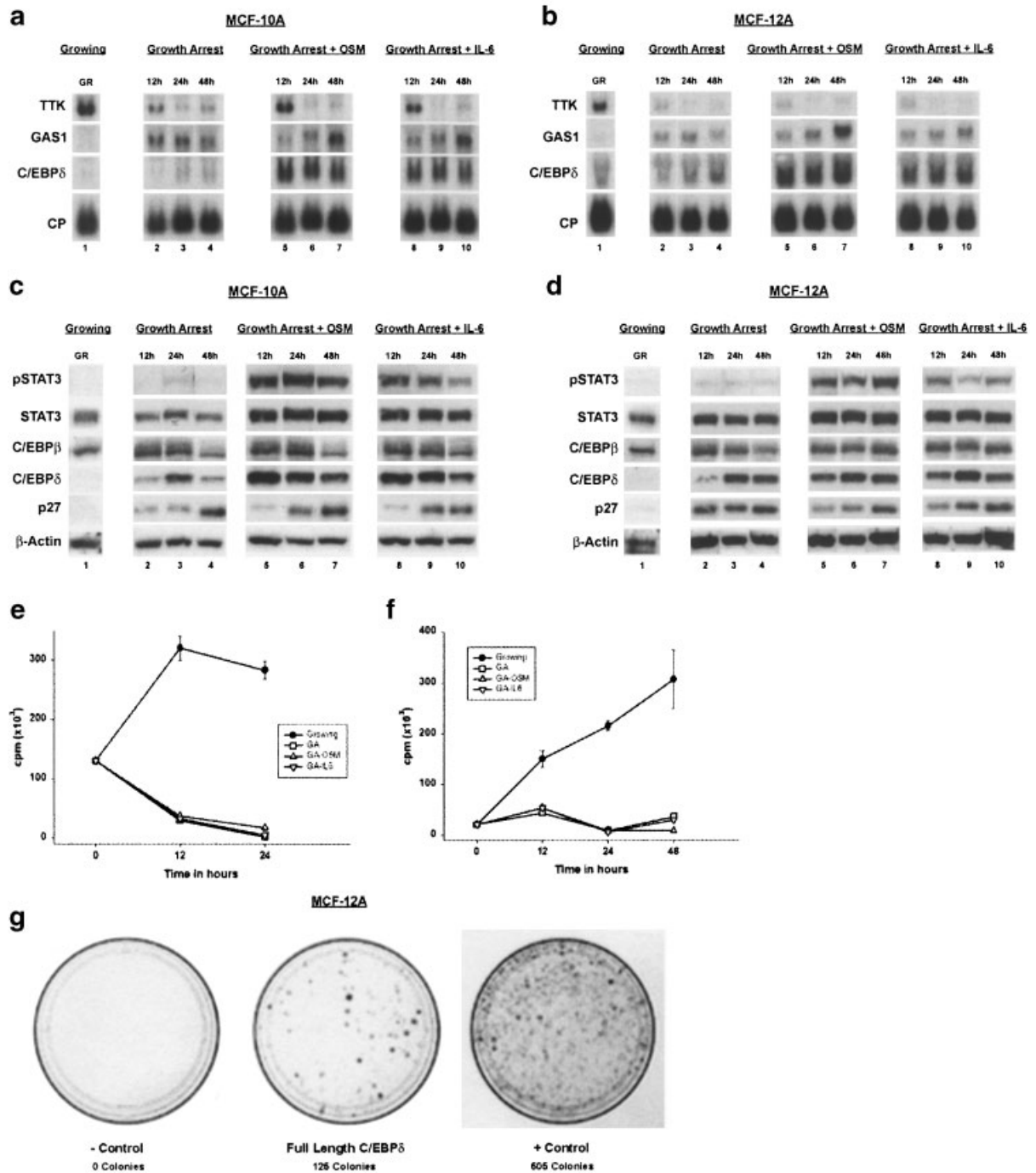
Fig. 2. Induction of C/EBPδ mRNA and protein and decrease in [³H]thymidine incorporation following serum and growth factor withdrawal and IL-6 family cytokine addition (IL-6 or OSM) in MCF-10A and MCF-12A human mammary epithelial cell (HMEC) lines. Near confluent plates were switched from complete growth media (CGM) to media containing only 0.1% FBS and no additional growth factors, with and without 50 ng/ml IL-6 or OSM. **a**, **b**: RNA was isolated from growing cells at 12, 24, and 48 h of growth arrest with or without IL-6 and OSM. Northern blots were analyzed for C/EBPδ expression and cell-cycle markers TTK (threonine tyrosine kinase) and GAS1 (growth arrest specific gene 1). CP (cyclophylin) was used as a loading control. **c**, **d**: Near confluent plates were growth-arrested with and without IL-6 and OSM, whole cell protein isolated at 12, 24, and 48 h timepoints, and Western blotted. Westerns were probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ, C/EBPβ, CDK inhibitor p27, and β-actin. **e**, **f**: [³H]Thymidine incorporation of MCF-10A and MCF-12A cells in growth media (GR), growth arrest media (GA), growth arrest media with 50 ng/ml OSM (GA-OSM) and growth arrest media with 50 ng/ml IL-6 (GA-IL6). **g**: Growth suppression of MCF-12A cells following C/EBPδ over-expression. MCF-12A cells were transfected with no construct (−, control), full length C/EBPδ construct (full length), or pcDNA3 (+, control) and cultured in G418 selection media. Plates were stained with crystal violet and photographed.

and growth factor withdrawal plus OSM or IL-6 significantly induced pSTAT3 levels (Fig. 2c,d, lanes 5−10). C/EBPδ protein levels increased twofold to threefold by 12 h of serum and growth factor withdrawal and remained elevated through the 48 h treatment period in both MCF-10A and MCF-12A cells (Fig. 2c,d, lane 1 vs. lanes 2−4). The combination of serum and growth factor withdrawal plus OSM or IL-6 significantly increased C/EBPδ protein levels compared to serum and growth factor withdrawal alone (Fig. 2c,d, lane 2 vs. lanes 5−10). We also assessed the levels of the cyclin dependent kinase inhibitor p27 under growing and GA conditions. p27 protein levels were undetectable in growing cells, however, p27 levels increased following both serum and growth factor withdrawal and IL-6 family cytokine treatment, consistent with a growth-arrest state. C/EBPβ protein was constitutively expressed in both MCF-10A and MCF-12A cells regardless of growth condition. [³H]Thymidine incorporation decreased following serum and growth factor withdrawal and cytokine treatment compared to untreated growing controls (Fig. 2e,f). Analysis of serum and growth factor withdrawal/cytokine-treated cultures by flow cytometry confirmed the increase of cells in G_0/G_1 (37% in growing cells vs. 75% in serum deprived/cytokine treated cells) (data not shown). To directly assess the role of C/EBPδ as a growth suppressor, we expressed C/EBPδ in MCF-12A cells and evaluated colony formation in a clonogenic assay (Fig. 2g). Ectopic C/EBPδ expression decreased colony formation by 80% compared to vector control (pcDNA3) transfected cells. These results demonstrate that increased pSTAT3 levels and increased C/EBPδ gene expression are linked to the G_0 growth inhibition in immortalized HMEC lines consistent with the results from studies with finite lifespan HMECs (Fig. 1, above). These results also demonstrate that C/EBPδ expression is sufficient to reduce MCF12A colony formation.

Serum and Growth Factor Withdrawal Plus IL-6 Family Cytokines Induces Variable Increases in pSTAT3 and C/EBPδ mRNA and Protein Levels, and Inconsistent GA of Human Breast Cancer Cell Lines MCF-7, MDA-MB-231, SK-BR-3, and T-47D

We next investigated the influence of serum and growth factor withdrawal and IL-6 family cytokine treatment on activation of the pSTAT3/C/EBPδ growth control pathway and growth inhibition in human breast cancer cell lines. Four human breast cancer cell lines were investigated—MCF-7, MDA-MB-231, SK-BR-3, and T-47D. All human breast cancer cell lines express detectable levels of TTK mRNA (S phase marker) when cultured in CGM ("Growing") (Fig. 3a, lanes 1, 5, 9, 13). Serum and growth factor withdrawal (GA) effectively reduced TTK mRNA levels in MCF-10A and MCF-12A immortalized HMEC lines but did not result in a significant reduction in TTK mRNA levels in any of the breast cancer cell lines (Fig. 3a, lanes 2, 6, 10, 14). TTK mRNA levels were reduced in MDA-MB-231 and T-47D cells by GA + OSM treatment and in MCF-7 cells by GA + IL-6 treatment (Fig. 3, lanes 3, 7, 16). Removal of serum and growth factors (GA) minimally induced C/EBPδ mRNA in the four breast cancer cell lines (Fig. 3a, lanes 2, 6, 10, 14), with the MDA-MB-231 and SK-BR-3 demonstrating slightly higher levels than the T-47D and MCF-7 cell lines. Significant induction of C/EBPδ mRNA levels was detected in GA plus OSM treated T47-D and GA plus OSM and GA plus IL-6 treated SK-BR-3 human breast cancer cell lines (Fig. 3a, lanes 7, 11, 12). Modest induction of C/EBPδ mRNA levels was detected in GA plus OSM and GA plus IL-6 treated MDA-MB-231 cells and GA plus OSM treated MCF-7 cells (Fig. 3a, lanes 3, 4, 15).

STAT3 was constitutively expressed in all four cell lines regardless of treatment (Fig. 3b, lanes 1−10). Phosphorylated (activated) STAT3 (pSTAT3) was not detected in growing human breast cancer cell lines (Fig. 3b, lane 1). Serum and growth factor withdrawal resulted in no detectable STAT3 activation (pSTAT3) in the T-47D and MCF-7 cell lines. Minimally detectable levels of pSTAT3 were present MDA-MB-231 and SK-BR-3 cells after 48 h (Fig. 3b, lanes 2−4). pSTAT3 was detected in GA plus OSM treated MDA-MB-231 cells, but not in GA plus IL-6 treated MDA-MB-231 cells (Fig. 3b, lanes 5−7 vs. 8−10). pSTAT3 levels were also higher in GA plus OSM treated T-47D cells compared to GA plus IL-6 treated T-47D cells (Fig. 3b, lanes 5−7 vs. 8−10). In SK-BR-3 cells, pSTAT3 levels were higher following GA plus IL-6 treatment compared to GA plus OSM treatment (Fig. 3b, lanes 8−10 vs. 5−7). MCF-7 cells exhibited modest pSTAT3 induction following GA plus OSM treatment and little or no pSTAT3 induction
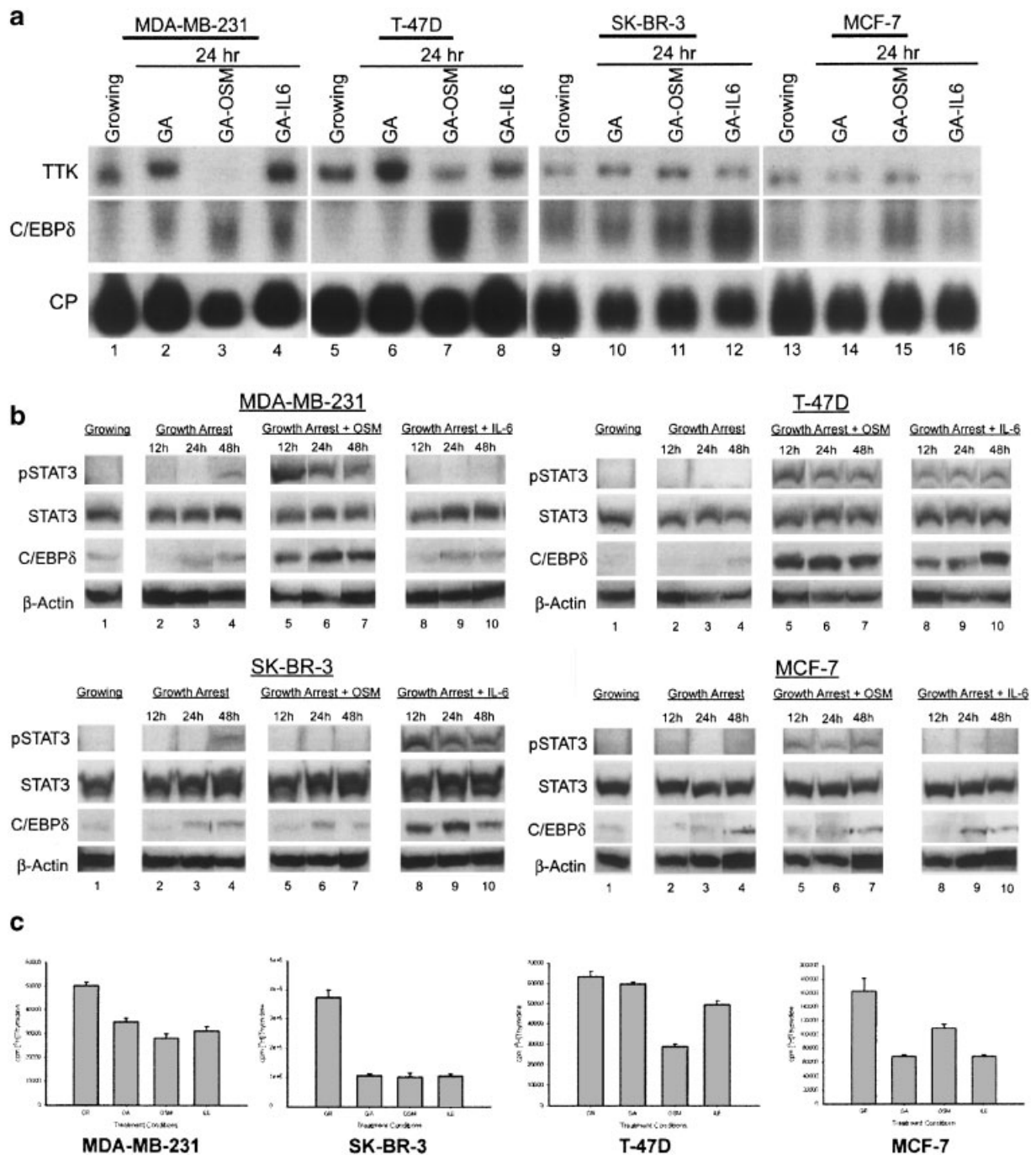
Fig. 3. Variable activation of pSTAT3, induction of C/EBPδ and minimal decrease in [³H]thymidine incorporation in human breast cancer cell lines MDA-MB-231, T-47D, SK-BR-3, and MCF-7. **a:** C/EBPδ mRNA induction following serum and growth factor withdrawal (growth arrest, GA) and IL-6/OSM treatment of human breast cancer cell lines, MDA-MB-231, T-47D, SK-BR-3, and MCF-7. Near confluent plates were deprived of serum and growth factors (GA) with and without the addition of 50 ng/ml IL-6 (GA-IL6) or OSM (GA-OSM) for 24 h. RNA was isolated, Northern blotted, and analyzed for C/EBPδ and growth marker TTK expression. CP was used as a loading control. **b:** STAT3 phosphorylation and C/EBPδ protein induction in breast cancer cell lines, MDA-MB-231, T-47D, SK-BR-3, and MCF-7, following serum and growth factor withdrawal and IL-6/OSM treatment. Near confluent plates were deprived of serum and growth factors with and without the addition of 50 ng/ml IL-6 (GA-IL6) or OSM (GA-OSM) for 12, 24, and 48 h and whole cell protein isolated. Western blots were probed with primary antibodies to STAT3, phosphoSTAT3, C/EBPδ, p27, and β-actin. **c:** [³H]Thymidine incorporation of near confluent MDA-MB-231, SK-BR-3, T-47D, and MCF-7 cells in growth media (GR), growth arrest media (GA), growth arrest media with 50 ng/ml OSM (OSM) or growth arrest media with 50 ng/ml IL-6 (IL6) for 24 h (48 h for SK-BR-3).

following GA plus IL-6 treatment (Fig. 3b, lanes 8–10).

Serum and growth factor withdrawal reduced [³H]thymidine incorporation into MDA-MB-231, SK-BR-3, and MCF-7 cells by 24 h (48 h for SK-BR-3) compared to growing controls. The reduction in [³H]thymidine incorporation in each cell line was proportional to the relative induction of C/EBPδ gene expression in the individual cell lines (Fig. 3a,b). For example, there was virtually no detectable induction of C/EBPδ mRNA or protein levels in response to serum and growth factor withdrawal in the T47-D human breast cancer cell line and also no reduction in [³H]thymidine incorporation was detected in the T47-D cell line (Fig. 3a, lane 5, 6; Fig. 3b, lanes 2–4, Fig. 3c). In general, OSM or IL-6 treatment resulted in C/EBPδ gene expression and reduced [³H]thymidine incorporation in all four cell lines (Fig. 3c). These results indicate that breast cancer cell lines respond relatively poorly to GA induction by serum and growth factor withdrawal. Serum and growth factor withdrawal plus IL-6 family cytokine treatment is associated with induction of the pSTAT3/C/EBPδ signal transduction pathway and variable growth inhibition in most human breast cancer cell lines.

Constitutive Akt Activation Contributes to the Lack of Growth Inhibition in the T-47D Breast Cancer Cell Line

Protein kinase B (PKB/Akt) is a serine/threonine kinase in the PI3K signaling pathway important in growth factor signaling, cell survival, cell-cycle progression, and motility [Downward, 1998]. Constitutive activation and the nuclear localization of Akt occurs frequently in primary breast tumors and breast cancer cell lines [Nicholson et al., 2003]. In human melanoma cells, constitutive activation of the PI3K/Akt pathway inhibits tyrosine phosphorylation of STAT3 and also negatively regulates STAT3's transcriptional activity.

To determine the Akt status in all four breast cancer cell lines, we assessed Akt levels and Akt activation (phosphorylation) under growing conditions (CGM) and GA (serum and growth factor withdrawal and serum and growth factor withdrawal plus IL-6 family cytokine treatment) (Fig. 4a). In the MCF-12A immortalized mammary epithelial cell line, phospho-Akt (pAkt) was detected at relatively low levels under growing conditions; pAkt levels were
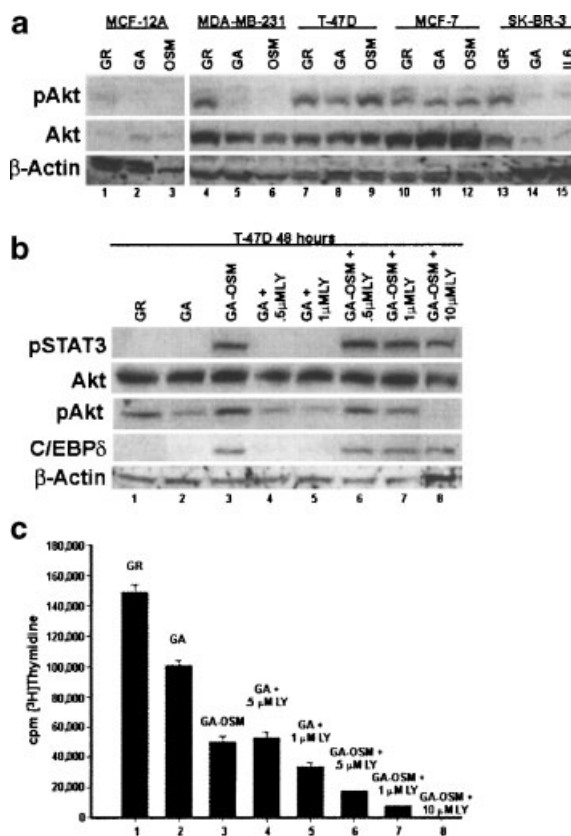

Fig. 4. Expression of pAkt in MCF-12A and MDA-MB-231, T-47D, MCF-7, and SK-BR-3 cell lines. Effect of Akt inhibitor LY 294,002 on T-47D cell growth arrest following serum and growth factor withdrawal and OSM addition. **a:** Near confluent plates were serum and growth factor deprived with or without the addition of 50 ng/ml OSM for 48 h. Whole cell protein was isolated from growing (GR), growth arrested (GA), and growth arrested with OSM (OSM) cells, Western blotted, and probed with primary antibodies to Akt, phospho-Akt (pAkt), and β-actin. **b:** Whole cell protein was isolated and Western blotted from near confluent T-47D cells under the following conditions: growing (GR), serum and growth factor withdrawal (GA), serum and growth factor withdrawal plus 50 ng/ml OSM (GA-OSM), serum and growth factor withdrawal plus either 0.5 or 1 µM LY 294,002, serum and growth factor withdrawal plus 50 ng/ml OSM plus either 0.5, 1, or 10 µM LY 294,002. Blots were probed with primary antibodies to pSTAT3, Akt, phospho-Akt (pAkt), C/EBPδ, and β-actin. **c:** [³H]Thymidine incorporation of T-47D cells under the same treatment conditions as described in (b).

undetectable under GA conditions (Fig. 4a, lanes 1–3). In the MDA-MB-231 and SK-BR-3 cell lines, pAkt levels declined as cells were switched from growing to GA conditions (Fig. 4a, lanes 4–6, 13–15). In the T-47D cell line, pAkt levels were slightly elevated under growing conditions, declined somewhat following serum and growth factor withdrawal, then increased with GA + OSM treatment (Fig. 4a, lanes 7–9). In the MCF-7 cell line, pAkt levels remained

relatively constitutive regardless of growth conditions (Fig. 4a, lanes 7–12). The amount of total Akt differed across the cell lines. Total Akt levels were relatively low in the MCF-12A. Total Akt levels were moderate in the MDA-MB-231 and SK-BR-3 cell lines, and in both cell lines total Akt levels appeared to decline somewhat under GA conditions (Fig. 4a, lanes 4–6, 13–15). Total Akt levels were constitutively elevated in the T-47D and MCF-7 cell lines (Fig. 4a, lanes 7–12). To determine the role of activated Akt (pAkt) in growth inhibitory signaling and growth inhibition, we blocked activation of the Akt signaling pathway in the T-47D cell line with LY 294,002 under GA and OSM-treated conditions. A dose-dependent reduction in pAkt levels was observed in both growth arrested (Fig. 4b, lane 2 vs. lanes 4–5) and OSM-treated (Fig. 4b, lane 3 vs. lanes 6–8) T-47D cells. T-47D total Akt levels did not vary across treatment groups. In agreement with previous results (Fig. 3b), pSTAT3 and C/EBPδ protein levels were not induced by serum and growth factor withdrawal conditions; however, pSTAT3 and C/EBPδ proteins were detected in OSM-treated T-47D cells (Fig. 4b, lanes 3, 6–8). LY 294,002 treatment did not affect GA + OSM induced pSTAT3 or C/EBPδ levels, except at the highest dose (10 μM) (Fig. 4b, lane 8).

T-47D [^{3}H]thymidine incorporation was reduced by ~30% following serum and growth factor withdrawal (GA) (Fig. 4c, lane 1 vs. lane 2). T-47D [^{3}H]thymidine incorporation was reduced by 67% in GA plus OSM treated cells (Fig. 4c, lane 1 vs. lane 3). Addition of LY 294,002 to GAM decreased [^{3}H]thymidine incorporation from a 65% reduction (0.5 μM) to a 78% (1.0 μM (Fig. 4c, lane 1 vs. lanes 4–5). Addition of LY 294,002 to GAM with OSM (GA plus OSM) reduced [^{3}H]thymidine incorporation by 88% (0.5 μM LY 294,002), by 95% reduction (1.0 μM LY 294,002) and 99.5% (10 μM LY 294,002) (Fig. 4c, lane 1 vs. lanes 6–8). These results are confirmed by flow cytometry (data not shown).

These results demonstrate that treatment with the IL-6 family cytokine OSM results in an increase in pSTAT3 levels and decreased proliferation of T-47D human breast cancer cells. The growth inhibitory effect of OSM can be enhanced by treatments that block Akt activation in T-47D cells. This suggests that growth inhibition is controlled by both the induction of growth-inhibitory signaling and the inhibition of growth-stimulatory signaling.

STAT3/C/EBPδ Growth Inhibition Is Independent of Estrogen Receptor Status

To determine whether the growth suppressive role of C/EBPδ was related to estrogen receptor status, the GA response of four breast cancer cell lines, MCF-7, T-47D, SK-BR-3, and MDA-MB-231, to serum and growth factor withdrawal and cytokine addition (Fig. 3c) was correlated with estrogen receptor status (Fig. 5a). One ER+ and one ER− cell line responded to STAT3/C/EBPδ growth inhibition (MCF-7 and SK-BR-3). One ER+ and one ER− cell line only minimally responded (T-47D and MDA-MB-231). This suggests that functionality of the STAT3/C/EBPδ growth inhibitory pathway does not depend on the presence or absence of the estrogen receptor. To confirm these findings, MCF-7 cells, which are known to be ER+, were growth arrested in charcoal-stripped serum for 72 h, followed by the addition of 17β-estradiol to induce a pure 'estrogen-induced' growth (Fig. 5b). Estrogen-induced growth was then suppressed by the addition of 4-OHT (Fig. 5c). C/EBPδ mRNA levels were not decreased by the addition of 17β-estradiol and did not increase after 4-OHT treatment (Fig. 5d), suggesting that estrogen receptor antagonists do not activate the Stat3/C/EBPδ growth inhibitory pathway. Studies with the pure anti-estrogen ICI 182,780 produced similar results (data not shown). These studies suggest that the C/EBPδ growth inhibitory pathway is not estrogen-regulated. Hence, determining methods for activation of the STAT3/C/EBPδ pathway could provide alternative treatment options for patients with unresponsive ER+ breast cancer and those with ER− breast cancer.

DISCUSSION

The goal of the present study was to investigate the role of C/EBPδ and its principal transcriptional regulator, pSTAT3, in the GA response of HMECs. In the initial experiments, we investigated the activation of the pSTAT3/C/EBPδ signal transduction pathway in primary, finite-lifespan HMECs. Stampfer et al. have extensively investigated growth regulatory pathways in primary HMECs [Stampfer et al., 1997; Romanov et al., 2001; Yaswen and Stampfer, 2001; Yaswen and Stampfer, 2002]. Their studies have demonstrated that growth factor receptor blockade and TGFβ treat-
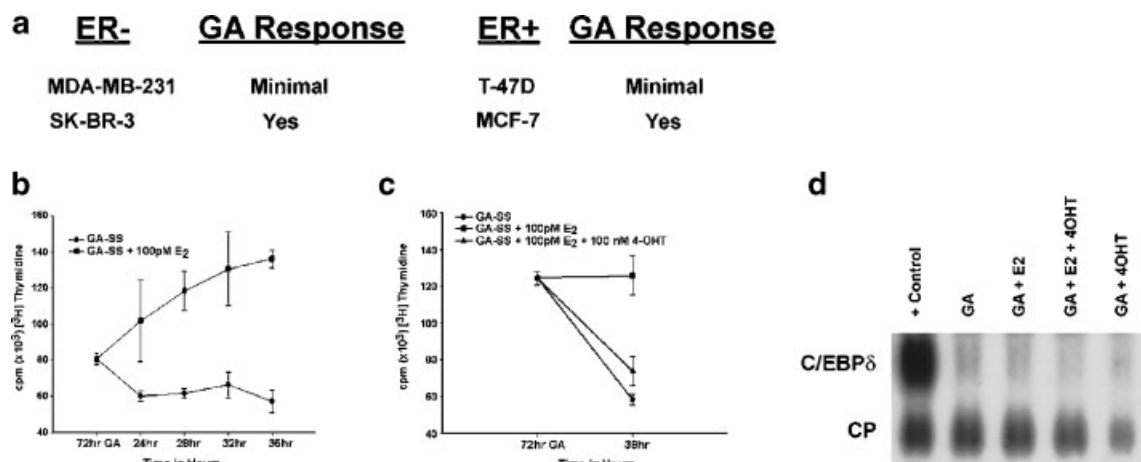
Fig. 5. Influence of estrogen receptor expression on C/EBPδ induction. **a**: Estrogen receptor status of MDA-MB-231, MCF-7, T-47D, and SK-BR-3 cells correlated with their ability to growth arrest following serum and growth factor withdrawal and IL-6 family cytokine addition. **b**: [³H]Thymidine incorporation of near confluent plates of MCF-7 cells initially growth arrested in media containing 0.1% charcoal-stripped serum for 72 h and then either maintained in growth arrest media (GA-SS) or having 100 pM 17β-estradiol (GA-SS + 100 pM E₂) added for 36 h. **c**: [³H]Thymidine incorporation of near confluent plates of MCF-7 cells initially growth arrested in media containing 0.1% charcoal- stripped serum for 72 h and then either maintained in growth arrest media (GA-SS), having 100 pM 17β-estradiol (GA + 100 pM E₂) added, or having 100 pM 17β-estradiol and 100 nM 4- hydroxytamoxifen (4-OHT) (GA + 100 pM E₂ + 100 nM 4-OHT) added for 38 h. **d**: Near confluent plates of MCF-7 cells were maintained in growth arrest media (0.1% stripped serum) alone for 72 h (GA), with the addition of 100 pM E₂ (GA + E₂), with the addition of 100 pM E₂ and 100 nM 4-OHT (GA + E₂ + 4-OHT), or with the addition of 100 nM 4-OHT (GA + 4-OHT). RNA was isolated and analyzed by Northern blot for C/EBPδ expression. CP was used as a loading control.

ment induce G_0 GA in primary, finite-lifespan HMECs [Stampfer et al., 1997]. In the present studies, we showed that serum and growth withdrawal and IL-6 family cytokine treatment also induce G_0 GA of primary, finite-lifespan HMECs (Fig. 1a–c). Our data also demonstrate that primary HMEC GA is associated with activation of the pSTAT3/C/EBPδ signal transduction pathway. These results are consistent with previous results from our laboratory demonstrating a role for the pSTAT3/C/EBPδ signal transduction pathway in the G_0 GA of HC11 mouse mammary epithelial cells [O'Rourke et al., 1999b; Hutt et al., 2000]. These results are also consistent with previous in vivo studies in which we demonstrated that C/EBPδ functions in mammary gland involution and nulliparous C/EBPδ knockout female mice exhibit aberrant mammary epithelial cell proliferation [Gigliotti and DeWille, 1999; Gigliotti et al., 2003]. Collectively, these results support a growth regulatory role for the pSTAT3/C/EBPδ signal transduction pathway in normal mammary epithelial cell biology.

Although our studies did not directly investigate the conversion of primary HMECs to immortalized HMECs, we did investigate the GA response of two immortalized HMEC cell lines, MCF-10A and MCF-12A. Both MCF-10A and MCF-12A cell lines growth arrested in response to GA treatments-serum and growth factor withdrawal alone or serum and growth factor withdrawal plus IL-6 family cytokines (Fig. 2e,f). In addition, growth-arrested MCF-10A and MCF-12A cells exhibited increased pSTAT3 levels, increased C/EBPδ mRNA and increased C/EBPδ protein levels (Fig. 2a–d). It is of interest that the immortalized MCF-10A and MCF-12A cell lines were not growth arrested by IL-6 family cytokine treatment when cultured in CGM, despite exhibiting increased pSTAT3 levels and increased C/EBPδ gene expression (data not shown). Stampfer and coworkers have demonstrated that one of the hallmarks of primary HMEC immortalization is loss of growth inhibition in response to specific GA treatments, such as TGFβ [Stampfer et al., 1997]. These present results indicate that growth in the presence IL-6 family cytokines, like growth in the presence of TGFβ, may be one of the growth control functions lost with HMEC immortalization.

The integration of signaling pathways is important in all phases of the cell cycle to

ensure appropriate cell-cycle progression, DNA replication, mitosis, etc. It is not surprising that the initiation and maintenance of G_0 GA involves the appropriate activation or deactivation of more than one signaling pathway. Inappropriate signaling by one pathway can modify the effect of another. While normal mammary epithelial cells respond to a reduction in growth factors or the addition of a growth-inhibitory cytokine by activating the pSTAT3/C/EBPδ pathway and entering G_0 GA, serum and growth factor withdrawal alone did not significantly activate the pSTAT3/C/EBPδ signal transduction pathway and only reduced growth in 2/4 human breast cancer cell lines studied (Fig. 3). Combining IL-6 family cytokine addition with serum and growth factor withdrawal consistently induced the pSTAT3/C/EBPδ signal transduction pathway and reduced growth in all four cell lines studied (Fig. 3). These results suggest that the pSTAT3/C/EBPδ signal transduction pathway and its role in mammary epithelial cell growth control become increasingly dysfunctional with the progression from immortalized to transformed cell lines. Not surprisingly, function of the pSTAT3/C/EBPδ signal transduction pathway in GA also appears to be dependent on the activation or inactivation of other cell signaling pathways in breast cancer cells. T-47D and MCF-7 cells have a luminal epithelial phenotype, are hormone sensitive and over-express E2F1 [Zhang et al., 2000; Lacroix and Leclercq, 2004]. SK-BR-3 cells are weakly luminal epithelial-like, are hormone insensitive and over-express Her-2/neu [Lacroix and Leclercq, 2004]. All three of these cell lines, however, are considered only weakly invasive [Zajchowski et al., 2001] and at least minimally respond to growth factor withdrawal and cytokine addition (Fig. 3c). The MDA-MB-231, on the other hand, overexpress FGF (fibroblast growth factor) and are highly invasive, "mesenchymal-like" cells [Zajchowski et al., 2001; Lacroix and Leclercq, 2004]. These characteristics could contribute to the inability of the MDA-MB-231 to GA under any of the treatment conditions studied (Fig. 3c). In addition, autocrine growth factors produced by human breast cancer cell lines may contribute to the loss of growth control by activating the PI3K/Akt growth signaling pathway and overriding cytokine-induced growth inhibitory signals. Many primary breast tumors and breast cancer cell lines exhibit constitutive activation

of the PI3K/Akt pathway (Fig. 4a) [Datta et al., 1999; Nicholson and Anderson, 2002; Nicholson et al., 2003]. The PI3K/Akt signaling pathway is important in cell survival, cell-cycle progression, and motility, and protects cells from apoptosis induced by growth factor depletion or extracellular matrix detachment [Downward, 1998; Datta et al., 1999; Nicholson et al., 2003]. While Akt phosphorylation status alone does not appear to play a significant independent role in breast cancer progression [Nicholson et al., 2003], it could have cooperative effects with disruptions in other signaling pathways. We demonstrate that both the activation of STAT3 and the inactivation of Akt are important in initiating GA. Normal mammary epithelial cells express high levels of pAkt during growth with no pAkt expression under GA conditions (Fig. 4a). In this study, breast cancer cells expressing high levels of activated Akt under both growing and GA conditions do not activate STAT3 and only minimally GA with the removal of serum and growth factors (Figs. 3c and 4a). In the T-47D cell line, [³H]thymidine incorporation is reduced by 30% with growth factor removal; most likely due to the reduction in media growth factors and slight decrease in phospho-Akt levels. Increasing phospho-STAT3 activation by combining growth factor reduction with OSM addition, reduces [³H]thymidine incorporation by 67% (Fig. 4c). Addition of 1 μM LY 294,002, an Akt inhibitor, without activation of STAT3 reduces [³H]thymidine incorporation by 65%; however, combined with STAT3 activation, the same 1 μM dose of LY 294,002 reduced [³H]thymidine incorporation by over 95% (Fig. 4c). In melanoma cells, constitutively activated Akt reduces both STAT3 tyrosine phosphorylation and STAT3 transcriptional activity [Krasilnikov et al., 2003]. Akt also blocks STAT3-mediated activation of p21 by inhibiting recruitment of CBP and RNA polymerase II to the promoter in HepG2 cells [Barre et al., 2003]. Reducing activated Akt levels in T-47D cells, however, did not appear to increase STAT3 phosphorylation or C/EBPδ induction at the doses of LY 294,002 used in this study (Fig. 4b).

Hormonal control and the abundance of estrogen/progesterone receptors are also important factors in cell-cycle progression of breast cancer cells. Receptor positive cells GA when treated with receptor antagonists while receptor negative cells do not. Estrogen (estradiol), a potent mitogen, has been shown to upregulate

p53 and induce hyperphosphorylation of Rb [Moudgil et al., 2001]. Synthetic anti-estrogens, such as tamoxifen and 4-OHT, induce G_0/G_1 GA in ER+ breast cancer cells by competitively binding the estrogen receptor, but also have been shown to inhibit proliferation in certain ER− cells by the induction of p21 and p27 and accumulation of hypo-phosphorylated Rb [Sutherland et al., 1983; Lee et al., 1999]. In this study, induction of the pSTAT3/C/EBPδ inhibitory pathway and G_0 GA were not affected by estrogen receptor status (Fig. 5a)—both ER+ and ER− cells growth arrested or only minimally growth arrested with serum and growth factor withdrawal or cytokine addition. Estrogen-induced growth in MCF-7 cells was inhibited by tamoxifen addition (Fig. 5b,c), as has been previously described [Taylor et al., 1984], however tamoxifen-induced GA did not increase C/EBPδ mRNA levels (Fig. 5d). The pSTAT3/C/EBPδ growth inhibitory pathway appears to initiate GA independently of the ER pathway. Hence, treatments which induce GA by activation of the pSTAT3/C/EBPδ pathway could be potential therapies in unresponsive ER+ or ER− breast cancer patients.

In conclusion, this study demonstrates that the pSTAT3/C/EBPδ signal transduction pathway plays an important role in the regulated GA of HMECs. This data is consistent with previous work from our laboratory in which activation of the pSTAT3/C/EBPδ signal transduction pathway has been shown to regulate mouse mammary epithelial cell GA in vivo and in vitro despite differences in the location and orientation of critical regulatory elements (Sp1 and STAT binding sites) between the mouse and human C/EBPδ promoter [Hutt et al., 2000]. These results demonstrate that the function of the pSTAT3/C/EBPδ signal transduction pathway is progressively disrupted in immortalized and transformed mammary epithelial cells. This "loss of function" pattern in the pSTAT3/C/EBPδ signal transduction pathway is consistent with recent SAGE studies by Polyak et al. in which loss of C/EBPδ gene expression was correlated with increased breast cancer progression [Alam et al., 1992]. Studies are ongoing in our laboratory to further investigate the expression of C/EBPδ in breast cancer patients. In addition, C/EBPδ gene promoter analysis and characterization of the downstream effectors of the pSTAT3/C/EBPδ signal transduction pathway are in progress.

REFERENCES

Alam T, An MR, Papaconstantinou J. 1992. Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. J Biol Chem 267:5021–5024.

Barre B, Avril S, Coqueret O. 2003. Opposite regulation of myc and p21waf1 transcription by STAT3 proteins. J Biol Chem 278:2990–2996.

Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA, Bender HG. 1997. Multistep carcinogenesis of breast cancer and tumour heterogeneity. J Mol Med 75:429–439.

Cao Z, Umek RM, McKnight SL. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538–1552.

Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: A play in three Akts. Genes Dev 13:2905–2927.

Downward J. 1998. Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10:262–267.

Gigliotti AP, DeWille JW. 1998. Lactation status influences expression of CCAAT/enhancer binding protein isoform mRNA in the mouse mammary gland. J Cell Physiol 174:232–239.

Gigliotti AP, DeWille JW. 1999. Local signals induce CCAAT/enhancer binding protein-delta (C/EBP-delta) and C/EBP-beta mRNA expression in the involuting mouse mammary gland. Breast Cancer Res Treat 58:57–63.

Gigliotti AP, Johnson PF, Sterneck E, DeWille JW. 2003. Nulliparous CCAAT/enhancer binding proteindelta (C/EBPdelta) knockout mice exhibit mammary gland ductal hyperlasia. Exp Biol Med (Maywood) 228:278–285.

Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, Larsen RJ, Asou H, Miller CW, Hoelzer D, Koeffler HP. 2002. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. Blood 99:1332–1340.

Greenlee RT, Murray T, Bolden S, Wingo PA. 2000. Cancer statistics, 2000. CA Cancer J Clin 50:7–33.

Halmos B, Huettner CS, Kocher O, Ferenczi K, Karp DD, Tenen DG. 2002. Down-regulation and antiproliferative role of C/EBPalpha in lung cancer. Cancer Res 62:528–534.

Harris TE, Albrecht JH, Nakanishi M, Darlington GJ. 2001. CCAAT/enhancer-binding protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and induces growth arrest independent of DNA binding. J Biol Chem 276:29200–29209.

Hurst HC. 1995. Transcription factors 1: bZIP proteins. Protein Profile 2:101–168.

Hutt JA, DeWille JW. 2002. Oncostatin M induces growth arrest of mammary epithelium via a CCAAT/enhancer-binding protein delta-dependent pathway. Mol Cancer Ther 1:601–610.

Hutt JA, O'Rourke JP, DeWille J. 2000. Signal transducer and activator of transcription 3 activates CCAAT enhancer-binding protein delta gene transcription in G_0 growth-arrested mouse mammary epithelial cells and in involuting mouse mammary gland. J Biol Chem 275:29123–29131.

Jung N, Yi YW, Kim D, Shong M, Hong SS, Lee HS, Bae I. 2000. Regulation of Gadd45gamma expression by C/EBP. Eur J Biochem 267:6180–6187.

Krasilnikov M, Ivanov VN, Dong J, Ronai Z. 2003. ERK and PI3K negatively regulate STAT-transcriptional activities in human melanoma cells: Implications towards sensitization to apoptosis. Oncogene 22:4092–4101.

Lacroix M, Leclercq G. 2004. Relevance of breast cancer cell lines as models for breast tumours: An update. Breast Cancer Res Treat 83:249–289.

Lee TH, Chuang LY, Hung WC. 1999. Tamoxifen induces p21WAF1 and p27KIP1 expression in estrogen receptor-negative lung cancer cells. Oncogene 18:4269–4274.

Lekstrom-Himes J, Xanthopoulos KG. 1998. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem 273:28545–28548.

Moudgil VK, Dinda S, Khattree N, Jhanwar S, Alban P, Hurd C. 2001. Hormonal regulation of tumor suppressor proteins in breast cancer cells. J Steroid Biochem Mol Biol 76:105–117.

Nicholson KM, Anderson NG. 2002. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 14:381–395.

Nicholson KM, Streuli CH, Anderson NG. 2003. Autocrine signalling through erbB receptors promotes constitutive activation of protein kinase B/Akt in breast cancer cell lines. Breast Cancer Res Treat 81:117–128.

O'Rourke J, Yuan R, DeWille J. 1997. CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem 272:6291–6296.

O'Rourke JP, Hutt JA, DeWille J. 1999a. Transcriptional regulation of C/EBPdelta in G(0) growth-arrested mouse mammary epithelial cells. Biochem Biophys Res Commun 262:696–701.

O'Rourke JP, Newbound GC, Hutt JA, DeWille J. 1999b. CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G_0 growth arrest and apoptosis. J Biol Chem 274:16582–16589.

Paine TM, Soule HD, Pauley RJ, Dawson PJ. 1992. Characterization of epithelial phenotypes in mortal and immortal human breast cells. Int J Cancer 50:463–473.

Porter DA, Krop IE, Nasser S, Sgroi D, Kaelin CM, Marks JR, Riggins G, Polyak K. 2001. A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res 61:5697–5702.

Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD. 2001. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 409:633–637.

Russo J, Russo IH. 2001. The pathway of neoplastic transformation of human breast epithelial cells. Radiat Res 155:151–154.

Soule HD, Maloney TM, Wolman SR, Peterson WD, Jr., Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC. 1990. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 50:6075–6086.

Stampfer MR, Bodnar A, Garbe J, Wong M, Pan A, Villeponteau B, Yaswen P. 1997. Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. Mol Biol Cell 8:2391–2405.

Sutherland RL, Green MD, Hall RE, Reddel RR, Taylor IW. 1983. Tamoxifen induces accumulation of MCF 7 human mammary carcinoma cells in the G_0/G_1 phase of the cell cycle. Eur J Cancer Clin Oncol 19:615–621.

Taylor CM, Blanchard B, Zava DT. 1984. Estrogen receptor-mediated and cytotoxic effects of the antiestrogens tamoxifen and 4-hydroxytamoxifen. Cancer Res 44:1409–1414.

Tenen DG. 2001. Abnormalities of the CEBP alpha transcription factor: A major target in acute myeloid leukemia. Leukemia 15:688–689.

Yaswen P, Stampfer MR. 2001. Epigenetic changes accompanying human mammary epithelial cell immortalization. J Mammary Gland Biol Neoplasia 6:223–234.

Yaswen P, Stampfer MR. 2002. Molecular changes accompanying senescence and immortalization of cultured human mammary epithelial cells. Int J Biochem Cell Biol 34:1382–1394.

Zajchowski DA, Bartholdi MF, Gong Y, Webster L, Liu HL, Munishkin A, Beauheim C, Harvey S, Ethier SP, Johnson PH. 2001. Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res 61:5168–5178.

Zhang SY, Liu SC, Al Saleem LF, Holloran D, Babb J, Guo X, Klein-Szanto AJ. 2000. E2F-1: A proliferative marker of breast neoplasia. Cancer Epidemiol Biomarkers Prev 9:395–401.

Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC. 2002. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci USA 99:207–212.