Prolactin Inhibits BCL6 Expression in Breast Cancer through a Stat5a-Dependent Mechanism

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Abstract

BCL6 is a transcriptional repressor that recognizes DNA target sequences similar to those recognized by signal transducer and activator of transcription 5 (Stat5). BCL6 disrupts differentiation of breast epithelia, is downregulated during lactation, and is upregulated in poorly differentiated breast cancer. In contrast, Stat5a mediates prolactin-induced differentiation of mammary epithelia, and loss of Stat5 signaling in human breast cancer is associated with undifferentiated histology and poor prognosis. Here, we identify the mammary cell growth factor prolactin as a potent suppressor of BCL6 protein expression in human breast cancer through a mechanism that requires Stat5a, but not prolactin-activated Stat5b, MEK-ERK, or PI3K-AKT pathways. Prolactin rapidly suppressed BCL6 mRNA in T47D, MCF7, ZR75.1, and SKBr3 breast cancer cell lines, followed by prolonged reduction of BCL6 protein levels within 3 hours. Prolactin suppression of BCL6 was enhanced by overexpression of Stat5a but not Stat5b, which mimicked the constitutively active Stat5a, but did not require the transactivation domain of Stat5a. Stat5 chromatin immunoprecipitation demonstrated physical interaction with a BCL6 gene regulatory region, and BCL6 transcript repression required histone deacetylase activity based on sensitivity to trichostatin A. Functionally, BCL6 overexpression disrupted prolactin induction of Stat5 reporter genes. Prolactin suppression of BCL6 was extended to xenotransplant tumors in nude mice in vivo and to freshly isolated human breast cancer explants ex vivo. Quantitative immunohistochemistry revealed elevated BCL6 in high-grade and metastatic breast cancer compared with ductal carcinoma in situ and nonmalignant breast, and cellular BCL6 protein levels correlated negatively with nuclear Stat5a \( r = -0.52; P < 0.001 \) but not with Stat5b. Loss of prolactin-Stat5a signaling and concomitant upregulation of BCL6 may represent a regulatory switch facilitating undifferentiated histology and poor prognosis of breast cancer.

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Introduction

Prolactin regulates growth and differentiation of breast epithelia during pregnancy and lactation (1, 2). Prolactin activates the prolactin receptor–associated Jak2 tyrosine kinase and downstream signaling proteins, including signal transducer and activator of transcription-5a (Stat5a) and Stat5b (3). Stat5a and Stat5b have 92% amino acid identity and share many characteristics, but are encoded by distinct genes that vary in tissue expression and display subtle differences in phosphorylation and DNA binding (3). Upon tyrosine phosphorylation of a conserved motif, Stat5 proteins form stable homodimers and heterodimers that translocate to the nucleus and bind to target DNA sequences. Prolactin activates both Stat5a and Stat5b in mammary epithelia of pregnant and lactating rodents (1, 4) and in many human breast cancer cell lines (5). Nonetheless, Stat5a null mice have a more pronounced lactational deficiency than Stat5b null mice (1, 6).

Studies in mice indicate that prolactin promotes mammary tumor initiation and growth via Stat5a (7–14). Several other prolactin-responsive signaling pathways have also been implicated in breast cancer growth and progression, including MEK-ERK, PI3K-AKT, and AP-1 pathways (11, 15–17). Evidence has also implicated prolactin and Stat5 in the maintenance of cell differentiation and suppression of invasive characteristics in breast cancer (18–21). Basal activation of Stat5 in healthy human breast epithelia is frequently lost in invasive and metastatic human breast cancer (19). Indeed, loss of active Stat5 in breast cancer correlated with poorly differentiated histology and poor prognosis (19, 22–24). Thus, a working model has been proposed in which prolactin-Jak2-Stat5 signaling promotes mammary tumor initiation but also maintains differentiation and suppresses progression of established breast cancer (25).
The proto-oncogene B-cell chronic lymphocytic leukemia/lymphoma 6 (BCL6) is a master regulator of B-lymphocyte development and facilitates proliferative expansion and blocks differentiation into plasma and memory cells (26). BCL6 is a zinc-finger protein and a potent transcriptional repressor (27). Intriguingly, the BCL6 consensus DNA-binding sequence resembles that of Stat5, and BCL6 competes for binding to many Stat5 DNA interaction sites (28–30). Emerging evidence points to a tumor-promoting role of BCL6 in breast cancer. First, BCL6 protein is elevated in human breast cancers, especially in high-grade, poorly differentiated cases (31, 32). Second, BCL6 is expressed in mouse mammary epithelia, primarily in virgin and pregnant animals, but is completely suppressed during lactation, a terminal differentiation stage that coincides with peak activation of Stat5a and Stat5b (33). Third, overexpression of BCL6 in immortalized mouse mammary EpH4 cells blocked cellular differentiation and promoted proliferation, supporting a differentiation-suppressive role of BCL6 in mammary epithelial cells (32).

Both negative and positive regulation of BCL6 by Stat5 has been reported. Stat5 suppressed BCL6 expression in B-cell lymphomas, adipocytes, and hepatocytes (28, 34, 35), but stimulated BCL6 in B lymphocytes (36) and in insulin-producing β-cells during pregnancy (37). A recent gene-profiling study of breast cancer cells indicated that prolactin inhibited expression of BCL6 mRNA, an effect that could be mimicked by a constitutively active Stat5a mutant (38). However, the study did not determine whether prolactin affected BCL6 protein levels or whether Stat5b or other prolactin pathways were involved. In fact, exposure of mammary epithelial cells to prolactin-containing differentiation media increased BCL6 mRNA but not protein (32). The present study provides novel evidence that prolactin effectively suppresses BCL6 protein and mRNA levels in human breast cancer through a mechanism that depends on Stat5a but not prolactin signaling via the Stat5b, MEK-ERK, or AKT pathways. The data are supported by experimental studies of prolactin-responsive human breast cancer cell lines in vitro and in vivo, as well as patient tumors ex vivo. In addition, correlative studies on a progression series of archival human specimens representing normal and malignant breast tissues further supported the conclusions.

Materials and Methods

Tissue culture. T47D, SKBr3, ZR75.1, and MCF7 cells [American Type Culture Collection (ATCC)] and surgical human breast tissue explants were cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 1 mmol/L sodium pyruvate. MDA-MB-231 cells (ATCC) and HEK293 cells (Invitrogen) were grown in DMEM containing 10% FBS and 1 mmol/L sodium pyruvate. Recombinant human prolactin (AFP795) was provided by Dr. A.F. Parlow (National Hormone and Pituitary Program, Torrence, CA). Confluent, serum-starved SKBr3 cells were incubated with DMSO, 10 μmol/L U0126 (Signagen), 10 μmol/L LY294002 (Signagen), or 500 nmol/L trichostatin A (TSA; Sigma) for 1 h before prolactin stimulation.

Luciferase assay. The BCL6 promoter gene construct (pGL3-BCL6-pr) was generated by PCR using BCL6-pr-f and BCL6-pr-r primers (Supplementary Table S1) to amplify the BCL6 regulatory region B of the BCL6 gene (34), digested with KpnI and HindIII and cloned into pGL3 vector. For BCL6 reporter assays, stably transfected T47D cells (T47D-BCL6-pr) were generated by cotransflecting pGL3-BCL6-pr and pcDNA3 (to provide neomycin selection; 10:1 ratio), and individual cell clones were selected with G418 (50 μg/mL). For Stat5 target gene reporter assays, T47D cells (1 × 105) were transiently cotransfected with either β-casein (39) or CIS (40) genomic reporter constructs and pCMV-SPOR6-BCL6 or pCMV-SPOR6 (Open Biosystems). After 12 h, cells were serum-starved in RPMI without FBS for 16 h and subsequently incubated with or without human prolactin (10 nmol/L) for 24 h. MDA-MB-231 cells were seeded at 1 × 103/24-well and transfected with combinations of 0.3 μg of DNA constructs for β-casein reporter (39), CIS reporter (40), pcDNA3-hPRLR (41), pCMV-SPOR6-BCL6, and pX-Mat-Stat5a. Transfections were equalized for total DNA with pcDNA3 empty vector. After 24 h, cells were incubated with vehicle control or prolactin (10 nmol/L) in DMEM containing 10% horse serum. Luciferase assays were performed 24 h after prolactin stimulation (BMG PolarStar Optima luminescence reader; BMG Technologies).

Lentiviral and adenoviral vectors. Lentivirus was produced in HEK-293 cells cotransfected with lentivectoral vectors carrying short hairpin RNAs (shRNA; Open Biosystems) for non-target control (SHC002). Stat5a [RHS-4533-NM-003142: TRCN0000019305 (5a2), TRCN0000019306 (5a3)], or Stat5b [RHS-4533-NM12448: TRCN0000019356 (5b3), TRCN0000019358 (5b6)], along with pCMV-dR8.2.dvpr and pCMV-VSV-G (Addgene [RHS-4533-NM12448: TRCN0000019305 (5a2), TRCN0000019306 (5a3)]), or Stat5b (RHS-4533-NM12448: TRCN0000019356 (5b3), TRCN0000019358 (5b6)], along with pCMV-dR8.2.dvpr and pCMV-VSV-G (Addgene cdx8454 and 8455; ref. 42). SKBr3 cells (4 × 105/T25) were infected with individual lentivirus and incubated for 48 h before exposure to prolactin. Cell lysates were subjected to immuno- blot and quantitative reverse transcriptase-PCR (qRT-PCR) analyses. Stat5a, Stat5b, Stat5a-Δ710F, and Stat5a-Δ713 adeno- virus preparations were prepared using double cesium chloride centrifugation (43) and used for gene delivery into SKBr3 cells (4 × 105/T25; multiplicity of infection = 5). After 24 h, cells were incubated with or without prolactin (10 nmol/L) in the absence of FBS for another 24 h and subsequently harvested for qRT-PCR analysis.

T47D xenograft tumors. T47D xenografts were performed as previously described (39). Briefly, nude mice implanted with 17β-estradiol pellets (0.72 mg; Innovative Research of America) were injected s.c. with 5 × 105 T47D cells into two dorsolateral sites. Once tumors averaging 0.5 cm had formed, mice were injected s.c. with either vehicle control (n = 10) or 5 μg/g body mass of human prolactin (n = 10) every 12 h for 48 h. Tumors were harvested and processed for immunohistochemistry and qRT-PCR.

Chromatin immunoprecipitation. Confluent SKBr3 cells were incubated with or without prolactin (10 nmol/L) for 1 h and exposed to 1% formaldehyde for 5 min. Reactions were terminated with 0.125 mol/L glycine.
Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 1% SDS] for 1 h and subsequently sonicated for 10 seconds (10x) on ice. Lysates were incubated with binding buffer [0.1% SDS, 1.1% Triton-X 100, 167 mmol/L NaCl, 16.7 mmol/L Tris-HCl (pH 8.1)] with a pan-Stat5 antibody (N20; Santa Cruz Biotechnology) overnight at 4°C, followed by capture with protein A–Sepharose (Amer sham) for 1 h. Samples were washed with binding buffer and resuspended in 100 μL of TE before immunoblot and qRT-PCR analyses.

Quantitative RT-PCR. qRT-PCR assays were performed with RNA isolated from cell lines and primary human breast tissues using RNeasy kit (Qiagen). cDNA was generated using iScript (Bio-Rad). Both cDNA and chromatin immunoprecipitation (ChIP) DNA were subjected to quantitative PCR using corresponding primers (Supplementary Table S1).

Immunoblotting. T47D and SKBr3 cell lysates were immunoprecipitated with 4 μL of rabbit Stat5a or Stat5b antibodies as described (39). Proteins were resolved by SDS-PAGE and immunoblotted with mouse pY-Stat5 (AX1, 1:10,000; Advantex), rabbit Stat5a (1:3,000), or rabbit Stat5b (1:1,500) antibodies followed by secondary horseradish peroxidase (HRP)–conjugated anti-mouse or anti-rabbit antibodies, respectively. BCL6 expression was detected in whole cell lysates with BCL6 antibody (1:1,000; Santa Cruz Biotechnology) followed by secondary HRP-conjugated anti-rabbit antibody. Densitometric analyses were performed using Chemidoc scanner and Quantity One software (Bio-Rad) on three independent experiments.

Immunohistochemistry. Immunohistochemistry and AQUA analyses were performed on sections containing either xenotransplant tissues or a tissue array constructed by cutting edge matrix assembly containing 14 deidentified breast carcinoma specimens [ductal carcinoma in situ (DCIS), primary invasive ductal carcinomas (IDC; grades 1–3), and lymph node metastases] and 40 normal breast tissues (Supplementary Data). Immunohistochemistry was performed as described previously (44) using Stat5a (Advantex, 1:8,000), Stat5b (Advantex, 1:4,000), pY-Stat5 (Epitomics, 1:200), and BCL6 (Santa Cruz Biotechnology, 1:50). AQUA analysis was performed using AQUA/PM2000 (HistoRx; ref. 45). Briefly, slides were scanned and fluorescent images were captured in three channels (FITC/Alexa-488, Cy5, or 4’,6-diamidino-2-phenylindole). AQUA scores for Stat5a, Stat5b, pY-Stat5, and BCL6 represent average signal intensities within the epithelial cell compartment as defined by cytokeratin-positive mapping.

Results

Prolactin suppresses BCL6 protein and mRNA levels in breast cancer cell lines. BCL6 protein levels in lysates of SKBr3 and T47D human breast cancer lines decreased rapidly within 3 hours of prolactin stimulation, whereas levels remained unchanged in untreated cells (Fig. 1A). By 6 hours and throughout the 48-hour time course, levels of BCL6 protein in both cell lines were markedly suppressed in the continued presence of prolactin. In parallel, levels of pY-Stat5 increased rapidly following prolactin receptor activation and remained elevated. Densitometry on four independent experiments confirmed the inverse relationship between pY-Stat5 and BCL6 proteins in breast cancer cells (Fig. 1B). Prolactin suppression of BCL6 protein levels in T47D and SKBr3 was associated with reduction in mRNA levels as revealed by qRT-PCR. BCL6 transcript levels were repressed as early as 1 hour of prolactin stimulation and reached maximum repression by 3 hours in both cell lines (Fig. 1C). In contrast, cytokine-inducible SH2 (CISH) mRNA, an established prolactin-stimulated gene (46), was markedly induced by prolactin in both SKBr3 and T47D cells (Fig. 1C). Marked inhibitory effect by prolactin on BCL6 mRNA levels was also observed in ZR-75.1 and MCF7 cells (Fig. 1D), suggesting a broad negative regulation by prolactin of BCL6 expression in human breast cancer lines. The rapid suppression of BCL6 transcript levels by prolactin is consistent with the ~30-minute half-life of BCL6 mRNA (34).

Prolactin suppression of BCL6 is dependent on Stat5a but not Stat5b, MEK/ERK, or AKT pathways. BCL6 mRNA and protein expression were examined in SKBr3 cells treated with vehicle or prolactin in the presence of MEK inhibitor U0126 or AKT inhibitor LY294002. Cells exposed to neither inhibitor displayed marked prolactin suppression of BCL6 and stimulation of CISH mRNA, effects that were not affected by MEK or AKT inhibitors (Fig. 2A, top). The two inhibitors were effective as judged by inhibition of prolactin-induced phosphorylation of ERK or AKT, but did not affect prolactin suppression of BCL6 protein levels as indicated in a representative protein blot and densitometric analyses of repeat experiments (Fig. 2A, middle and bottom, respectively).

To examine the requirement of Stat5 signaling for prolactin-induced BCL6 repression, shRNA sequences that targeted either Stat5a (shRNA-5a2) or Stat5b (shRNA-5b6) or a non-target control shRNA were introduced into SKBr3 cells by lentiviral delivery, and cells were subsequently treated with prolactin for 6 hours. Selective knockdown of Stat5a significantly reversed both prolactin suppression of BCL6 mRNA levels and stimulation of CISH mRNA expression, whereas selective knockdown of Stat5b did not (Fig. 2B, top). Prolactin suppression of BCL6 protein levels was also reversed by shRNA-5a2 as well as to a lesser but statistically significant extent by a second independent Stat5a-targeted shRNA, shRNA-5a3 (Fig. 2B, middle and bottom). Two shRNA constructs, 5b3 and 5b6, targeting Stat5b effectively knocked down Stat5b but did not affect prolactin suppression of BCL6 protein, consistent with mRNA data (Fig. 2B, middle and bottom).

Conversely, overexpression of Stat5a but not Stat5b in SKBr3 cells using adenoviral gene delivery suppressed basal levels of BCL6 and further enhanced prolactin suppression of BCL6 (Fig. 2C, top). The lack of efficacy of Stat5b could not be attributed to differences in expression (Supplementary Fig. S1). Furthermore, Stat5a-Δ713, which lacks the transactivation domain, retained the ability to mediate prolactin suppression of BCL6 (Fig. 2C, bottom left). Stat5a-Δ713 acts as a dominant-negative mutant for transactivation function.
and suppressed both basal and prolactin-induced CISH transcript levels (Fig. 2C, bottom right). Importantly, Stat5a-Δ713 was at least as effective as Stat5a in enhancing prolactin suppression of BCL6 mRNA levels. Finally, the constitutively active Stat5a-S710F mimicked prolactin suppression of BCL6 in the absence of prolactin and further suppressed BCL6 in response to prolactin (Fig. 2C, bottom left). In conclusion, prolactin suppression of BCL6 could be reversed by knockdown of Stat5a but not Stat5b or by disruption of MEK-ERK or AKT pathways.

Prolactin-activated Stat5 directly binds and functionally inhibits the BCL6 regulatory region by a TSA-sensitive mechanism. Stat5 ChIP assays in SKBr3 cells revealed prolactin-inducible Stat5 binding to the exon I region of the BCL6 gene (region B), which contains four adjacent canonical GAS (IFN-γ-activated Sequence) sites (Fig. 3A) previously shown to be regulated by Stat5 in B-cell lymphoma lines (34). Anti-Stat5 ChIP and qPCR revealed that capture of the BCL6 response region almost exclusively occurred when Stat5 was activated, although Stat5 protein was captured equally well in cell lysates from prolactin-treated or untreated cells (Fig. 3B). In fact, activation of Stat5 was associated with >10-fold enrichment for BCL6 DNA (Fig. 3C). Likewise, the genomic promoter region of CISH, a known Stat5 target gene, was also significantly enriched upon Stat5 immunoprecipitation in prolactin-treated cells, but not the glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–negative control DNA.
To test whether the interaction between Stat5 and the BCL6 regulatory sequence was associated with transcriptional repression of BCL6, a genomic BCL6 luciferase reporter was generated that contained the regulatory region B with the four GAS sites (34). When tested in transient transfection assays, prolactin consistently stimulated this reporter gene (data not shown), in agreement with previous analysis of this regulatory genomic element in isolation outside of chromatin.
context (34). However, when stably transfected into T47D cells, 2 of 10 clones consistently showed prolactin repression of the BCL6 luciferase reporter gene by ∼50% (Fig. 3D), whereas the other clones did not respond to prolactin (data not shown). This observation suggested that prolactin repression of BCL6 is dependent on chromatin context and may require additional cofactors. In fact, prolactin-induced repression of BCL6 required HDAC activity as revealed by reversal upon pretreatment of cells with TSA, a histone deacetylase inhibitor that inactivates HDAC class I and II (ref. 47; Supplementary Fig. S2). In the absence of TSA, prolactin effectively inhibited BCL6 mRNA expression, stimulated expression of CISH, and derepressed the BCL6 target gene, BLIMP1 (26). TSA effectively blocked prolactin repression of BCL6 but did not affect basal levels of BCL6 (Supplementary Fig. S2A and S2B). Consistent with the histone deacetylase (HDAC) requirement for prolactin repression of BCL6, the associated prolactin derepression of the BCL6-target gene, BLIMP1 (26), was also sensitive to TSA (Supplementary Fig. S2C). In contrast, prolactin stimulation of CISH mRNA levels remained intact (Supplementary Fig. S2D), a result consistent with the lack of requirement for HDAC for transcriptional activation by Stat5 of CISH. Collectively, ChIP assays and the reporter gene analyses provided evidence of direct involvement of Stat5 in occupying the regulatory region of the BCL6 gene and suggested critical involvement of HDAC activity for gene suppression.

**BCL6 interferes with Stat5-induced gene transcription.** Whereas Stat5a suppressed BCL6 protein expression, BCL6 conversely interfered with prolactin-Stat5 signaling in breast cancer. Overexpression of BCL6 in T47D cells completely blocked prolactin-induced expression of β-casein and CIS reporter gene constructs (Fig. 4A and B), indicating that BCL6 effectively disrupts at least some of the Stat5-induced genes in breast cancer cells. Stat5a induction of β-casein and CIS reporter genes was also disrupted by BCL6 in MDA-MB-231 breast cancer cells. Because our MDA-MB-231 cells do not express appreciable levels of prolactin receptor or Stat5, prolactin receptor and Stat5a cDNAs were also co-transfected. Overexpression of BCL6 completely blocked prolactin-induced expression of both Stat5 target genes in MDA-MB-231 cells (Fig. 4C). We conclude that BCL6 disrupts prolactin induction of Stat5-regulated reporter genes in both T47D and MDA-MB-231 cells.

**Prolactin inhibits BCL6 expression in human breast cancer in vivo and ex vivo.** We tested whether BCL6 expression was suppressed by prolactin in vivo using T47D xenotransplants and ex vivo using freshly isolated explant cultures of human surgical breast cancer tissues. For xenotransplant experiments, T47D tumor-bearing mice were...
treated with either PBS control \((n = 10)\) or human prolactin \((n = 10)\) for 48 hours. Immunohistochemistry revealed an inverse relationship between levels of nuclear pY-Stat5 and cellular BCL6 protein in xenotransplant tumors (Fig. 5A). Without prolactin treatment, Stat5 was inactive and BCL6 expression was detectable in the majority of T47D tumor cells. In contrast, tumors in prolactin-treated animals displayed high levels of nuclear pY-Stat5 and markedly reduced BCL6 protein levels. In addition, qRT-PCR analysis showed high levels of BCL6 transcripts in untreated control tumors and at least 4-fold reduction of BCL6 transcripts in prolactin-stimulated tumors, consistent with the observed in vitro prolactin suppression of BCL6 (Fig. 5B). Conversely, control tumors expressed low levels of CISH mRNA that were stimulated up to 8-fold by prolactin treatment.

Human primary breast cancer tissue explants in short-term ex vivo cultures were also examined, extending the effect of prolactin on BCL6 to more clinically relevant conditions. Human breast cancer tissue explants from two patients were exposed ex vivo to either vehicle or prolactin for 1 hour before being subjected to immunohistochemical or qRT-PCR analyses. Specimen 1 responded to prolactin by increased levels of pY-Stat5 (Fig. 5C), whereas specimen 2 had no detectable pY-Stat5 in response to prolactin. qRT-PCR assays revealed that prolactin suppressed BCL6 expression 2-fold in prolactin-responsive specimen 1 but not in prolactin-unresponsive specimen 2 (Fig. 5D). Consistent with Stat5 activation, CISH mRNA was stimulated 2-fold by prolactin in specimen 1 but not in specimen 2 (Fig. 5D). Collectively, these data further extended prolactin suppression of BCL6 expression in human breast cancer to both in vivo and ex vivo conditions.

**Cellular levels of BCL6 protein are negatively correlated with levels of nuclear-localized Stat5a but not Stat5b in human breast cancer tissues.** A breast cancer progression array containing 40 normal and 140 malignant breast tissues, including DCIS, IDC, and metastases, was analyzed by automated quantitative immunohistochemistry (45) for levels within the epithelial compartment of cellular BCL6 protein, nuclear-localized pY-Stat5, nuclear-localized Stat5a protein, and nuclear-localized Stat5b protein (Fig. 6A–C). Overall, whereas cellular levels of BCL6 increased, levels of nuclear-localized pY-Stat5 gradually decreased over the progression series from normal breast to metastatic lesions (Fig. 6B, left). A weak negative correlation between levels of BCL6 and nuclear-localized tyrosine phosphorylated Stat5 \((r = -0.23, P < 0.014; \text{Fig. }6B, \text{right})\) was observed in the clinical specimens. However, when Stat5a and Stat5b proteins were analyzed separately, a strong negative correlation was observed between levels of cellular BCL6 and nuclear Stat5a \((r = -0.52, P < 0.001)\) but not with nuclear Stat5b (Fig. 6C). Instead, a weak positive correlation was noted between cellular BCL6 and nuclear Stat5b \((r = 0.27; P = 0.006)\). The observed selective negative correlation between cellular BCL6 and nuclear Stat5a but not Stat5b is consistent with the observed selective mechanistic role of Stat5a in prolactin suppression of BCL6 based on the in vitro cell line data.

**Discussion**

The present study provides novel evidence of prolactin suppression of BCL6 protein levels in human breast cancer and suggests a mechanism that selectively involves Stat5a despite robust parallel activation of Stat5b, ERK, and AKT by prolactin in breast cancer cell lines. Prolactin inhibited BCL6 protein expression through rapid suppression of BCL6 mRNA, an effect that could be reversed by shRNA-mediated suppression of Stat5a but not Stat5b. A strong negative correlation between protein levels of cellular BCL6 and nuclear Stat5a, but not Stat5b, in a progression material of normal and malignant breast tissues supported the selective role of Stat5a as a suppressor of BCL6, as suggested by the in vitro data and provided clinical relevance to the observations. Furthermore, several lines of evidence indicated that the effect was mediated by repressor activity and direct binding of Stat5a to regulatory elements in the BCL6 gene based on \((a)\) ChIP, \((b)\) rapid reduction of BCL6 mRNA levels within minutes of Stat5a activation, \((c)\) requirement for HDAC activity as determined by sensitivity to TSA, and \((d)\) the lack of requirement for the transactivation domain of Stat5a. Furthermore, a genomic BCL6 DNA fragment containing the

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**Figure 4.** BCL6 blocks prolactin-induced Stat5 target gene expression in breast cancer. A, luciferase assays demonstrating that overexpression of BCL6 abolished prolactin-stimulated β-casein and CIS reporter genes in T47D cells. B, corresponding immunoblots of BCL6 and pY-Stat5 proteins. C, luciferase assays demonstrating that BCL6 overexpression abolished prolactin induction of β-casein and CIS reporters in MDA-MB-231 cells expressing prolactin receptor and Stat5a.
Figure 5. Prolactin suppression of BCL6 expression in vivo. A, representative immunohistochemistry of pY-Stat5 and BCL6 proteins in T47D xenograft tumors in mice treated with or without prolactin for 48 h. B, corresponding qRT-PCR analyses of BCL6 mRNA levels in T47D xenograft tumors. C, immunohistochemistry of two surgical human breast cancer tissue explants treated ex vivo with or without prolactin (100 nmol/L) for 1 h. Specimen 1 but not specimen 2 is responsive to prolactin stimulation as measured by pY-Stat5 induction. D, qRT-PCR quantified CISH and BCL6 mRNA levels in response to prolactin in specimens 1 and 2.
Stat5-binding elements coupled to a luciferase reporter gene could restore Stat5-dependent repression when stably transfected into breast cancer cells. However, repression seemed to be chromatin context dependent because not all of the stably transfected clones revealed repression. Finally, functional involvement of BCL6 as a direct negative regulator of Stat5-induced gene transcription supported the concept that elevated BCL6 may enhance the effects of reduced Stat5 signaling during breast cancer progression.

The observation that shRNA targeting of Stat5a but not Stat5b reversed prolactin suppression of BCL6 suggests a unique repressor capacity of prolactin-activated Stat5a that is not mimicked by prolactin-activated Stat5b in breast cancer cells. Although Stat5a and Stat5b generally recognize the same primary GAS sites (3), the greater ability of Stat5a to form N-domain–dependent tetramers on tandem GAS sites (48) provides one possible Stat5a-selective mechanism. Further analyses are therefore needed to map in detail which of

Figure 6. AQUA immunohistochemical quantification of BCL6 and pY-Stat5 protein levels in human breast tissues. A, representative immunofluorescent images of normal human breast tissue or primary breast cancer stained for pY-Stat5 (red) or BCL6 (red) and cytokeratin (green) and DNA (blue). Case 1, case 2, and normal tissue were selected from a progression series to show a range of BCL6 and pY-Stat5 levels. B, AQUA quantification of pY-Stat5 and BCL6 levels in a progression array of normal, DCIS, primary invasive breast cancer (grades 1–3), and metastases (left). Correlation analyses of levels of cellular BCL6 protein and nuclear-localized pY-Stat5 in the human breast tissues (right). C, correlation analyses between levels of cellular BCL6 protein and nuclear Stat5a protein (left) or nuclear Stat5b protein (right).
the regulatory GAS sites of the BCL6 gene are required for Stat5a-mediated repression of BCL6, whether Stat5a preferentially binds to this region, and to identify the exact molecular complexes responsible for prolactin suppression of BCL6 mRNA. The transcriptional corepressor SMRT can bind to the coiled-coil domain of Stat5a and Stat5b activated by interleukin-3 and provide repressor functions (49), and future work will determine the requirement for SMRT for prolactin-dependent repression of BCL6.

Previously, the observed loss of nuclear-localized, tyrosine phosphorylated Stat5 protein in invasive breast cancer was associated with poor prognosis and raised the possibility that Stat5 inhibits cell invasion and metastasis. In established breast cancer, active Stat5 is associated with more differentiated histology and experimental evidence suggests that Stat5a promotes differentiation and suppresses invasive features (19, 20, 23). Our present results demonstrating that prolactin-activated Stat5a negatively regulates BCL6 expression provide a new mechanism by which Stat5 may control differentiation of normal and malignant breast epithelia. BCL6 functions as a tumor-promoting factor by blocking differentiation and stimulating cell cycle progression in lymphomas. Consistent with this action, poorly differentiated and rapidly growing breast cancers exhibited elevated levels of BCL6 protein. Overexpression of BCL6 in the mammary cell line EpH4 inhibited cellular differentiation and promoted growth by increasing cell proliferation and reducing apoptosis (32, 50). Correspondingly, in the present study, overexpression of BCL6 in breast cancer cell lines abolished prolactin-induced expression of Stat5 reporter genes [5-casein and CIS. Ongoing studies will address the impact of BCL6 for prolactin regulation of breast cancer cell biology in vitro and in vivo.

In summary, the present work has revealed a novel negative regulatory interaction placing the proto-oncogene BCL6 within the prolactin-Jak2-Stat5a signaling network in human breast cancer. The resulting upregulation of BCL6 may exacerbate the biological consequences associated with loss of Stat5a signaling in breast cancer due to the suppressive effect of BCL6 on Stat5 target gene induction. Further work is now warranted to determine the diagnostic and therapeutic implications for human breast cancer of the mutually negative crosstalk between Stat5a and BCL6 signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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