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Reciprocal Effects of STAT5 and STAT3 in Breast Cancer

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Abstract

Breast cancer is often associated with inappropriate activation of transcription factors involved in normal mammary development. Two related transcription factors, signal transducer and activator of transcription (STAT) 5 and STAT3, play important and distinct roles in mammary development and both can be activated in breast cancer. However, the relative contribution of these STATs to mammary tumorigenesis is unknown. We have found that primary human breast tumors displaying activation of both STATs are more differentiated than those with STAT3 activation alone and display more favorable prognostic characteristics. To understand this difference, we have analyzed the effect of these STATs on gene regulation and phenotype of mammary carcinoma cells. STAT5 and STAT3 mediate opposing effects on several key target genes, with STAT5 exerting a dominant role. Using a model system of paired breast cancer cell lines, we found that coactivation of STAT5 and STAT3 leads to decreased proliferation and increased sensitivity to the chemotherapeutic drugs paclitaxel and vinorelbine compared with cells that have only STAT3 activation. Thus, STAT5 can modify the effects of STAT3 from the level of gene expression to cellular phenotype and analysis of the activation state of both STAT5 and STAT3 may provide important diagnostic and prognostic information in breast cancer. (Mol Cancer Res 2009;7(6):OF1–11)

Introduction

Mammary development occurs through precise activation of a variety of transcription factors. Inappropriate or constitutive activation of many of these transcription factors is found in breast cancer and may contribute directly to its pathogenesis (1). In particular, signal transducers and activators of transcription (STAT), a family of transcription factors that play important roles in many cellular functions, are often activated inappropriately in cancer (2). STATs are latent transcription factors that reside in the cytoplasm. Upon activation by tyrosine phosphorylation, STATs dimerize, translocate to the nucleus, bind to DNA, and modulate transcription, thereby regulating cellular functions such as survival, proliferation, and differentiation (3). Two closely related STAT family members, STAT5 and STAT3, play distinct roles in mammary development and both have been found to be activated in breast cancer (4).

STAT5, which encompasses two highly homologous proteins, STAT5a and STAT5b, is activated late in pregnancy by prolactin to promote terminal differentiation and milk production (5-8). STAT5 has also been found to be constitutively activated in a subset of breast cancers, generally in well-differentiated tumors (9, 10). In addition, the level of circulating prolactin, which signals principally through STAT5, correlates with risk of breast cancer in both premenopausal and postmenopausal women (11). Murine models have also supported a role for STAT5 in mammary tumorigenesis. Mice that express a constitutively activated form of STAT5 develop mammary carcinomas, whereas mice that lack STAT5a are protected against mammary tumors induced by transforming growth factor α (6, 12, 13). Taken together, these data implicate STAT5 as playing a central role in both normal and neoplastic mammary function.

STAT3 also plays a critical role in mammary development, although one that is distinct from STAT5. Once lactation has ceased, leukemia inhibitory factor (LIF) activates STAT3 to promote involution of the mammary gland, allowing remodeling to a prepregnancy-like state (14-18). STAT3 is often constitutively activated in breast cancer, but unlike STAT5, it is often activated in invasive and metastatic tumors (19). Reduction of STAT3 expression by RNA interference in breast cancer cell lines inhibits tumor formation in mice, further supporting the importance of STAT3 in breast cancer pathogenesis (20). Therefore, both STAT5 and STAT3 play important roles in mammary development and breast cancer.

Not only do STAT5 and STAT3 have distinct roles in mammary tissue, but there is evidence that they can exert opposing effects. In murine models, constitutive activation of STAT5 prevents STAT3 activation and delays involution (6). Furthermore, STAT5 activation reduces LIF-induced apoptosis of mammary epithelial cells (21). In addition, although either STAT5 or STAT3 can be activated in breast cancer, this usually occurs in distinct histologic and biological subtypes (9, 10, 19, 22). Taken together, these findings suggest that STAT5 and STAT3

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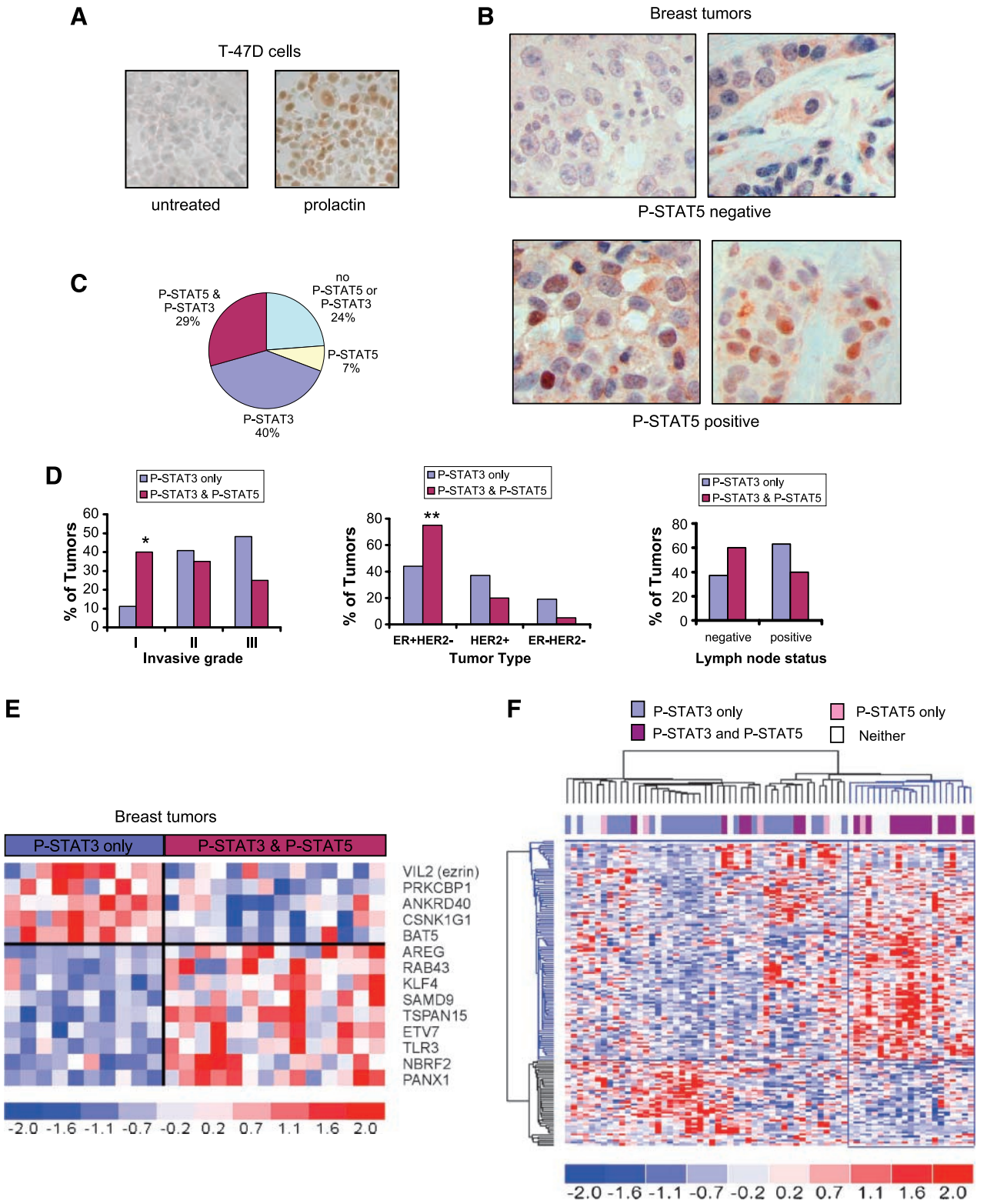
may have opposing roles in mammary function and breast cancer biology.

To define the roles of STAT5 and STAT3 in breast cancer, we analyzed the characteristics of primary human breast cancers displaying activation of one or both of these proteins, determined the consequence of activation of these STATs on the biology of breast cancer cell lines, and defined the effects of these STATs on regulating gene expression.



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Results

Primary Human Breast Cancers with Activation of STAT5 and STAT3 Are More Differentiated than Those with STAT3 Activation Alone

Given that STAT5 and STAT3 have each been reported to be activated in breast tumors and have distinct roles in normal mammary function, we wanted to determine the characteristics of tumors displaying activation of these proteins alone or in conjunction. Staining tissue microarrays for phosphorylated STAT3 has been shown to have high specificity and reproducibility (22). To test the specificity of the phospho-STAT5–specific antibody in immunohistochemistry, T-47D cells were either untreated or stimulated with prolactin, then fixed and stained. Untreated cells showed no staining, whereas the prolactin-treated cells showed nearly 100% nuclear staining (Fig. 1A). This confirmed that the phospho-STAT5 antibody could detect phosphorylated STAT5 with high specificity and reproducibility in immunohistochemistry. We then stained breast cancer tissue microarrays with antibodies specific for phosphorylated STAT5 (Fig. 1B) or phosphorylated STAT3 (22) and analyzed for nuclear staining. Of the tissues stained, 68 tumors had interpretable data for both phosphorylated STAT5 and phosphorylated STAT3. Of these, STAT5 and STAT3 were both activated in 29% of the breast tumors, STAT3 was solely activated in 40%, and STAT5 was solely activated in 7% (Fig. 1C). Because a high proportion of tumors had activation of both STATs, we wanted to determine the differences in phenotype between tumors with activation of both STATs and those with activation of only STAT3. Compared with tumors in which STAT3 was activated alone, tumors displaying activation of both STAT5 and STAT3 were more likely to be low grade (Fig. 1D). Furthermore, cancers with activation of both STAT5 and STAT3 were more likely to be estrogen receptor (ER)-positive HER2-negative tumors and less likely to overexpress HER2 or be negative for both ER and HER2 (i.e., basal-like) than tumors containing STAT3 activation alone (Fig. 1D). In addition, tumors with activation of STAT5 and STAT3 were more likely to be lymph node negative than tumors with only STAT3 activation (Fig. 1D). The small number of tumors displaying STAT5 activation alone precluded statistically meaningful comparisons with this population. However, these findings suggest that activation of STAT5 may moderate the effect of activated STAT3 in human breast cancers.

We thus considered the possibility that STAT5 activation modulated gene expression in these human tumors. Using microarray analysis, we compared the expression of genes in tumors displaying activation of both STAT5 and STAT3 with tumors having activated STAT3 alone. We controlled for potentially confounding effects of estrogen receptor expression, HER2 amplification, and tumor grade by restricting our analysis to the 24 ER-positive HER2-negative tumors of low or intermediate grade. This consisted of 10 tumors with STAT3 activation alone and 14 tumors with activation of both STAT3 and STAT5. Comparison of mean gene expression levels between the two groups using *t* statistics identified 153 genes with at least 1.2-fold differential expression ($P < 0.05$), composed of 114 genes that showed increased expression with STAT5 activation and 39 genes that showed decreased expression (Supplementary Tables S1 and S2, and representative genes, Fig. 1E). Hierarchical clustering of all 68 tumors using this list of 153 differentially expressed genes was able to accurately group tumors according to the activation state of STAT5 ($P = 0.0001$) and the combination of activated STAT5 and STAT3 ($P = 0.000016$; Fig. 1F). This shows that STAT5 activation is associated with a distinct gene expression pattern in human breast tumors containing activated STAT3.

STAT5 Does Not Globally Inhibit STAT3 Function

Because tumors with activation of both STAT5 and STAT3 were more differentiated and displayed more favorable prognostic characteristics than tumors with activation of STAT3 alone, we hypothesized that STAT5 was inhibiting STAT3 signaling. To test this, we used T-47D cells, which are ER positive and resemble the tumor type that most often contains STAT5 and STAT3 activation. T-47D cells were stimulated with prolactin to activate STAT5 or oncostatin M (OSM) to activate STAT3, separately or simultaneously, and STAT5 and STAT3 phosphorylation was then analyzed by immunoblot. Prolactin, which only activated STAT5, had no effect on the magnitude of STAT3 phosphorylation induced by OSM; similarly, oncostatin MOSM had no effect on the magnitude of STAT5 phosphorylation induced by prolactin (Fig. 2A). Because STAT activation also occurred in tumors that were HER2 positive, albeit less often than ER-positive tumors, we also analyzed SK-BR-3 cells, which are HER2 positive. As with the T-47D cells, prolactin activated only STAT5 and OSM activated only STAT3; neither cytokine affected the other pathway (Fig. 2A). This effect is not unique to OSM, as treatment with LIF yielded comparable results (Fig. 2A). Therefore, the activation of STAT5 does not directly affect STAT3 activation. We next considered the possibility that STAT5 was broadly inhibiting STAT3-dependent gene activation. To assess this, T-47D cells were transfected with a STAT3-responsive luciferase reporter plasmid. Following transfection, STAT5 and STAT3 were activated separately or simultaneously with prolactin to activate STAT5 and OSM to activate STAT3. Cells in which STAT3 was

FIGURE 1. Activation of both STAT5 and STAT3 in breast cancer is associated with a distinct phenotype compared with tumors with activation of only STAT3. **A.** T-47D cells were untreated or treated with prolactin and stained with an antibody to phosphorylated STAT5 antibody (*brown*) and counterstained with hematoxylin (*blue*). **B.** Human breast tumors were stained with a phospho-STAT5 antibody and scored for the presence or absence of nuclear phosphorylated STAT5. **C.** Distribution of phosphorylation status of STAT3 and STAT5 in primary breast tumors. **D.** Distribution of tumor grade, tumor type, and lymph node status based on the phosphorylation status of STAT5 and STAT3. *, $P < 0.025$; **, $P < 0.01$. **E.** Gene expression patterns are distinct in tumors with activation of both STAT5 and STAT3 from those with STAT3 activation alone. A subset of differentially expressed genes (fold change >1.2, $P < 0.05$) were grouped from tumors containing phosphorylated STAT3 only and tumors with concomitant phosphorylation of STAT3 and STAT5. To minimize extraneous differences, all tumors were ER positive and low grade (1 and 2). Relative gene expression is shown by a color scale: red, higher expression; blue, lower expression. Columns, tumors; rows, specific genes. **F.** Hierarchical clustering of tumors based on expression of differentially regulated genes (Supplementary Tables S1 and S2) can accurately group tumors according to the activation state of STAT5 alone or in conjunction with STAT3. STAT activation status of each tumor is shown at the top of the heat map.

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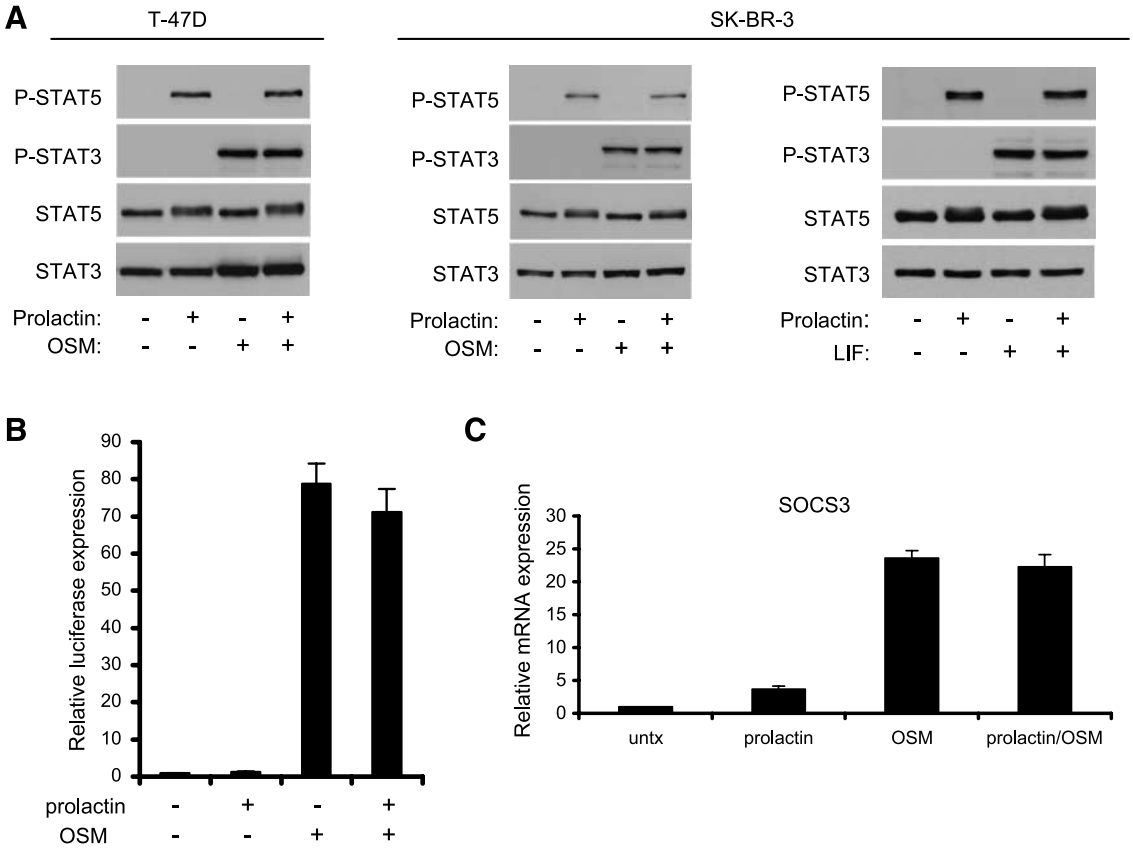

FIGURE 2. STAT5 does not globally inhibit STAT3 signaling. **A.** T-47D cells or SK-BR-3 cells were stimulated for 15 min with the indicated cytokines alone or in combination, and analyzed by immunoblot with the indicated antibodies. **B.** T-47D cells were transfected with a STAT3-dependent luciferase reporter, and then stimulated for 6 h with prolactin, OSM, or the combination of prolactin and OSM, after which luciferase activity was quantitated. Values were normalized to untreated cells. **C.** T-47D cells were stimulated as in **B** for 2 h, after which RNA was harvested. *SOCS3* mRNA was analyzed by quantitative reverse transcription-PCR (qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase.

activated with OSM showed a prominent induction of luciferase expression, whereas the activation of STAT5 with prolactin showed no effect. When STAT5 and STAT3 were activated simultaneously, luciferase expression was comparable with that seen in cells in which STAT3 was activated alone (Fig. 2B). This finding suggests that STAT5 does not generally affect STAT3-mediated gene regulation. To further dissect the effect of STAT5 on STAT3-mediated gene expression, we examined the well-characterized STAT3 target gene *SOCS3*. *SOCS3* showed enhanced expression with activation of either STAT5 or STAT3 (Fig. 2C); however, STAT5 activation did not inhibit the induction mediated by STAT3. These data show that STAT5 does not globally affect STAT3 signaling.

STAT5 and STAT3 Oppositely Regulate BCL6 in Breast Cancer Cells

Because STAT5 activation does not alter global STAT3 function, we considered the possibility that the differences in tumor phenotype reflected opposite regulation of specific target genes. One candidate is *BCL6*, a transcriptional repressor that blocks mammary differentiation and shows increased expression in some types of breast cancer (23, 24) and has also been shown to be regulated by STATs (22, 25). To determine whether STAT5 and STAT3 oppositely regulate *BCL6* expression in breast cancer, T-47D cells were treated with prolactin, which induces tyrosine phosphorylation of STAT5 (Fig. 3A). Prolactin treatment resulted in prominent induction of the well-characterized STAT5 target gene *CIS* (Fig. 3A). By contrast, prolactin led to a significant repression of *BCL6* mRNA (Fig. 3A). Therefore, STAT5 activation can promote increased expression of certain target genes and simultaneous repression of BCL6, as has been previously reported in hematopoietic cell lines (25). Similar results were seen with SK-BR-3 cells in which STAT5 activation was induced by treatment with prolactin or epidermal growth factor (Supplementary Fig. S1A and B). This suggested that STAT5 down-regulates *BCL6* expression in breast cancer cells.

Given the contrasting roles played by STAT5 and STAT3 in mammary epithelium, we next determined the effect of STAT3 on *BCL6* expression in breast cancer cells. T-47D cells were stimulated with OSM, which resulted in prominent phosphorylation of STAT3 (Fig. 3B) but not STAT5. In contrast to stimuli that activate STAT5, OSM treatment resulted in increased *BCL6* mRNA expression (Fig. 3B). Similar results were seen with SK-BR-3 cells stimulated with LIF or OSM to induce STAT3 activation (Supplementary Fig. S1C and D).

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Because these cytokines may mediate changes in gene expression through non-STAT pathways, we next used RNA interference to determine whether STAT5 and STAT3 were necessary for these changes in *BCL6* expression. Reducing STAT5 levels nearly completely abrogated the ability of prolactin to repress BCL6 expression (Supplementary Fig. S2A). Similarly, reducing STAT3 levels almost completely abolished LIF-mediated induction of *BCL6* (Supplementary Fig. S2B), demonstrating that these STATs are necessary for this response. To determine if STAT5 is sufficient to repress *BCL6* expression, a constitutively active mutant of STAT5, STAT5a1*6, was introduced into SK-BR-3 cells. Reflecting the physiologic function of STAT5a1*6, expression of the STAT5-responsive gene *CIS* showed increased expression. By contrast, *BCL6* expression was repressed by nearly 80% (Fig. 3C). Conversely, introduction of a constitutively active form of STAT3, STAT3C, resulted in up-regulation of BCL6 mRNA expression (Fig. 3D). Thus, STAT5 is sufficient to down-regulate BCL6 expression, whereas STAT3 is sufficient to up-regulate BCL6 expression. Taken together, these data suggest that, consistent with their distinct effects in mammary biology, STAT5 and STAT3 exert opposite effects on expression of a key gene in breast cancer cell lines.

STAT5 Opposes STAT3 Function on BCL6

Having shown that STAT5 and STAT3 oppositely regulate *BCL6* expression, we next determined the effects of concomitant activation of these proteins on *BCL6* expression. STAT5 and STAT3 were activated simultaneously in SK-BR-3 cells by stimulation with prolactin and LIF. *BCL6* expression was down-regulated upon simultaneous activation of STAT5 and STAT3 (Fig. 4A). Similar results were obtained when SK-BR-3 cells (Fig. 4A) and T-47D cells (Fig. 4B) were stimulated with prolactin and OSM. This showed that STAT5 activation not only inhibited the STAT3-induced up-regulation of *BCL6*, but STAT5 was also dominant over STAT3 because *BCL6* expression was down-regulated even when STAT3 was activated.

Transiently Activated STAT5 Is Dominant Over Constitutively Active STAT3

We have shown that STAT5 is dominant over STAT3 on *BCL6* expression when both are transiently activated; however, STAT3 is often constitutively activated in tumors. Therefore, we wanted to determine if STAT5 was dominant over constitutively active STAT3. MDA-MB-468 cells, which contain high levels of tyrosine phosphorylated STAT3 (Fig. 5A), were left untreated or treated with prolactin. This resulted in STAT5 phosphorylation (Fig. 5A) and down-regulation of *BCL6* expression (Fig. 5B), demonstrating that STAT5 is dominant over STAT3 at the level of gene expression, even in cells containing constitutive STAT3 activation. To determine if further activation of STAT3 can overcome the repressive effects of STAT5, MDA-MB-468 cells were treated with prolactin and OSM (or

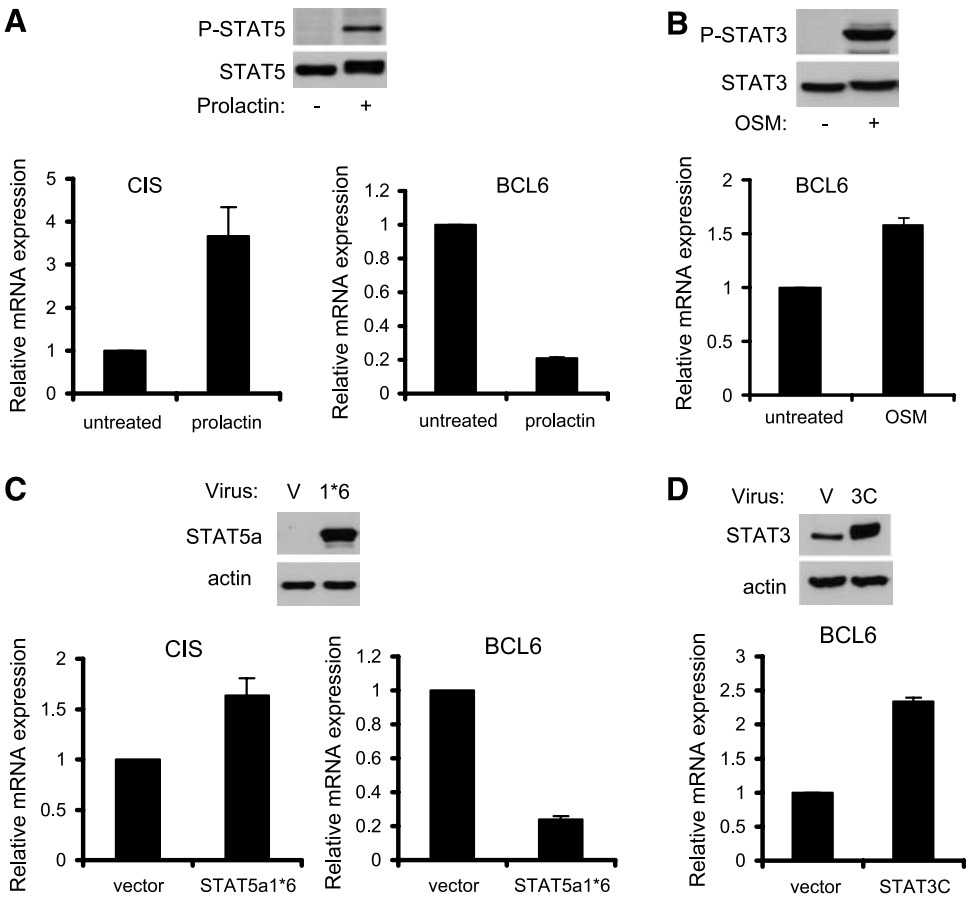

FIGURE 3. STAT5 and STAT3 oppositely regulate *BCL6* expression. **A.** T-47D cells were stimulated with prolactin for 15 min and analyzed by immunoblot for phosphorylated STAT5 (*top*) or were stimulated with prolactin for 2 h and *CIS* and *BCL6* mRNA expression was analyzed by qPCR (*bottom*). **B.** T-47D cells were stimulated with OSM for 15 min and analyzed by immunoblot for phosphorylated STAT3 (*top*) or were stimulated for 90 min and *BCL6* mRNA expression was analyzed by qPCR (*bottom*). **C.** SK-BR-3 cells were infected with virus containing vector (*V*) or the constitutively active mutant STAT5a1*6 (*1*6*), and were analyzed by immunoblot for STAT5a expression (*top*), or were analyzed by qPCR for *CIS* and *BCL6* mRNA expression (*bottom*). **D.** SK-BR-3 cells were infected with virus containing vector (*V*) or the constitutively active mutant STAT3C (*3C*) and analyzed by immunoblot for STAT3 expression (*top*), and *BCL6* mRNA expression was analyzed by qPCR (*bottom*).

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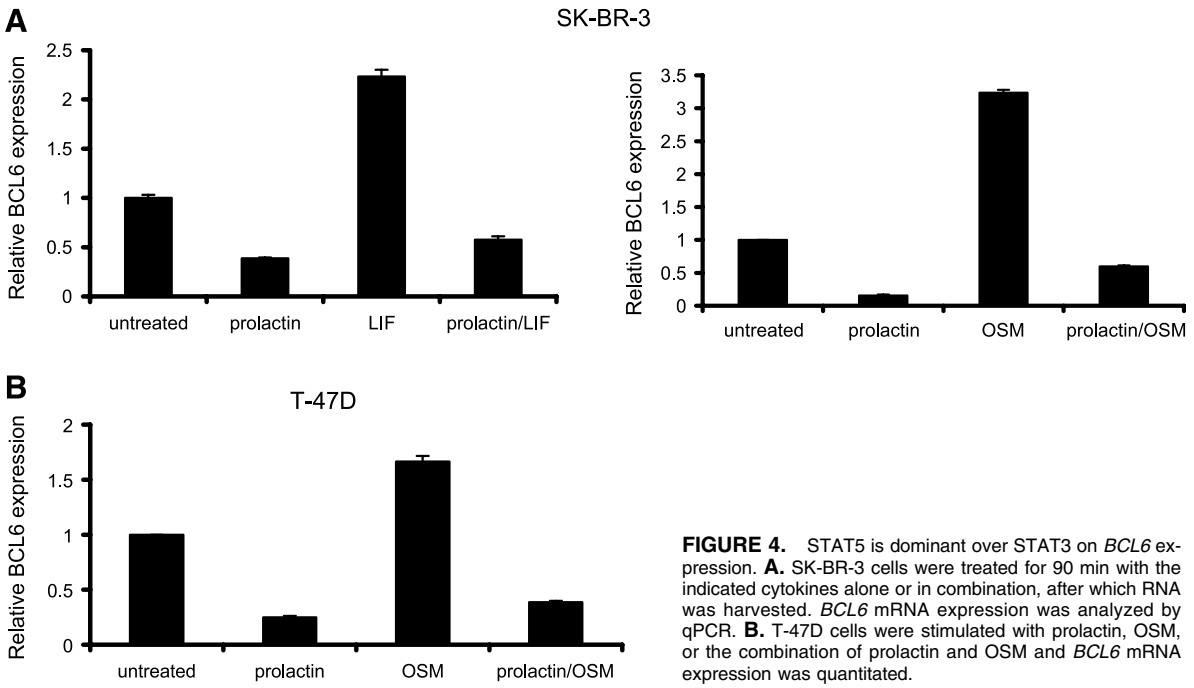

FIGURE 4. STAT5 is dominant over STAT3 on *BCL6* expression. **A.** SK-BR-3 cells were treated for 90 min with the indicated cytokines alone or in combination, after which RNA was harvested. *BCL6* mRNA expression was analyzed by qPCR. **B.** T-47D cells were stimulated with prolactin, OSM, or the combination of prolactin and OSM and *BCL6* mRNA expression was quantitated.

LIF) separately and simultaneously. Stimulation with OSM (Fig. 5C) or LIF (Supplementary Fig. S3A) resulted in enhanced phosphorylation of STAT3; however, this did not prevent STAT5 from promoting down-regulation of BCL6 (Fig. 5D; Supplementary Fig. S3B), suggesting that STAT5 is dominant over both constitutively active and stimulated STAT3 on BCL6 expression.

Constitutive STAT5 Activation Is Dominant Over Constitutively Active STAT3

To more closely mimic the conditions in a tumor in which both STATs are activated constitutively, we wished to generate cells that chronically expressed an activated form of both STAT3 and STAT5. To achieve this, we used MDA-MB-468 cells, which display constitutive STAT3 activation. These cells are ER, PR, and HER2 negative and resemble basal-like tumors, a tumor type most likely to display STAT3 activation alone (Fig. 1D). In fact, we have not identified breast cancer cell lines that are ER/PR positive and which display constitutively active STAT3 (data not shown). We introduced STAT5a1*6 into MDA-MB-468 cells and selected cells stably expressing this activated form of STAT5. Analysis of three different pools showed that chronic STAT5 activation resulted in modest reduction of STAT3 phosphorylation and total STAT3 expression (Fig. 6A and data not shown). This may reflect the fact that chronic activation of STAT5 results in the up-regulation of *SOCS3*, which may inhibit STAT3 activation, thereby establishing a new equilibrium (Supplementary Fig. S4).

Constitutive expression of activated STAT5 resulted in up-regulation of the validated STAT5 target gene *CIS* (Fig. 6B). Importantly, chronic STAT5 activation significantly reduced the expression of *BCL6* (Fig. 6B), demonstrating that STAT5 is dominant over STAT3 on *BCL6* expression when both are chronically activated. Taken together with the previous findings, these data show that both transient and chronic STAT5 activation are dominant over STAT3 function on expression of *BCL6*.

Chronic STAT5 Activation Alters the Biology of Breast Cancer Cells Containing Constitutively Activated STAT3

To validate that this MDA-MB-468 model of STAT activation reflected the biology of the primary breast cancers, we analyzed mRNA levels for a subset of genes that were differentially expressed between tumors containing activation of both STAT5 and STAT3 and tumors with STAT3 activation alone. Of the nine chosen genes identified as being up-regulated in tumors displaying activation of both STATs (Fig. 1E), all but two (*SAMD9* and *TSPAN15*) were also up-regulated to varying levels in MDA-MB-468 cells in which STAT5 was activated (Fig. 7A, *left* and data not shown). Of the five chosen genes that showed lower expression in tumors with concomitant activation of STAT3 and STAT5 (Fig. 1E), all five showed decreased expression in the MDA-MB-468 cells displaying activation of STAT5 as well as STAT3 (Fig. 7A, *right*). This indicated that at the level of gene expression, this model system closely mirrored the findings in primary breast cancers.

Because tumors containing activation of both STATs have better prognostic features than tumors that contain activation of only STAT3, we hypothesized that activation of STAT5 in breast cancer cell lines containing activated STAT3 would modulate the phenotype of these cells. To address this, we first analyzed proliferation. MDA-MB-468 cells containing constitutively activated STAT5a1*6 (in addition to activated STAT3) grew slower than cells infected with the vector control (Fig. 7B). This shows that STAT5 can modulate an important phenotype of breast cancer cells containing activated STAT3.

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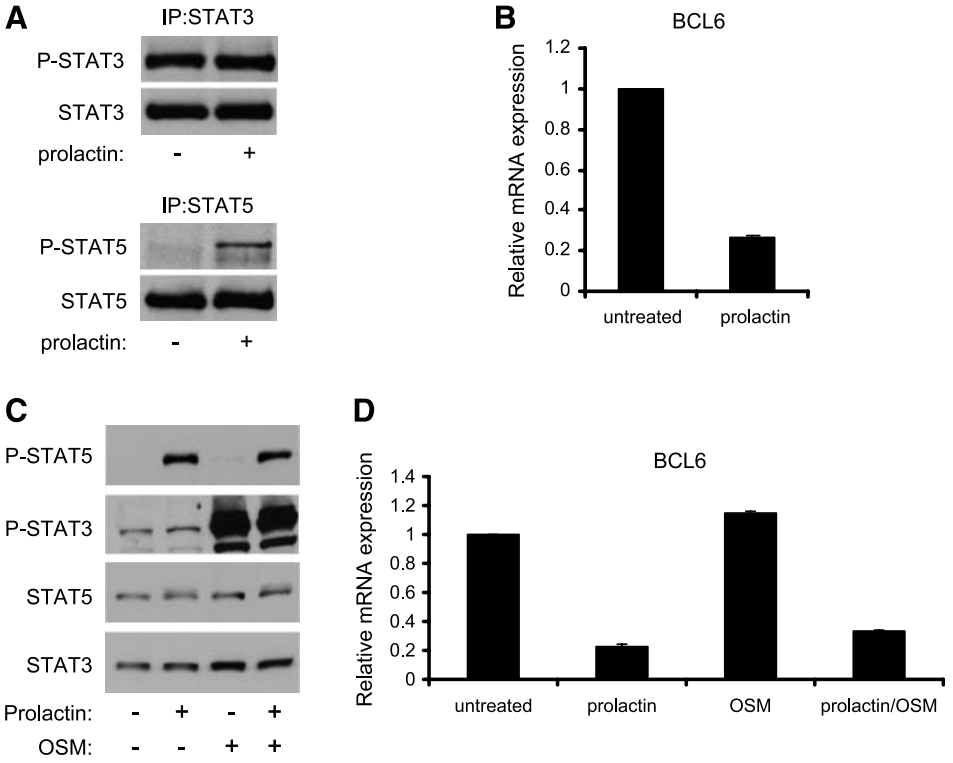

FIGURE 5. Transient STAT5 activation is dominant over constitutively active STAT3 on *BCL6* expression. **A.** MDA-MB-468 cells were stimulated with prolactin for 15 min, after which cell lysates were prepared. Immunoprecipitation was done to STAT5 or STAT3 and analyzed by immunoblot with the indicated antibodies. **B.** MDA-MB-468 cells were stimulated with prolactin for 90 min and *BCL6* mRNA was analyzed by qPCR. **C.** MDA-MB-468 cells were stimulated for 15 min with the indicated cytokines alone or in combination, and analyzed by immunoblot with the indicated antibodies. **D.** MDA-MB-468 cells were treated for 90 min with the indicated cytokines and *BCL6* mRNA expression was analyzed by qPCR.

STAT3 activation can also lead to resistance to chemotherapy and radiation, likely due to the up-regulation of prosurvival genes such as *survivin* (26, 27). Specifically, STAT3 has been shown to promote resistance to paclitaxel in ovarian cancer cells (28). Thus, we next determined if activated STAT5 could affect the response of breast cancer cells containing constitutively active STAT3 to chemotherapeutic agents. Stable pools of MDA-MB-468 cells expressing STAT5a1*6 or empty vector were treated with increasing doses of paclitaxel, a microtubule stabilizer, vinorelbine, a microtubule destabilizer, and doxorubicin, a topoisomerase II inhibitor. Whereas paclitaxel and vinorelbine treatment reduced the viability of both control and STAT5a1*6-expressing MDA-MB-468 cells, the cells with activated STAT5 were approximately twice as sensitive to the inhibitory effects of paclitaxel and vinorelbine at low micromolar concentrations (Supplementary Fig. S5). Both cell lines were equally sensitive to doxorubicin (data not shown). Consistent with this enhanced sensitivity to paclitaxel and vinorelbine,

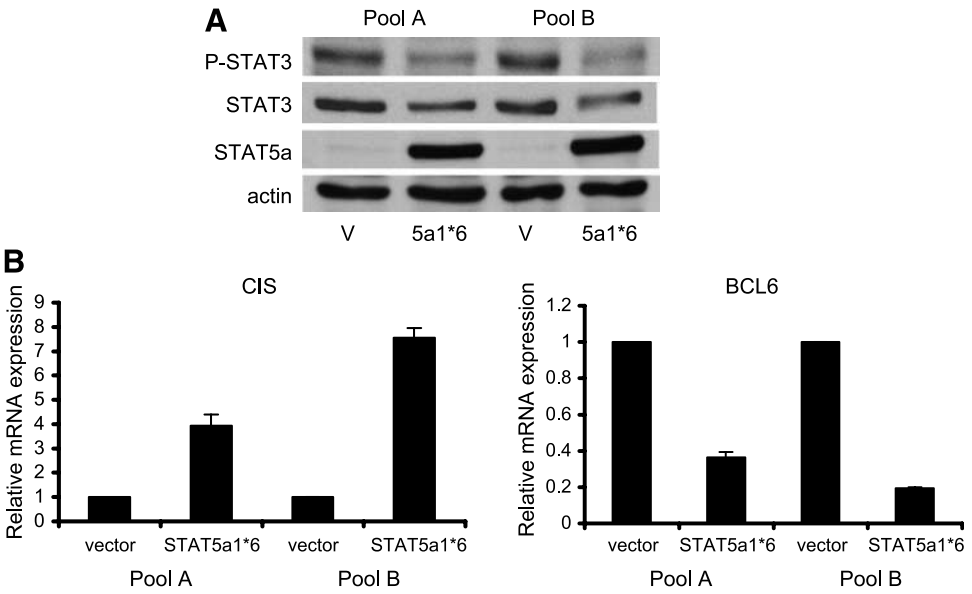

FIGURE 6. Constitutive STAT5 activation is dominant over constitutively active STAT3 on *BCL6* expression. **A.** MDA-MB-468 cells stably expressing vector alone (*V*) or constitutively active STAT5a1*6 (*5a1*6*) were analyzed by immunoblot with the indicated antibodies. **B.** MDA-MB-468 cells stably expressing STAT5a1*6 were analyzed for *CIS* and *BCL6* expression by qPCR.

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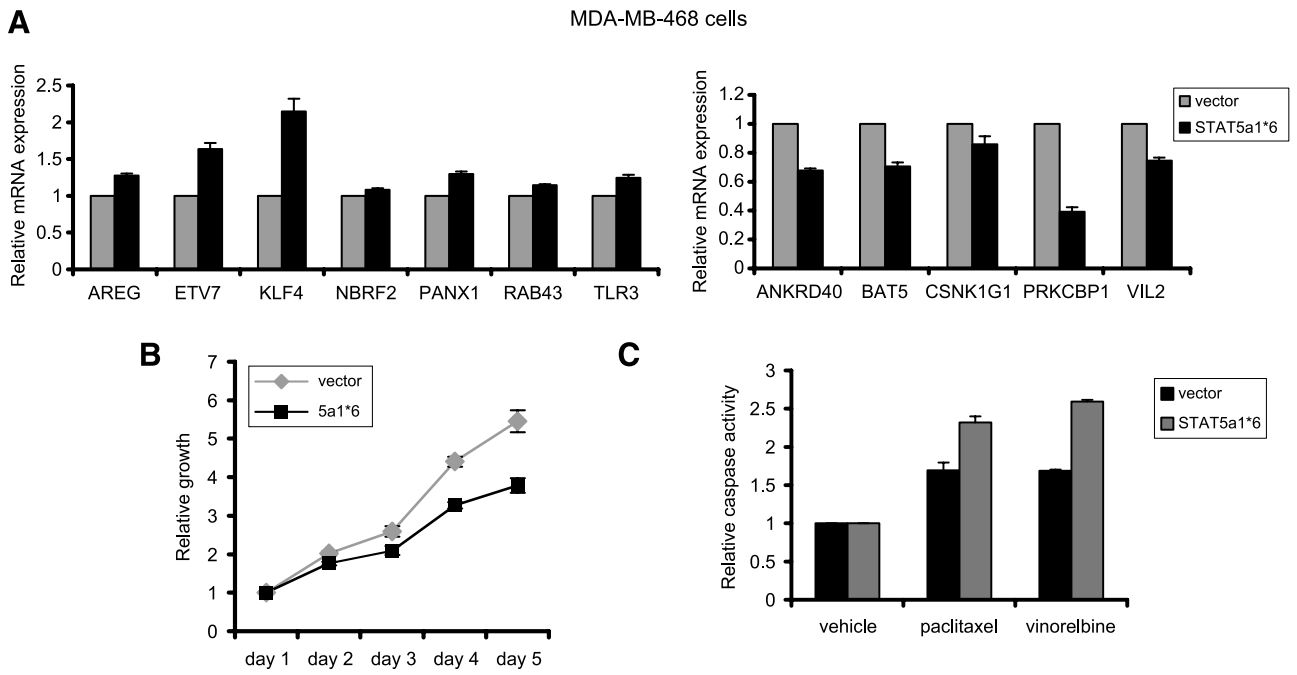

FIGURE 7. Chronic STAT5 activation affects the phenotype of breast cancer cells containing constitutively active STAT3. **A.** mRNA was quantitated by qPCR from MDA-MB-468 cells stably expressing STAT5a1*6 (containing activated STAT3 and STAT5) or vector alone (containing activated STAT3 only) for a subset of genes identified as being differentially expressed in breast tumors. **B.** Cell growth analysis was done on stable pools of MDA-MB-468 cells expressing vector or STAT5a1*6 using ATP bioluminescence, normalizing values to day 1 readings. **C.** MDA-MB-468 cells stably expressing STAT5a1*6 were treated with vehicle (DMSO), 5 μmol/L paclitaxel, or 5 μmol/L vinorelbine for 24 h. Caspase activity was quantitated using CaspaseGlo and normalized to each vehicle-treated control.

both of these drugs promoted increased apoptosis, as measured by caspase activation, in cells with activation of both STAT5 and STAT3 (Fig. 7C). Taken together, these data show that breast cancer cells with chronic activation of both STAT5 and STAT3 have less aggressive features than cells containing activated STAT3 alone.

Discussion

We have shown that STAT5 and STAT3 have opposing roles in breast cancer on three levels. First, STAT5 and STAT3 oppositely regulate a subset of target genes, in which the repression of gene expression mediated by STAT5 is dominant over the increased expression mediated by STAT3. Second, chronic activation of STAT5 affects the phenotype of breast cancer cells containing constitutively active STAT3 such that coexpression of activated STAT5 leads to a decrease in proliferation and increased sensitivity to the chemotherapeutic agents paclitaxel and vinorelbine. Finally, human breast tumors displaying activation of both STATs are more differentiated than tumors that contain only STAT3 activation.

Although STAT5 and STAT3 are highly homologous, they mediate distinct effects in mammary physiology. Many genes are regulated in a parallel fashion by both transcription factors, such as *bcl-xl, mcl-1*, and *cyclin D1* (29). However, STAT5 and STAT3 also modulate distinct subsets of genes (21). Although STATs were identified as activators of transcription, it is becoming increasingly clear that at least a subset of genes can be repressed by STATs. One gene showing reciprocal regulation by STAT5 and STAT3 is *BCL6*, a transcriptional repressor. BCL6 can block cellular differentiation in both hematopoietic and epithelial cells (23, 30). Reflecting this function, increased expression of BCL6 has been found in high-grade ductal carcinomas and invasive breast cancers (23, 24). Thus, the regulation of expression of this gene may be a critical factor in mammary tumorigenesis.

The fact that STAT5 mediates the repression of *BCL6* expression is not surprising given the fact that STAT5 is a key mediator of the effects of prolactin, a hormone necessary for the differentiation of mammary epithelium, ultimately leading to lactation (31, 32). Many of the target genes of STAT5, which was initially defined as "mammary gland factor" for its critical role in this process, are milk proteins such as β-casein and whey acidic protein (33, 34). Thus, the ability to down-regulate an inhibitor of differentiation such as *BCL6* is consistent with this function of STAT5. However, prolactin can also increase proliferation and survival of mammary epithelial cells so that constitutive activation of STAT5 could be associated with the promotion of neoplastic cell growth as well. In fact, in murine models, STAT5 has been shown to promote mammary tumors and loss of STAT5 delays tumor formation (6, 12, 13). In addition, serum prolactin levels show a positive correlation with the risk of developing breast cancer in both premenopausal and postmenopausal women (11). However, this effect is strongest for tumors that express the estrogen receptor and/or the progesterone receptor, which are generally more differentiated. This mirrors the finding in the present study that STAT5 activation generally occurs in more differentiated tumors that express the

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estrogen receptor (Fig. 1). Thus, because prolactin can promote proliferation, survival, and differentiation, it is not surprising that STAT5 activation is a component of mammary tumorigenesis, but may be associated with tumors that are more differentiated and less aggressive.

The role of STAT3 in the mammary gland is multifaceted as well. STAT3 target genes, including *BCL6*, have been implicated in promoting pluripotency and maintaining cells in an undifferentiated state. For example, the pluripotency of murine embryonal stem cells can be maintained by LIF-induced STAT3 activation (35, 36). In addition, STAT3 target genes promote cell cycle proliferation, survival, migration, and angiogenesis (37). Thus, the observation reported in multiple studies that STAT3 is activated in primary breast cancers, particularly high-grade tumors, is consistent with this role of STAT3 (19, 22). However, in the normal development of the mammary gland, STAT3 is necessary for the cell death that occurs during the involution and remodeling process after lactation ceases (14, 15). Thus, it is clear that STAT3 is a key regulator in both normal mammary epithelium and in breast cancer.

The dominant effect of STAT5 over STAT3 is not restricted to modulating gene expression but also extends to other aspects of the biology of breast cancer cells in which both transcription factors are activated. In addition to the decreased proliferation and increased sensitivity to paclitaxel and vinorelbine seen *in vitro* (Fig. 7), primary breast cancers with activation of both STATs are lower grade and more likely to be ER positive and HER2 negative than those displaying activated STAT3 alone (Fig. 1). The presence or absence of STAT5 activation may explain the diversity of phenotypes of breast cancers displaying activation of STAT3, with tumors containing activated STAT3 alone being more likely to be high grade and those with activation of both STATs being low grade.

Although distinct cells of origin may explain some component of breast cancer heterogeneity, it is unlikely to be the sole explanation for the differences between tumors containing activated STAT5 and STAT3 versus those containing activated STAT3 alone. Using a model system in which STAT3 is either activated alone or in conjunction with STAT5 in an identical genetic background, we identified similar changes in gene expression as seen in primary human tumors displaying activation of one or both of these transcription factors (Fig. 7A). This shows that the differential gene expression is not entirely due to different tumor cell types and that STAT5 activation directly affects the transcriptional profile of breast tumors that contain STAT3 activation.

Interestingly, *BCL6* was not a gene that showed significant differential regulation between the tumors with activation of STAT5 and STAT3 versus those displaying activation of STAT3 (Supplementary Table S3). This may reflect the limited size of this data set as well as the fact that a number of other transcription factors known to play an important role in breast cancer pathogenesis, including p53, progesterone receptor, and NF-κB, can also regulate *BCL6* expression (38-40). This may have attenuated the ability to detect the effects of STAT3 and STAT5 in these samples. However, *BCL6* clearly plays a role in differentiation of mammary tumors and remains a good model for the reciprocal effects of STAT5 and STAT3 on gene expression. Similarly, negative regulators of STAT signaling, such as *CIS* or *SOCS3*, may be inactivated by methylation or deletion in cancers and this may also attenuate STAT-dependent differences in expression detected in tissue microarrays (Supplementary Table S3).

The reciprocal effects of STAT5 and STAT3 on breast cancer cells also provide an opportunity for therapeutic intervention. A number of approaches have been used recently to inhibit STAT3 function for therapeutic purposes (41-43). There is also evidence that small molecules can specifically enhance the function of STAT family members (44). Therefore, given the potentially beneficial role of STAT5 activity in opposing STAT3 function and possibly promoting differentiation, activation of STAT5 may be a useful strategy to treat aggressive tumors alone or in combination with STAT3 inhibitors. Thus, pharmacologic STAT modulators, perhaps in conjunction with chemotherapeutic agents, may be a rational molecular strategy for treating these forms of breast cancer.

In this work, we have shown that two highly related transcription factors oppositely regulate a subset of target genes. This may explain, at least in part, how STAT5 and STAT3 promote distinct effects in normal mammary function. In addition, we have shown that whereas both STATs can be activated in breast cancer, they are associated with distinct phenotypes. Furthermore, STAT5 exerts a dominant effect over STAT3 in terms of gene expression, cellular phenotype, and breast cancer tumor type. Therefore, analysis of the activation status of both STAT5 and STAT3 in breast tumors may be important in understanding breast cancer pathogenesis, may aid in diagnosis and prognosis, and may be useful in identifying targeted therapeutic approaches for the treatment of breast cancer.

Materials and Methods

Immunohistochemistry

T-47D cells were washed in PBS, scraped, and centrifuged. Cell pellets were fixed in 10% formalin and embedded in paraffin. Human breast tumor cohorts were described previously (22, 45, 46). Tissue microarrays contained two representative 0.6-mm cores of each breast tumor and several cores of representative normal breast tissue. STAT5 phosphorylation was determined by immunohistochemistry using an antibody specific for tyrosine phosphorylated STAT5 (Cell Signaling). This antibody has been validated independently as being specific to phosphorylated STAT5 in immunohistochemistry on breast tumors (9). For phospho-STAT5 immunohistochemistry, only nuclear reactivity was considered positive; the proportion of tumor cells staining positive for phospho-STAT5 ranged from only a few cells to most of the tumor cells. Results for phospho-STAT3 immunohistochemistry on these tumors was reported previously (22). *P* values were determined using the χ^2 test.

Gene Expression Array Analysis

Expression array data determined using Affymetrix U133p2.0 microarrays were available for each of the tumors in the tissue microarray. This represented a subset of previously published array data (ref. 47; GEO accession no. GSE3744). Comparisons were made between tumors in which both STAT3 and STAT5 were activated versus tumors displaying activation of STAT3 alone as determined by immunohistochemical staining. One hundred fifty-three nonredundant RefSeq validated

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genes were identified that differed by >1.2-fold between the two groups with lower 90% confidence bound and a *P* value of <0.05 for testing the alternative hypothesis that there is no difference in expression of these genes between the two groups. Gene filtering, group comparisons, and clustering analyses were done using the dCHIP software (48).

Cell Lines and Stimulations

T-47D (American Type Culture Collection), MDA-MB-468 (kindly provided by Myles Brown, Dana-Farber Cancer Institute), and 293 cells were maintained in DMEM containing 10% FCS. SK-BR-3 cells (kindly provided by Lyndsay Harris, Dana-Farber Cancer Institute) were maintained in RPMI 1640 with 10% FCS. Cells were stimulated with 100 ng/mL prolactin, 10 ng/mL OSM (R&D Systems), 10 ng/mL LIF (Chemicon), or 50 ng/mL epidermal growth factor (Sigma).

Immunoblots

Immunoblots and immunoprecipitations were done as described (49) using antibodies toward phospho-STAT5 and phospho-STAT3 from Cell Signaling; STAT5a, STAT5, and STAT3 from Santa Cruz Biotechnology; STAT5b (Zymed); and tubulin and actin from Sigma.

Reporter Gene Assays

T-47D cells (5×10^4) were transfected in duplicate with 1 μg of the STAT3-dependent reporter m67-luc (kindly provided by J. Bromberg, Memorial Sloan-Kettering) and 0.1 μg phRL-tk (Promega) as a transfection control, using Lipofectamine 2000 (Invitrogen). Sixteen hours after transfection, cells were stimulated for 6 h. Luciferase activity was measured as described (25).

Reverse Transcription-PCR

RNA was harvested using the RNeasy Mini kit from Qiagen. cDNA was generated using the TaqMan first strand kit from Applied Biosystems. Quantitative reverse transcription-PCR was done as described (25), using the indicated primers (Supplementary Table S4). For experiments analyzing *CIS* expression, DNase treatment (Qiagen) was carried out according to the manufacturer's protocol. This was done to remove any genomic DNA contaminants, because the intron spanned by the primers is relatively small.

Short Interfering RNA

Cells (5×10^5) were transfected with short interfering RNA from Dharmacon, Inc. Cells were transfected with 50 nmol/L siSTAT5a and 50 nmol/L siSTAT5b or 100 nmol/L siControl using Lipofectamine 2000 according to the manufacturer's protocol. Medium was added 5 h after transfection and exchanged 24 h after transfection. Forty-eight hours after transfection, cells were harvested for mRNA analysis or immunoblotting.

Viral Production and Infections

Cells (293) were transfected with VSV-G and gag-pol–expressing vectors using Lipofectamine 2000. Six hours after transfection, the medium was exchanged. Twenty-four hours after transfection, the supernatant was collected. A 1:1 ratio of viral supernatant was added to cells with 8 μg/mL polybrene and incubated for 16 h, after which the medium was replaced. For transient infections, RNA and protein were isolated 24 h after medium replacement. For stable integration, selection was begun 24 h after medium replacement. For plncx2 and plncx2-STAT5a1*6 vectors, MDA-MB-468 cells were selected in 1 mg/mL G418 for 14 d. Three pools were generated by infecting cells at distinct times. For introduction of RNA interference vectors, SK-BR-3 cells were infected with retrovirus containing pRetroSuper (pRS) or pRetroSuper-STAT3i (22) and selected in 750 ng/mL puromycin. Cells remained under selection for all experiments.

Viability Assays

MDA-MB-468 cells (3×10^3) containing plncx2 (vector) or plncx2-STAT5a1*6 (STAT5a1*6) were plated in quadruplicate. Twenty-four hours after plating, cells were left untreated or were treated with vehicle, paclitaxel, vinorelbine, or doxorubicin (NovaPlus, Dana-Farber Cancer Institute Pharmacy). ATP was measured daily for proliferation assays or 48 h after drug treatment using Celltiter-Glo (Promega) and quantitated on a Luminoskan luminometer. Proliferation assays were normalized to values on day 1 and cytotoxicity assays were normalized to cells treated with vehicle. Data are representative of at least two different experiments in multiple different pools.

Caspase Activation Assays

MDA-MB-468 cells (3×10^3) containing plncx2 (vector) or plncx2-STAT5a1*6 (STAT5a1*6) were plated in duplicate. Twenty-four hours after plating, cells were left untreated or were treated with vehicle, paclitaxel, vinorelbine, or doxorubicin. Twenty-four hours later, caspase activity was measured using CaspaseGlo (Promega) and quantitated on a Luminoskan luminometer. Data are representative of all three pools.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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