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Review

Roles and Regulation of Stat Family Transcription Factors in Human Breast Cancer

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Stats (for signal transducers and activators of transcription) are a family of transcription factors that regulate cell growth and differentiation. Their activity is latent until phosphorylation by receptor-associated kinases. A sizable body of data from cell lines, mouse models, and human tissues now implicates these transcription factors in the oncogenesis of breast cancer. Because Stat activity is modulated by several post-translational modifications and protein-protein interactions, these transcription factors are capable of integrating inputs from multiple signaling networks. Given this, the future utilization of Stats as prognostic markers and therapeutic targets in human breast cancer appears likely. ***(Am J Pathol 2004, 165:1449–1460)***

Since their identification 12 years ago,[1] the Stat (for signal transducers and activators of transcription) family of transcription factors have been recognized as critical integrators of cytokine and growth factor receptor signaling required for cell growth, survival, differentiation, and motility. A role for two members of this family, namely Stat3 and Stat5, in the pathogenesis of human breast cancer is increasingly appreciated. As presented below, several lines of evidence from breast cancer cell lines, mouse models, and primary human tissues have implicated these transcription factors in mammary oncogenesis. With these findings as a backdrop, this review will explore the multiple mechanisms through which Stat activity is regulated, mechanisms that provide new targets of potential prognostic and therapeutic utility.

Stats exist within the cytoplasm in a latent or inactive state. After cell-surface receptor activation by ligand, Stats are activated by receptor-associated tyrosine kinases (Figure 1). Significant in this regard are the Jak family (Jaks 1 to 3 and Tyk2) of tyrosine kinases that are rapidly (within 1 minute) activated by autophosphorylation, presumably triggered by ligand-induced receptor dimerization/multimerization. In turn, activated Jak kinases phosphorylate the receptor to which it has bound, and receptor-associated signaling proteins. Receptor phosphorylation enables Stat docking to this complex, via binding of a Stat SH2 domain to a receptor phosphotyrosine residue. This event permits Stat phosphorylation by the juxtaposed Jaks and other receptor-associated kinases. Phosphorylation of a tyrosine residue present in the Stat C-terminus triggers its release from receptor, and homo (and in some cases hetero-)-dimerization of phosphorylated Stat proteins briskly ensues. Dimerized Stats rapidly translocate to the nucleus, where binding to gene promoters bearing cognate DNA-binding sequences occurs. Once bound, Stats engage several elements of the transcriptional apparatus, stimulating gene expression. In the context of breast cancer, Stat activation has been found to occur both *in vitro* and *in vivo* after the binding of ligand to several receptors implicated in the pathogenesis of breast cancer; a listing of receptor-Stat affiliations is presented in Table 1.

Identification and Functional Role of Stats

The Stat family of transcription factors was first identified through the careful analysis of the molecular requirements of interferon (IFN)-triggered gene expression.[2] Both IFN-α and IFN-γ trigger gene expression within 15 to 30 minutes of IFN receptor stimulation; analysis of the promoter regions of these IFN-activated genes revealed that specific motifs (the GAS motifs) with a consensus DNA sequence of TT(C/A)YNR(G/T)AA were required for this expression. These motifs were used in DNA affinity pull-down of cell lysates; coupled with biochemical purification, these approaches enabled the isolation and sequencing of the first Stats (Stat1α, Stat1β, and Stat2).[1] Using DNA-binding sequences obtained from the β-casein promoter, Stat5 was subsequently purified, se-

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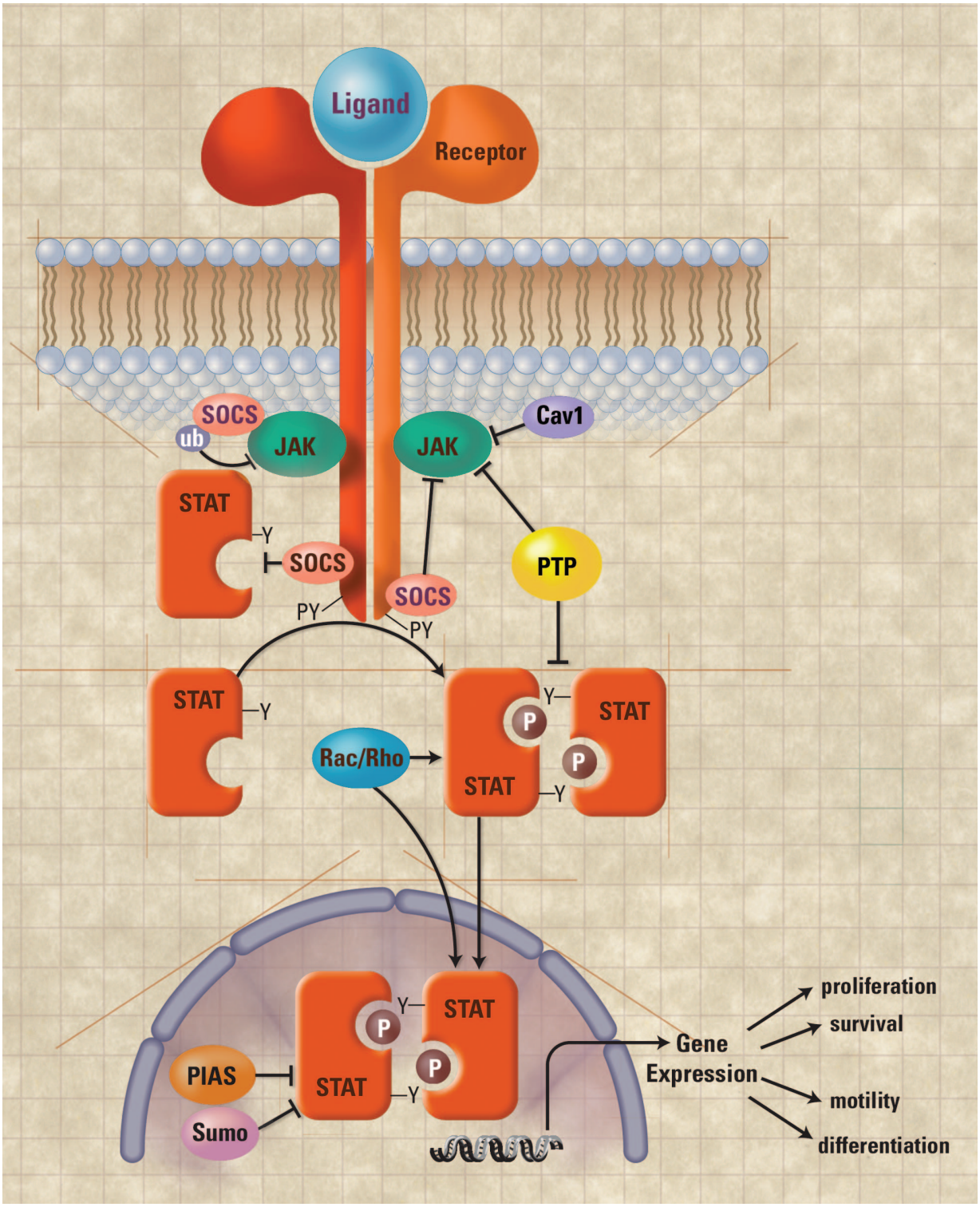

Figure 1. Activation and regulation of Stat-mediated transcription. After ligand-induced receptor dimerization, activated Jak sequentially phosphorylates receptor and Stat. This induces the release and dimerization of phosphorylated Stat, enabling its translocation into the nucleus and subsequent DNA binding. As indicated in the figure, Stat activity is regulated at many levels; events that stimulate Stat activity are noted with **lines** terminating in **arrowheads**, whereas inhibitory events are marked with **lines** ending with **bars**. Abbreviations: ub, ubiquitin; pY, phosphotyrosine; PTP, phosphatase. For the sake of simplicity some Stat regulatory events (ie, serine phosphorylation, protein-protein interactions, and so forth) have been omitted.

quenced, and cloned in a manner similar to Stats 1 and 2.[3] In contrast, Stat3 was identified by cDNA library screen with the SH2 domain of Stat1.[4] As previous studies had demonstrated that IFN-induced gene expression was inhibited by tyrosine kinase inhibitors, it was quickly recognized that the targets of this phosphorylation were the Stats. As noted above, tyrosine phosphorylation is required for Stat dimerization and nuclear translocation.

Table 1. Stat Activation Mediated by Cell-Surface Ligand/Receptor Complexes Relevant to Breast Cancer

Stat3	Stat5
Epidermal growth factor	Epidermal growth factor
Platelet-derived growth factor	Platelet-derived growth factor
Hepatocyte growth factor	Prolactin
Insulin-like growth factor	Growth hormone
Insulin	Erythropoietin

Additional analysis revealed that Stats' members were activated by several disparate cell surface receptors (Table 1).[5] Contemporaneously, with the recognition of Stats as transcription factors, came the identification and characterization of the Jak family of tyrosine kinases.[6–8] The signaling connection between the Jak and Stat families, however, was not known *a priori*; the breakthrough for this connection was provided by somatic cell genetics.[9] Using mutagenesis and complementation approaches, cell clones defective in IFN-induced signaling regained IFN-responsiveness when Jaks or Stats were reintroduced, establishing linkage between these two families. Subsequent analysis with intact, nonmutagenized cells demonstrated the critical, transient association between Jak and Stat family members, required for Stat phosphorylation and activation.[2,6]

Stat Structure/Function

Extensive structural mutagenesis studies have revealed that the Stat proteins consist of numerous distinct functional domains (Figure 2).[2,10] The N-terminal domain has been found necessary for the interaction of Stats 3 and 5 with a number of co-activators, such as CBP/p300, c-Jun, and Nmi;[11] in addition, this domain is necessary for higher-order associations between Stat5 dimers (ie, tetramerization) that contribute to enhanced Stat5 activity on certain promoters.[12] A central DNA-binding domain is required for recognition of cognate binding sequences. This domain is coupled by a flexible linker to a SH2 domain that is necessary for the recognition of phosphotyrosine residues that contribute to Stat dimerization and the recognition of cell-surface receptor binding sites. Between the SH2 motif and the C-terminal transactivation domain resides a conserved tyrosine residue whose phosphorylation is required for the dimerization of all Stat family members. Aside from its critical role in coordination with the transcriptional apparatus, the transactivation domain contains a serine residue(s) that also regulates the activity of this domain.

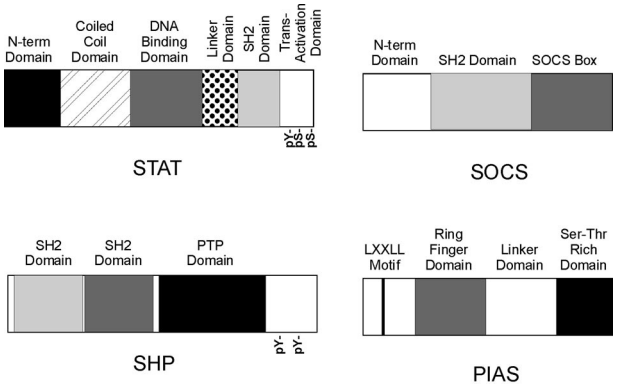

Figure 2. Structural maps of the Stat, SOCS, SHP, and PIAS protein families. Phosphorylated tyrosine and serine residues implicated in protein function are, respectively, designated pY and pS. The phosphatase domain is noted in the C-terminus of SHP (PTP). The signature leucine-rich motif conserved in the PIAS family is designated in the N-terminus of PIAS3 (LXXLL).

The structure of DNA-engaged STAT dimers has been resolved at the crystallographic level for both Stats 1 and 3.[13,14] It should be noted that the data from these crystal structures is limited because they do not include either the functionally critical N-terminal domain or the C-terminal transactivation motif. On a topological level the crystal structures of Stats 1 and 3 resemble a nutcracker, with the interactions between the respective Stat phosphotyrosine/SH2 domains serving as the hinge and the DNA acting as the nut. These structures have revealed a twofold axis of dyadic symmetry surrounding the DNA-binding sites used in these studies. The structures are similar and consist of four domains: a four α-helical bundle within the N-terminus that is presumed to contribute to protein-protein interactions, an eight-stranded β-barrel in which the DNA-binding domain resides, an α-helical connector, and a classic SH2 domain. The Stat homodimer envelopes the bound DNA with distinct amino acid residues contacting both the phosphate moieties and sugar residues of the DNA backbone, as well as specific nucleotides. Interestingly, the engaged DNA is actually bent by 40° as the result of this interaction. The C-terminal phosphotyrosine residues are engaged by their counterparts' SH2 motif, providing a pivot point for DNA binding.

Role of Stats in Normal Mammary Biology

Mouse Models

During pregnancy, the expression and activity of the Stat family is variably modulated, with the expression and activity of Stat5 closely linked to the onset of lactation, whereas the expression and activity of Stats 1 and 3 are associated with the virgin animal and involution.[15] Gene targeting studies have revealed phenotypes for Stats 3 and 5a during mammary differentiation; the Stat5a knockout demonstrates profound loss of alveolar maturation and milk production;[16] indeed the mammary phenotype of the Stat5a$^{-/-}$ mouse is similar to that observed in both the prolactin (PRL) and prolactin receptor (PRLr) knockout mice.[17,18] In contrast, Stat3 mice with a conditional knockout of Stat3 within the mammary gland demonstrate delayed mammary involution after cessation of pregnancy.[19] These data would suggest that Stat5 plays a critical role in lobuloalveolar proliferation, differentiation, and expansion, whereas Stat3 regulates lobuloalveolar apoptosis during pregnancy, lactation, and involution.

Role of Stats in Breast Cancer

Data from Human Tissues—Insights into Biology

Several studies have used different approaches to assess the expression, localization, phosphorylation, and DNA binding ability of Stats 3 and 5 in breast tissues. Immunohistochemical approaches have successfully examined total and phosphorylated Stat 3 and 5 levels in breast tissues, and correlated this expression with intranuclear localization. In this context, both the phosphorylation and nuclear localization of Stats 3 and 5 are presumed to correlate with the level of transcriptional activity of these proteins. The advantage of an immunohistochemical approach is its ease in application to large numbers of patient specimens. The disadvantage to this approach is the presumption that phosphorylation and nuclear localization equate to transcriptionally active Stat; as discussed below there are several mechanisms through which intranuclear Stat function can be repressed. Alternatively, the level of Stat DNA binding can be measured by electrophoretic mobility shift analysis (EMSA); although this is perhaps a better measure of Stat activation, it is time consuming and not amenable to small tissue samples. Ideally, both immunohistochemical analysis and EMSA would be compared on the same cohort of human samples; unfortunately, only one study has made such direct comparison.

Significant elevations in the DNA-binding activity of both Stat3 and Stat5[15,20] were noted in a small sample of malignantly transformed breast tissues when compared to normal tissues. Although this pioneering study provided some of the first evidence for alterations in Stat DNA-binding activity in breast cancer, it was limited by its lack of normalization to total Stat protein levels (ie, the EMSA analysis was not normalized), small patient cohort, and lack of associated outcomes analysis.

At the immunohistochemical level, two studies have examined Stat3 localization and phosphorylation. The first study[21] examined 62 malignant breast cancers and found increased levels of nuclear localized Stat3 in comparison to normal surrounding tissues; no association with clinicopathological or outcomes data were provided. The second study[22] assessed a microarray containing tissues from 346 node-negative breast cancer patients with both anti-Stat3 and anti-phospho-Stat3 immunohistochemistry and correlated these results with clinicopathological and survival data. This study revealed that nuclear Stat3 and phospho-Stat3 was noted in 23% and 44% of patients, respectively; however, a rationale for the discrepancy between these percentages was not commented on. In addition, the study found that nuclear phospho-Stat3 expression was correlated with a modest, but statistically significant, improvement in patient survival both at 5 and 20 years. Multivariate analysis of these data revealed that this parameter was statistically more predictive of outcome than tumor size, nuclear grade, age, estrogen/progesterone receptors and Her2 status, and Ki-67 positivity.

A small study examining the expression of phosphorylated Stat5 in human breast tissues by immunohistochemical approaches has revealed significant intranuclear Stat5 in both normal and lactating human breast tissues; parallel studies in mice confirmed that a basal level of Stat5 phosphorylation can be noted in normal virgin, pregnant, and lactating mammary glands.[23] This phosphorylation may be because of the local production of PRL by breast tissues.[24,25] Whether the phosphorylated Stat5 in the nonpregnant, normal breast is actually bound to DNA and transcriptionally active (or bound to a repressor such as PIAS3, see below) remains to be determined. In another study, immunohistochemical analysis of 83 primary human breast cancers for both Stat5 and tyrosine-phosphorylated Stat5 revealed phosphorylation and nuclear localization in 76% of malignancies examined; no associations with several recognized prognostic markers (tumor size, nodal and estrogen receptor status, percent S phase, and so forth), save for histological differentiation.[26] This study also confirmed the presence of a basal level of Stat5 phosphorylation in normal breast tissues. A more recent study examining more than 1300 breast cancers has found a strong association between Stat5 nuclear localization/phosphorylation and improved overall and disease-free survival.[27]

Only a single study to date has examined the relationship between Stat phosphorylation and DNA binding status[28] *in vivo*. This study, which evaluated Stats 1, 3, and 5, found a strong correlation between Stat 1 and 3 phosphorylation (as measured by immunoblot analysis) and DNA binding (as measured by EMSA) in human tissues, but could not detect Stat5 phosphorylation (as noted above), possibly secondary to antibody difficulties. Although the study found a correlation between Stat1 activation/phosphorylation and survival (but not Stats 3 or 5), the numbers of this study were small (68 samples including both node-negative and node-positive), the data were not normalized to total Stat content, and Stat phosphorylation was not assessed by immunohistochemistry (ie, the contribution of inactive Stat from the surrounding stromal tissues may have been significant). As a final note, no study to date has reported on Stat localization or phosphorylation in the nonepithelial compartment of normal or malignant breast tissues, or in the various histological subtypes of breast cancer.

Data from Mouse Models of Breast Cancer

In vivo, the effect of hemizygous loss of Stat5a in a SV40-T antigen transgenic mouse model of mammary cancer has been tested after cross-breeding. The resultant progeny ($Stat5a^{+/-}$ + Tag) demonstrated a significant reduction in the percentage of tumor-bearing mice, as well as decreased tumor size, delayed first tumor appearance, and increased apoptotic indices.[29] The role of Stat3 in murine mammary cancer models remains to be determined, and awaits cross-breeding of the conditional Stat3 mammary knockout mouse with other murine models of mammary cancer.[19]

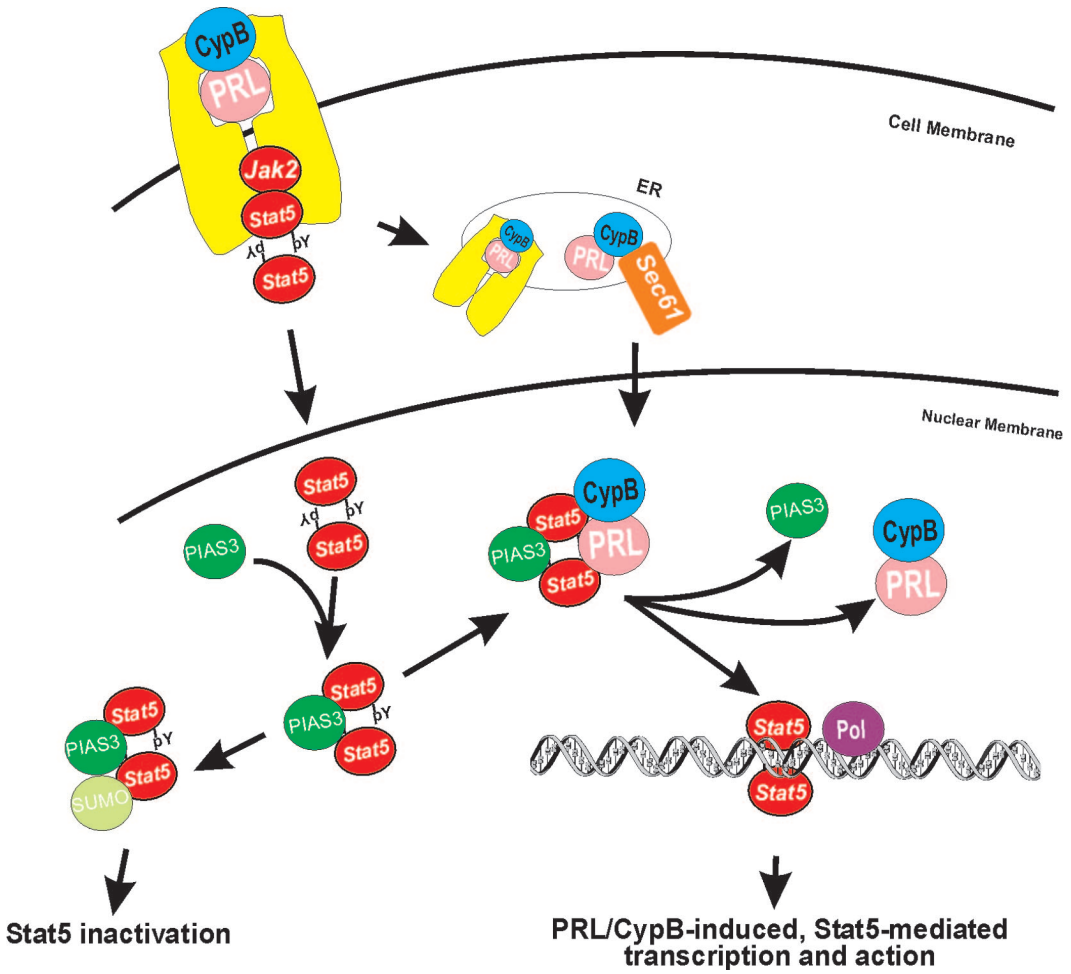

Figure 3. Mechanism for PRL/CypB induction of Stat5-induced gene expression. After receptor-mediated endocytosis, the transport of PRL into the nucleus is facilitated by its interaction with CypB. On entry into the nucleus, this complex interacts with Stat5 inducing the release of a repressor of its DNA-binding activity, namely PIAS3. The PRL/CypB-mediated release of PIAS3 from Stat5 results in significantly enhanced gene expression. Abbreviations: Pol, RNA polymerase II transcriptional apparatus; Sec61, ER transporter apparatus.

Data from Cell Lines—Insights into Function

Stat3 tyrosine phosphorylation and DNA-binding activity of several breast cancer lines has been found to be elevated; conversely, pharmacological or dominant-negative inhibition of Stat3 activity has been found to block the proliferation and survival of breast cancer cells *in vitro*.[30–32] Stat5 has been demonstrated to stimulate the transcriptional activity of the cyclin D locus.[33] Transfection of a constitutively activated Stat5 resulted in growth factor-independent growth of the murine Ba/F3 pro-B cell line[34] and overexpression of Stat5b has been found to potentiate v-src-mediated transformation of NIH-3T3 cells.[35] As noted below, repression of Stat5 DNA-binding activity by PIAS3 inhibits breast cancer cell growth.[36] Collectively, these studies have argued for a significant role for Stats in breast cancer proliferation, survival, and differentiation.

The mechanisms through which the Stats promote these protumorigenic events has been an active area of investigation; several lines of evidence indicate that Stats 3 and 5 can activate the transcription of genes associated with cell-cycle progression, cell survival and transformation, and angiogenesis, as elaborated in recent reviews.[37,38] Several reports have implicated Stat3 and Stat5 activity in the up-regulation of expression of the cell cycle-regulatory proteins cyclin D1 and D2 in several nonmammary cell lines. However, only a single report to date has suggested that Stats may have similar effects in breast epithelial cells.[39] Regulation of anti-apoptotic members of the Bcl-2 family, specifically Bcl-X_L, has also been associated with Stat3 and Stat5 activity[37,40] in several nonmammary cell lines. Stat 3- and Stat5-induced transcription has also been implicated in c-*myc* proto-oncogene overexpression;[40] like Bcl-X_L, little data currently demonstrates whether the activity of the Stats contributes to the regulation of these proteins in mammary epithelial cells. Stat3 activity has also been recently implicated in up-regulated vascular endothelial growth factor expression, an event that may further serve to promote tumor progression.[41] Although collectively these data are intriguing from a mechanistic standpoint, further research is required because little data exists regarding Stat-mediated gene up-regulation in malignant breast tissues; furthermore, the specific contributions of the cyclins, Bcl-

X_L, c-*myc*, and vascular endothelial growth factor during the pathogenesis of human breast cancer remain to be completely elucidated.

Parallels in Prostate

Many signaling pathways thought to contribute to breast neoplasia are also activated in prostate cancer, and Stats 3 and 5 are no exception. One study of 42 human prostate carcinomas with matched normal controls found significant elevations of Stat3 tyrosine phosphorylation and DNA-binding activity in the malignant specimens;[42] no correlations with Gleason grade or prostate-specific antigen levels were noted. Interestingly, surrounding normal tissues associated with prostate cancers were also noted to have elevated levels of Stat3 activity, suggesting that Stat3 activation may precede frank histopathologically detectable prostate cancer. Similarly, 65% of human prostate cancers were noted to have nuclear localized, tyrosine-phosphorylated Stat5.[43] Introduction of a dominant-negative Stat construct into the human prostate cancer cell lines CWR22Rv and LnCap was also found to induce cellular apoptosis.[43] Interestingly, Stat5a knockout mice demonstrated acinar cyst formation and cell degeneration reminiscent of benign prostatic hypertrophy, however, epithelial hyperplasia or increased prostate size were not noted.[44] These data suggest, that, as in the mammary gland, Stat5 contributes to the maintenance of normal tissue architecture and function in the prostate.

Regulation of Stat Function

Tyrosine Phosphorylation

Stat activation requires C-terminal tyrosine phosphorylation by a receptor-associated Jak kinase.[1,45] This phosphorylation event occurs adjacent to the transcriptional activation domain within Stats (at position 705 in Stat3 and Y694 and Y699 in Stat5a and Stat5b, respectively) and induces dimerization/multimerization and nuclear translocation of the Stat complex where it engages its cognate DNA-binding sequence, resulting in promoter transactivation under appropriate conditions.[2,9,13,46,47] Tyrosine phosphorylation is necessary for Stat activity; replacement of this tyrosine residue at Y694/699/705 results in an inactive Stat incapable of nuclear translocation or transactivation. However, other tyrosine kinase-signaling pathways may also impact Stat activation because both Stats 3 and 5 can be phosphorylated by Src family members.[32,35,48] Such Src-mediated phosphorylation by itself, however, does not always results in Stat activation because only Src-induced phosphorylation of Stat5b, but not Stat5a, induced nuclear translocation.[48] However, it is clear that Stats are downstream of Src-family-mediated signaling because transcriptionally inactive Stat3 blocks Src-mediated transformation.[32] As noted above, enhanced levels of Stat3 and Stat5 tyrosine phosphorylation have been noted in human breast cancers.[20,26]

Serine Phosphorylation

All Stat family members, with the exception of Stat2, undergo serine phosphorylation after receptor-mediated signaling. Serine phosphorylation at residue 727 of Stats 1 and 3 results in a significant up-regulation of the transcriptional activity of these Stats.[49,50] This event appears to be mediated by several converging kinases including MAPK, p38, JNK, and protein kinase Cδ.[51–53] Serine phosphorylation of Stat3 appears essential *in vivo* for postnatal survival and growth because knock-in of a mutant Stat3 cDNA bearing an alanine for serine replacement at position 727 into Stat3 knockout mice failed to compensate the noted phenotype.[54] As discussed at greater length below, both of the small GTP binding proteins Rac and Rho are capable of up-regulating Stat3 serine phosphorylation and transactivation; indeed dominant-negative Rac inhibits Stat3 activity, and conversely, dominant-negative Stat3 blocks mutant oncogenic RhoA-induced cell transformation.[55,56]

In contrast, serine phosphorylation at positions 725 and 779 in Stat5a and position 730 in Stat5b down-regulates the transcriptional activity of Stat5.[53,57,58] Serine phosphorylation of both Stat5a and Stat5b appears to be increased during late pregnancy and lactation.[57] The phosphorylation of serine 725 and 779 of Stat5a is co-operative and mediated by both MAPK and non-MAPK signaling pathways; however, the suppressive effects of this phosphorylation appears to be mitigated in part by co-stimulation of glucocorticoid receptors in MCF7 breast cancer cells.[57] Despite these tantalizing data regarding the serine phosphorylation of Stats 3 and 5, the exact levels of serine phosphorylation of these transcription factors during the pathogenesis of human breast cancer remains to be determined.

Dephosphorylation

Several phosphatases, notably SHP1, SHP2, CD45, PTP1B, and TCPTP[59] have been demonstrated to regulate Jak kinase activity, and many of these have also been found in association with the Stat family. A structural example of a SHP phosphatase is presented in Figure 2; typically, receptor-associating phosphatases contain tyrosine-binding SH2 motifs, a phosphatase domain, and regulatory tyrosine residues that undergo variable phosphorylation. TCPTP has been found to associate with Stat5a and Stat5b within the nucleus and induce their dephosphorylation and inactivation; interestingly the phosphatase activity of TCPTP is not required for the inactivation of Stat5-mediated gene expression (ie, the association of TCPTP in this regard may be sufficient).[60] The overexpression of PTP1B also has similar effects on Stat5a and Stat5b phosphorylation and activity; however, this phosphatase is found within the cytoplasm.[61] SHP2 has been found in association with Stat5a,[62,63] and migrates with Stat5 as a complex into the nucleus. Interestingly, the intact phosphatase activity of SHP2 is required for Stat5 phosphorylation, suggesting that this phosphatase is not directly involved in the dephosphorylation/deactivation of Stat5, but instead indirectly with the acti-

vation of this Stat. SHP1 has also been reported to associate with Stat5b in the liver.[64] Although the effects of TCPTP, PTP1B, and SHP2 on Stat activity have been reported in mammary epithelial cell lines, these studies have singularly used overexpression methodologies. Furthermore, to date no study has examined either the expression or activity of these phosphatases in the context of primary human neoplasms. Thus, additional studies examining the role of the phosphatases during the pathogenesis of human breast cancer may prove highly illuminating.

SOCS Proteins

The SOCS (suppressors of cytokine signaling) family consists of eight members comprised of SOCS1 to SOCS7 and CIS (cytokine-inducible SH2 domain proteins). Each family member demonstrates three domains: a poorly conserved amino terminus, a central phosphotyrosine-binding SH2 domain, and a conserved, carboxy terminal SOCS-box motif, that may mediate posttranslational ubiquitination (Figure 2).[65–67] In resting cells, SOCS proteins are expressed at low levels; after receptor-mediated Stat family signaling, SOCS levels dramatically increase within 20 to 40 minutes. This increase is principally mediated at the transcriptional level,[68,69] however posttranslational mechanisms may also contribute (ie, phosphorylation) to SOCS protein stability.[70] SOCS proteins serve as classic negative counterregulatory inhibitors of Stat activation. This occurs by several mechanisms. SOCS1 blocks Stat phosphorylation and activation by directly binding to phosphorylated JAK, whereas SOCS3 inhibits JAK activity by first binding receptor. In contrast, CIS blocks Stat activation by blocking Stat-binding sites on receptors.[71–73] In addition, the ability of the SOCS box to engage elements of the ubiquitination pathway may significantly contribute to the down-regulation of receptor activated Jak kinase.[74] Analysis of mice bearing knockouts of specific SOCS family members has revealed dysregulation of cytokine and growth hormone signaling *in vivo*.[59] Modulation of both CIS and SOCS3 levels in mammary epithelial cells has been found to differentially regulate Stat5 signaling.[75] In parallel, knockout of a single SOCS1 allele resulted in correction of the lactational deficiency presented in PRLr$^{+/-}$ heterozygous mice; in contrast knockout of SOCS1 in an IFN-$\gamma^{-/-}$ mouse resulted in accelerated lobuloalveolar development and precocious lactation.[76] Together these knockout data suggest that SOCS1 has a considerable role in dampening PRL-activated Stat signaling in mammary tissues. A single report examining small numbers of primary human breast cancer has found elevations in SOCS family transcripts and protein,[77] an event that may contribute to elevated Stat family signaling in breast cancer.

Caveolin

Caveolins are necessary and sufficient for the formation of caveola that serve to concentrate and internalize lipids and cell surface receptor-signaling complexes. Recently, the observation that caveolin-1 shares homology with the SOCS family, led to the hypothesis that this protein may also function to down-regulate Jak-Stat signaling.[78] Indeed, when examined, caveolin-1 was found to associate specifically with Jak2 and down-regulate Jak2-Stat5a signaling. When caveolin-1 knockout mice were generated, these animals during pregnancy were found to have accelerated development of the lobuloalveolar compartment of the mammary gland, premature milk production, and enhanced phosphorylation of Stat5a.[78] These finding indicate that caveolin-1 may function in a manner analogous to the SOCS family. Additional matings of the caveolin-1$^{-/-}$ mice with the mammary tumor-prone polyoma middle T transgenic mice, revealed that the offspring had significant acceleration in the development of dysplastic mammary lesions.[79] As loss and/or mutation of the *caveolin-1* genomic locus has been reported in breast cancer, these findings suggest that caveolin-1, acting through the Jak-Stat pathway may contribute to the pathogenesis of human breast cancer.

Rac/Rho

Recent evidence has indicated that the Rho family of small GTP-binding proteins may influence Stat activation at multiple levels. Overexpression studies have indicated that the Rho family member Rac1 can directly bind to Stat3;[55] use of both constitutively active and dominant-negative forms of Rac suggested that Rac activity may influence both the tyrosine and serine phosphorylation of Stat3, as well as its activity. This regulation may occur at multiple levels because data has also suggested that Rac can influence Jak2 activity,[55] stress-activated kinase 4,[80] and the autocrine elaboration of cytokines, such as interleukin-6,[81] that also may indirectly influence Stat phosphorylation and activity. Similarly, RhoA can modulate the tyrosine and serine phosphorylation status and activity of Stat3 through the tyrosine kinases Jak2 and Src, and the serine kinase JNK.[56] It is uncertain whether RhoA directly interacts with Stat3, and it is unclear whether the actions of either RhoA or Rac are exerted purely at the cytoplasmic level or whether these two GTP-binding proteins accompany Stat3 into the nucleus. Recent data has also indicated that like Stat3, Stat5 may also be activated by RhoA.[82] These data suggest that RhoA may increase the tyrosine and decrease the serine phosphorylation levels of Stat5 and contribute to the process of epithelial to mesenchymal transition. In addition, recent data from our laboratory has indicated that the guanine nucleotide exchange factor, Vav2, can bind Stat5 directly and up-regulate the activity of both Rac1 and Stat5 (Miller SL, DeMaria JE, Freier D, Riegal AM, Clevenger CV, submitted for publication). Several lines of evidence now indicate that both Rac1 and RhoA protein expression is elevated in breast malignancies, particularly those of high-grade or advanced stage;[83–85] preliminary evidence suggests these proteins are not mutated and that the mechanisms of their overexpression is post-transcriptional.[85] Thus, the activation of small GTP-binding proteins may provide an additional mechanism for

additional signal integration and activation of the Stat5 complex.

Ligand/CypB

Classic theory dictates that peptide hormones regulate Stat family activity at a distance via cell-surface receptors, ie, by receptor-mediated activation of Jak2 and MAPK, which in turn phosphorylate and regulate Stat family activity. A growing body of evidence, however, has indicated an intranuclear function for polypeptide hormones with relevance to breast cancer, including PRL, growth hormone, epidermal growth factor, nerve growth factor, platelet-derived growth factor, fibroblast growth factor, interleukin 5, and insulin.[86] In parallel with the cell-surface activation of Stat, these hormones are endocytosed and retrotranslocated to the nucleus after receptor binding.[87–89] The relationship between the nuclear actions of polypeptide hormones and Stats had been unclear until the recent discovery by my laboratory of the role of the peptidyl prolyl isomerase cyclophilin B (CypB) in the nuclear transport and function of PRL.[90] Our studies revealed that complex between PRL and CypB exists in human serum. After receptor-mediated transport of the PRL/CypB, this complex is retrotransported to the nucleus via a N-terminal nuclear localization sequence present in CypB (Figure 3). Within the nucleus the PRL/CypB complex directly interacts with Stat5. PRL/CypB acts as a transcriptional inducer of Stat5 by facilitating the interaction of this transcription factor with DNA by inducing the release of a repressor of Stat5, namely PIAS3.[36] The actions of the PIAS family of proteins with respect to Stat activity are discussed below. By inducing the release of this Stat repressor, intranuclear PRL can directly potentiate the activity of Stat5. These observations would suggest that considerable parallels exist between steroid and peptide hormones in their respective genomic and nongenomic actions, and like steroid hormones, may suggest that polypeptide ligands contribute to their signaling specificity through their intranuclear functions. Although preliminary data from our laboratory[90] also indicate that CypB can potentiate the activity of growth hormone, its role with respect to other polypeptide hormones implicated in the pathogenesis of breast cancer remain unclear. Whether such mechanisms contribute to the regulation of Stat3 activity in breast tissues also remains to be determined.

Precedent studies have demonstrated ubiquitous expression of CypB. Although the expression of PRL has been classically associated with the pituitary, the local autocrine/paracrine elaboration of this hormone has been documented at multiple sites, including breast tissues.[24,25] Recent unpublished studies in our laboratory have also found appreciable PRL within the nucleus of breast malignancies. If the intranuclear actions of PRL contribute to the growth of PRL-responsive tissues, then interference with this pathway may prove useful in the treatment of breast cancer. To test this hypothesis, an enzymatically inactive mutant of CypB was synthesized by recombinant technique and introduced into the culture medium of human breast cancer cells. This treatment resulted in a significant inhibition of the growth of such cells,[36] at concentrations 100- to 1000-fold less than any previously reported PRL antagonist.[91] Collectively, these data would suggest that pharmacological manipulation of intranuclear actions of polypeptide hormones may inhibit Stat activity and be of therapeutic utility in the treatment of breast cancer.

PIAS/Sumoylation

The PIAS (for peptide inhibitors of activated Stats) family of proteins (PIAS1, PIAS3, PIASx, and PIASy) have been found to bind Stat family members and block their binding to DNA and/or transcriptional activity.[92–94] PIAS proteins demonstrate three regions of protein homology: a signature N-terminal LXXLL motif, thought to contribute to nuclear receptor interactions, a central ring finger domain, whose function was unknown, until recently (see below), and a serine-rich C-terminus (Figure 2). Unlike the cytokine-induced expression of the SOCS/CIS family, PIAS proteins are constitutively expressed within the nucleus and appear to act as constitutive repressors of Stat activity.[94] Each PIAS member associates and modulates the function of distinct subset of transcription factors, ie, PIAS1 binds to Stat1 and p53;[93,95] PIAS3 associates with Stats 3, 5a, 5b, and Gfi;[36,92,96] PIASx with the androgen receptor;[97] and PIASy with LEF1.[98] While the nature of the interaction between PIAS proteins and transcription factors is undergoing elucidation, recent evidence examining the PIAS1-Stat1 association indicates that the N-terminal domain of Stat1 binds to the region between the ring finger and serine/threonine-rich domains of PIAS1, a region termed the linker domain.[99] How the PIAS family modulates transcription factor function has been uncertain, however, recent data have indicated that PIAS proteins serve as small ubiquitin-like modifier (SUMO) E3 ligases.[95,98,100,101]

Sumoylation is the process whereby one of three SUMO (for small ubiquitin-like modifiers) peptides (termed SUMO1 to SUMO3), consisting of ~100 amino acids (ie, comparable in length to ubiquitin) is added to a consensus sumoylation site (ΨKXE) within a peptide. Like ubiquitin conjugation, sumoylation requires an E1-activating enzyme and an E2 conjugase.[101] Although this process can occur without an E3 ligase, it is not efficient. The existence of mammalian E3 ligases was not recognized until two separate studies using yeast two-hybrid approaches suggested that PIAS family members were such SUMO ligases. These studies revealed that PIAS1 and PIASy *in vivo* and *in vitro* promoted the sumoylation of p53 and LEF1, respectively.[95,98,102] Unlike ubiquitin conjugation, sumoylation appears to modify protein function not through degradation, but instead by altering function, localization, or extent of ubiquitylation.[100] PIAS-induced sumoylation requires the ring finger motif of PIAS because deletion or modification of this motif eliminates sumoylation of PIAS-binding partners.[98,102] Interestingly, the functional effects of PIAS association and PIAS-induced sumoylation appear to be distinct. For instance, removal of sumoylation sites in LEF1 did not alter its targeting to nuclear bodies, whereas deletion of the ring

motif in PIASy did block such targeting.[98] Loss of the ring finger domain from PIAS1 did not block p53-mediated transcription in one study,[103] although other studies have suggested that this effect may be cell-type specific.[102] These data suggest that functional modulation by PIAS proteins may result as a consequence of PIAS interaction, subsequent sumoylation, or both in a given cellular context.

Collectively, it appears that either the direct association of PIAS family members, or the SUMO conjugation they induce, can significantly modify the function of transcription factors that include members of the Stat family. Recent data from our laboratory has indicated that PIAS3 overexpression in breast cancer cell lines can significantly modulate Stat5-mediated gene expression and induce cellular apoptosis.[36] The extent of PIAS and SUMO family expression in breast tissues remains unclear, although preliminary evidence from our laboratory suggests that dysregulation of PIAS expression does occur in human breast cancers.

Transcriptional Co-Regulators

The activity of Stats 3 and 5 has been shown to be up-regulated by its interaction with transcription factors/co-activators including CBP, Nmi, CPAP, c-jun, SMRT, and the glucocorticoid receptor[53,94,104–109] and histone deactylases.[110] Although the association of some of these proteins, such as CBP, with Stat family proteins is thought to be rate limiting to transactivation, the precise function and expression of most of these proteins with respect to Stat5 activity in breast cancer remains to be determined.

Future Opportunities—From the Lab to the Bedside

The central role that Stats may play in breast cancer relates to their intrinsic ability to integrate the incoming signals from activated receptors and their associated signal transduction networks. As such they are ripe for additional studies, both as prognostic markers and therapeutic targets.

As noted above, preliminary data indicate that the levels of Stat3 and Stat5 expression and/or phosphorylation may relate to the differentiation and/or favorable outcome. As in many initial studies, limitations as to sample size, outcome follow-up, and experimental design found in the immunohistochemical and EMSA studies with Stats 3 and 5 to date will require additional follow-up investigation. Because Stat transcriptional activity may be the central measure of the biological function of Stats 3 and 5, additional assays other than measurement of Stat phosphorylation and DNA binding may need to be applied to clinical specimens. Furthermore, the analysis of Stat activity will need to be targeted to the pluripotent tumor stem cells that may require Stat activity for survival and/or growth.[111]

With the development of high-throughput screening assays for Stat-protein interaction and Stat transcriptional activity,[112] the ability to screen sizable libraries for small pharmacological inhibitors of Stats has become a distinct possibility. It is conceivable that an effective inhibitor of Stat function may interrupt its ability to interact with DNA, alter its posttranslational modifications (phosphorylation/sumoylation), or block/enhance its interaction with the transcriptional co-activators or co-repressors.[113] Given the existent data from cell lines and animal models overviewed above, it is probable that a future pharmacological agent targeting Stat function will be of use in the treatment of human breast cancer.

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