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Progesterone receptor promotes degradation of STAT2 to inhibit the interferon response in breast cancer

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ABSTRACT

Type I (IFNα/β) interferon signaling represents a critical transduction pathway involved in recognition and destruction of nascent tumor cells. Downregulation of this pathway to promote a more immuno-suppressed microenvironment contributes to the ability of tumor cells to evade the immune system, a known Hallmark of Cancer. The present study investigates the progesterone receptor (PR), which is expressed in the vast majority of breast cancers, and its ability to inhibit efficient interferon signaling in tumor cells. We have shown that PR can block the interferon signaling cascade by promoting ubiquitination and degradation of STAT2. Targeting STAT2 is critical, as we show that it is an essential protein in inducing transcription of interferon-stimulated genes (ISG); shRNA-mediated knockdown of STAT2 severely abrogates the interferon response in vitro. Importantly, we were able to reverse this inhibition by treating with onapristone, an anti-progestin currently being investigated in breast cancer clinical trials. Additionally, we have found that an interferon-related gene signature (composed of ISGs) is inversely correlated with PR expression in human tumors. We speculate that PR inhibition of interferon signaling may contribute to creating an immunosuppressed microenvironment and reversal of this through anti-progestins may present a novel therapeutic target to promote immune activity within the tumor.

Introduction

Hanahan and Weinberg revolutionized our understanding of cancer biology through the issuing of the "Hallmarks of Cancer" close to two decades ago, with a vital update occurring 10 y later.[1,2] Among these updated hallmarks-i.e. characteristics shared by virtually all tumors-was 'evasion of the immune system.' As such, our understanding of how the immune system is involved in all steps of the tumor life cycle (initiation, progression, metastasis, etc.) has grown exponentially and allowed for the development of specific immune-targeting therapies.[3,4]

Multiple signaling pathways involved in the immune response are responsible for identification and elimination of malignantly transformed cells.[4] Among these key signaling mechanisms are a diverse group of cytokines called interferons. Type I interferons (IFNα/β) are cytokines canonically released by immune cells during viral infection and act through binding their cognate transmembrane receptor (IFNαR1/2). This binding leads to dimerization of the receptor, cross-phosphorylation of receptor associated Janus Activated Kinases (JAK1 and TYK2), and recruitment of Signal Transducer and Activator of Transcription family members (STAT1 and STAT2) that are then subsequently phosphorylated. Phosphorylated STAT1 and STAT2, in conjunction with Interferon Regulatory Factor 9 (IRF9), form a transcriptional complex that translocates to the nucleus, binds DNA at interferon-stimulated response elements (ISRE), and activates transcription of a diverse subset of Interferon Stimulated Genes (ISGs).[5] While canonically a pathway activated in response to viral infection, growing evidence has implicated type I interferons as crucial mediators in tumor detection and destruction.[6-8] Immune "cold" tumors – those deficient in lymphocyte infiltration/activity – characteristically lack efficient interferon signaling, justifying the usage of Stimulator of Interferon Genes (STING) agonists to bolster activity within this pathway.[9] As such, induction of interferon signaling is a key step in bridging the innate and adaptive immune systems to mount an effective, anti-tumorigenic response.

Breast cancer represents the second leading cause of cancer-related death in women within the United States.[10] Of the over 250,000 new cases diagnosed annually in the United States, the vast majority of these express the estrogen (ER) and progesterone (PR) receptors.[11] PR is a ligand-activated nuclear receptor that functions as a transcription factor, regulating a variety of genes involved in proliferation and differentiation in the reproductive tract and mammary gland.[12] Clinical trial data emerging over the last few decades have suggested a role for PR, independent of ER, in the development of breast cancer.[13,14] In light of these data, there is

clinical impetus for identifying how PR is signaling in breast cancer in order to better inform treatments for patients with hormone receptor (ER/PR) positive cancer.

Our previous studies have concluded that PR can inhibit type I interferon signaling at multiple steps along the cascade, including prevention of STAT1 phosphorylation, DNA binding, and ISG transcription.[15,16] Because of the biological importance of interferon signaling (prevention of infection and disease spread), compensatory mechanisms have been discovered in the absence of a functional STAT1 complex. In some instances, STAT2 is able to propagate the interferon response without STAT1, thus suggesting that multiple antagonistic mechanisms are necessary to fully hinder interferon signaling.[17] The present study investigates PR's ability to inhibit STAT2 signaling through a mechanism distinct from PR's ability to inhibit STAT1. Through inhibition of both STAT1 and STAT2, PR effectively shuts down interferon signaling, a vital pathway for the identification and destruction of nascent tumor cells.

Methods

Cell lines and treatments

T47D and BT474 cells were acquired from ATCC and cultured in DMEM (Cellgro) supplemented with 5% FBS and 1% penicillin/streptomycin. PR, STAT1, and STAT2 shRNA knockdown cells were created using viral particles (GE/Dharmacon) targeting three different regions of each respective gene. Viral transduction protocol was followed as per manufacturer's instructions. Transduced, stable cell line pools expressing NS, PR, STAT1, or STAT2 shRNA were created in T47D cells following 14 d of selection in 2.5 ug/ml Puromycin (MP Biomedicals). Target shRNA sequences are listed in Supplementary Table 1. RFP and STAT1 overexpressing cells were created using lentiviral ORF particles (GE/Dharmacon). Transduced, stable cell line pools overexpressing RFP (control) or STAT1 were created in T47D cell lines stably expressing NS or STAT2 shRNA following 14 d of selection in 15 ug/mL Blasticidin S HCL (Corning). Sequences are shown in Supplementary Table 1. Cells were treated with the following reagents where indicated: R5020 (10 nM, Sigma), human rIFN-α (1000 IU/mL, IFN-α2A, SPR4594; Sigma-Aldrich, vehicle-H20), onapristone (10uM, provided through an MTA with Context Therapeutics), cycloheximide (200ug/mL, Calbiochem), and MG132 (5uM, Selleck Chemical).

Western blot analysis

Western blot analysis was performed as previously described.[15,16] Primary antibodies for immunoblotting are as follows: PR (sc-7208; Santa Cruz Biotechnology), phospho-STAT2 (4441; Cell Signaling), Total STAT2 (sc-1668; Santa Cruz Biotechnology), IFIT3 (sc-393512; Santa Cruz Biotechnology), IFIT1 (14769; Cell Signaling), STAT1 (9172; Cell Signaling), Ubiquitin (3933; Cell Signaling), and beta-tubulin (2128; Cell Signaling). Densitometry to quantify immunoblots was performed using ImageJ.

Co-immunoprecipitation

Co-immunoprecipitation experiments were performed as previously described.[15] In brief, 1000ug of protein from isolated protein lysates were incubated with 2 mg of STAT2 (SC-1668, Santa Cruz Biotechnology) or control IgG antibody and rocked overnight at 4°C. Protein G agarose (Roche Diagnostics) was added for the final 2 h of incubation time. Beads were washed three times in supplemented RIPA buffer, bound protein eluted in Laemmli sample buffer, then subjected to immunoblotting as described above.

Quantitative RT-PCR

RNA isolation, cDNA generation, and qRT-PCR were performed as previously described.[15,16] qPCR primer sequences are listed in Supplementary Table 1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed using the ChIP-IT Express Kit (Active Motif) according to the manufacturer's instructions. Isolated chromatin was sheared using sonication and sheared chromatin was IP'd overnight using the following primary antibodies (or species-specific IgG control): STAT1 (ab47425; abcam), STAT2 (sc-1668; Santa Cruz Biotechnology), and IRF9 (76684; Cell Signaling). IP'd chromatin was analyzed using qRT-PCR with primers designed to amplify specific ISG promoters. Data shown as percentage IP'd DNA over input DNA. ChIP-qPCR primer sequences are listed in Supplementary Table 1.

TCGA analysis

Gene expression data for the 50-gene IRDS signature[18] (full list of genes in IRDS shown in Supplementary Table 2) were extracted from TCGA breast[19] 'Provisional' dataset and associated clinical metadata were accessed via the cBio portal.[20] The IRDS signature score was calculated by summing the normalized log2 Z-scores of the expression data from the 50 genes. Clinical metadata were used to stratify signature scores by ER/PR status, and the degree of PR expression by IHC. PGR gene expression was also extracted from the TCGA dataset via the cBio portal.[20]

Statistical analyses

Statistical significance for all experiments was determined using an unpaired Student's *t*-test unless otherwise specified. A *p* value ≤0.05 is considered statistically significant. The Delta method was used to calculate standard deviation for the ratio of two variables using their individual standard deviations, as seen when plotting fold relative RNA expression data between two treatment groups/cell lines.[21]

Results

PR and STAT2 interact without affecting STAT2 phosphorylation

As we have previously shown that PR interacts with STAT1, we proposed that PR may be interacting with multiple proteins in the type I interferon signaling pathway to inhibit efficient signal transduction. To test whether PR was interacting with STAT2, we utilized co-immunoprecipitation in T47D cells (ER/PR-positive human breast cancer). Following treatment with the synthetic PR ligand, 10 nM R5020, we found an increase in the formation of a PR:STAT2 complex when compared to the vehicle control (Figure 1(a)). Importantly, this was independent of STAT1, as STAT1 was not involved in PR:STAT2 complex formation (Supplementary Figure 1). Like other signal transduction pathways, type I interferon signaling is heavily regulated through the concerted addition and removal of post-translational modifications such as phosphorylation, acetylation, ubiquitination, etc.[22] To identify whether PR interacting with STAT2 impeded phosphorylation of STAT2, we treated with IFNα for 0–30 min in the presence or absence of PR ligand (R5020) and found no differences in STAT2 phosphorylation with PR activation (Figure 1(b)). These data suggest that the interaction between PR and STAT2 does not affect interferon-induced STAT2 phosphorylation.
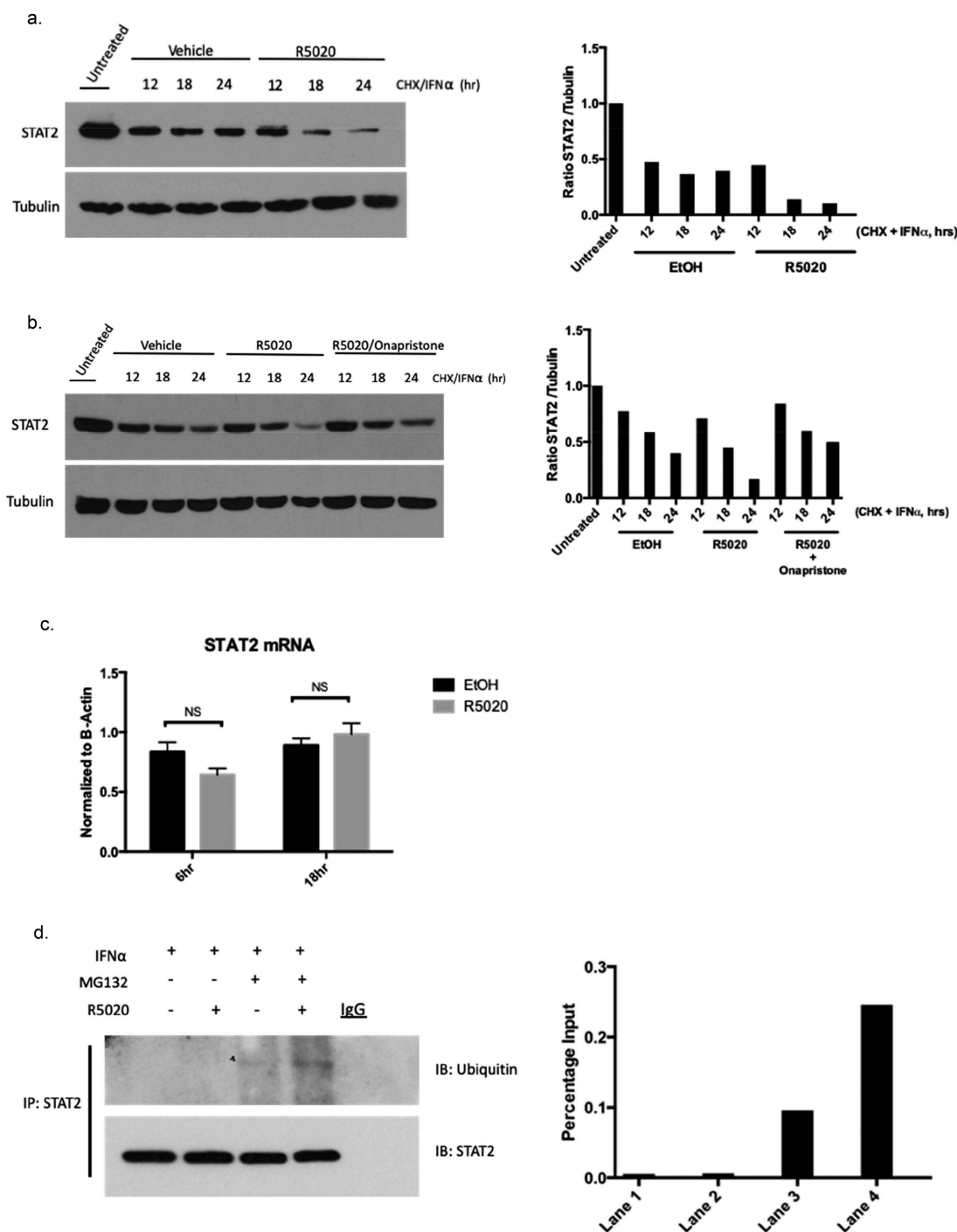
PR activation promotes STAT2 ubiquitination and subsequent protein degradation

Multiple mechanisms exist to inhibit effective interferon signaling in the context of a viral infection – blocking the production of the interferons themselves, prevention of phosphorylation of JAK/STAT proteins, promotion of JAK/STAT degradation, etc.[23] We observed that phosphorylation of STAT2 was unaffected (Figure 1(b)), so we investigated how PR activation affects STAT2 protein turnover. To investigate whether PR is affecting STAT2 expression in this manner, we utilized the inhibitor cycloheximide (CHX) which prevents translation and allows for the observation of protein turnover. We treated T47D cells with CHX and IFNα at varying time points (0–24 h) in the presence or absence of PR ligand and found that STAT2 protein was destabilized/degraded at a higher rate when PR was activated with ligand (Figure 2(a), Supplementary Figure 2). We observed this phenotype in an additional PR+ breast cancer cell line, BT474 (Supplementary Figure 3) Onapristone, a potent PR antagonist, is currently being evaluated in a surgical window of opportunity trial in treatment naïve, PR-positive breast cancers (Onward 203, ONAWA trial). Additionally, onapristone is currently being used in a Phase I clinical trial for treatment of patients with late-stage breast cancer.[24] When treating with onapristone, we were able to effectively reverse
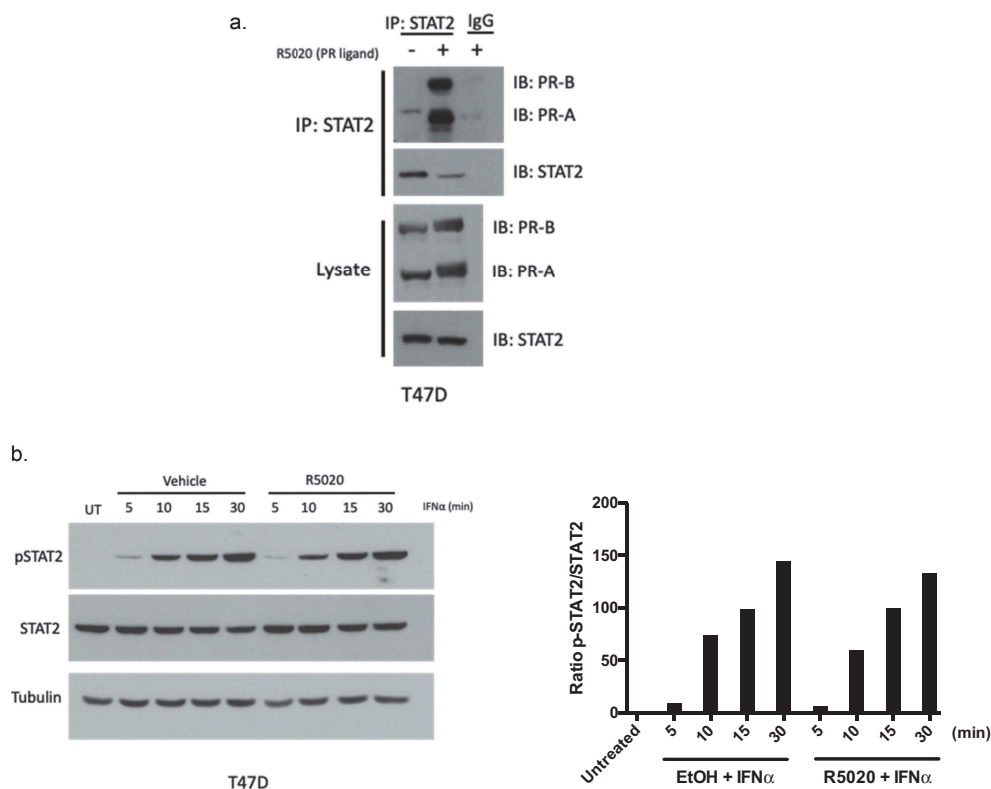
Figure 1. PR and STAT2 interact without affecting STAT2 phosphorylation.
(a) STAT2 was immunoprecipitated (IP) from T47D whole cell lysate treated with vehicle (EtOH) control or R5020 (10 nM, 1 hr) followed by immunoblotting with PR-specific antibody. Antibody for PR recognizes both isoforms (PR-A and PR-B), as labeled in Co-IP and input lysate blots. Mouse-specific IgG used as a control for the IP. (b) T47D cells were treated with IFNα (1000 IU/mL, or vehicle [H20] in UT condition) for 0–30 min in the presence of vehicle (EtOH) or R5020 (10 nM). Isolated protein lysate then analyzed for phosphorylated STAT2 (or total STAT2). Beta-tubulin shown as loading control. Densitometry of the ratio of *p*-STAT2/total STAT2, as determined using ImageJ analysis, is shown to the right of the immunoblot. These experiments were performed in triplicate, and a representative experiment for each is shown.

Figure 2. PR activation promotes STAT2 ubiquitination and subsequent protein degradation.

(a) T47D cells were treated with cycloheximide (CHX, 200ug/mL) and IFNα (1000 IU/mL, or vehicle [H20] in UT condition) in the presence of either vehicle control or R5020 (10 nM) for 12–24 hours. Isolated protein lysates were then analyzed to assess STAT2 degradation. Beta-tubulin shown as a loading control. Densitometry of the ratio of STAT2/B-Tubulin, as determined using ImageJ analysis, is shown to the right of the immunoblot. (b) T47D cells were treated with CHX (200ug/mL) and IFNα (1000 IU/mL, or vehicle [H20] in UT condition) and either vehicle (EtOH), R5020 (10 nM), or a combination of R5020 and Onapristone (10uM) for 12–24 h. Isolated protein was then analyzed to assess STAT2 degradation. Beta-tubulin shown as a loading control. Densitometry of the ratio of STAT2/B-Tubulin, as determined using ImageJ analysis, is shown to the right of the immunoblot. (c) T47D cells treated with vehicle (EtOH) or R5020 (10 nM) for 6 or 18 h. Isolated mRNA was then analyzed for STAT2 using quantitative RT-PCR. Gene values were normalized to an internal control (beta-actin). Error bars represent standard deviation (SD) between biological triplicates. NS = non-significant as determined using a Student's t-test. (d) T47D cells were pretreated with MG132 (5uM) (or with vehicle control) and IFNα (1000 IU/mL) for 1 h then co-treated with R5020 (or vehicle) for 6 h. Whole cell lysates were subjected to IP with STAT2-specific antibody and immunoblotted for ubiquitin. Rabbit-specific IgG used as control for IP. Densitometry of the ratio of Ubiquitin/STAT2, as determined using ImageJ analysis, is shown to the right of the immunoblot. These experiments were performed in triplicate, and a representative experiment for each is shown.

the ability of PR to promote STAT2 turnover (Figure 2(b)). This illustrates 1) this effect on STAT2 degradation is PR dependent, and 2) a drug already in clinical development for breast cancer prevents PR-mediated STAT2 degradation.

Importantly, we did not see an effect on STAT2 mRNA in response to R5020 treatment indicating that this regulation is exclusively post-translational (Figure 2(c)). Increased STAT2 turnover in the presence of PR ligand suggests that PR may be

promoting ubiquitination of STAT2 protein, effectively targeting the protein for proteasomal degradation. To test this, we used the proteasome inhibitor MG132 to observe the accumulation of ubiquitinated protein. Following pre-treatment with MG132 and IFNα and subsequent treatment with R5020, we found that when PR is activated, we observed an increased population of ubiquitinated STAT2 (Figure 2(d)). Taken together, these data suggest that PR interaction with STAT2 promotes ubiquitination, thereby targeting it for degradation.

STAT2 is essential for type I interferon signaling

Much of the current knowledge regarding interferon signaling has focused on the role of STAT1, as it is a major component of multiple interferon-regulated transcriptional complexes. Emerging data, however, has illuminated the essential role for STAT2 in promotion of type I interferon signaling during viral infection.[25,26] The dependence on STAT2 to propagate the immune response in breast cancer, however, has been understudied. To tease apart how loss of STAT proteins affect ISGF3 component DNA binding, we first utilized T47D cells stably expressing either non-silencing (NS) or STAT1 shRNA (mRNA to confirm STAT1 knockdown with shRNA shown in Figure 3(a), protein in Figure 5(b)). Using chromatin immunoprecipitation (ChIP) assays, we found that in cells lacking STAT1, STAT2, and IRF9 were still able to bind DNA at Interferon Stimulated Gene (ISG) promoters (ISREs) in response to IFNα (Figure 3(b), Supplementary Table 3); recruitment at select representative ISGs is shown. To then test the dependence upon STAT2, we used shRNA to stably knockdown STAT2 (mRNA shown in Figure 4(a), protein in Figure 5(b)). Contrary to what occurred when STAT1 was knocked down, we observed abrogated recruitment of STAT1 and IRF9 to ISG promoters, suggesting STAT2 is necessary to promote efficient DNA binding of other ISGF3 components (Figure 4(b), Supplementary Table 4). These data suggest that STAT2, not STAT1, is essential in mediating the interferon response.

We next measured ISG transcription as a readout of activated interferon signaling in both shRNA knock down (STAT1 and STAT2) cell lines. We found that knocking down STAT1 did not affect ISG transcription (Figure 5(a)), indicating compensatory mechanisms involving STAT2 can effectively overcome the lack of STAT1 in breast cancer cells. Studies in viral models have shown that STAT2 can direct prolonged, constitutive interferon activation in the absence of a functional STAT1 complex.[25,27] We observe a similar phenotype in our breast cancer cells as exemplified by increased recruitment of STAT2 and IRF9 to ISREs and increased ISG transcription when STAT1 is knocked down. Conversely, knocking down STAT2 severely hindered the ability of these cells to respond to IFNα, further demonstrating the indispensability of STAT2 in the propagating the interferon signaling cascade (Figure 5(a)). Finally, observing ISG protein output, we compared the interferon response between cells expressing NS, PR, STAT1, or STAT2 shRNA. We found that knocking down PR increased the response to IFNα (consistent with previous data from our laboratory[15,16]). Furthermore, IFNα response in STAT1 shRNA cells was comparable to the NS

control, and STAT2 knockdown completely eliminated ISG protein production (Figure 5(b)). As STAT1 itself is an ISG whose expression is dampened in STAT2 shRNA cells (Figure 5(b)), we performed a STAT1 re-expression experiment in these cells. Cells without STAT2, but with overexpression of STAT1 via lentiviral transduction, exhibited a similar phenotype in both STAT1 DNA binding and ISG protein production when compared to STAT2 shRNA alone (Supplementary Figure 4). These data indicate that the hinderance of the interferon response is solely a result of loss of STAT2. These data taken together establish the essential role of STAT2 in the type I interferon response in breast cancer, and therefore underscore the consequences of PR-mediated degradation of STAT2 protein.

PR status in human tumors is correlated with decreased interferon gene signature

To investigate how PR status affects interferon signaling in human breast cancers, we analyzed data acquired from The Cancer Genome Atlas (TCGA). Our data suggest PR promotes STAT2 degradation and thereby inhibits proper interferon signaling. Because this regulation occurs post-translationally and not at the gene/transcript level, we found that STAT2 gene expression was expectedly not significantly different between PR-positive and PR-negative tumors (data not shown). While protein expression data is limited in TCGA, we can instead identify changes in gene signatures among PR-positive and PR-negative breast cancers as a readout for active interferon signaling. We utilized TCGA breast cancer gene expression datasets (TCGA Breast, provisional[19]) and assessed differences in expression of an established interferon gene signature (that includes the ISGs shown herein,[18] full list of genes shown in Supplementary Table 2) to identify any differences between PR-positive and PR-negative breast cancers (Figure 6(a)). We found that PR expression was significantly associated with a decrease in this interferon gene signature when analyzing all tumors in this TCGA dataset. Moreover, when stratifying PR-positive breast cancers based upon level of expression (< or >20% PR-positive cellular expression), we found that expression of the interferon-related gene signature is inversely correlated with level (% positivity) of PR in ER/PR-positive breast tumors; tumors with higher percentage PR-positivity exhibited lower expression of the interferon gene signature (Figure 6(b)). Moreover, we see a negative correlation (spearman correlation coefficient [$r = -0.1$; $p = .008$]) between PR (PGR) mRNA levels and expression of the interferon gene signature in the TCGA dataset containing all ER-positive tumors. Cumulatively, these data show that PR expression in human breast tumors correlates with decreased interferon signaling. These data suggest that PR positivity promotes an immunosuppressive phenotype as measured by decreased interferon signaling; a potential mechanism by which early malignancies progress to clinically relevant tumors.

Discussion

In the present study, we have shown that PR interacts with STAT2. While this interaction does not affect STAT2 phosphorylation, we do observe an increase in STAT2 ubiquitination and degradation when PR is activated by ligand. Previous studies in virally infected cells have discovered
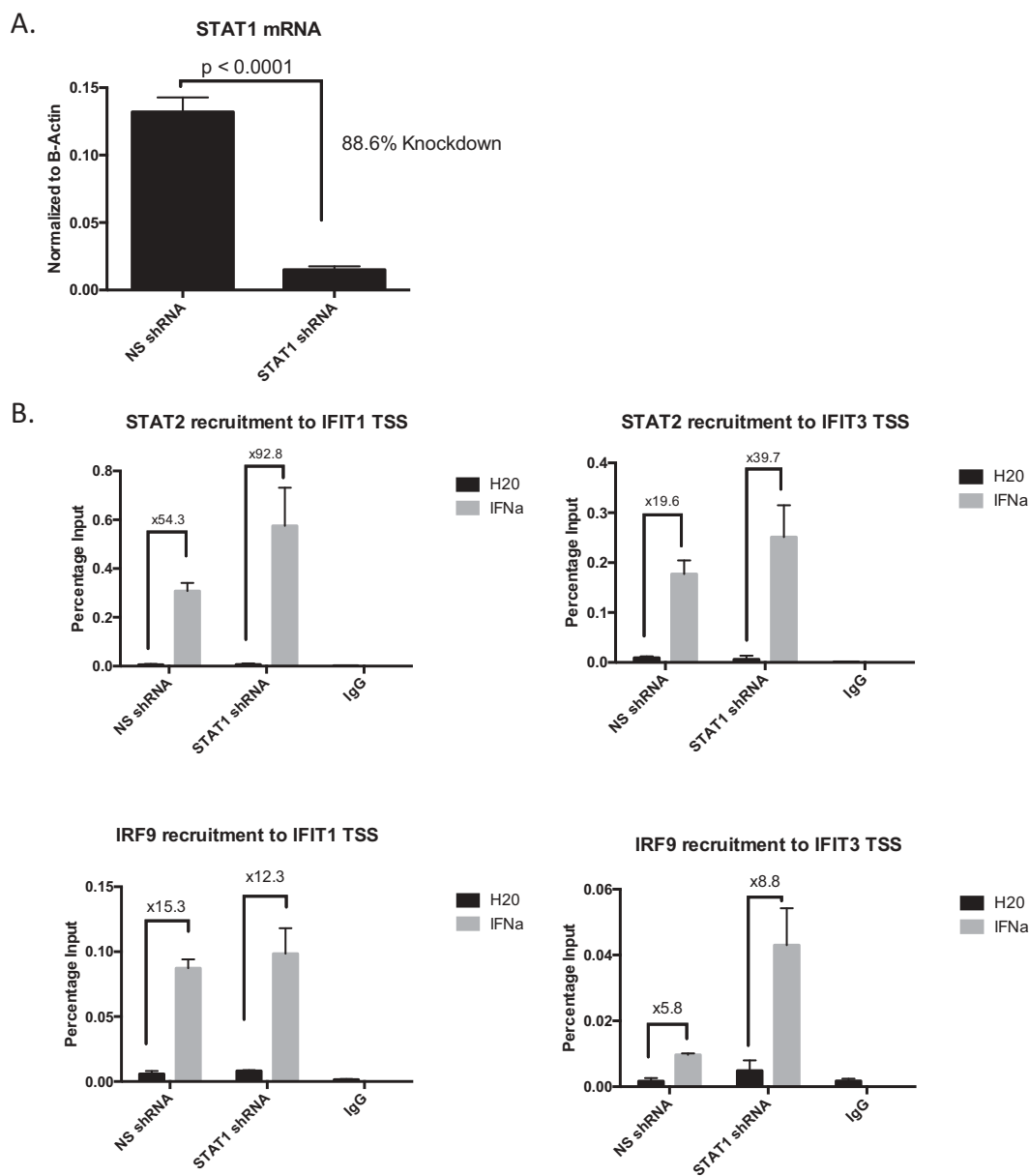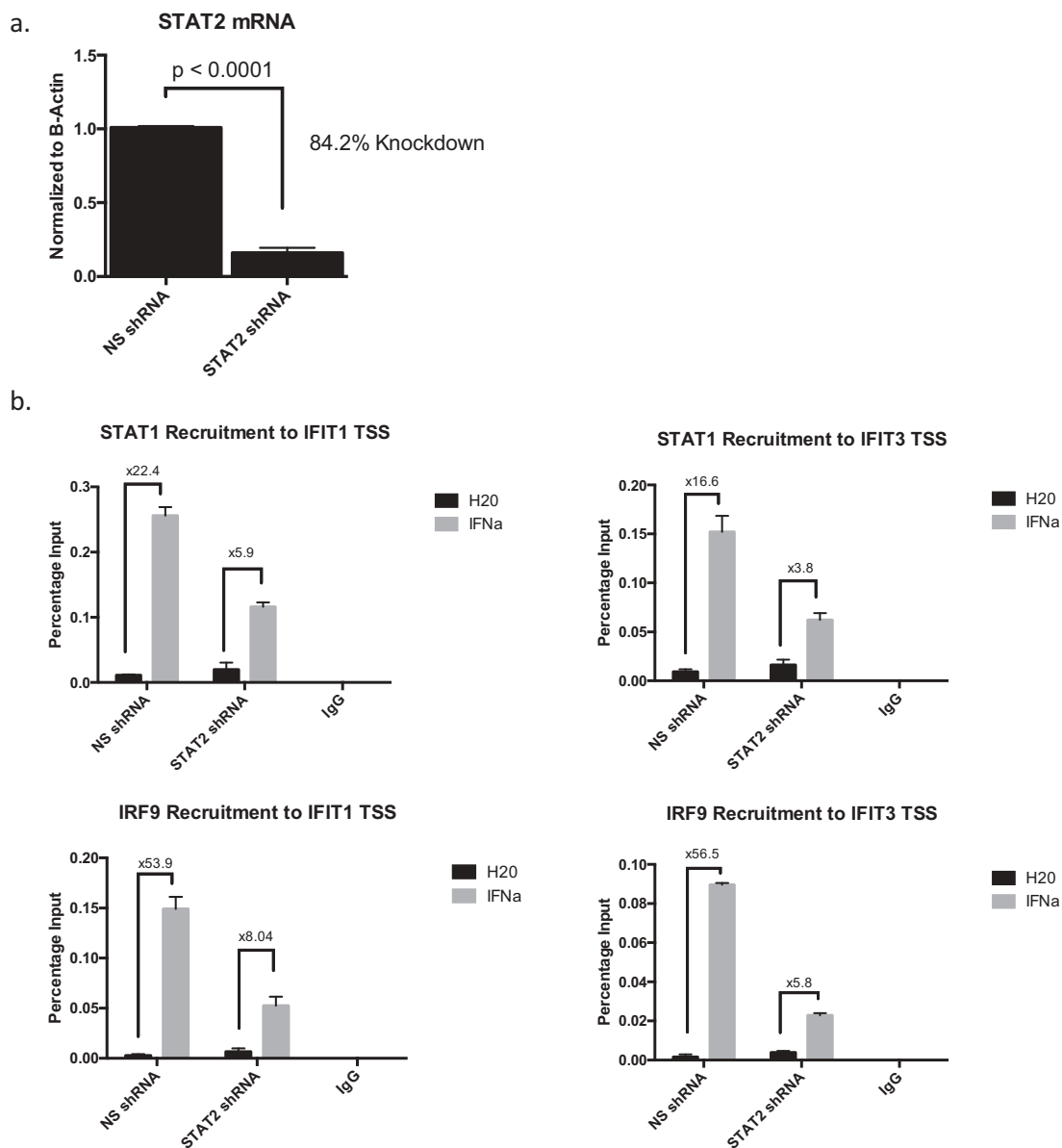
Figure 3. Interferon-induced DNA binding is maintained in the absence of STAT1.

(a) RNA isolated from T47D cells stably expressing either Non-silencing (NS) or STAT1 shRNA then subjected to quantitative RT-PCR to assess efficiency of knockdown. Gene values were normalized to an internal control (beta-actin). Error bars represent SD between biological triplicates. Statistical significance ($p < .05$) determined using a Student's t-test. (b) T47D-NS and T47D-STAT1 shRNA cells lines were treated with vehicle (H20) or IFNα (1000 IU/mL) for 4 h then fixed lysates were subjected to ChIP analysis using antibodies specific for STAT2 and IRF9 (or species-specific IgG control). Isolated DNA was then analyzed using qRT-PCR using primers designed to amplify the IFIT1 and IFIT3 promoters (TSS = Transcriptional Start Site). Percentage recruitment of STAT2 and IRF9 shown as ChIP'd DNA over input DNA. Numbers above bar indicate fold recruitment over vehicle control for each cell line. These experiments were performed in triplicate, and a representative experiment for each is shown. Results from replicate experiments are shown in Supplementary Table 3.

that in the absence of a functional STAT1 complex, compensatory STAT2-dependent signaling mechanisms are employed to maintain active interferon signaling.[17,25-29] A recent study established the indispensability of STAT2 in interferon signaling in Hela cells and our work has shown a similar vital role of STAT2 in breast cancer.[30] As our previous study examined PR's ability to inhibit STAT1 functionality in breast cancer, we have exhibited a mechanism by which breast cancer cells attempt to overcome this inhibition. By inhibiting both STAT1 and STAT2 functionalities, PR is able to fully abrogate the interferon response, as

exemplified through significantly decreased ISG transcription (Figure 7). Data from our previous studies, as well as analyzed TCGA data in the present study, have shown that this PR-dependent downregulation of ISGs is seen in human tumors as well.

For patients with ER/PR-positive breast cancer, the current standard of care primarily involves ER-targeted therapies like aromatase inhibitors or selective estrogen modulators/down regulators (i.e. fulvestrant, tamoxifen).[31] While such therapies have proven to be effective in these patients, they are accompanied by undesirable side effects which can hinder compliance.

Figure 4. ISGF3 binding is severely hindered in the absence of STAT2.

(a) RNA isolated from T47D cells stably expressing either Non-silencing (NS) or STAT2 shRNA then subjected to quantitative RT-PCR to assess efficiency of knockdown. Gene values were normalized to an internal control (beta-actin). Error bars represent SD between biological triplicates. Statistical significance ($p < .05$) determined using a Student's t-test. (b) T47D-NS and T47D-STAT2 shRNA cells lines were treated with vehicle (H20) or IFNα (1000 IU/mL) for 4 h then fixed lysates were subjected to ChIP analysis using antibodies specific for STAT1 and IRF9 (or species-specific IgG control). Isolated DNA was then analyzed using qRT-PCR using primers designed to amplify the IFIT1 and IFIT3 promoters (TSS = Transcriptional Start Site). Percentage recruitment of STAT1 and IRF9 shown as ChIP'd DNA over input DNA. Numbers above bar indicate fold recruitment over vehicle control for each cell line. These experiments were performed in triplicate, and a representative experiment for each is shown. Results from replicate experiments shown in Supplementary Table 3.

Furthermore, many patients, even when compliant, will still relapse.[32,33] Our increased understanding of immune signaling in tumorigenesis has led the development of multiple immunotherapies – checkpoint inhibitors (CTLA-4, PD-L1), CAR-T therapy, vaccination, etc.[34] These innovative approaches to cancer treatment have proven to be effective in several tumor types but have been largely disappointing in luminal breast cancer.[35] Tumors more likely to respond to these therapies are considered immune "hot" – higher mutational burden, cytotoxic lymphocyte infiltration, increased antigen presentation, etc.[36] Breast cancer, particularly hormone receptor-positive breast cancer, lacks immunogenicity and therefore is immune "cold".[37] Identifying

a mechanism by which immune signaling is hindered in PR-positive breast cancer, and finding a way to target and convert to a "hot" environment could open up the possible therapies these patients will respond to and vastly improve clinical outcomes.[38] The data shown herein illustrates that PR is repressing the interferon response, which we speculate may contribute to the promotion of an immunosuppressed microenvironment. Additionally, data from this study and previous studies from our lab have illustrated a ligand-independent effect of PR downregulating interferon response, further reinforcing a potent effort in inhibiting this particular immune response.[15,16] It is essential to further define how PR suppresses immune signaling, as it may be
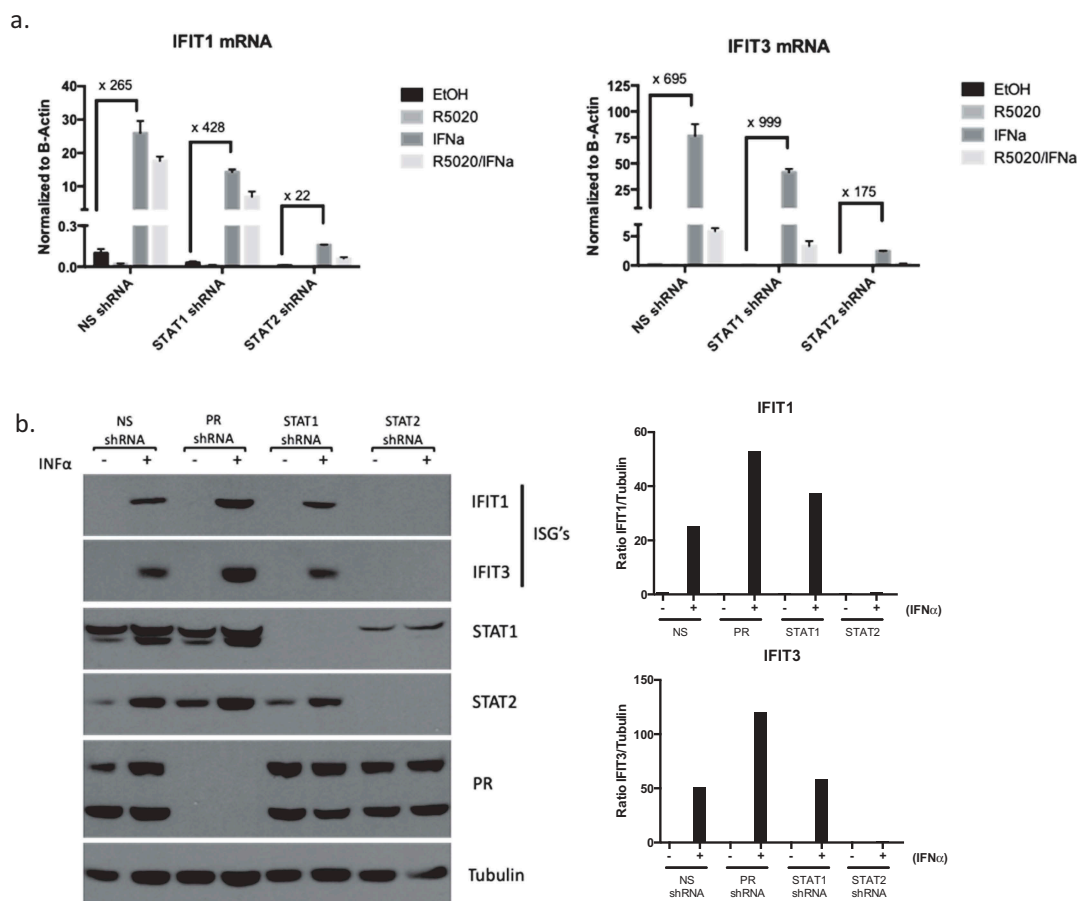
Figure 5. STAT2 is essential for efficient ISG transcription.

(a) T47D-NS, T47D-STAT1 (Figure 3(a)), and STAT2 shRNA cells (Figure 4(a)) were treated with vehicle, R5020 (10 nM), INFα (1000IU), or a combination of both for 6 h. RNA was isolated and subjected to qRT-PCR to analyze ISG (IFIT1, IFIT3) transcription. Gene values were normalized to an internal control (beta-actin). Error bars represent SD between biological triplicates. Fold induction (IFNα/Vehicle) labeled above bar to illustrate the effect of STAT1 and STAT2 knockdown on interferon response. (b). T47D cells stably expressing NS, PR, STAT1, or STAT2 shRNA were treated with IFNα (or vehicle, H20) for 6 h then isolated protein was analyzed using western blotting to assess ISG protein expression. Beta-tubulin shown as loading control. These experiments were performed in triplicate, and a representative experiment for each is shown. Densitometry of the ratio of IFIT1/B-Tubulin and IFIT3/B-Tubulin, as determined using ImageJ analysis, is shown to the right of the immunoblot.

a promising therapeutic target to prime tumors for immune-directed therapies and current experiments are underway to further characterize this connection.

Pre-clinical and clinical data have shown an essential role for type I interferons in bridging the innate and adaptive immune responses to promote dendritic cell (DC) priming and cytotoxic T cell recruitment/activation.[8] In particular, mice lacking the interferon receptor (IFNαR) show defects in CD8α+ DC ability to cross-present to cytotoxic CD8 + T cells; lymphocytes established to be vital in tumor rejection.[39] Interferon stimulated genes (ISGs), are a diverse group of proteins produced in response to active interferon signaling. Previous studies have shown that many of these proteins are involved in lymphocyte trafficking, inhibition of proliferation and cell motility, antigen presentation, and apoptosis.[40]

Additionally, PD-L1 expression is induced on immune cells in response to interferons.[41] As PD-L1 expression is necessary in determining whether a patient will respond to anti-PD-1/PD-L1 directed checkpoint inhibitors, the implications of inhibiting interferon signaling in the tumor microenvironment cannot be overstated.[42] The upregulation of PD-L1 on immune cells is not

unique to type I interferons, as crosstalk exists between type I and type II (i.e. INFgamma) signaling via STAT1-dependent mechanisms.[43] Current studies are underway to identify the impact of PR and progesterone on type II interferon signaling. Finally, a study recently published by Anurag et al., showed that endocrine therapy-resistant tumors exhibited an inverse correlation between PR expression and immune checkpoint expression, further illustrating the role of PR in promoting an immunosuppressive microenvironment.[44] The initial data herein and previous studies from our lab have demonstrated a concerted effort of PR to decrease interferon signaling in breast cancer cells which warrants further investigation into hormone mediation of the immune response in tumor development.

There is precedence for utilizing ubiquitination of STAT2 to inhibit type I interferon signaling. Multiple viruses employ viral proteins to function as E3 ubiquitin ligases to promote degradation of STAT2, thereby effectively inhibiting interferon signaling and allowing for the progression of infection.[45–47] Much like viruses that need to overcome type I interferon signaling to replicate within their hosts, transformed cells need to downregulate or inhibit this pathway to
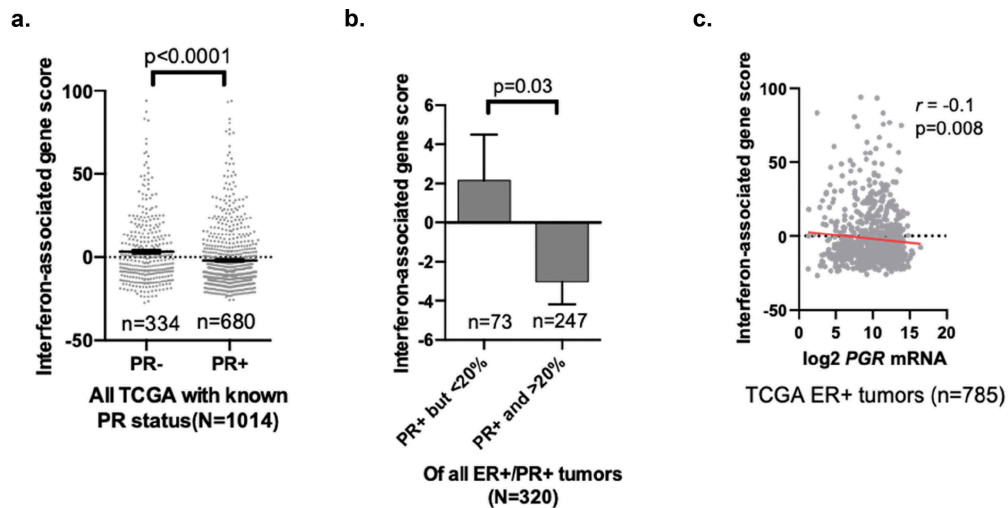
Figure 6. PR status in human tumors is correlated with decreased interferon gene signature.

TCGA (Breast, provisional[19]) data analyzed for expression of interferon gene signature.[18] Breast tumor data stratified based on ER/PR status (a) or on degree of PR positivity as determined by IHC (b). Gene signature score calculated by summing the normalized log2 Z-scores of the expression data from 50 interferon-related genes. Statistics were determined using a two-sided t-test. (c) A Pearson's correlation was tested between the 50 interferon-associated gene signature and *PGR* gene expression across clinically ER+ tumors.
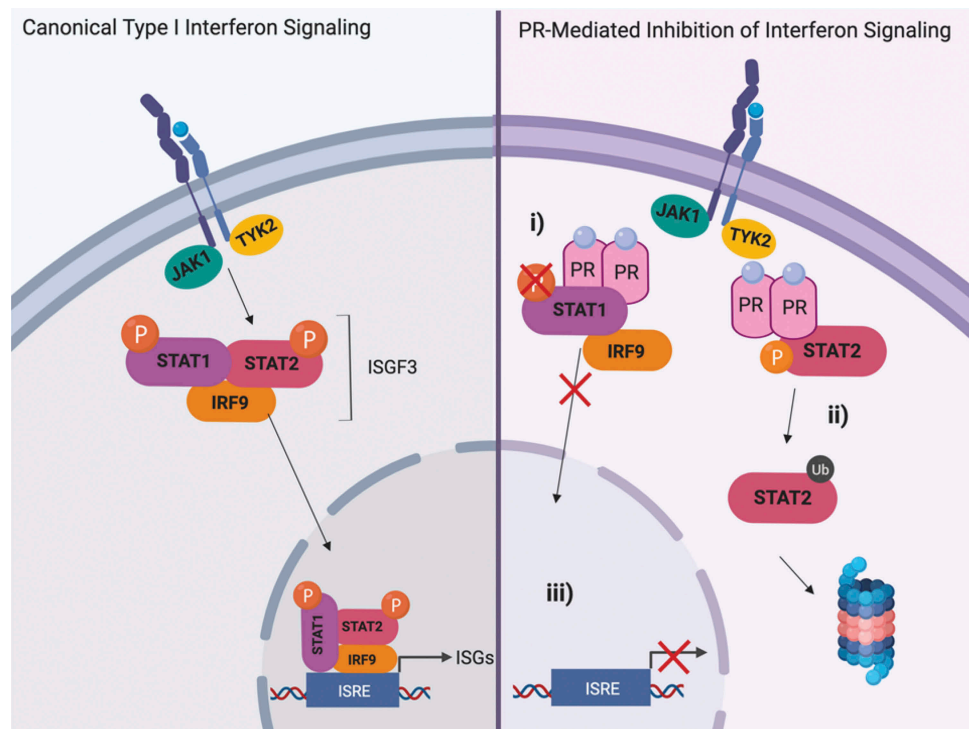
Figure 7. PR inhibits type I interferon signaling by targeting both STAT1 and STAT2.

Summary of PR-mediated inhibition of type I interferon signaling through multiple mechanisms. Previous study showed that PR inhibits STAT1's ability to be efficiently activated (i),[15] but this is not sufficient to fully shut down interferon signaling (Figures 3 and 5). STAT2 compensates for loss of STAT1 functionality and PR intervenes by promoting STAT2 ubiquitination and degradation (ii) (Figure 2). Without STAT1 and STAT2, PR effectively shuts down interferon signaling in the tumor cell, thus inhibiting a crucial signal transduction pathway necessary for immune recognition (iii).

avoid immune detection. Immune evasion allows for nascent tumor cells to avoid destruction by cytotoxic immune cells and progress to palpable tumors. Interestingly, mutations in STAT1 and STAT2 are not common in breast cancer.[19] As the majority of breast cancers are PR-positive and our data have shown PR inhibits these pathways, genomic mutations of these proteins would not be necessary to establish an immunosuppressive, tumorigenic microenvironment; PR inhibition of STAT1 and STAT2 activity circumvents the need for loss/mutation of these proteins.

We have identified multiple ways in which PR attacks the interferon signaling pathway, which we speculate may offer a potential mechanism by which nascent breast cancer cells (the majority of which are PR-positive) evade immune

detection and effectively escape immune-mediated destruction. We have also demonstrated that treatment with onapristone, a potent anti-progestin can reverse the ability of PR to promote STAT2 degradation. Because of this, it is imperative to investigate further whether targeting PR signaling could offer an advantage as a chemopreventative agent to prime immunosuppressed tumors to respond to other types of therapy. Current studies are underway to further characterize PR's role in immune escape and how this affects tumor formation and progression *in vivo*.

Declaration of interest statement

In accordance with Taylor & Francis policy and my ethical obligation as a researcher, I am reporting that Justin Balko, a co-author on this manuscript, receives research support from Genentech/Roche, Bristol Myers Squibb, and Incyte Corporation, has received consulting/expert witness fees from Novartis, and is an inventor on provisional patents regarding immunotherapy targets and biomarkers in cancer. I have disclosed these interests fully to Taylor & Francis, and I have in place an approved plan for managing any potential conflicts arising from this involvement.

Funding

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