Progesterone Receptor-Mediated Inhibition of Type I Interferon Signaling in Breast Cancer

Katherine R. Walter
M.S., Medical University of South Carolina, 2015
B.Sc., Rockhurst University, 2013

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Committee Co-Chair: Danny Welch, PhD

_____
Committee Co-Chair: Christy Hagan, PhD

_____
Chad Slawson, PhD

_____
Joseph Fontes, PhD

_____
Nikki Cheng, PhD

Date Defended: 27 March 2020

The dissertation committee for Katherine R. Walter certifies that
this is the approved version of the following dissertation:

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_____
Committee Co-Chair: Danny Welch, PhD

_____
Committee Co-Chair: Christy Hagan, PhD

_____
Graduate Director: Joan Lewis-Wambi, PhD

Date Approved: 4 April 2020

Abstract

Breast Cancer is an extremely heterogenous disease that is the second leading cause of cancer-related death among women in the United States. Of those diagnosed, the majority of breast cancers are hormone receptor positive, characterized by the dual expression of the estrogen (ER) and progesterone (PR) receptors. Much of what is currently known regarding hormonal regulation of breast cancer development focuses on estrogen-dependent signaling and, as such, many of the current standard of care therapies target this signaling axis. Despite being relatively effective, many will relapse on these first- and second-line therapies.

The progesterone receptor and its role in breast cancer development is less established. A ligand-activated, nuclear receptor, PR signals through binding of progesterone and regulating the transcription of a variety of genes involved in mammary gland development and pregnancy. The role of PR as a transcriptional activator is well-characterized. Its role as a transcriptional repressor, however, is less understood. Herein, we discover a novel role for PR as a potent repressor of type I interferon signaling in breast cancer. We establish that PR activation leads to the decreased activity of STAT1, a key player in the interferon signaling axis. We also demonstrate that compensatory mechanisms exist in the absence of a functional STAT1 complex, as interferon signaling remains active in STAT1 knock down cells. STAT2 is able to circumvent PR-mediated inhibition of STAT1 to propagate the interferon response. PR, however, promotes STAT2 ubiquitination and degradation, thereby abrogating interferon signaling with a "one, two punch" approach. These interactions between PR and interferon signaling components serve to decrease transcription of interferon stimulated genes (ISGs) and stifle this immune response.

It is significant that the interferon response is hindered in breast cancer as this particular immune pathway is integral in clearing early transformed cells in the body. PR dampening this pathway may offer an explanation as to why hormone receptor positive tumors are immune "cold" and present a mechanism by which early tumor cells are able to escape immune-mediated destruction and progress to clinically relevant, PR-positive tumors.

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Christy Hagan for her continued support—personal and professional—throughout my graduate school journey. Her "take no prisoners" attitude in what is often a male-dominated field has been inspirational in building up my own confidence. Because of Christy, I can honestly say I have become not only a better scientist, but a more assertive and confident person. I truly mean it when I say I "knocked it out of the park" when selecting my mentor.

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To my family: thank you for your unwavering support and love. Not many people would agree to be bored out of their minds and sit through not one, but TWO defenses; your presence means more than you know. My family has always been my outlet, whether it be for a good distraction, people to vent to, or a group to share in the celebratory moments. I love you all.

To my friends: Wednesday night kickball games (special shout out to the championship Pounders), weekend brewery crawls, and chiefs watch parties were only a few of the many things that helped me maintain my sanity throughout graduate school. I am certain that I would not have been able to accomplish all I did without the support I received from my people.

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Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Statistics and trends

In the United States, it is estimated that close to 300,000 new cases of breast cancer will be diagnosed in 2020, with over 40,000 deaths attributed to the disease. A substantial health burden, breast cancer represents the most commonly diagnosed and second leading cause of cancer-related death in women.[1] Breast cancer prevalence is undeniably high, as it is estimated that 1 in 8 women will be diagnosed with the disease at some point in their lifetime. Incidence of the disease has been slowly increasing in the past decade with mortality in post-menopausal women incrementally decreasing. These alterations can be attributed to increased availability of screening technologies and earlier diagnoses, leading to better prognoses in patients.[2]

1.1.2 Hormone receptor positive breast cancer

Despite being enveloped under a single term, breast cancer encompasses a group of extremely heterogeneous diseases with varying prognoses and therapeutic options.

A key step in the diagnosis of breast cancer involves the characterization of tumors based on the expression of three different cellular receptors: estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2).[3] Deciphering a tumor's receptor status is crucial in the formation of a patient's treatment plan, as receptor expression allows for targeted therapies that disrupt tumorigenic signaling, resulting in inhibition of cancer growth and survival.[3]

The vast majority of invasive breast cancers are ER-positive.[4] These tumors are generally less aggressive and therefore have better prognoses than other breast cancer subtypes.[5] Because the role of estrogenic signaling in the development and progression of breast cancer has been widely studied and well-established, therapies developed for these patients chiefly target this

signaling axis.[6] These include selective estrogen receptor modulators, such as tamoxifen, which interrupt the receptor's ability to affect gene regulation, and aromatase inhibitors which decrease circulating estrogen. Despite their effectiveness, some tumors never respond to these therapies, and of those tumors that do initially respond, many will become hormone independent and acquire resistance during treatment.[7] Of note, the majority of these ER-expressing tumors also express PR. The role of progesterone in breast cancer, however, is not fully understood and has only relatively recently become a topic of study.

1.1.3 Progesterone receptor signaling

Progesterone is a steroid hormone that elicits its effects by binding to the progesterone receptor. A lipid soluble compound, progesterone freely diffuses through the plasma membrane into the cytoplasm where PR is bound to a complex of chaperone and co-chaperone proteins.[8] When progesterone binds, PR dissociates from these proteins and localizes to the nucleus where it dimerizes and binds to specific sequences of DNA (progesterone response elements, PRE) or to other transcription factors through tethering interactions **(Figure 1)**. These interactions recruit transcriptional cofactors that can regulate the transcription of a variety of genes involved in mammary gland development, stem cell maintenance, and pregnancy.[9]

Like PR, ER is also a steroid activated nuclear receptor and therefore signals in a very similar manner. It affects transcription of a unique subset of genes by binding to estrogen response elements (ERE) on DNA. Interestingly, among these estrogen-regulated genes is PR.[4] Because ER controls PR expression, the presence of PR in breast cancer is seen as a positive prognostic marker rather than a therapeutic target. This logic stems from the assumption that PR expression indicates an active ER transcriptional complex, suggesting that the tumor will be responsive to anti-estrogen therapies.[8]

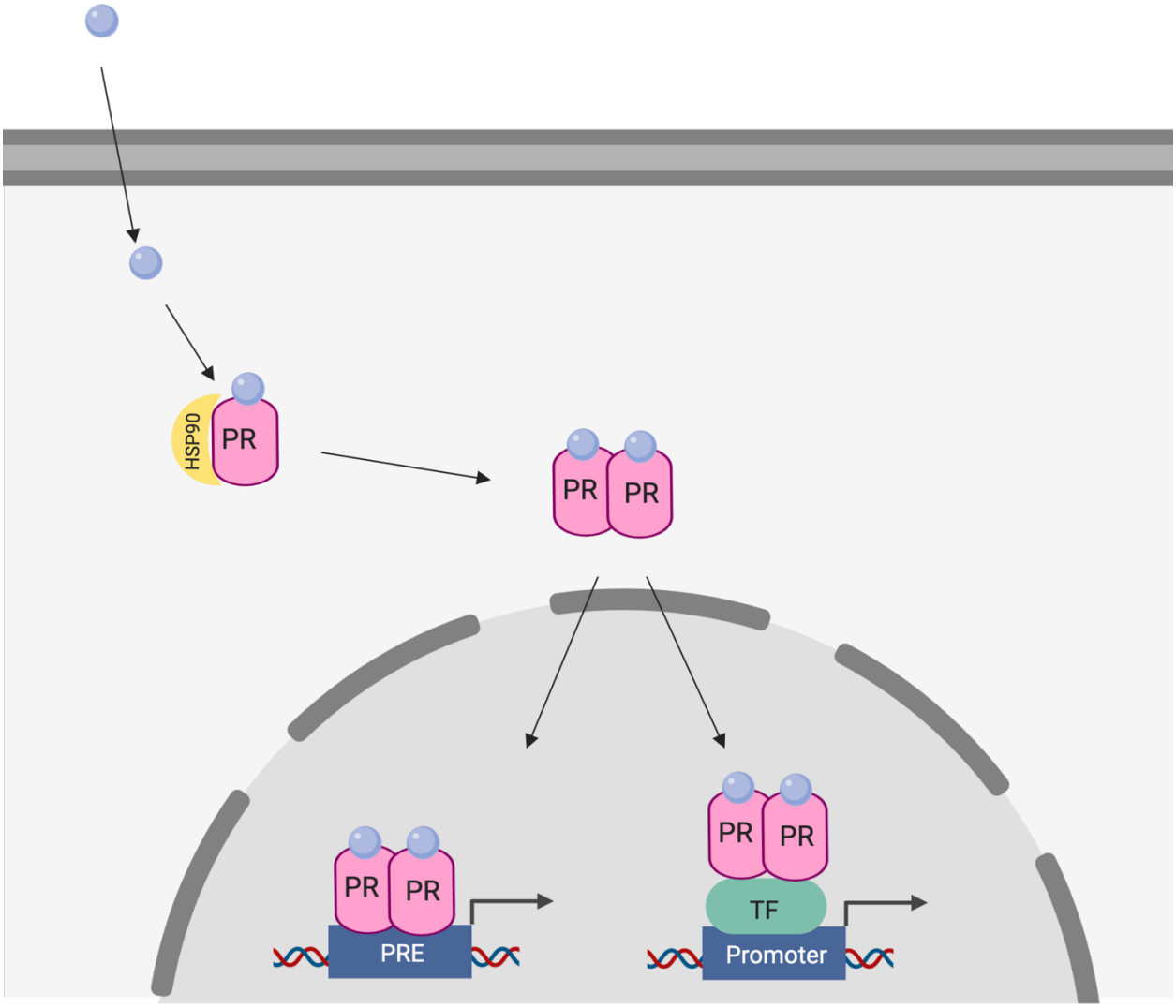

Figure 1. Progesterone Receptor Signaling

Schematic depicting canonical signaling mechanisms of the progesterone receptor. The hormone, progesterone, binds the receptor leading to dimerization, nuclear translocation, and either direct DNA binding (PREs) or tethering interactions with other transcription factors.

1.1.4 Progesterone and breast cancer

Hormone replacement therapy (HRT) is a common treatment for menopausal symptoms. The treatment regimen generally contains an estrogen component and, for women who have not undergone a hysterectomy previously, a progestin (synthetic progesterone) component. This is added to combat hyperplasia in the uterus driven by estrogen.[10] The progestin component of HRT has been a topic of study as a result of compelling data from two independent clinical trials published over a decade ago, suggesting a role in breast cancer development.

The Women's Health Initiative (WHI) trial recruited over 16,000 postmenopausal women in the United States and gave them HRT containing either placebo or estrogen plus medroxyprogesterone acetate (MPA), a synthetic progestin. Women were treated daily and breast cancer incidence was recorded.[11] Interestingly, the study was stopped prematurely because of the health risks associated with the hormonal treatment and the investigators found a higher incidence of breast cancer in the estrogen plus progestin group. Additionally, a separate arm of the study recruited over 10,000 women with prior hysterectomies and treated them with estrogen only (no progesterone necessary). They found a greater incidence of breast cancer in the estrogen plus progestin arm than in the estrogen only arm, indicating that the progestin component was pro-tumorigenic.[12]

Another trial that took place in the United Kingdom was the Million Women Study which utilized self-reporting to identify different types of hormone replacement therapies and how each affected cancer development.[13] The study found that relative risk of breast cancer significantly increased with the addition of synthetic progestins to HRT when compared to estrogen only.

These data taken together suggest that the progestin component, an activator of PR, is the driver behind breast cancer development and has created a clinical impetus in deciphering the function of PR signaling in this context.

1.2 Tumor immunology

1.2.1 Cancer immunoediting hypothesis

Hanahan and Weinberg's "The Hallmarks of Cancer" (i.e. characteristics shared by all tumors), transformed the way researchers approach targeting tumor development and progression.[14,15] Among these hallmarks is the ability of tumors to evade immune intervention. Robert Schreiber and his group built on the already existing theory of immunosurveillance—the idea that the immune system is constantly monitoring for tumor outgrowth—and illustrated that surveillance was only part of the role immunity plays in carcinogenesis.[16] The Cancer Immuoediting hypothesis demonstrated that the immune system plays dual roles in the process of tumor development (**Figure 2**). On the one hand, the immune system does indeed eliminate nascent tumor cells as was proposed in the original immunosurveillance theory. Additionally, however, the immune system can also shape the immunogenicity (i.e. immunoediting) of tumor cells and in that manner promote tumor outgrowth. The Immunoediting hypothesis was established and described in three distinct steps.[16,17]

1) **Elimination** (**Figure 2,** left)—concerted effort by the innate and adaptive immune systems to detect tumor specific antigens and 'danger signals' on transformed cells and eliminate these cells before any considerable outgrowth is apparent.

2) **Equilibrium** (**Figure 2,** middle)—Tumor cell variants that are not eliminated in the first step are held in a dormant phase by the immune system while simultaneously being "edited" by adaptive immune cells, thereby altering the tumor cell immunogenicity.

3) **Escape** (**Figure 2,** right)—Cells with low immunogenicity (i.e. immunosuppressed) circumvent immune intervention and escape from the equilibrium phase to progress to clinically relevant tumors.

This step-wise process can terminate at any point—i.e. effective elimination prevents tumor outgrowth entirely (1), equilibrium can persist through the lifetime of the host (2), or escape occurs and tumor progression ensues (3).[16]

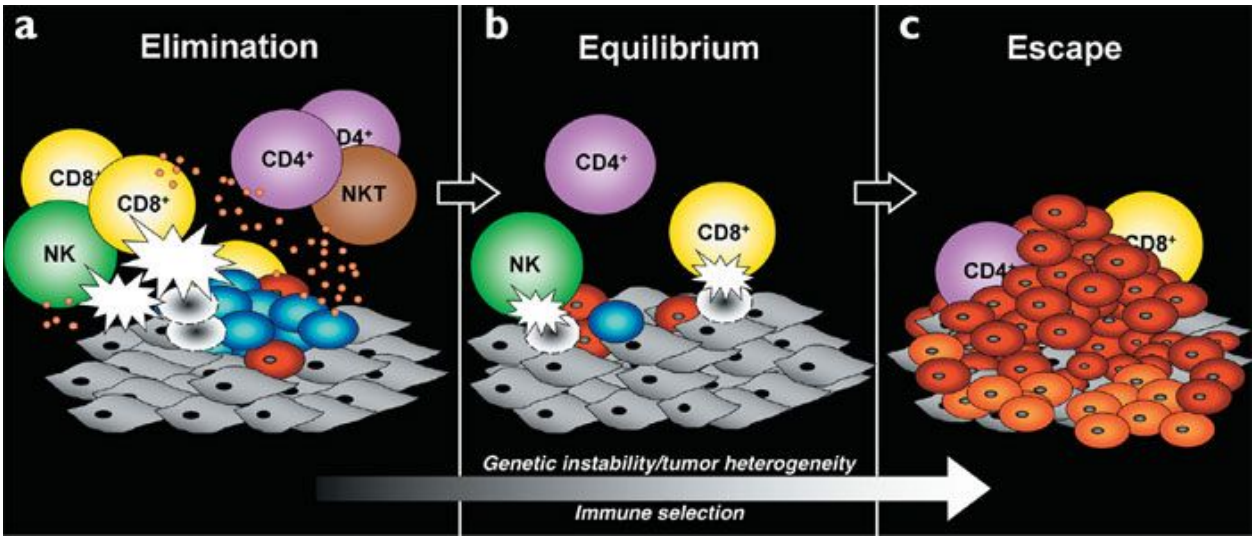

Figure 2. Cancer Immunoediting Hypothesis

Interactions between the immune system and developing tumors are divided into three steps: 1) Elimination, 2) Equilibrium, and 3) Escape. Tumor outgrowth requires dampened immunogenicity on cells to escape immune intervention. This figure was originally published in: Dunn, G., Bruce, A., Ikeda, H. et al. "Cancer immunoediting: from immunosurveillance to tumor escape." *Nat Immunol* 3**,** 991–998 (2002). https://doi.org/10.1038/ni1102-991. Reproduced with full permissions from Springer Nature and the Copyright Clearance Center (License #4771450513109)

1.2.2 Type I interferon signaling

As alluded to in the previous section, cancer cells need to evade immune intervention to progress to clinically relevant tumors. Among the immune mechanisms necessary for tumor elimination to occur is functional type I interferon signaling.[18] Type I interferons (IFNα/ß) are cytokines canonically produced in response to viral infection. These cytokines bind to a transmembrane receptor (IFNαR1/IFNαR2) leading to receptor dimerization and phosphorylation of the Janus Activated Kinases, JAK1 and TYK2. Phosphorylation of these proteins leads to phosphorylation of Signal Transducer and Activator of Transcription proteins, STAT1 and STAT2. Phosphorylated STAT1 and STAT2 interact with Interferon Regulator Factor 9 (IRF9) to form the Interferon Stimulated Gene Factor 3 (ISGF3) transcriptional complex. ISGF3 translocates to the nucleus and binds DNA at Interferon Stimulated Response Elements (ISREs) to turn on transcription of a diverse subset of genes known as Interferon Stimulated Genes (ISGs) (**Figure 3**)[19].

Despite being a signaling pathway chiefly known for preventing viral infection, type I interferons have been established as indispensable in the elimination phase of cancer immunoediting.[20] Among multiple studies demonstrating this connection, Dunn et al. showed that mice lacking the interferon receptor (IFNαR) were significantly more susceptible to both carcinogen-induced and transplanted tumors.[21] Additionally, STAT1knockout mice were also more susceptible to tumorigenesis suggesting that functionality in this pathway is imperative in eliminating nascent tumor cells.[22]

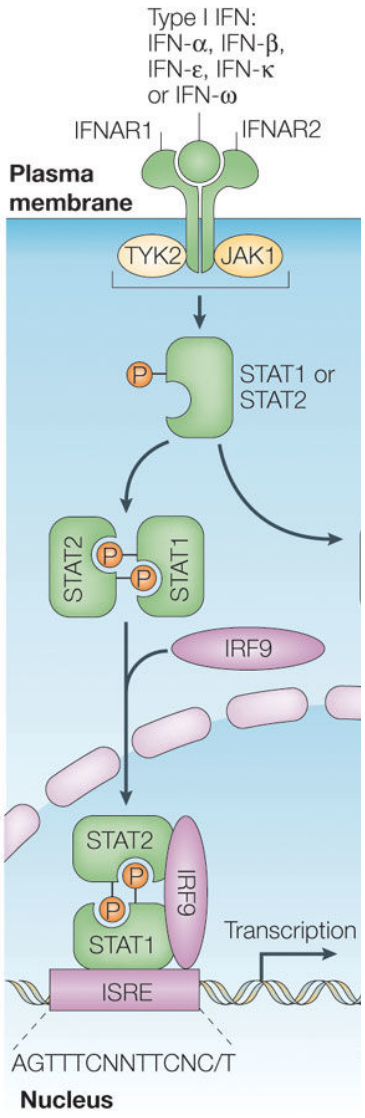

Figure 3. Type I Interferon Signaling

Schematic depicting canonical type I interferon signaling and the formation and subsequent DNA binding of ISGF3 complex at ISRE sites on DNA. This figure was originally published in: Platanias, L. "Mechanisms of type-I- and type-II-interferon-mediated signaling." *Nat Rev Immunol* 5, 375–386 (2005). https://doi.org/10.1038/nri1604. Reproduced with full permission from Springer Nature and the Copyright Clearance Center (License #4773780985125).

1.3 Project rationale and hypothesis

The majority of all breast cancers at the time of diagnosis are PR-positive, yet the role of progesterone in tumorigenesis has yet to be fully elucidated. Additionally, hormone receptor positive cancers are notoriously immune "cold," meaning they have limited immunogenicity. The aim of this study is to identify the link between the hormone, progesterone, and immunosuppression in breast cancer. We have found that progesterone and PR function to limit the functionality of type I interferon signaling in breast cancer. This body of work lays the foundation for the overarching hypothesis of this project: progesterone and PR, through inhibition of the type I interferon response, provide a mechanism by which nascent tumor cells evade immune intervention and progress to clinically relevant tumors.

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Chapter 2: Interferon-Stimulated Genes are Transcriptionally Repressed by PR in Breast Cancer

This chapter has previously been published in whole without any adaptations since publication and is reprinted here with permission from Walter KR, Goodman ML, Singhal H, et al. Interferon-Stimulated Genes Are Transcriptionally Repressed by PR in Breast Cancer. Mol Cancer Res. 2017;15(10):1331–1340.

2.1 Introduction

There is an emerging role for the ovarian steroid hormone, progesterone, and its receptor, the progesterone receptor (PR), in the development of breast cancer.[1-3] Clinical data have shown increased breast cancer incidence in women taking post-menopausal hormone replacement therapy (HRT) whose combined regimens included estrogen and progestins; this increased risk was not present in women taking estrogen-only HRT.[4] Although much debate continues regarding the role for native progesterone in breast cancer development, it is clear that PR, alone or in combination with the estrogen receptor (ER), affects the transcriptional landscape of breast cancer.[5-8]

PR is a ligand-activated transcription factor that binds DNA either directly through progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors. These interactions, together with the recruitment of transcriptional co-regulators, lead to transcriptional activation or repression of PR target genes. In the breast, these PR-dependent gene programs can drive proliferation, cell survival, and mammary stem cell self-renewal (reviewed in Ref. 3).[3] The mechanisms for PR-dependent transcriptional activation have been well studied, while PR-mediated transcriptional repression, especially direct repression in response to ligand, remains less understood.

Interferon signaling is a critical response of the innate immune system, which typically occurs following pathogen detection. Following interferon (IFN; types I–III) production and binding to their cognate receptors, a signaling cascade mediated by JAK/STAT (Janus Activated Kinase/Signal Transducer and Activator of Transcription) is initiated, culminating in a transcriptional response whose gene products aid the cell in responding to a pathogenic threat. The genes activated in response to interferons are collectively called interferon-stimulated genes,

or ISGs (reviewed in Ref. 9).[9] In response to type I interferons, such as IFN-alpha (IFNα), a heterodimeric receptor (IFNαR1 [interferon-alpha receptor 1] and IFNαR2) complex is activated/auto-phosphorylated in response to ligand, promoting JAK1/Tyrosine Kinase 2 (TYK2)-dependent phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2, together with IFN-regulatory factor 9 (IRF9), form a transcriptional complex referred to as IFN-stimulated gene factor 3 (ISGF3) which binds to DNA-sequences within ISG promoter regions, referred to as IFN-stimulated response elements (ISREs). Binding of ISGF3 to ISREs leads to transcription of ISGs, preparing the cell with a diverse response to viral infection. Interferon-signaling activation and ISG expression has also been detected in human tumors, independent of viral infection. The role of interferon signaling in human tumors is not well known, and remains an area of intense research.[10] The cancer immunoediting hypothesis postulates that IFN-signaling is an early step required for immune-recognition and subsequent destruction of nascent tumors by immune modulatory cells.[11] Alterations in immune surveillance, recognition, and destruction may have dramatic effects contributing to the development of clinically overt tumors. This effect appears to be most prominent in the early stages of tumor development. The opposite phenotype is seen in advanced, therapy-resistant tumors: evidence of interferon signaling (high ISG levels) is associated with a range of tumor types, including breast, that have escaped chemotherapy, radiation, or immunotherapy.[12] Herein, we present evidence of a novel role for PR in mediating transcriptional repression of ISGs. This newly described activity of PR has significant clinical implications for immune evasion and development of PR-positive human breast tumors.

2.2 Materials and Methods

2.2.1 Cell lines and constructs

T47D-co, T47D-Y, T47D-YB, T47D-YA, and Hela-PR cells have been previously described [13,14] and were a generous gift of Dr. Carol Lange (University of Minnesota). T47D cells (unmodified) were obtained from ATCC, and cultured as recommended. Cell line authentication is currently underway. MDA-MB-231 cells were maintained in Minimum Essential Media (MEM; CellGro) supplemented with 5% FBS, 1% Penicillin/Streptomycin, 1% non-essential amino acids, and 6 ng/ml insulin (cMEM). BT549 cells were maintained in RPMI-1640 Media (CellGro) supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% non-essential amino acids, and 6 ng/ml insulin. The PR-mDBD construct was a generous gift of Dr. Kathryn Horwitz (University of Colorado). Cells were treated with the following reagents (when applicable): R5020 (10nM; Sigma), human recombinant interferon-alpha (Sigma-Aldrich, SPR4594).

2.2.2 Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed using the javaGSEA desktop software; the c2 Molecular Signatures Database (MSigDB) version 5.2 was queried.[15,16] Dataset files (GEO accession number:GSE46850) were developed based on normalized Illumina expression intensities from cells that constitutively express wt PR-B (T47D-YB cells), as published in.[13] Specifically, the log2 transformed expression values were compared for two phenotypes: T47D-YB EtOH (vehicle) and T47D-YB R5020. GSEA was executed using the default settings, except the permutation type was set to Gene_set with 1000 permutations, and the metric for ranking genes was set to Diff_of_Classes, because normalized expression data was log2 transformed. Leading Edge analysis was performed on the 54 gene sets from MSigDB c2 analysis (PR-B EtOH vs R5020) that achieved FDR values ≤ 0.05.

2.2.3 Tumor explants

Tumor explant processing and treatments were done as previously described in [8]. Raw FASTQ data was re-analyzed using the VIPER RNA-seq pipeline (https://bitbucket.org/cfce/viper/) at Center for Functional Cancer Epigenetics (CFCE) at the Dana-Farber Cancer Institute. For visualizing gene expression data, we used heatmap.2 function of gplots package in R programming language.

2.2.4 Immunoblotting

Immunoblotting/Western blotting was performed as previously described.[13,14] Membranes were probed with primary antibodies recognizing total PR (Santa Cruz Biotechnology, sc-7208 or ThermoScientific, MS-298-P), IRF9 (Cell Signaling, 76684), IFIT1 (Cell Signaling, 14769), IFIT3 (Santa Cruz Biotechnology, sc-393512), OAS1 (Cell Signaling, 14498), and ERK (Cell Signaling, 9102). All Western blotting experiments were performed in triplicate, and representative experiments are shown.

2.2.5 siRNA/shRNA

ON-TARGETplus SMARTpool (a pool of 4 individual siRNAs) designed to target human PR was purchased from Dharmacon (L-003433-00), as were Non-silencing (NS) siRNA controls (D-001810-01-05) and the suggested DharmaFect 1 Transfection Reagent (T-2001-03). For siRNA experiments, the cells were plated at a density of 3×10^5 cells per well in a 6-well plate. 24 hours following plating, T47D-co cells were transfected with 25nM NS or PR siRNA per manufacturer's siRNA transfection protocol. At 48 hours post-transfection, the cells were starved in serum/phenol red-free IMEM (Gibco, A10488-01). At 72hr post-transfection the cells were treated with EtOH or 10nM R5020 for 6hr. Subsequent RNA isolation and qPCR experiments were carried out and analyzed as described below. PR shRNA knockdown cells were created

using viral particles (GE/Dharmacon) targeting three different regions of human PR. Viral transduction protocol was followed as per manufacturer's instructions. Transduced, stable cell line pools expressing NS or PR shRNA were created in T47D-co or T47D-YB cells following 14 days of selection in 2.5 ug/ml Puromycin (MP Biomedicals). Target si/shRNA sequences are listed in Supplementary Table 1.

2.2.6 Luciferase transcription assays

pGL4.45[luc2P/ISRE/Hygro] luciferase construct (Promega) was stably integrated in HeLa and HeLa-PR cells using Hygromycin selection. Luciferase assays were performed as previously described [17] using the Dual Luciferase Reporter Assay (Promega). Starved cells were treated for 18hr with 10nM R5020 and/or 10,000IU/ml interferon-alpha.

2.2.7 Real-time Quantitative PCR (qPCR)

RNA isolation, cDNA creation, and qPCR were performed as previously described,[13,14] with modifications noted here and in the Figure legends. qPCR was performed using the Faststart Essential DNA Green Master (Roche) on a Roche LightCycler96. Relative concentrations were quantified using the LightCycler96 (Roche, Software 1.1, Absolute Quantification Analysis), using a 6-point standard curve. Primer sets are listed in Supplementary Table 1. Relevant genomic sequence information is based on GR37 hg19.

2.2.8 ChIP assays

ChIP was performed using the ChIP-IT Express Kit (Active Motif) according to manufacturer's instructions using sonication for chromatin shearing. Lysates were immunoprecipitated (IP) overnight (18hr) with the following antibodies: PR (Santa Cruz Biotechnology, sc-7208), STAT2 (Santa Cruz, sc-476), IRF9 (Santa Cruz Biotechnology, sc-10793), H3K27ac1 (Cell Signaling, 8173), or an equal amount of negative control mouse or rabbit IgG. Resulting DNA was

analyzed using qPCR as described above, and data is represented as a percentage of input DNA. *In silico* analysis was performed using MatInspector (Genomatix) to identify potential PRE-binding sites. Primer sets are listed in Supplementary Table 1.

2.2.9 Statistics

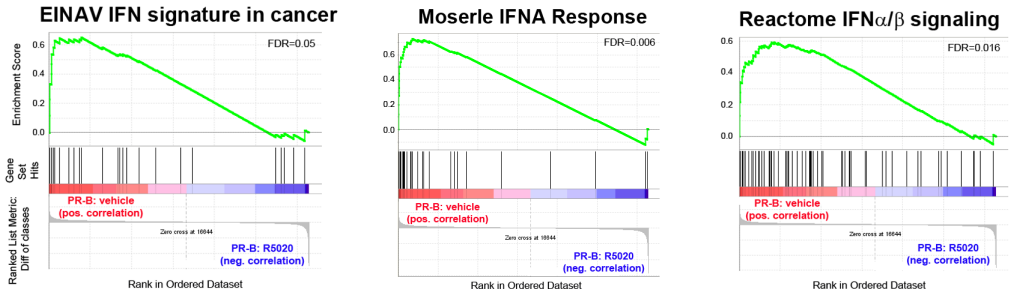
Statistical significance for all experiments was determined using an unpaired Student's *t*-test, unless otherwise specified. A p value ≤ 0.05 is considered statistically significant. The Delta method was used to calculate standard deviation for the ratio of two variables using their individual standard deviations, as seen when plotting fold relative RNA expression data between two treatment groups/cell lines.[18]

2.3 Results

2.3.1 Interferon gene sets enriched in PR-regulated transcriptional data set

Gene Set Enrichment Analysis (GSEA) is a powerful computational tool for comparing gene expression datasets of interest (i.e. genes regulated by PR) to published gene sets culled from the literature.[15,16] Using GSEA, we analyzed our previously published microarray dataset from ligand (synthetic progestin; R5020)-treated T47D breast cancer cells stably expressing full length PR (GEO:GSE46850).[13] GSEA revealed that multiple interferon-related gene sets (examples shown in **Fig 4A**) were significantly enriched in the absence of ligand; this enrichment was lost in cells treated with ligand, representing a negative correlation between ligand treatment and enrichment with interferon-related gene sets. Nearly 20% of the top significantly-regulated gene sets (54 sets with a false discovery rate [FDR] ≤ 0.05) are interferon-related gene sets (select sets shown in **Fig 4B**). As enrichment in these gene sets is lost in progestin-treated cells, these data imply that interferon gene programs are repressed/lost in response to ligand. Leading Edge (LE) analysis is a component of GSEA that allows one to identify individual genes that are present in multiple highly significant gene sets (i.e. core genes that drive the enrichment of a particular gene set). LE analysis identified multiple genes that are transcriptional targets of interferon-signaling pathways (i.e. IFITs, IRF7, OAS1/2, STAT1 and MX2) whose regulation is lost in ligand-treated cells (**Fig 4C**). These genes, classically activated by interferons, are collectively referred to as interferon-stimulated genes (ISGs). Cumulatively, these computational data suggest that interferon gene programs are negatively regulated by progestins.

A.



B.

Gene Set Name	Number of Genes in List	FDR
HECKER_IFNB1_TARGETS	92	0.000
BENNETT_SYSTEMIC_LUPUS_ERYTHEMATOSUS	31	0.001
MOSERLE_IFNA_RESPONSE	31	0.006
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	63	0.015
BOSCO_INTERFERON_INDUCED_ANTIVIRAL_MODULE	76	0.017
RADAEVA_RESPONSE_TO_IFNA1_UP	52	0.045
EINAV_INTERFERON_SIGNATURE_IN_CANCER	27	0.049

C.

Top 30 ranking leading edge genes	
IFIT1	KIAA1324
CXCL10	RSAD2
IFI44L	FOXA1
IFIT3	PLAU
STAT1	TFF3
TNSF10	OASL
IFI44L	ERBB2
RHOB	EIF2AK2
ESR1	AGR2
CCL2	BIK
IRF7	SIDT1
IFIT2	RND1
OAS2	MX2
GATA3	CCL20
SPDEF	TJP3

Figure 4. Interferon signaling enriched in PR data set

(A). GSEA comparing gene expression datasets obtained using the Illumina Microarray data set published in Hagan et al, 2013. Vehicle vs R5020 (synthetic PR ligand) are compared in T47D cells stably expressing full length PR. Select gene sets are shown from the C2 MSigDB. (B). Summary of interferon-related gene sets that are included in the most significant (FDR < 0.05; 54 gene sets in total) gene sets from MSigDB C2 GSEA analysis. (C). Top 30 ranking genes as identified using Leading Edge (LE) analysis on 54 gene sets referred to in (B). Shading denotes genes that are interferon-signaling related and/or interferon-stimulated genes (ISGs).

2.3.2 ISGs are downregulated by progestins in human breast cancer

We used a human tumor-explant model, previously described by Singhal et al,[8] to determine if progestin-dependent ISG downregulation occurs in human breast tumors. Sliced portions of ER/PR-positive tumors from eight patients were obtained during surgical breast cancer resection. These tumor samples were grown in an *ex vivo* culture system in media containing vehicle (EtOH) or R5020 (24–48hr). RNA-seq was performed on cultures of each tumor sample; these data were analyzed for ISG repression following R5020 treatment. The heatmap in **Figure 5** shows fold expression (R5020/vehicle), for RNA-seq data obtained from all eight patients, for select ISGs. There is a clear trend towards transcriptional repression for ISGs in patient *ex vivo* tumor explants. These data indicate that ISGs are regulated by progestins not only in human breast cancer cell lines (**Fig 4**), but, importantly, in human breast tumors as well (**Fig 5**).

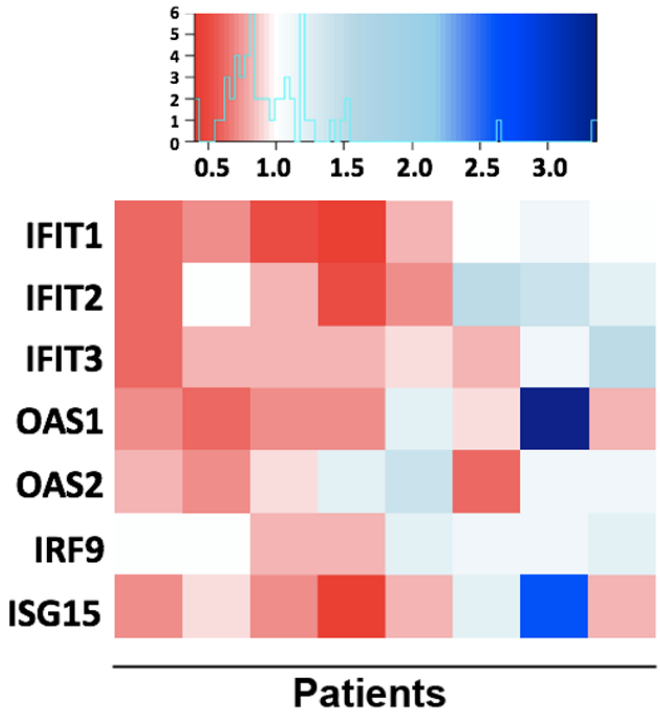


Figure 5. Progestin-dependent ISG repression in human breast tumors

Human breast tumors were grown ex vivo for 24–48hr in media containing vehicle or 10nM R5020, followed by RNA-seq. Heat map represents fold RNA expression values for R5020/vehicle for seven ISGs, in each of eight patient samples. Red shading indicates downregulation of RNA expression in R5020-treated tumors, blue represents gene upregulation in response to R5020 treatment, white represents no change.

2.3.3 ISG repression is PR-dependent

To further characterize the mechanism through which progestins regulate ISG expression, we studied progestin-dependent ISG repression in multiple breast cancer cell line models. A brief note about our most frequently used model system: PR is an important target gene of ER and, as such, PR expression is regulated by estrogen in most tissues.[19,20] In order to differentiate between the effects of ER/estrogen and PR/progesterone, our laboratory uses PR-positive (T47D-co) and PR-null (T47D-Y) variants of the ER/PR+ breast cancer cell line, T47D.[21] T47D-co cells endogenously express both isoforms of PR, PR-A and PR-B, without the need for exogenously added estrogen, allowing us to study the function of PR without the confounding effects of estrogen. T47D-Y cells can also be used to reintroduce single isoform variants or mutants of PR, such as PR-A (T47D-YA cells) and PR-B (T47D-YB cells) or DNA-binding mutant PR (T47D-PRB-mDBD). We have published extensively using these cell line models to define isoform- and phosphorylation-specific PR gene regulation and protein-protein interactions,[13,14,22-24] and this cell line model remains a powerful and well-established system for studying PR activity.[5]

To characterize regulation of ISGs by progestins, we analyzed RNA levels of ISGs selected from the LE gene list in T47D-co breast cancer cells. T47D-co cells were treated for 6hr with synthetic progestins (R5020 or medroxyprogesterone acetate [MPA]), native progesterone, or vehicle (EtOH); isolated RNA was used for quantitative real-time PCR (qPCR) analysis. For the ISGs we assayed, six of which are shown in **Figure 6A**, expression was transcriptionally repressed (2- to 10-fold) by all progestins, both synthetic and native. Individual gene validation (such as those shown in **Fig 6A**, and data not shown) and microarray data mining [13] showed that this transcriptional repression is conserved for a large cohort of ISGs. Progestin-dependent ISG transcriptional downregulation can be mediated via both isoforms of PR, as T47D-Y cells stably

expressing either PR-B or PR-A can both repress ISG RNA levels, although PR-B appears to have greater transcriptional repressor activity on the ISGs assayed (Supplement, **Figure 11A**). Additionally, because the T47D-co cells are unique in that exogenous estrogen is not needed for PR expression, we verified that ISG transcripts were repressed following progestin treatment in unmodified, parental T47D cells grown in an estrogenic environment (T47D-ATCC; Supplement, **Figure 11B**). To determine if repression of ISGs by PR is cell-type specific, we stably expressed PR-B in HeLa cells (normally PR-null) and observed ISG transcriptional repression in response to progestin treatment (Supplement, **Figure 11C**). Finally, this transcriptional repression was associated with a concomitant decrease in ISG protein levels. ISG proteins were assayed from T47D-co breast cancer cells treated with ligand (R5020; 18hr); decreased protein levels were observed for all ISGs assayed via Western blotting (**Fig 6B**). Cumulatively, these data suggest that ligand-activated PR promotes the downregulation of ISG RNA and protein levels.

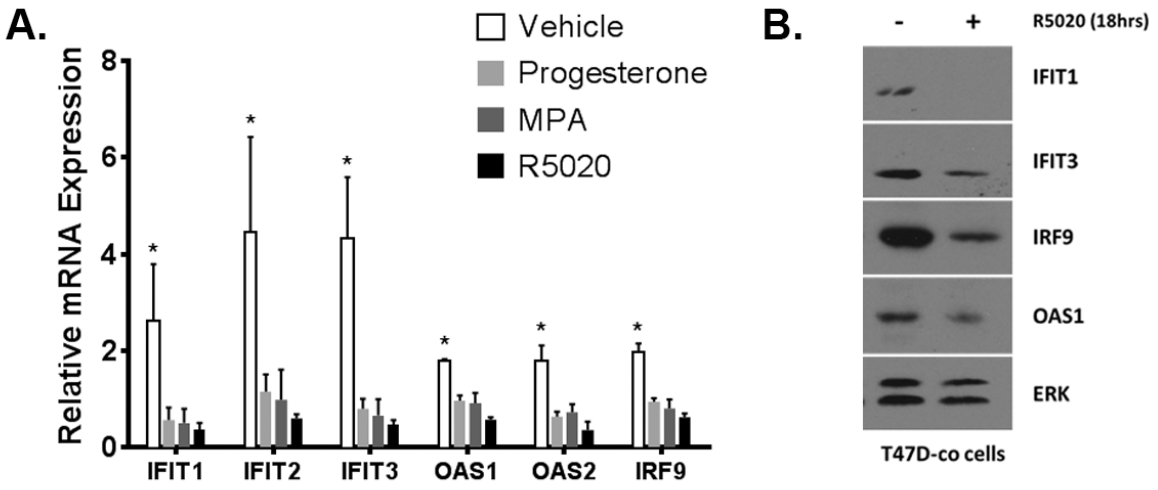


Figure 6. ISGs are repressed by ligand-activated PR

(A). T47D PR-positive breast cancer cells (T47D-co) were starved for 18hr in serum-free media, followed by treatment with 10nM R5020, 10nM medroxyprogesterone acetate (MPA), 100nM native progesterone, or vehicle for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (β-actin). Error bars represent standard deviation (SD) between biological triplicates. Asterisks represent statistical significance between the vehicle treated groups and all treatment groups (R5020, MPA, and progesterone); $p < 0.05$, as determined using an unpaired Student's *t*-test. This experiment was performed in triplicate, and a representative experiment is shown here. (B). T47D-co cells were starved for 18hr in serum-free media and then treated with 10nM R5020 or vehicle for 18hr. Protein lysates were analyzed via Western blotting. ERK represents the loading control.

To determine whether ISG transcriptional repression following progestin treatment is dependent upon PR, we assayed ISG transcript levels in cells where PR expression was knocked-down using a pool of four siRNAs. T47D-co cells transiently transfected with non-silencing (NS) or PR siRNA were treated with vehicle or R5020 for 6hr, and RNA was analyzed using qPCR. ISG transcriptional repression in response to ligand was lost in cells with PR knockdown (**Fig 7A**; PR knockdown efficiency shown in **Fig 7B**). These data were repeated in cells stably expressing PR shRNA (Supplement, **Fig 12**). Surprisingly, basal levels (vehicle treated, in the absence of progestin) of ISGs were markedly higher in cells lacking PR (compare vehicle bars between NS and PR siRNA for each gene shown in **Fig 7A**). Basal levels of select ISGs were increased 1.5 to 3.5-fold when PR expression was knocked-down using siRNA (**Fig 7C**). These data indicate that PR has ligand-independent functions that appear to maintain low levels of ISG expression. Moreover, when ISG expression was assayed in two PR-negative (triple negative, lacking ER/PR/HER2) breast cancer cell lines, we saw a similar phenotype: no ISG repression in response to ligand and high basal levels of ISGs (Supplement, **Fig 13**). Together, these data indicate that PR attenuates ISG expression, even in the absence of ligand, suggesting a concerted biological program aimed at PR-dependent down-regulation of ISGs.

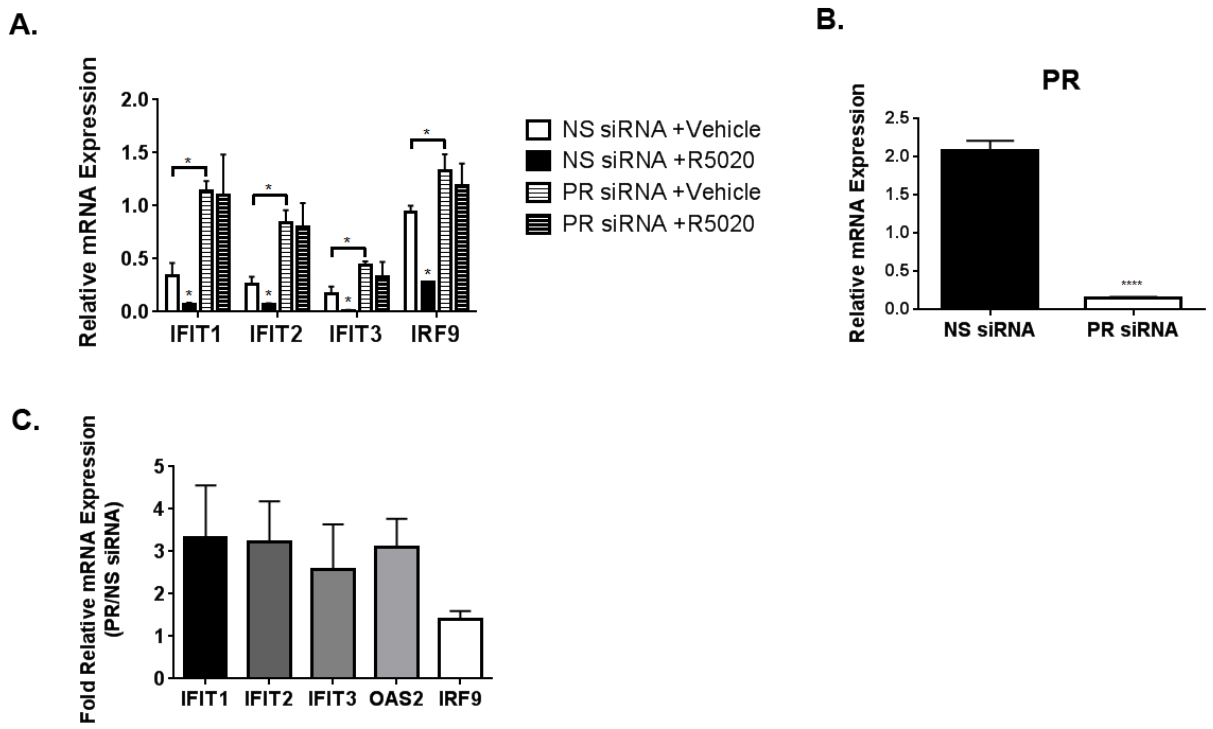


Figure 7. ISG repression is PR-dependent

(A). T47D-co cells were transfected with non-silencing (NS) or a pool of four PR siRNAs. 48hr following siRNA transfection, cells were starved for 18hr, followed by 10nM R5020 or vehicle for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (β-actin). Error bars represent SD between biological triplicates. Asterisks represent statistical significance between the vehicle and R5020-treated groups; $p < 0.05$, as determined using an unpaired Student's t-test. This experiment was performed in triplicate, and a representative experiment is shown here. (B). PR knockdown efficiency by siRNA was determined using relative PR levels between the NS and PR siRNA transfected cells. Asterisks represent statistical significance; $p < 0.0001$. (C). Fold increases in basal (no ligand) ISG expression between PR and NS siRNA transfected cells. Error bars represent the SD for the ratio of two variables using their individual standard deviations, calculated using the Delta method.

2.3.4 Interferon-activation of ISGs is repressed by ligand-activated PR

Most typically, ISGs are activated in response to interferons in specialized cell types of the innate immune system, such as dendritic cells. However, all cell types have the capacity to produce and respond to interferons (including fibroblasts and epithelial cells), as long as the proper receptors (IFNARs) and transcriptional machinery are intact.[10] Although our T47D-co cells do not secrete endogenous interferons (measured using highly-sensitive ELISA assays designed to detect picomolar concentrations; data not shown), they retain their response to exogenous treatment with IFNα by transcriptionally upregulating ISGs (**Fig 8**). Interestingly, when we co-treated cells with IFNα and progestin (R5020), we found that progestin-treatment attenuated the IFNα-induced ISG transcriptional response, reducing it to near baseline levels (**Fig 8**). We concluded from these data that ligand-activated PR can repress ISG transcripts in response to IFNα.

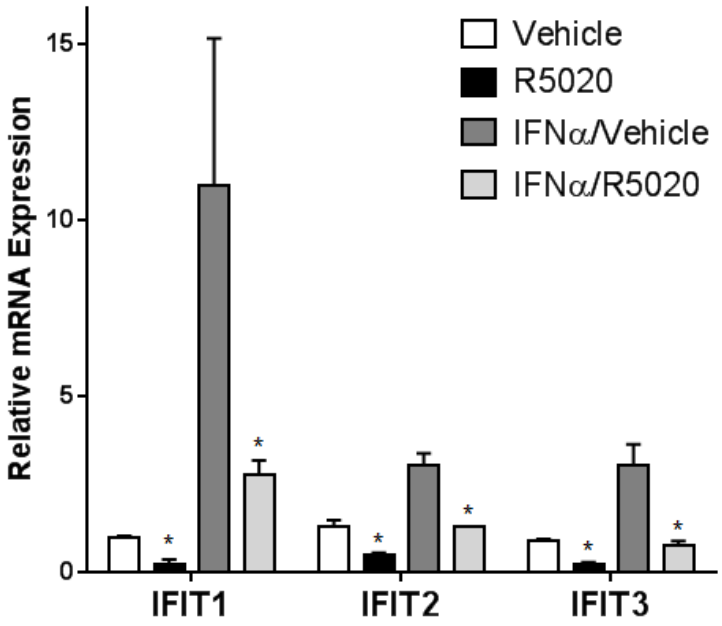


Figure 8. Interferon-activated ISG expression is repressed by ligand-activated PR

Following an 18hr starvation in serum-free media, cells were treated for 18hr with IFNα (20 IU/ml) or vehicle (water) for 18hr, followed by R5020 (10nM) or vehicle (EtOH) for 6hr. Isolated RNA was analyzed for expression of IFIT1, IFIT2, and IFIT3. Gene expression values were normalized to an internal control (β-actin). Error bars represent SD between biological triplicates. Asterisks represent statistical significance between the respective vehicle and R5020-treated groups; $p < 0.05$, as determined using an unpaired Student's t-test. This experiment was performed in triplicate, and a representative experiment is shown here.

2.3.5 PR DNA-binding needed for ISG repression

There are many potential mechanisms through which PR can mediate transcriptional repression of target genes.[25] To determine if DNA-binding by PR is required to mediate ISG transcriptional repression, we employed a well characterized DNA-binding domain (DBD) mutant of PR (PR-mDBD). This mutant contains a single point mutation at Cys587, located within the first zinc finger of the PR DBD, which abolishes PR's ability to bind DNA.[26] Using T47D-PRB-mDBD cells, we measured the capacity of mDBD PR to repress ISG transcription. T47D cells stably expressing wt PR-B (T47D-YB, described above) robustly repress ISG transcription in response to ligand; this effect is lost in T47D-PRB-mDBD cells (**Fig 9A**). These data suggest PR binding to DNA is required for ISG transcriptional repression. IFNα-activated ISG transcription is regulated through ISREs, DNA sequences where the ISGF3 complex (STAT1, STAT2, and IRF9) binds and activates transcription. To test if PR repressed transcription via direct or indirect binding to ISRE sequences, we used an ISRE-luciferase reporter construct stably expressed in Hela or Hela-PR cells. ISRE-linked luciferase activity was unaffected by the presence of PR or its ligand in either cell line (**Fig 9B**); no changes were seen basally or in response to IFNα treatment. These data imply that PR does not repress ISG transcription by binding to or blocking the ISRE promoter element directly.

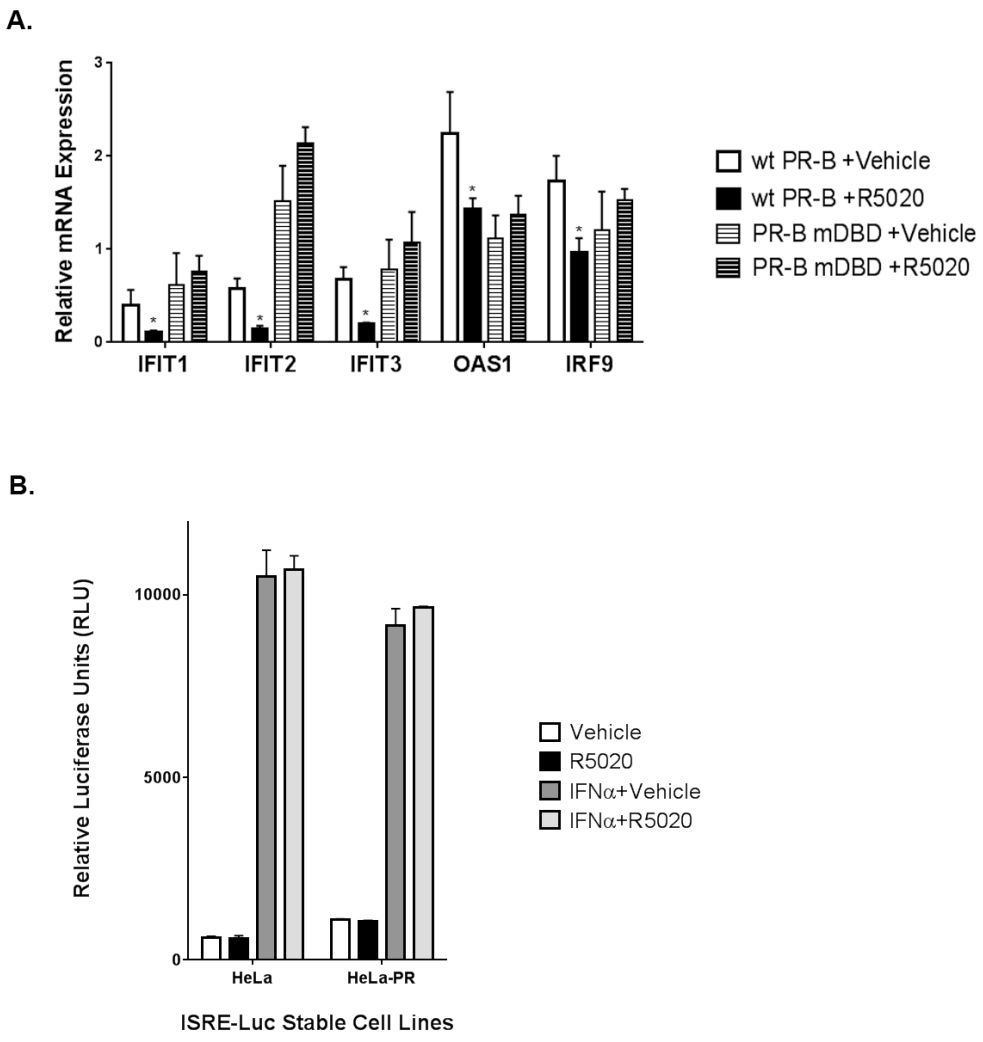


Figure 9. PR DNA-binding required for ISG transcriptional repression

(A). Following an 18hr starvation in serum-free media, T47D-YB or T47D-PRB-mDBD cells were treated for 6hr with vehicle or 10nM R5020 for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (β-actin). Error bars represent SD between biological triplicates. Asterisks represent statistical significance; $p < 0.05$, as determined using an unpaired Student's t-test. (B). HeLa and HeLa-PR cells that were stably transfected with an ISRE-luciferase were starved for 24hr, followed by an 18hr co-treatment with interferon-alpha (IFNα) (or water) or 10nM R5020 (or EtOH). Luciferase assays were performed as described in the 'Materials and Methods' section. Error bars represent SD of biological replicates. The experiments in (A) and (B) were performed in triplicate, and representative experiments are shown here.

2.3.6 PR is recruited to ISG enhancers

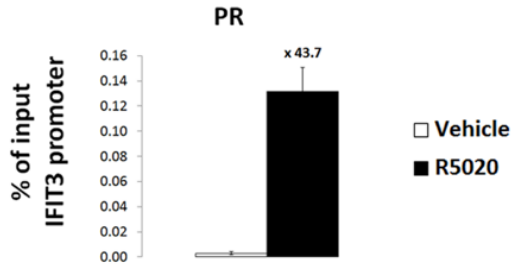
To determine if PR is directly recruited to promoters/enhancers that regulate ISG transcription, we used chromatin-immunoprecipitation (ChIP) to detect PR binding at ISG-regulatory regions. As mentioned above, ISGs are regulated by promoter proximal ISREs, typically within 100bp of ISG transcriptional start sites (TSS).[27] Using published ChIP-seq data for ligand-activated PR binding,[6] we identified that all ISGs we assayed exhibited PR binding in their promoter/enhancer regions. It is well documented that nuclear receptors often bind in intragenic or distal (> 40kb away from the TSS) enhancer regions of the genes they regulate;[28-32] PR binding to ISG enhancers falls well within this range (−18kb to +5kb). We identified individual PREs within these binding sites using *in silico* analysis. **Figure 10A** (left) highlights the position of the PREs and ISREs (ISREs defined in [27] or through ENCODE STAT1/2 ChIP-seq binding data [33-35] in relation to the TSS for select genes assayed via ChIP-qPCR for PR and ISGF3 (STAT1/STAT2/IRF9) binding. Using PR ChIP-qPCR analysis following R5020 treatment, we showed robust PR recruitment to enhancer regions of multiple ISGs (**Fig 10A** and Supplement, **Fig 14**); recruitment of PR to the IFIT3 promoter (**Fig 10A**) is shown. All subsequent ChIP-qPCR results will be shown for the IFIT3 promoter as a representative example of protein recruitment to all ISG enhancer regions assayed and summarized in **Fig 10A**.

Although STAT1/STAT2/IRF9 historically comprise the ISGF3 transcription factor complex, IRF9 and STAT2 complexed together (in the absence of STAT1) can recapitulate ISRE-mediated transcription.[36] We therefore focused on how PR activation alters STAT2/IRF9 recruitment to ISG promoter regions. We observed robust recruitment of STAT2 and IRF9 to ISREs following treatment with IFNα (**Fig 10B**). Interestingly, interferon-stimulated STAT2/IRF9 recruitment was decreased in the presence of PR ligand. Basal STAT2/IRF9

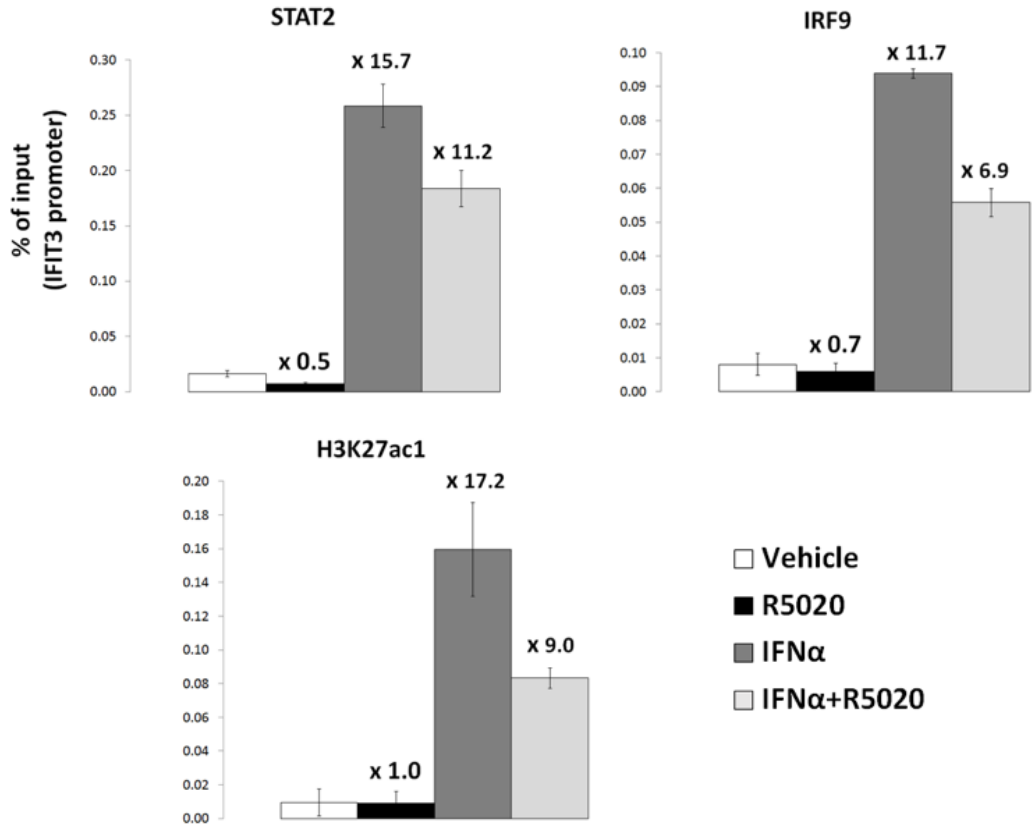
occupancy in the absence of interferon is too low (at/above IgG levels) to generate a consistently robust signal from ChIP-qPCR. However, a trend exists towards STAT2/IRF9 loss when comparing vehicle and R5020 in the absence of IFNα (**Fig 10B** – compare vehicle and R5020 bars). This observation (loss of STAT2/IRF9) is potentiated when IFNα treatment, in combination with PR ligand, is used. Moreover, we see a potent increase in H3K27ac1, a histone mark indicative of activated transcription and open chromatin, following treatment with interferon. Treatment with PR ligand decreases the levels of H3K27ac1, suggesting that recruitment of PR (and loss of STAT2/IRF9) leads to active repression of these enhancers. Finally, in T47D-co cells where PR expression was knocked-down using shRNA directed against PR (or a non-silencing control; NS), recruitment of STAT2/IRF9 was potently enhanced in response to IFNα, indicating that the presence of PR (ligand-independent) decreases ISG promoter occupancy of ISGF3 components (**Fig 10C**). Cumulatively, these data suggest that PR blocks/decreases efficient STAT2/IRF9 recruitment to ISRE regulatory regions, thereby decreasing transcription of ISGs. The ligand-dependent loss of STAT2/IFR9 occurs when the PRE and ISRE sequences are both located in the proximal promoter regions (as is the case with IFIT3), as well as when the PR binding sites occurs upstream of the ISRE (data not shown). The details regarding how PR recruitment destabilizes STAT2/IRF9 DNA binding/recruitment are the subject of our ongoing experiments.

A.

Gene name	ISRE location (respective to TSS)	PRE location (respective to TSS)	Ligand-dependent PR binding confirmed by ChIP
IFIT1	-100bp	-18kb	✓
IFIT2	-80bp	-18kb/+5kb	✓
IFIT3	-100bp	0kb (at TSS)	✓
OAS1	-30bp	-11kb	✓
OAS2	-15bp	0kb (at TSS)	✓



B.



C.

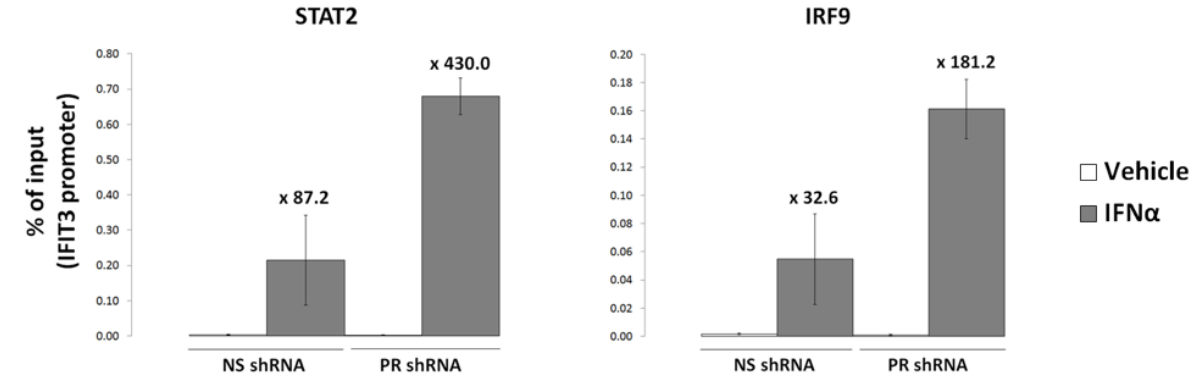


Figure 10. PR diminishes recruitment of STAT2/IRF9 to ISG promoters

(A). Left: table summary of ISG promoters/enhancers assayed by ChIP-qPCR for PR, STAT2, and IRF9 recruitment. Location of interferon-stimulated response elements (ISREs) and progesterone receptor response elements (PREs) from the transcriptional start site (TSS) of each gene is shown in kilobases (kb). Right: T47D-co cells were serum-starved for 18hr. Cells were then treated with 10nM R5020 or vehicle for 30min. Fixed lysates were subjected to ChIP with antibodies against PR or a species-specific IgG (control; not shown), and qPCR was performed on the isolated DNA using primers designed to amplify the IFIT3 promoter. A percentage of ChIP'd DNA over input DNA is shown. (B). T47D-co cells were serum-starved for 18hr. Cells were then treated with 10nM R5020, 1000IU/ml IFNα, or a co-treatment of both (or appropriate vehicle controls). Fixed lysates were subjected to ChIP with antibodies against STAT2, IRF9, H3K27ac1 or a species-specific IgG (control; not shown), and qPCR was performed on the isolated DNA using primers designed to amplify the IFIT3 promoter. A percentage of ChIP'd DNA over input DNA is shown. (C). ChIP experiments were performed as in (B), but T47D-co NS and PR shRNA cells were used, with only IFNα treatment. All ChIP experiments were performed in triplicate; a representative experiment is shown here. Fold-recruitment in treated conditions (R5020, IFNα, or combo), as compared to vehicle treatment, is displayed above each bar. Error bars represent SD of technical replicates.

2.4 Discussion

Herein, we demonstrate that PR transcriptionally represses ISGs in human breast cancer. This represents a novel class of genes previously unknown to be regulated by PR. Although regulation of ISGs has been well defined in response to interferons, PR-dependent repression contributes a novel layer of ISG regulation. We show PR-mediated ISG repression in breast cancer cell lines, as well as in tumor explants from breast cancer patients. Additionally, ISG repression exists in response to PR ligand, as well as in unliganded (basal) conditions. These data indicate a concerted PR-dependent effort aimed at maintaining low ISG levels in breast cancer.

The cancer immunoediting hypothesis highlights that the innate and adaptive immune responses work together to flag early neoplastic lesions for immune-mediated elimination.[11] An early mediator of this elimination process is activation of type I interferon-signaling.[10] Thus, suppression of type I interferon-signaling may help developing tumors evade the critical early steps of immune recognition and subsequent clearance, allowing nascent tumors to progress. In vivo studies support this notion. For example, female STAT1 knockout mice, lacking a key upstream activator of type I interferon-signaling, develop mammary gland adenocarcinomas.[37] We show that PR represses the end product of interferon-signaling, ISG transcription, suggesting that progesterone, working through PR, may be a key player in tumor immune evasion. A number of previous observations support the importance of the mechanism we described, in the development of breast cancer. For example, 70% of breast cancers are ER/PR-positive at the time of diagnosis, and recent data has suggested a positive correlation between PR positivity in benign, normal breast tissue and risk for the development of breast cancer.[38] Moreover, >90% of the mammary gland tumors that form in the STAT1-deficient mice are ER/PR-positive and have gene expression profiles that mimic the luminal ER/PR-positive molecular subtype of breast

cancer.[37] These data suggest there is pro-tumorigenic crosstalk between PR and interferon-signaling pathways.

Downregulation of multiple components of interferon-signaling have been correlated with increased tumor incidence and metastasis. Mice lacking IFNAR, the IFNα-binding receptor that initiates JAK/STAT-signaling which culminates in ISG transcription, have enhanced tumor development, impaired ability to reject syngeneic/allogenic tumors, and accelerated metastasis in a spontaneous mouse mammary gland tumor model.[39,40] A recent report shows that IFNAR inactivation in tumor-associated stroma leads to an immune-privileged niche for developing colon cancers [41]. Type I interferons, in particular, and dendritic cells have been shown to be critical to tumor cell rejection, and therefore immune surveillance.[42] Moreover, interferon regulatory factor 7 (IRF7) and its downstream targets (many of which are ISGs shown herein to be repressed by PR), are suppressed in the aforementioned mammary gland tumor metastasis model; restoration of IRF7-signaling suppressed bone metastasis.[39] Finally, expression of an interferon-gene signature in a cohort of breast cancer patients was correlated to a lower frequency of breast cancer metastasis.[43] Cumulatively, these data suggest mounting evidence that interferon signaling is correlated with tumor development and metastasis. Conversely, high/increased ISG expression has been linked to poor prognosis in more advanced tumors. An interferon-related DNA damage resistance signature (IRDS) has been defined by Weichselbaum and Minn that is associated with chemotherapy, radiation, and most recently, immunotherapy resistance in multiple tumor types, including breast.[12,44-46] Therefore, ISG expression, and subsequently the effect of ISGs on tumor growth, may vary by tumor stage (early vs late). Other nuclear receptors, most notably the glucocorticoid receptor (GR), have been implicated in modulating interferon and inflammatory responses. Inhibitory interactions between GR and

AP1/NF-kB have been shown to inhibit a broad range of inflammatory functions, largely through a mechanism termed transrepression. In this mechanism, GR tethers to DNA-bound transcription factors/regulatory proteins and affects co-factor recruitment and subsequent gene regulation (reviewed in Ref. 47).[47] Of note, GR has been shown to repress ISG transcription in a cell-type specific manner in macrophages by squelching away an ISGF3 co-factor, GRIP1 (glutamate receptor interacting protein 1), needed for interferon-dependent activation of ISGs in macrophages.[48] In this instance, the repression was independent of GR binding to DNA, though ligand-activated GR effectively repressed ISRE-luciferase activity. This is in contrast to our findings (**Fig 9B**) where PR ligand treatment was not able to repress an ISRE-luciferase construct, implying that PR-dependent ISG repression occurs via a mechanism independent of co-factor squelching. Our data suggest that PR-mediated repression of ISGs in breast cancer cells occurs through a different mechanism than that proposed for GR-mediated ISG repression in macrophages, perhaps indicating cell type- and nuclear receptor-specific regulation. Recent work, however, suggests that select GR-dependent gene repression events may require direct GR binding to DNA through GR response elements (GREs). These GREs are in close proximity to AP1 and NF-kB binding sites. GRE-mediated binding of GR leads to recruitment of GRIP1, the aforementioned ISGF3 co-factor and known GR co-repressor, leading to repressive changes in the chromatin and subsequent gene repression.[49] This mechanism has similarities to the mechanism we propose herein for PR-mediated, and PRE-dependent, ISG transcriptional repression. Because we observe decreased recruitment of ISGF3 components (STAT2 and IRF9) to ISRE promoter sequences following PR activation and recruitment of PR to PREs, we favor a model where protein displacement or steric competition occurs between PR and STAT2/IRF9. We recognize that transcriptional repression is complex, and a combination of mechanisms may

contribute to PR-dependent ISG repression. Our future work is focused on detailing this mechanism(s).

Although PR gene activation has been well characterized, the mechanisms through which PR represses transcription remain poorly understood. Various mechanisms have been put forth for PR-mediated transcriptional repression, and are similar to what has been well characterized for GR-mediated repression (squelching of co-factors, recruitment of co-repressors, chromatin remodeling; reviewed in [25]). A recent study details ligand-dependent recruitment of PR, an HP1γ-LSD1 repressive complex, and BRG1 to repressed target gene promoters/enhancers. This repressive complex leads to ligand-dependent changes in chromatin architecture that result in transcriptional repression.[50] Although we observed a decrease in activating histone marks at ISG enhancers following treatment with PR ligand (**Fig 10B**), preliminary data show that knockdown of BRG1, LSD1 or HP1γ had no effect on PR-dependent transcriptional repression of ISGs (not shown), suggesting an alternative (non- HP1γ/LSD1-dependent) mechanism for PR-mediated ISG transcriptional repression. Experiments to investigate PR-dependent recruitment of other chromatin modifiers to ISG enhancers are currently underway.

In summary, our results show novel PR-dependent repression of ISG transcription. These data have significant implications for the regulation of interferon-signaling in breast cancer, and provide a putative mechanism through which nascent breast cancers may avoid immune surveillance. Future directions will be aimed at understanding what effect PR-dependent downregulation of ISGs has on breast cancer development and progression.

2.5 Supplemental Materials

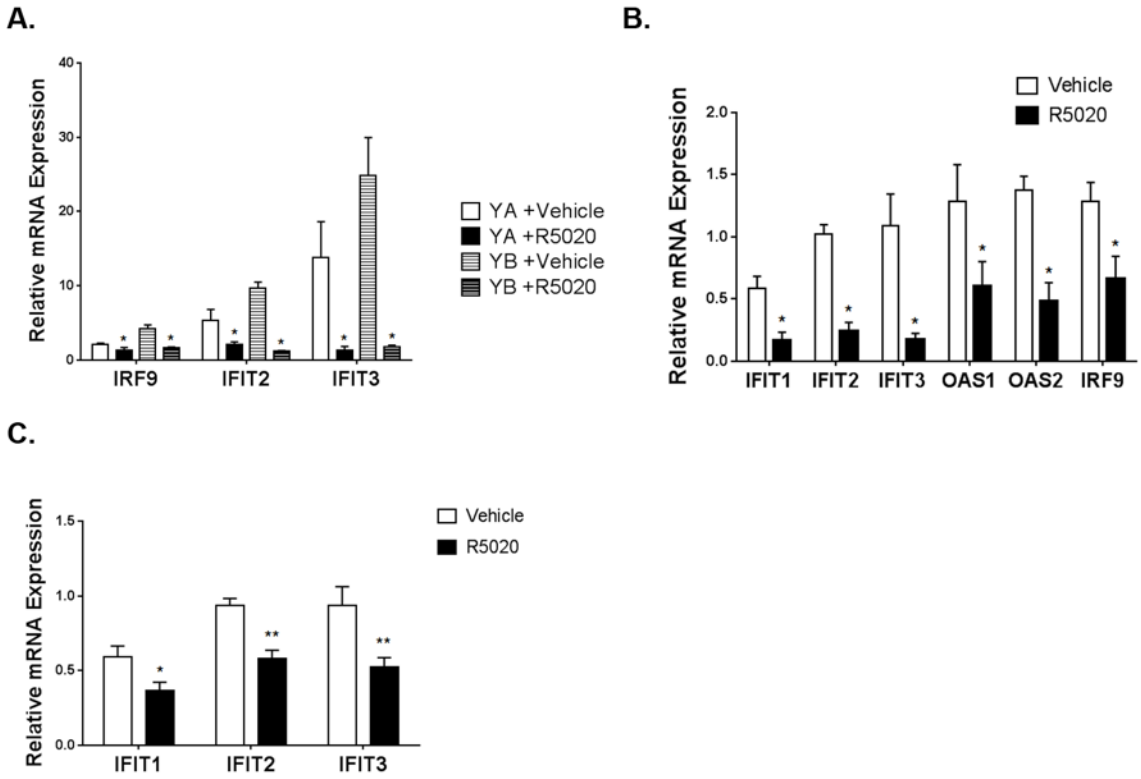


Figure 11. PR represses ISGs in multiple cell line models

(A). T47D-Y cells stably expressing PR-A (YA) or PR-B (YB) were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 (or vehicle) for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (B-actin). (B). Parental, unmodified T47D cells (T47D-ATCC) were treated with 10nM R5020 (or vehicle) for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (B-actin). (C). HeLa cells stably expressing PR-B were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 (or vehicle) for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (B-actin). Error bars represent standard deviation (SD) between triplicate biological replicates. Asterisks represent statistical significance between the vehicle and R5020-treated groups; $p < 0.05$, as determined using an unpaired Student's *t*-test. All experiments were performed in triplicate, and representative experiments are shown here.

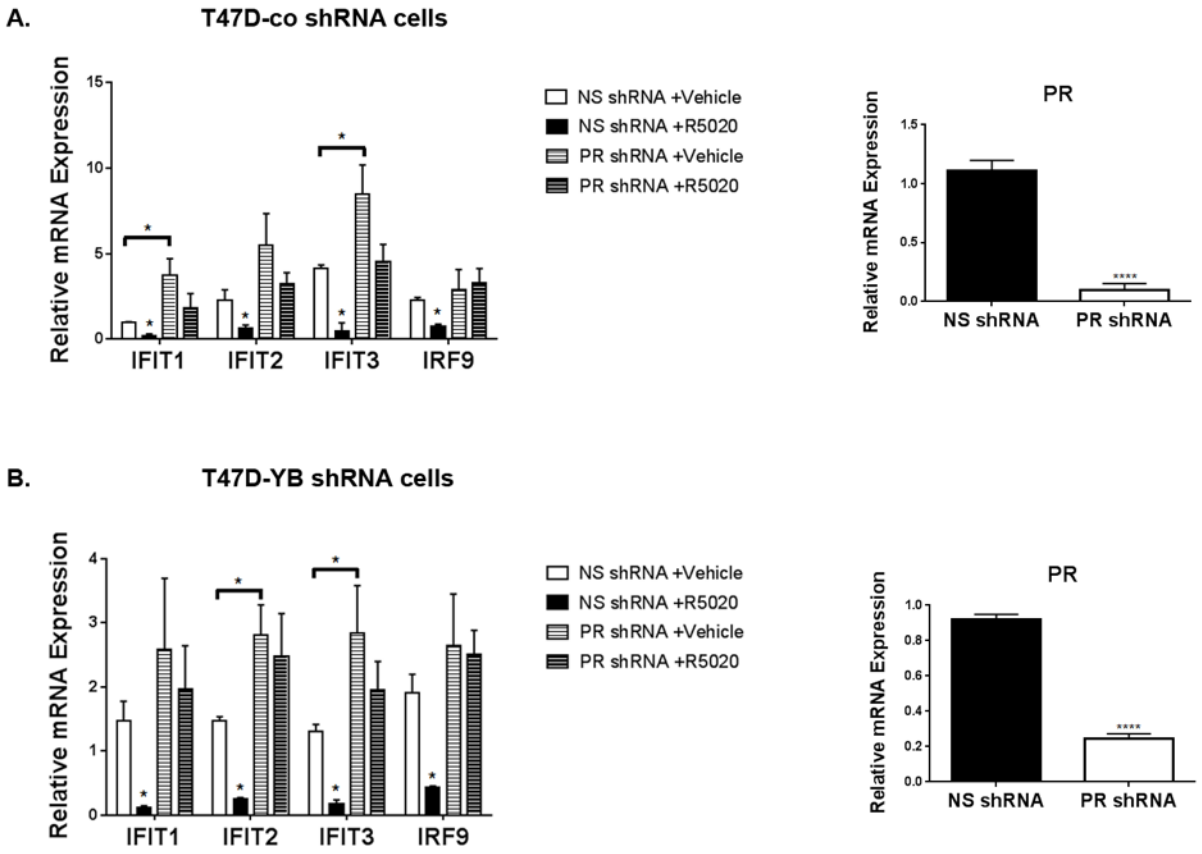


Figure 12. ISG repression is PR-dependent

(A). Left: T47D-co cells stably expressing NS or PR shRNA were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 (or vehicle) for 6hr. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (B-actin). Error bars represent SD between triplicate biological replicates. Asterisks represent statistical significance between the vehicle and R5020-treated groups, or vehicle-treated groups; $p < 0.05$, as determined using an unpaired Student's *t*-test. Right: PR knock-down efficiency by shRNA was determined using relative PR levels between the NS and PR shRNA T47D-co stable cell lines. Asterisks represent statistical significance; $p < 0.0001$. (B). Experiments were performed and analyzed as in (A), but with T47D-YB NS and PR shRNA stable cell lines. The experiments in (A) and (B) were performed in triplicate, and a representative experiment is shown here.

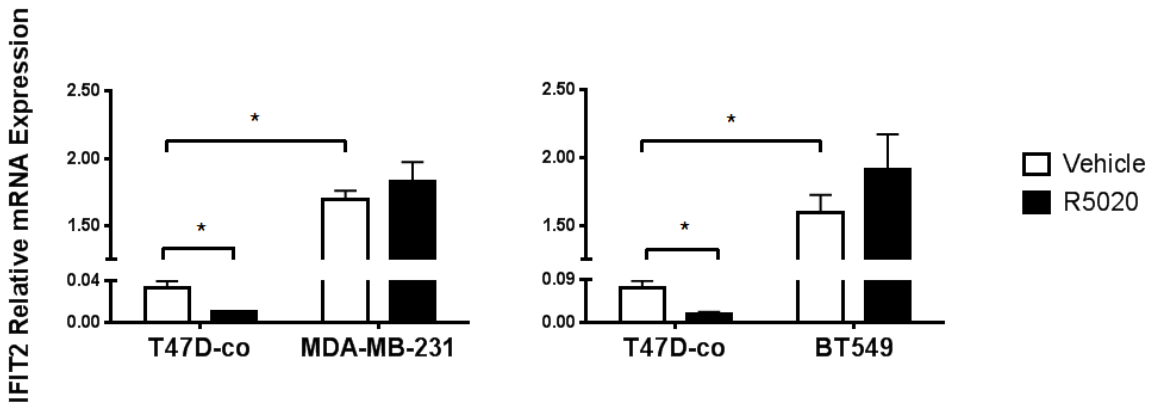


Figure 13. ISG repression not observed in PR-negative breast cancer cell lines

T47D-co and two triple negative (lacking ER/PR/HER2) breast cancer cell lines (left: MDA-MB-231 and right: BT549) were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 or vehicle for 6hr. Isolated RNA was analyzed for ISG RNA levels; IFIT2 is shown here as a representative ISG example. Gene values were normalized to an internal control (B-actin). Error bars represent SD between triplicate biological replicates. Asterisks represent statistical significance; $p < 0.05$, as determined using an unpaired Student's *t*-test. This experiment was performed in triplicate, and a representative experiment is shown here.

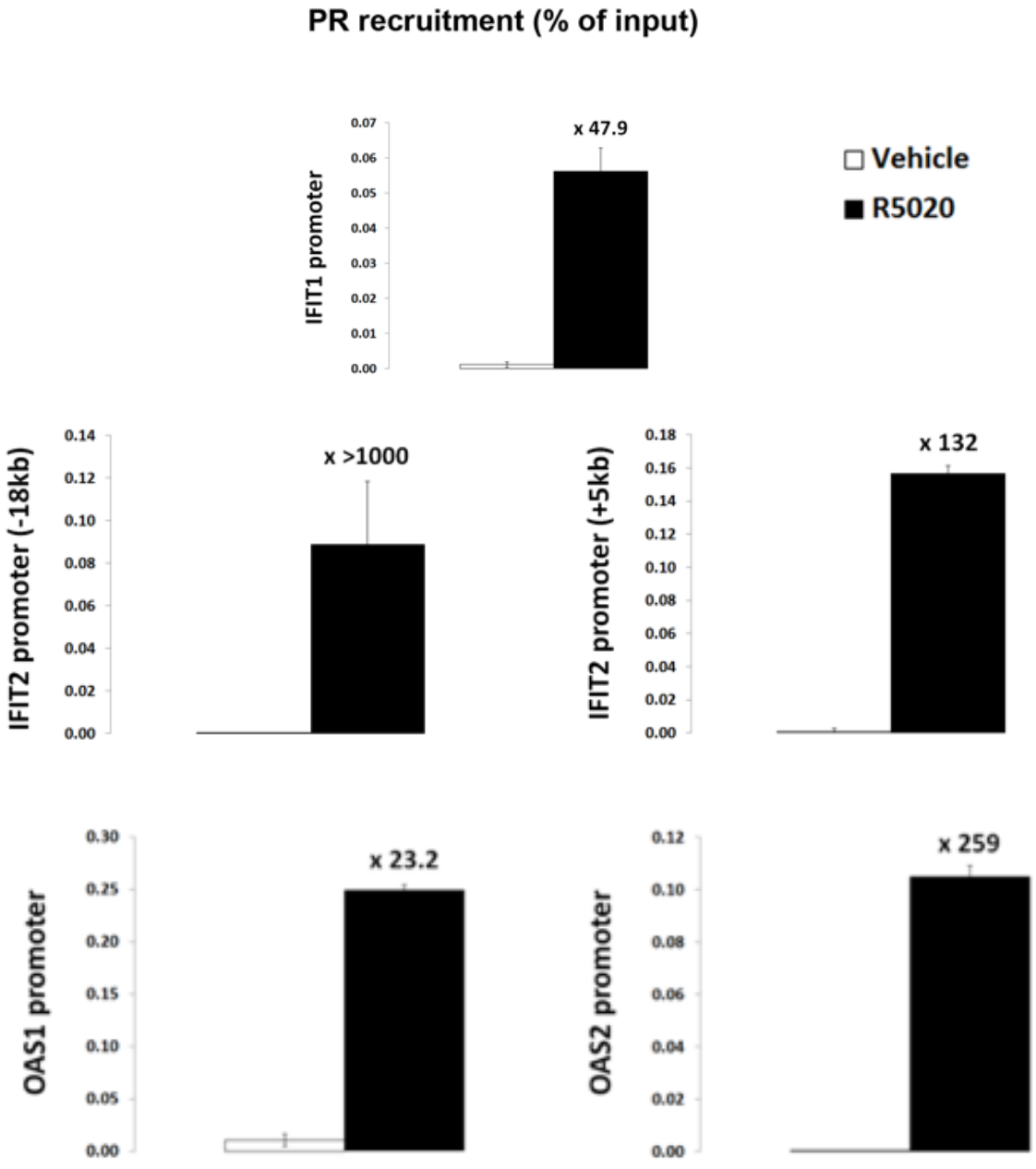


Figure 14. PR is recruited to ISG enhancers

T47D-co cells were serum-starved for 18hr. Cells were then treated with 10nM R5020 or vehicle for 30min. Fixed lysates were subjected to ChIP with antibodies against PR or a species-specific IgG (control; not shown), and qPCR was performed on the isolated DNA using primers designed to amplify ISG enhancers (as outlined in Fig 7A). A percentage of ChIP'd DNA over input DNA is shown. All ChIP experiments were performed in triplicate; a representative experiment is shown here. Fold-recruitment in treated conditions (R5020), as compared to vehicle treatment, is displayed above each bar. Error bars represent SD of technical replicates.

Table 1. Primer and si/shRNA sequences

qPCR IFIT1-F	GATCAGCCATATTTCATTTTGAATC	
qPCR IFIT1-R	GAAAATTCTCTTCAGCTTTTCTGTG	
qPCR IFIT2-F	AAGAGGAAGATTTCTGAAGAGTGC	
qPCR IFIT2-R	TCTCCAAGGAATTCTTATTGTTCTC	
qPCR IFIT3-F	GGAACAGCAGAGACACAGAGG	
qPCR IFIT3-R	ACACTGTCTTCCTTGAATAAGTTCC	
qPCR OAS1-F	GTGTGTGTCCAAGGTGGTAAAG	
qPCR OAS1-R	ATTTAACTGATCCTGAAAAGTGGTG	
qPCR B-actin F	AGAAAATCTGGCACCACACC	
qPCR B-actin R	GCTGGGGTGTTGAAGGTCTC	
qPCR OAS2-F	CAAAAGAAGCAAAGGAATGGTTATC	
qPCR OAS2-R	ATGGATCCTAGCTCCACAACTTC	
qPCR IRF9-F	GAGAGGAAGGAGGAAGAGGATG	
qPCR IRF9-R	TCCCAATGTCTGAATGGACTG	
qPCR PR-F	GTCAGTGGGCAGATGCTGTA	
qPCR PR-R	TGCCACATGGTAAGGCATAA	
CHIP IFIT3 PRE-F	CTCATGACTGCCCTCTGTGTC	
CHIP IFIT3 PRE-R	CTTCAGTATTTACTTGAGGCAGACAG	
CHIP IFIT3 ISRE-F	TGTAAGTTGAGTTTCTTACTGTGCAG	
CHIP IFIT3 ISRE-R	AACCCCTCAAACATCTTACAAATGC	
CHIP IFIT2(up) PRE-F	GATGGAAGCCTTTAGCAATCC	
CHIP IFIT2(up) PRE-R	ACATGCTTGTTAAGAACGTTTCAG	
CHIP IFIT2(down) PRE-F	AATGTATGGGAAAAGAAAGTTACTGG	
CHIP IFIT2(down) PRE-R	ACAGACACGGAAGAGATTATCATTG	
CHIP IFIT2 ISRE-F	CAGACCCATCTTTAAGTGAAGCAC	
CHIP IFIT2 ISRE-R	GCTGAGTTGTGATCAGAGAAAGAAG	
CHIP IFIT1 PRE-F	TTCTCCAATGCTTAAGTTTCATTTC	
CHIP IFIT1 PRE-R	GAGAGAGAGAGAGAGTGTGTGTGTG	
CHIP IFIT1 ISRE-F	ACAAGACAGAATAGCCAGATCTCAG	
CHIP IFIT1 ISRE-R	GAGCAAAGAAATCCTTACCTCATGG	
ChIP OAS1 PRE-F	GATTACATCGGTGAGTGTTCCTAAC	
ChIP OAS1 PRE-R	AAGAATAGCAATGACCTCAACTCTC	
CHIP OAS1 ISRE-F	GTCAGCAGAAGAGATAAAAGCAAAC	
CHIP OAS1 ISRE-R	GGTATTTCTGAGATCCATCATTGAC	
CHIP OAS2 PRE-F	GCATAATACCCACTTCAGAGTATCG	
CHIP OAS2 PRE-R	TGTGGAGAGGATAGAAGAAAATCG	
CHIP OAS2 ISRE-F	CTGGGTGCCTATTATGTGCGAG	
CHIP OAS2 ISRE-R	ATTTTACAGAGTCAGAGACTGAGGC	
PGR siRNA pool oligo #1	GGACGUGGAGGGCGCAUAU	
PGR siRNA pool oligo #2	GAGAUGAGGUCAAGCUACA	
PGR siRNA pool oligo #3	ACAUAUUGAUGACCAGAUA	
PGR siRNA pool oligo #4	GCACCUGAUCUAAUACUAA	
PGR shRNA target clone# V2LHS_239356	CAGATAACTCTCATTCAGT	
PGR shRNA target clone# V3LHS_355882	AGGTCAAGACATACAGTTG	
PGR shRNA target clone# V3LHS_355883	ACAGCGTTTCTATCAACTT	

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Chapter 3: Progesterone Receptor Attenuates STAT1-Mediated IFN Signaling in Breast Cancer.

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3.1 Introduction

Tumor escape from the surveilling immune system is a critical first step in tumor development and essential for tumor progression.[1] The cancer immunoediting hypothesis postulated by Schreiber et al.[2] highlights that the innate and adaptive immune responses work together to "flag" early neoplastic lesions for immune-mediated elimination. An early mediator of this elimination process is activation of type I IFN signaling.[3-5] Thus, suppression of IFN signaling may help developing tumors evade the critical early steps of immune recognition and clearance.

IFN signaling encompasses the mechanism through which IFNs (type I–III) are made and released by the host cell in response to a viral infection. IFNs are cytokines that bind their corresponding cell surface receptor, allowing the JAK-STAT signaling cascade to initiate. Type I IFNs, such as IFN-α/β, bind their cognate IFN-α/β receptor complex, which is associated with tyrosine kinases. The activation of both JAK1 and tyrosine kinase 2 (TYK2) results in the downstream phosphorylation of both STAT1 and STAT2. The phosphorylated STAT1–STAT2 heterodimer associates with a third transcription factor, IFN regulatory factor 9 (IRF9), leading to the formation of the IFN-stimulated gene (ISG) factor 3 (ISGF3) complex. The ISGF3 complex then translocates to the nucleus and binds DNA sequences referred to as IFN-stimulated response elements (ISREs) within the ISG promoter regions, leading to ISG transcription. Collectively, the IFN-induced production of these ISGs limits the spread of virally infected cells by promoting an antiproliferative and/or proapoptotic response in addition to the activation of immune clearance mechanisms.[6]

IFN responsiveness and signaling play a critical role during viral infections; however, emerging data suggest an important role for IFN in tumorigenesis through modulation of

immune surveillance. Independent of viral infection, both IFNs and ISG expression have been found in human tumors.[4,7,8] Although the effects of IFN signaling and ISG phenotypes vary in cancer biology, it remains clinically relevant to gain a deeper understanding of IFN signaling regulation in this context.[4,9] We have previously shown that the progesterone receptor (PR), a steroid-activated nuclear receptor implicated in breast cancer, can transcriptionally repress ISGs in breast cancer via decreased recruitment of ISGF3 components to ISREs in response to progestins.[10] In this study, we present a mechanism whereby PR, in the absence of its activating ligand, can attenuate IFN signaling through decreased STAT1 activation/phosphorylation. The potent loss in IFN signaling sensitivity may contribute to the escape of malignant cells from the surveilling immune system.

3.2 Materials and Methods

3.2.1 Cell lines and constructs

T47D-co, T47D-Y, and T47D-YB have been previously described and were a generous gift of Dr. C. Lange (Minneapolis, Minnesota).[11,12] MCF7[13] and T47D-co short hairpin RNA (shRNA) cells have been previously described.[10] Cells were treated with the following reagents (when applicable): R5020 (10 nM; Sigma-Aldrich) and human rIFN-α (IFN-α2A, SPR4594; Sigma-Aldrich).

3.2.2 Rapid immunoprecipitation mass spectrometry of endogenous proteins

Rapid immunoprecipitation (IP) mass spectrometry (MS) of endogenous proteins (RIME) experiments were performed as previously described[14-16] in R5020-treated (10 nM for 60 min) T47D-YB or T47D-Y cell lines that were cross-linked with 1% formaldehyde for 10 min. IPs were performed using a PR Ab (20 μg, sc-7208; Santa Cruz Biotechnology). The peptide samples were analyzed on a Dionex Ultimate 3000 UHPLC system coupled with the LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). For the separation of the peptides, a multistep gradient elution was used: mobile phase (A) was composed of 2% acetonitrile, 0.1% formic acid, and 5% DMSO, and mobile phase (B) was composed of 80% acetonitrile, 0.1% formic acid, and 5% DMSO. The gradient elution method at flow rate 300 nl/min was as follows: for 65 min, gradient up to 45% (B); for 10 min, gradient up to 95% (B); for 10 min, isocratic 95% (B); for 5 min, down to 5% (B); and for 10 min, isocratic equilibration 5% (B) at 40°C. The full scan was performed in the Orbitrap in the range of 400–1600 m/z at 60K resolution. The tandem MS scan was performed with collision-induced dissociation energy 30% and exclusion duration 30 s. The raw data were processed in Proteome Discoverer 1.4 using the Sequest HT search engine. The node for Sequest HT included the following parameters: precursor mass

tolerance, 20 ppm; fragment mass tolerance, 0.5 Da; and dynamic modifications, which were oxidation of M (+15.995 Da) and deamidation of N and Q (+0.984 Da). Significant peptides were filtered at a false discovery rate (FDR) <1%, and specific interactors were considered if they were identified in both RIME replicate experiments in T47D-YB cells but not in the T47D-Y (PR-null) cell line.

3.2.3 Co-IP

For co-IP experiments, cell lysates were collected in RIPA buffer (supplemented with protease/phosphatase inhibitors) and incubated on ice for 30 min. Cell lysates containing equivalent protein concentrations (1000 μg) were incubated overnight at 4°C with 2 μg of appropriate Ab or control IgG. Protein G agarose (Roche Diagnostics, Indianapolis, IN) was added for the final 2 h of incubation time. Immune complexes were washed three times with supplemented RIPA buffer, resuspended in Laemmli sample buffer containing 2-ME, boiled for 5 min, and subjected to Western blotting analysis.

3.2.4 Immunoblotting

Immunoblotting/Western blotting was performed as previously described.[10-12] Membranes were probed with primary Abs recognizing total PR (sc-7208; Santa Cruz Biotechnology or MS-298-P; Thermo Fisher Scientific), STAT1 (9172; Cell Signaling), p-STAT1 (7649; Cell Signaling), p-TYK2 (68790; Cell Signaling), TYK2 (14193; Cell Signaling), STAT2 (4594; Cell Signaling), IRF9 (10793; Santa Cruz), IFIT1 (14769; Cell Signaling), IFIT2 (sc-390724; Santa Cruz Biotechnology), IFIT3 (sc-393512; Santa Cruz Biotechnology), OAS1 (14498; Cell Signaling), topoisomerase II-α (12286; Cell Signaling), and β-tubulin (2128; Cell Signaling). All Western blotting experiments were performed in triplicate, and representative experiments are shown.

3.2.5 Breast cancer tissue microarray

Tissue microarrays (TMAs) were constructed from archival formalin-fixed, paraffin-embedded samples of invasive mammary carcinoma (43 patients) as well as matched benign breast tissue (35 patients). These samples were identified from the pathology departmental archives of the University of Kansas Medical Center from 1997 to 2011. Based on review of the original pathology reports, the invasive mammary carcinomas were typed as invasive ductal carcinoma (39 patients), metaplastic carcinoma (1 patient), invasive mammary carcinoma with ductal and lobular features (1 patient), invasive ductal carcinoma with a minor lobular component (1 patient), and invasive lobular carcinoma (1 patient). Using the semiautomated TMArrayer (Pathology Devices, Westminster, MD), TMA paraffin blocks were assembled with 2.0-mm cores.

3.2.6 Immunohistochemistry

Phospho-STAT1 (no. 8826; Cell Signaling), STAT1 (no. 14994; Cell Signaling), and PR (no. M3568; Dako) Abs were used for immunohistochemical (IHC) staining according to the following procedure: 4-μm paraffin sections were mounted on Fisherbrand Superfrost slides and baked for 60 min at 60°C then deparaffinized. Epitope retrieval was performed in a Biocare Decloaking Chamber (pressure cooker) under pressure for 5 min, using pH 6 citrate buffer followed by a 10-min cooldown period. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min, followed by incubation with phospho-STAT1 (1:800), STAT1 (1:3200), or PR (1:2000) primary Ab for 30 min (PR) or 45 min (p-STAT1, STAT1), followed by Mach 2 HRP Polymer (Biocare Medical) for 30 min (p-STAT1, STAT1) or Envision+ Anti-Mouse (Dako) for 30 min (PR) and DAB+ chromogen (Dako) for 5 min. IHC staining was performed using the IntelliPATH FLX Automated Stainer at room temperature. A light hematoxylin counterstain was

performed, following which, the slides were dehydrated, cleared, and mounted using permanent mounting media. A pathologist then scored the slides according to the intensity of staining (0 = no staining, 1 = mild intensity, 2 = moderate intensity, 3 = strong intensity), percentage of positive cells, and the subcellular localization of the staining. A second pathologist blindly scored the slides to identify any discordant results. For statistical analysis, the Wilcoxon rank-sum test was used to assess the differences in the phospho-STAT1 and STAT1 intensities between two groups of subjects (PR-positive versus PR-negative tumors).

3.2.7 Subcellular fractionation

Subcellular fractionation studies were performed as described previously,[17] with modifications noted in the figure legend.

3.2.8 RNA sequencing

Approximately 500 ng total RNA was used for stranded total RNA sequencing (RNA-Seq) library preparation by following the KAPA Stranded RNA-Seq Kit with RiboErase (HMR) sample preparation guide. The first step in the workflow involved the depletion of rRNA by hybridization of cDNA oligonucleotides, followed by treatment with RNase H and DNase to remove rRNA duplexed to DNA and original DNA oligonucleotides, respectively. Following rRNA removal, the RNA was fragmented into small pieces using divalent cautions under elevated temperature and magnesium. The cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers. This was followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. Strand specificity was achieved by replacing dTTP with dUTP in the second strand marking mix. The incorporation of dUTP in second-strand synthesis effectively quenches the second strand during amplification because the polymerase used in the assay will not incorporate past this nucleotide. These cDNA fragments

then went through an end repair process, the addition of a single "A" base, and then ligation of the adapters. The products were then purified and enriched with PCR to create the final RNA-Seq library. RNA-Seq libraries were subjected to quantification process, pooled for cBot amplification, and subsequently sequenced with 50-bp single-end sequencing run with an Illumina HiSeq 3000 platform. After the sequencing run, demultiplexing with Bcl2fastq2 was employed to generate the FASTQ file for each sample.

RNA-Seq data processing was performed as previously described.[18] Briefly, RNA-Seq FASTQ files were aligned to hg19 using TopHat (version 2.0.14), and only uniquely mapped reads were used for further downstream analysis.[19] Expression levels of genes were measured with normalized counts of reads by their respective lengths using the Cufflinks 2.0.2 package, followed by their distribution analysis as fragments per kilobase million unit.[20] These data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus[21] and are accessible through Gene Expression Omnibus series accession number GSE126517 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126517).

3.2.9 Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed using the javaGSEA desktop software; the c2 Molecular Signatures Database (MSigDB) version 5.2 was queried.[22,23] Dataset files were developed based on normalized Illumina expression intensities from PR-null and PR-positive T47D cells (as described in the *RNA sequencing* section). Specifically, the log2-transformed expression values were compared for two phenotypes: T47D PR null (IFN-α/vehicle) and T47D PR positive (IFN-α/vehicle). GSEA was executed using the default settings, except the permutation type was set to Gene_set with 1000 permutations, and the metric for ranking genes was set to Diff_of_Classes, because normalized expression data were log2 transformed. Leading

edge (LE) analysis was performed on the 29 gene sets from MSigDB c2 analysis that achieved FDR values ≤0.05.

3.2.10 Real-time quantitative PCR

RNA isolation, cDNA creation, and quantitative PCR (qPCR) were performed as previously described,[10-12] with modifications noted in this article and in the figure legends. qPCR was performed using the Faststart Essential DNA Green Master (Roche) on a Roche LightCycler 96. Relative concentrations were quantified using the LightCycler 96 (software 1.1, Absolute Quantification Analysis; Roche) using a six-point standard curve.

3.2.11 Chromatin IP assays

Chromatin IP (ChIP) was performed using the ChIP-IT Express Kit (Active Motif) according to manufacturer's instructions, using sonication for chromatin shearing. Lysates were IP overnight (18 h) with the following Abs: STAT1 (sc-346; Santa Cruz), STAT2 (sc-476; Santa Cruz), IRF9 (sc-10793; Santa Cruz), or an equal amount of negative control mouse or rabbit IgG. Resulting DNA was analyzed using qPCR as described above, and data are represented as a percentage of input DNA.

3.2.12 Statistical analysis

Statistical significance for all experiments was determined using an unpaired Student *t* test, unless otherwise specified. A *p* value ≤0.05 is considered statistically significant. The delta method was used to calculate SD for the ratio of two variables using their individual SD, as seen when plotting fold-relative RNA expression data between two treatment groups and cell lines.[24]

3.3 Results

3.3.1 PR interacts with STAT1

Our laboratory has a long-standing interest in defining how PR affects the activity of other transcription factors, particularly through protein–protein interactions. To identify novel protein binding partners of PR, we used an IP/MS approach called RIME. This technique, pioneered by the Carroll laboratory to identify estrogen receptor (ER)–interacting proteins, has been a powerful tool to study cross-talk between steroid receptors and other nuclear proteins.[14,15] PR is an important target gene of ER, and, as such, PR expression is regulated by estrogen in most tissues.[25,26] To differentiate between the effects of ER/estrogen and PR/progesterone, our laboratory uses PR-positive (T47D-co) and PR-null (T47D-Y) variants of the ER/PR$^+$ breast cancer cell line T47D.[27] T47D-co cells endogenously express both isoforms of PR, PR-A, and PR-B, without the need for exogenously added estrogen, allowing us to study the function of PR without the confounding effects of estrogen. T47D-Y (PR-null) cells can be used to determine the effect of PR isoform variants or mutants, such as PR-A (T47D-YA cells) and PR-B (T47D-YB cells). We have published extensively using these cell line models to define isoform- and phosphorylation-specific PR gene regulation and protein–protein interactions,[10-12,28-30] and this cell line model remains a powerful and well-established system for studying the transcriptional activity of PR.[31]

We used T47D-YB (stably expressing the full-length PR-B isoform) and PR-null (T47D-Y) cells as a model system to study PR–protein interactions using RIME. Briefly, T47D-YB and T47D-Y cells were cross-linked following treatment with a synthetic PR ligand (R5020) or vehicle (ethyl alcohol [EtOH]). PR was IP from isolated nuclei, and PR-interacting proteins were identified using MS. Among the top 30 identified PR-interacting proteins was STAT1 (**Fig.**

15A); peptide sequence coverage for PR and STAT1 is shown in **Fig. 15B**. The interaction between PR and STAT1 was validated using co-IP in T47D-YB cells (cell line used for the RIME experiments): an interaction between PR and STAT1 could be detected in vehicle-treated cells, and this interaction was slightly increased in cells treated with R5020 (**Fig. 15C**, left). The interaction between PR and STAT1 was also observed in unmodified T47D-co cells, endogenously expressing both isoforms of PR (**Fig. 15C**, right); both PR-A (lower band) and PR-B (upper band) appear to interact with STAT1, suggesting that the interaction between PR and STAT1 is not unique to the full-length PR-B isoform. Interestingly, the interaction between STAT1 and PR-B is increased following treatment with PR ligand, as compared with the STAT1/PR-A interaction. These data suggest that the STAT1/PR-B interaction is primarily regulated by ligand, whereas the STAT1 interaction with PR-A may be a more basal interaction, unaffected by ligand. Moreover, an interaction between PR and STAT1 was also shown in MCF7 cells, an additional PR-positive breast cancer cell line (Supplement, **Fig 22A**). Cumulatively, these data show that PR and STAT1 interact, and this interaction is potentiated when PR is activated by ligand.

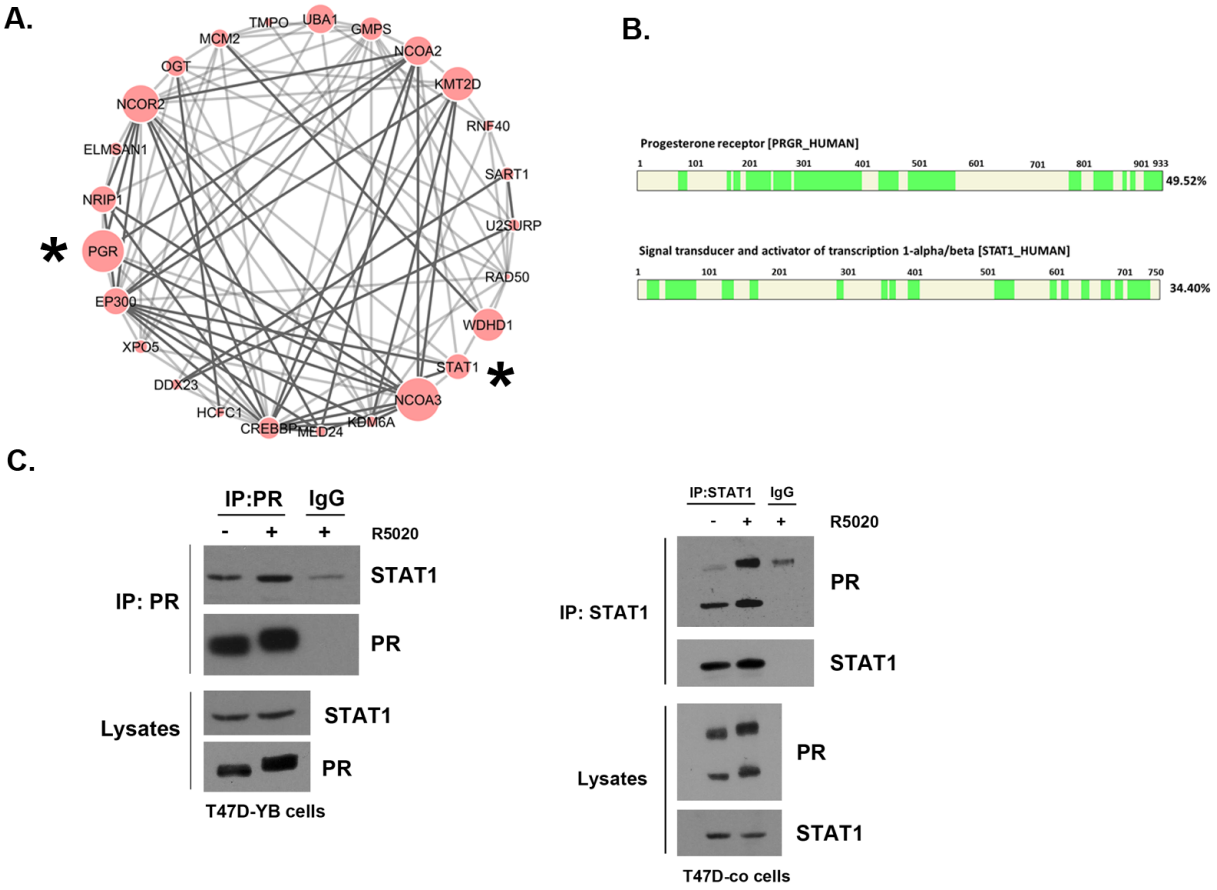


Figure 15. PR interacts with STAT1

(A) Search Tool for Retrieval of Interacting Genes/Proteins (STRING) network of the top 30 PR interactors identified in RIME. The size of the node increases proportionally to the number of identified peptides, and thick edges denote high-confidence STRING interactions (0.7–0.99). Asterisk used to denote location of PGR and STAT1 on STRING diagram. (B) Sequence coverage of PR and STAT1 in both replicate RIME experiments. Green highlights high-confidence peptides at FDR <1%. PR and STAT1 have been identified by 27 and 19 unique peptides, respectively. (C) Left: PR was IP from starved T47D-YB cell lysates (with or without R5020; 60 min), and the resulting associated protein complexes were analyzed by Western blotting. Bottom panels represent total input cell lysates. Species-specific (rabbit or mouse) IgG was used as a control for the IP. Right: STAT1 was IP from T47D-co cell lysates (with or without R5020), and the resulting associated protein complexes were analyzed by Western blotting. Bottom panels represent total input cell lysates. PR-B (upper) and PR-A (lower) isoforms are both recognized by the PR Ab. Species-specific IgG was used as a control for the IP; the band visible in the IgG only lanes represents nonspecific binding between PR/STAT1 and the IgG Ab. These experiments were performed in triplicate, and a representative experiment is shown.

3.3.2 PR attenuates IFN-induced STAT1 phosphorylation

STAT1 is a key mediator of the innate immune response, typically in response to viral infection. However, IFN activation in cancer cells, independent of viral infection, has been widely reported.[4] In response to IFN-α stimulation, STAT1 and STAT2 are phosphorylated by JAK1 and TYK2. Phosphorylated STAT1/2 form heterodimers that bind with IRF9 to form a transcriptional complex referred to as ISGF3. This transcriptional complex binds to ISREs throughout the genome, activating transcription of a cohort of genes essential for mounting the cellular antiviral response.[5] To determine if the interaction between PR and STAT1 affects the function of STAT1, we looked at STAT1 phosphorylation in response to IFN-α treatment. T47D cells that are positive (T47D-co) or negative (T47D-Y) for PR expression were treated for 0–2 h with IFN-α. Cells lacking PR expression (PR null) phosphorylated STAT1 on an earlier time course and at a greater magnitude in response to IFN-α, as compared with PR-positive cells (**Fig. 16A**, left and quantification of **Fig. 16A**, right). There are two isoforms of STAT1, STAT1α (larger) and STAT1β (smaller); both isoforms appear to be equally affected by the presence/absence of PR. Moreover, treatment of PR-positive cells (T47D-co) with PR ligand (R5020) even further attenuated phospho-STAT1 in response to IFN-α treatment (**Fig. 16B**, left and quantification of **Fig. 16B**, right). Decreased STAT1 phosphorylation in the presence of activated PR was repeated in MCF7 cells (Supplement **Fig 22B** and **22C**). These data suggest that both the presence (PR negative versus PR positive) and activation of PR (with or without ligand) decreases the levels of phospho-STAT1 in response to IFN treatment, likely because of the interaction between PR and STAT1 (see **Fig. 15C**). STAT2 phosphorylation, another downstream effector of IFN-α signaling, was unaffected by the presence or activation of PR following IFN-α treatment (data not shown). Finally, to determine if the decrease in IFN-induced

phosho-STAT1 following treatment with PR ligand was restricted to a particular cellular compartment, we used subcellular fractionation experiments to assay phospho-STAT1. We observed similar decreases in phospho-STAT1 following treatment with IFN and PR ligand in the nuclear and cytoplasmic fractions, as well as the whole cell lysate, following treatment with PR ligand (**Fig 16C**).

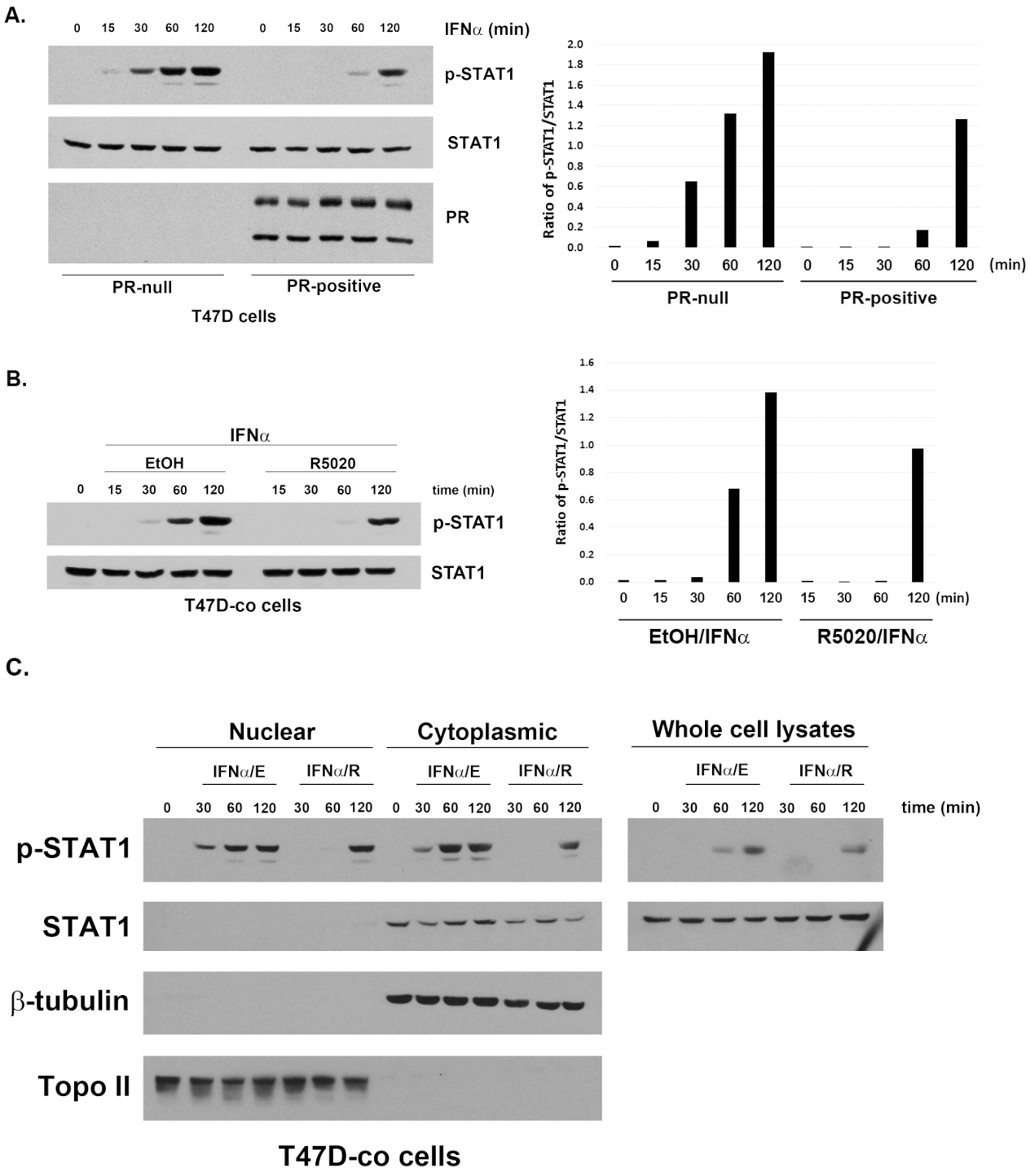


Figure 16. PR attenuates IFN-induced STAT1 phosphorylation

(A) T47D cells that are PR negative (T47D-Y) or PR positive (T47D-co) were treated with IFN-α for 0–2 h. Isolated protein lysates were analyzed by Western blotting. Densitometry of the ratio of p-STAT1/total STAT1, as determined using ImageJ analysis, is shown to the right of the

immunoblot. (B) T47D-co cells were starved for 18 h in serum-free media, followed by treatment with IFN-α and R5020 or vehicle (EtOH) for 0–2 h. Isolated protein lysates were analyzed by Western blotting. Densitometry of the ratio of p-STAT1/total STAT1, as determined using ImageJ analysis, is shown to the right of the immunoblot. (C) T47D-co cells were treated as in (B). Whole cell lysates were subjected to nuclear/cytoplasmic fractionation, and resulting subcellular lysates were analyzed by Western blotting. β-tubulin (cytoplasmic) and topoisomerase II (topo II; nuclear) are shown as fractionation markers. These experiments were performed in triplicate, and a representative experiment is shown.

3.3.3 Regulation of p-STAT1/STAT1 is disrupted in PR-positive human breast tumors

To determine if there is a correlation between PR positivity and phospho-STAT1 levels in human breast tumors, we analyzed a previously described, custom-designed breast tumor TMA.[13] In brief, this breast cancer TMA is composed of specimens collected from 39 breast cancer patients seen at the University of Kansas Medical Center, 21 of which were PR positive and 18 of which were PR negative. We stained this breast TMA with Abs that recognize phospho-STAT1 and total STAT1, and staining intensities were blindly scored by a clinical pathologist. Interestingly, PR-positive tumors had lower phospho-STAT1 staining intensity when compared with their PR-negative counterparts. Although statistical analysis (Wilcoxon rank-sum test) did not show statistically significant differences in the intensities (likely because of the limited numbers of samples on the TMA), there is a clear trend toward lower staining intensity in PR-positive tumors (**Fig. 17A**, left). The trend between lower phospho-STAT1 staining in PR-positive tumors appears to be specific for phosphorylated STAT1, as no trend or statistical significance exists for total STAT1 staining intensity between PR-positive and PR-negative tumors (**Fig. 17A**, right). Select examples from the TMA are shown in **Fig. 17B**. As STAT1 itself is an ISG regulated by IFN signaling[32], higher activation of STAT1 (measured via phosphorylation) is normally positively correlated with total STAT1 levels, representing a positive-feedback regulatory loop. A scatter plot (**Fig. 17C**) of p-STAT1 and STAT1 staining intensities for each tumor highlights the positive correlation between p-STAT1 and total STAT1 in PR-negative tumors (blue line; $r = 0.7188$, $p = 0.0291$); this correlation is not present in PR-positive tumors (red line; $r = 0.0291$, $p = 0.9364$). These data suggest that PR disrupts the activation of STAT1, as measured via p-STAT1 levels (**Fig. 17A**), and the correlation between p-STAT1 and total

STAT1 (**Fig. 17C**). Cumulatively, these data suggest that PR positivity affects phospho-STAT1 levels in human breast tumors in addition to human breast cancer cell lines.

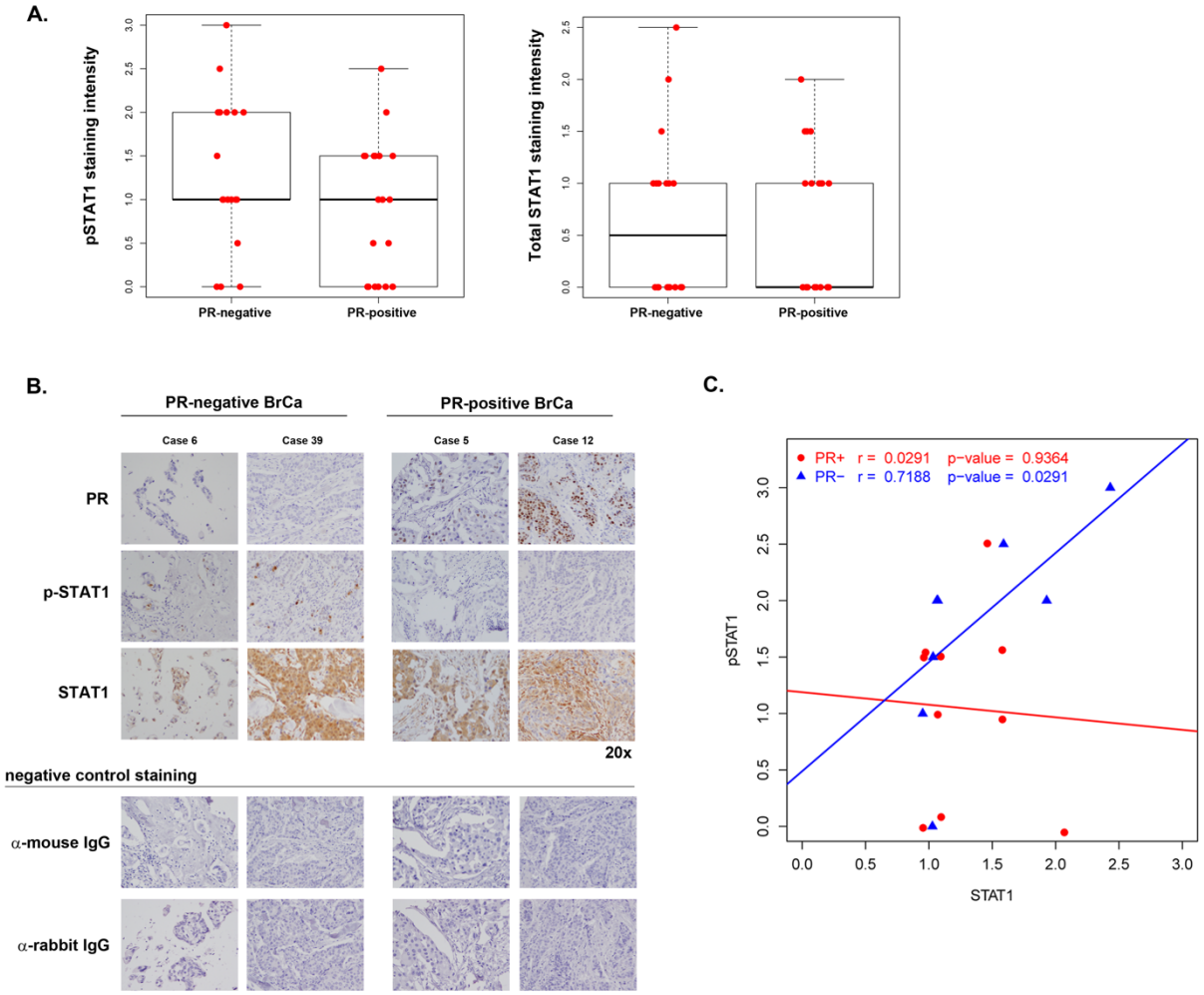


Figure 17. Regulation of p-STAT1/STAT1 is disrupted in PR-positive human breast tumors

(A) TMA analysis was performed using IHC staining with phospho-STAT1 (p-STAT1), STAT1, and PR Abs. Boxplots and stripcharts show the distribution of p-STAT1 (left) and STAT1 (right) staining intensities in PR-negative and PR-positive breast cancer samples. (B) Select PR-negative (left) and PR-positive (right) breast cancer (BrCa) cases stained for PR, p-STAT1, and total STAT1 are shown at original magnification ×20. Negative control isotype-only control staining is shown. (C) Scatter plot of pSTAT1 and STAT1 with different colors for PR positive (red) and PR negative (blue), with trend lines for each. The Spearman correlation coefficient and p values for each group are shown on top. Only tumors with >0 total STAT1 staining were included in the analysis.

3.3.4 IFN-induced TYK2 activity is attenuated by activated PR

Interestingly, phospho-STAT1 following treatment with IFN-γ (IFNγ−type II IFN signaling ligand) was unaffected by PR activation (data not shown), suggesting that the effect of PR on STAT1 phosphorylation is exclusive to pathways/proteins involved in type I IFN signaling. TYK2 is the primary kinase responsible for phosphorylating STAT1 in response to IFN-α (reviewed in Ref. 6)[6], whereas JAK1 and JAK2 are responsible for STAT1 phosphorylation following IFN-γ. Because TYK2 is specific to type I IFN signaling, we sought to determine how TYK2 activation was impacted by PR. We measured phospho-TYK2 (indicative of TYK2 activation) following treatment with IFN-α in the presence/absence of PR activation. In T47D-co cells treated with PR ligand, IFN-α–induced TYK2 phosphorylation was attenuated (**Fig. 18A**, left and quantification of **Fig. 18A**, right). Importantly, JAK1 and JAK2 phosphorylation was unaffected by PR activation (data not shown), thus reinforcing the specificity of this effect to type I IFN signaling. As such, like PR and STAT1, we identified an interaction between PR and TYK2 that was increased in response to PR ligand (**Fig. 18B**). Again, similar to PR-STAT1, the PR-TYK2 interaction in response to PR ligand appears to be primarily driven by PR-B (larger isoform, upper band). Together, these data suggest that an interaction between PR and TYK2 leads to decreased TYK2 activation, which subsequently translates to decreased STAT1 phosphorylation when PR is present/activated.

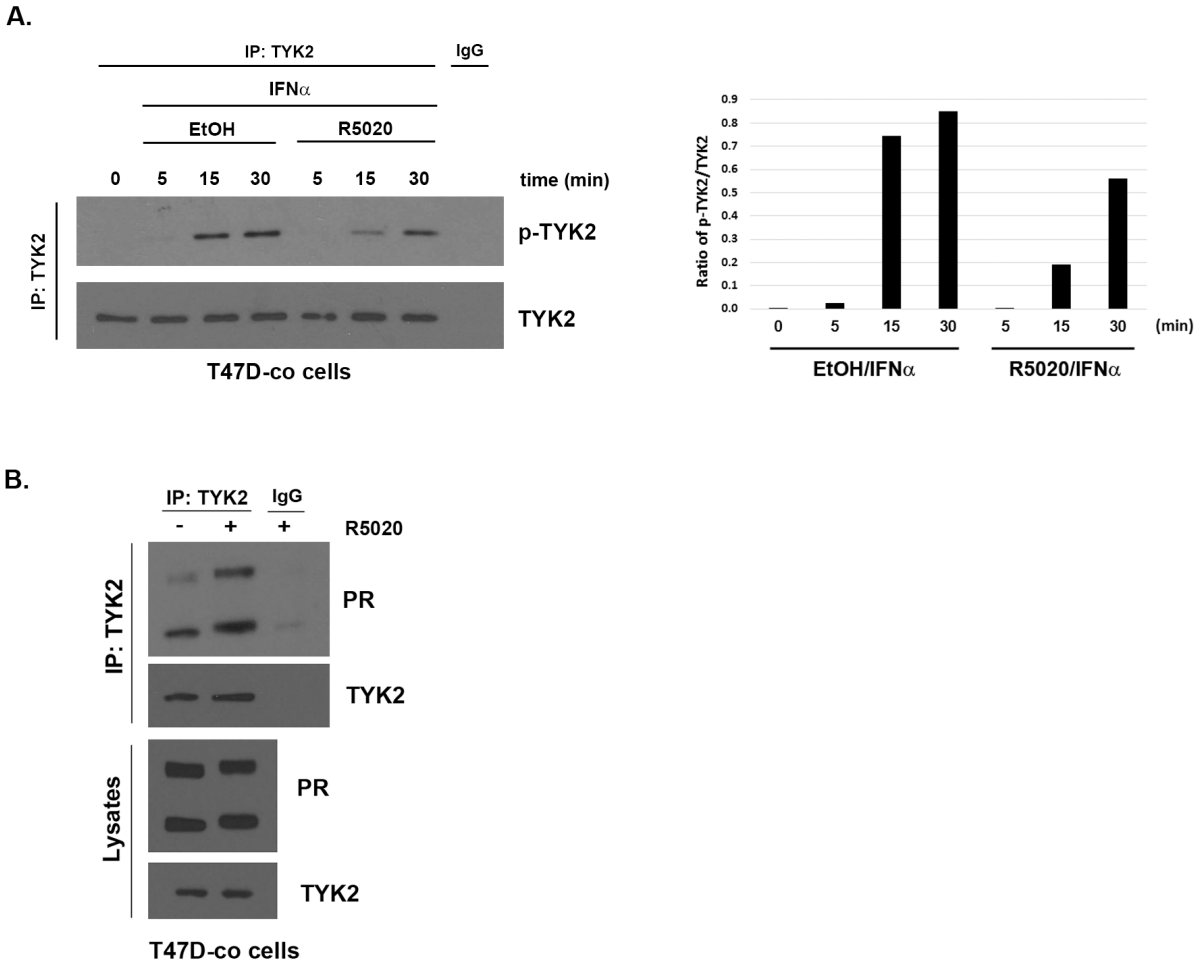


Figure 18. TYK2 phosphorylation is attenuated by activated PR

(A) T47D-co cells were starved for 18 h in serum-free media, followed by treatment with IFN-α and R5020 or vehicle (EtOH) for 0–30 min. Isolated protein lysates were IP with the total TYK2 Ab, and the resulting protein complexes were blotted with phospho-TYK2 Ab. Species-specific IgG was used as a control for the IP. Densitometry of the ratio of p-TYK2/total TYK2, as determined using ImageJ analysis, is shown to the right of the immunoblot. (B) TYK2 was IP from T47D-co cell lysates (with or without R5020; 60 min), and the resulting associated protein complexes were analyzed by Western blotting. Bottom panels represent total input cell lysates. PR-B (upper) and PR-A (lower) isoforms are both recognized by the PR Ab. Species-specific IgG was used as a control for the IP. These experiments were performed in triplicate, and a representative experiment is shown.

3.3.5 PR activation disrupts the ISGF3 complex

Following STAT1 phosphorylation, a transcriptional complex is formed containing STAT1, STAT2, and IRF9. Formation of this complex is key to transcriptional activation of genes turned on in response to IFN treatment, such as ISGs. To determine if PR, because of the interaction between PR and STAT1, affects the formation or integrity of the ISGF3 complex, we used co-IP assays to interrogate the interactions within the ISGF3 complex. In T47D-co cells treated with IFN-α, the interaction between STAT2 and STAT1 decreased with the addition of PR ligand (R5020; **Fig. 19**). A decreased interaction was also observed between IRF9 and STAT2 in the presence of R5020 (**Fig. 19**). These data suggest that PR activation decreases the integrity of the ISGF3 complex, either through promoting disassembly of the complex or preventing efficient assembly. Cumulatively, these data suggest that the interaction between PR and STAT1 disrupts the functionality of STAT1 through decreased phosphorylation (**Figs. 16, 17**) and disruption of STAT1-containing protein complexes (**Fig. 19**).

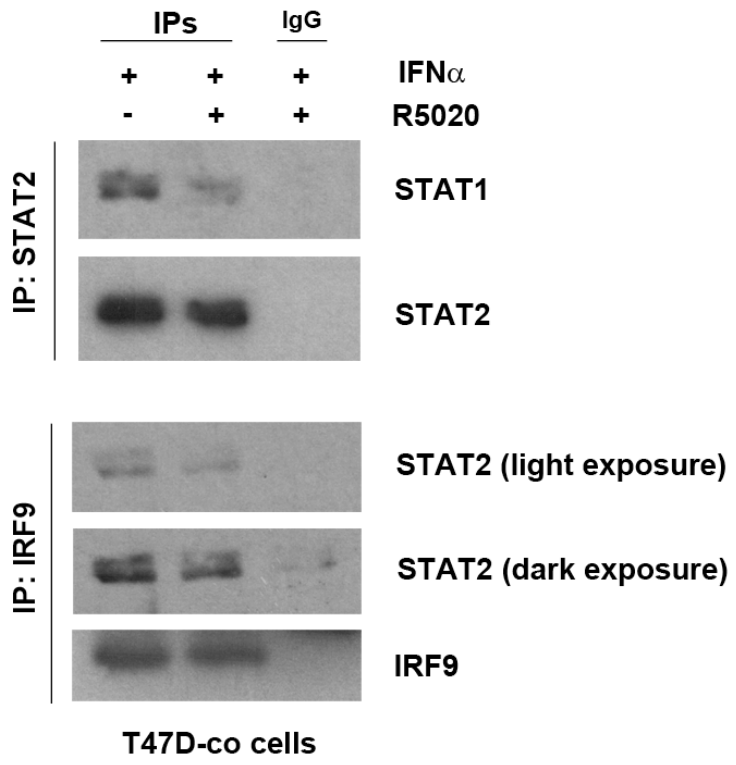


Figure 19. PR activation disrupts the ISGF3 complex

STAT2 and IRF9 (or a species-specific IgG control) was IP from IFN-α–treated (2 h), starved T47D-co cell lysates (with or without R5020; 60 min), and the resulting associated protein complexes were analyzed by Western blotting. This experiment was performed in triplicate, and a representative experiment is shown.

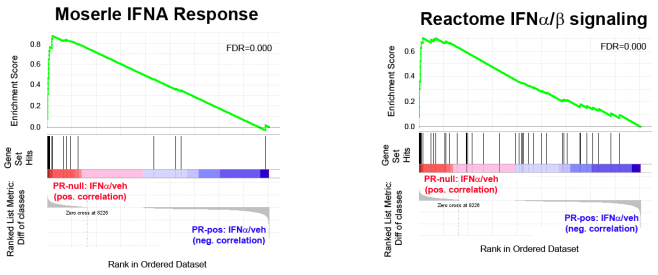
3.3.6 PR decreases ISG transcriptional response and protein levels

The end point of type I IFN signaling is transcriptional activation of ISGs, genes that canonically orchestrate the cellular response to viral pathogens. As PR presence attenuated STAT1 phosphorylation in response to IFN treatment and promoted the dissociation of the ISGF3 complex, we performed RNA-Seq on IFN-treated PR-positive and PR-negative breast cancer cells to determine how the presence of unliganded PR affects global IFN-activated transcriptional programs. T47D cells that are positive (T47D-co) or negative (T47D-Y) for PR expression were treated with IFN-α (or vehicle) for 18 h. GSEA revealed that multiple IFN-associated gene sets were enriched in the PR-null RNA-Seq dataset; this enrichment was lost in cells expressing PR.[22,23] The top significantly regulated gene sets in PR-null cells (as compared with PR-positive cells) are shown in **Fig. 20A**; select examples of enrichment are shown in **Fig. 20B**. LE analysis, a component of GSEA that allows the identification of core genes that drive the enrichment of a particular gene set, identified multiple genes that are transcriptional targets (ISGs) of IFN signaling pathways (i.e., IFITs, MX1, OASs) whose regulation is enriched in PR-null cells but lost in PR-positive cells (**Fig. 20C**). Using qPCR, we validated a core set of ISGs and confirmed on the individual gene level that IFN activation of IGSs is transcriptionally attenuated (ranging from 50 to 85% reduction) in T47D cells expressing PR, as compared with PR-null cells (**Fig. 20D**). Protein expression for select ISGs mimics the same phenotype; IFN activation of ISG protein levels is higher in PR-negative cells as compared with PR-positive cells (**Fig. 20E**). Cumulatively, these data suggest that unliganded PR attenuates the transcriptional response to IFN-α through the downregulation of ISG RNA and protein.

A.

Gene Set Name	Number of Genes in List	FDR
MOSERLE_IFNA_RESPONSE	27	0.000
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	54	0.000
BROWNE_INTERFERON_RESPONSIVE_GENES	51	0.000
ZHANG_INTERFERON_RESPONSE	17	0.000
EINAV_INTERFERON_SIGNATURE_IN_CANCER	21	0.000
RADAEVA_RESPONSE_TO_IFNA1_UP	44	0.000
HECKER_IFNB1_TARGETS	83	0.001
SANA_RESPONSE_TO_IFNG_UP	62	0.001
BENNETT_SYSTEMIC_LUPUS_ERYTHEMATOSUS	24	0.001
DER_IFN_ALPHA_RESPONSE_UP	54	0.001
KRASNOSELSKAYA_ILF3_TARGETS_UP	32	0.001
BOSCO_INTERFERON_INDUCED_ANTIVIRAL_MODULE	60	0.002
DAUER_STAT3_TARGETS_DN	43	0.004
DER_IFN_BETA_RESPONSE_UP	77	0.016

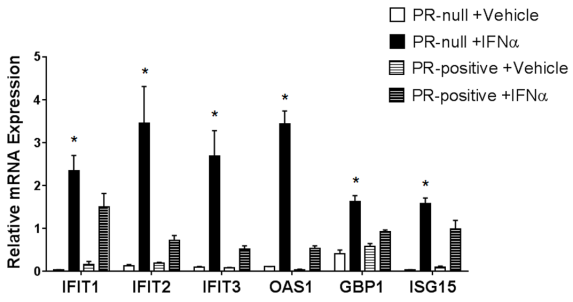
B.



C.

Top 20 ranking leading edge genes	
MX1	IFI44L
IFIT1	IFIT2
OAS1	IFI27
ISG15	XAF1
OAS2	GBP1
IFIT3	RSAD2
IFI16	DDX60
IFI44	IRF7
IFI35	MX2
OASL	OAS3

D.



E.

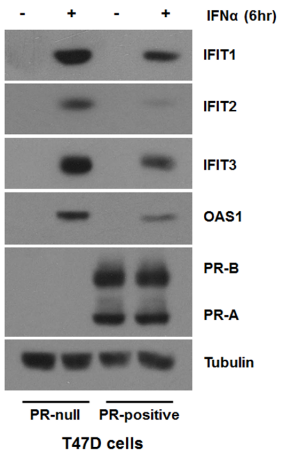


Figure 20. PR decreases ISG transcriptional response and protein levels

(A) RNA-Seq was performed on RNA isolated from IFN-treated (20 IU/ml IFN-α for 18 h) T47D PR-null or PR-positive cell lines. GSEA was performed using the c2 MSigDB collection, comparing RNA-Seq gene expression datasets obtained from IFN-treated PR-null and PR-positive cells. Shown are the top most-significantly enriched gene sets (FDR < 0.05); select enrichment examples are shown in (B). (C) Top 20 ranking genes as identified using LE analysis on 29 gene sets referred to in (A). (D) T47D PR-null or PR-positive cell lines were treated as in (A). Isolated RNA was analyzed for multiple ISGs using qPCR. Gene values were normalized to an internal control (β-actin). Error bars represent SD between biological triplicates. Asterisks (*) represent statistical significance between groups; $p < 0.01$, as determined using an unpaired Student t test. (E) Cells were treated with 20 IU/ml IFN-α for 6 h, and isolated protein was analyzed via Western blotting with respective Abs. The experiments presented in (D) and (E) were performed in triplicate, and a representative experiment is shown.

3.3.7 ISGF3 recruitment to ISREs is attenuated in PR-positive cells

ISGs are transcriptionally activated following ISGF3 (composed of STAT1, STAT2, and IRF9) binding to ISRE sequences in the proximal promoter regions of these genes. To determine if the interaction between PR and STAT1 may affect recruitment of the ISGF3 complex to ISREs, we used ChIP assays to measure STAT1, STAT2, and IRF9 recruitment in T47D-co cells expressing nonsilencing or PR shRNA [previously described[10] and Supplement **Fig. 23**]. In response to IFN-α treatment, ISGF3 components (STAT1, STAT2, and IRF9) were potently recruited to ISRE sequences of the IFIT genes (**Fig. 21**). Significantly, in cells lacking PR expression (PR shRNA), ISGF3 components exhibited more robust recruitment to ISREs, compared with cells expressing PR (nonsilencing shRNA). These data indicate that the interaction between PR and STAT1 decreases DNA recruitment of the transcriptional complex required for ISG transcription, thereby leading to a decrease in ISG RNA levels.

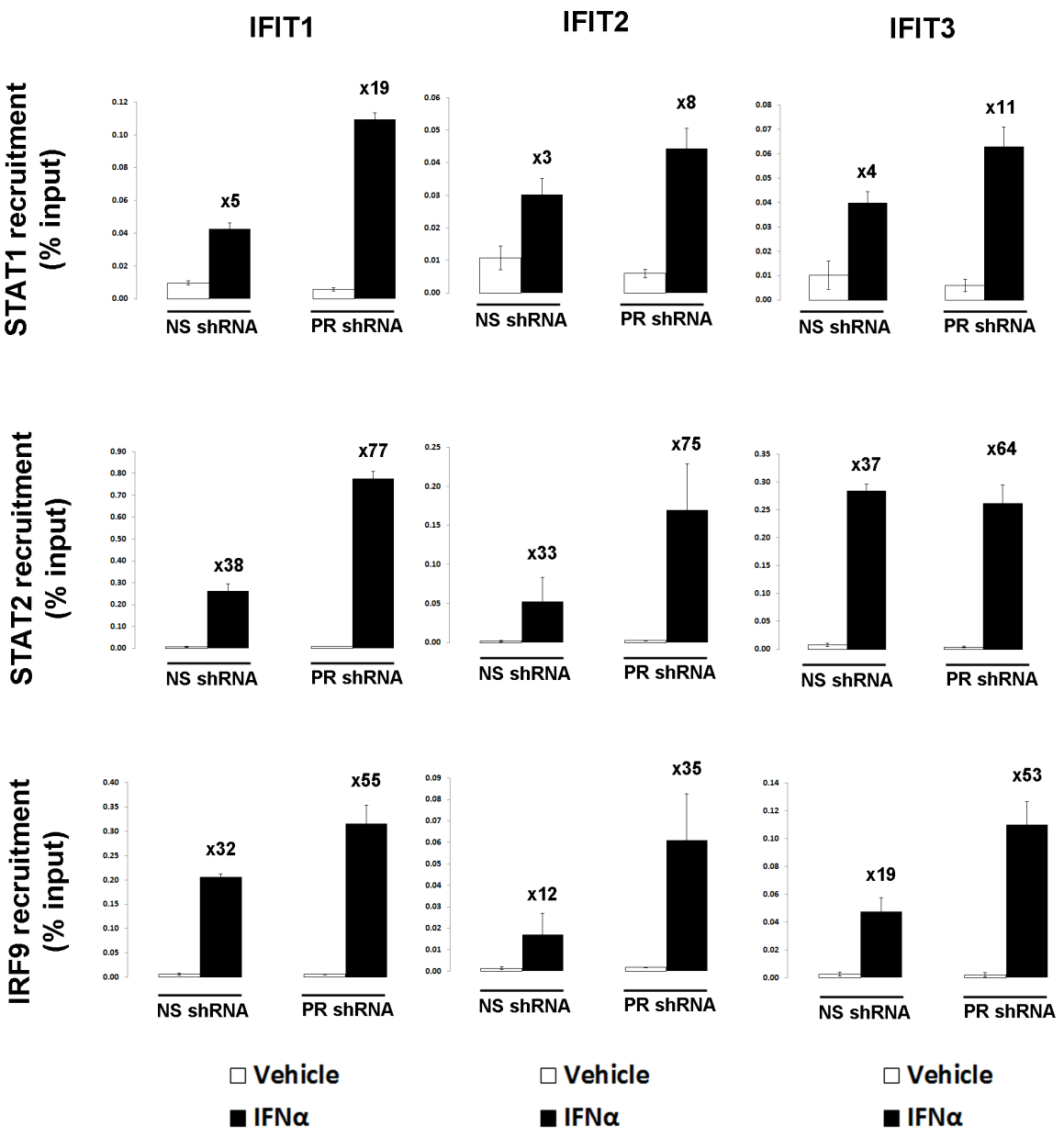


Figure 21. PR decreases ISGF3 recruitment to ISG promoters.

T47D-co nonsilencing (NS) and PR shRNA cells were serum starved for 18 h and then treated with 1000 IU/ml IFN-α (or vehicle) for 4 h. Fixed lysates were subjected to ChIP with Abs against STAT1, STAT2, IRF9, or a species-specific IgG (control; data not shown), and qPCR was performed on the isolated DNA, using primers designed to amplify select ISG promoters. A percentage of ChIP DNA over input DNA is shown. All ChIP experiments were performed in triplicate; a representative experiment is shown. Fold recruitment in IFN-α–treated conditions, as compared with vehicle treatment, is displayed above each bar. Error bars represent SD of technical replicates.

3.4 Discussion

The data presented in this study support a model whereby PR positivity can influence the potency of type I IFN signaling through an interaction with STAT1. We show that PR positivity leads to a decrease in IFN-stimulated STAT1 phosphorylation, a collapse of the ISGF3 transcriptional complex, decreased recruitment of STAT1 to ISG enhancers, and, subsequently, lower levels of ISG RNA and protein. The inhibitory effects of PR on IFN signaling are seen in the absence of PR ligand (ligand independent) and are further potentiated when PR ligand is added. These observations are clinically relevant, as we see decreased phospho-STAT1 in PR-positive breast tumors compared with their PR-negative counterparts. These data regarding the actions of unliganded (ligand independent) PR, together with our recently published data showing PR ligand-dependent transcriptional repression of ISGs,[10] suggest a novel, multifactorial role for PR in attenuation of IFN signaling.

IFN signaling has been classically defined as a critical part of the innate immune system's response to viral infection. However, there is an emerging role for IFNs in aiding the immune system in detection and clearance of early malignancies. The cancer immunoediting hypothesis, first put forth by Robert Schreiber, highlights that immunoediting can both prevent and promote cancer progression, clearing early tumors but helping shape the immunogenicity of more-developed tumors.[2,33] The three stages of cancer immunoediting are elimination, equilibrium, and escape. The elimination phase involves recognition and destruction of nascent tumors before they can advance to tumors of biological relevance. There is a clear role for type I IFNs in this process.[34] Both tumor cells and host immune cells can produce and are responsive to IFNs, and this plays a key role in tumor cell elimination. As such, alterations in IFN production, as well as the cellular response to IFNs, can have dramatic effects on recognition and clearance

of developing tumors. Multiple studies suggest this is the case, showing increased tumor formation in mice lacking key components of the IFN response pathway, such as IFN receptors (IFNAR and IFGAR) and key IFN signaling molecules (JAK2 and STAT1) [these data are thoroughly reviewed in Ref. (34)].[34] Situations in which the cellular response to IFN signaling is decreased or abrogated, such as that seen in the PR-positive breast cell line models shown in this study, may have a significant impact on the efficacy of immune-mediated tumor elimination. Decreased IFN/STAT1-signaling, as mediated by PR, may aid early PR-positive tumors in evading immune surveillance, allowing for the development of clinically relevant tumors.

There is a growing body of evidence to suggest that STAT1 is involved in breast tumorigenesis, and this has been extensively reviewed [Refs. (35–37)].[35-37] Significantly, STAT1 knockout mice develop mammary gland adenocarcinomas, 90% of which are ER/PR positive.[38] The molecular signatures of these tumors overlap with human luminal A/B tumors, which are predominantly ER/PR-positive tumors. Further, dysregulation of the JAK2/STAT1 signaling axis leads to the survival and proliferation of luminal progenitor cells, the precursors to ER/PR-positive tumors, in the murine mammary gland.[39] These data implicate STAT1 loss as an early event in the development of mammary gland tumors. Our data suggest that one putative mechanism for STAT1 loss (i.e., loss of function) is through the PR–STAT1 interaction and subsequent decreased STAT1 signaling, presented in this study. Cumulatively, our data, together with previously published data, suggest PR–STAT1 cross-talk is critical to the development of ER/PR-positive tumors.

These data discussed thus far present a role for STAT1 as a tumor suppressor in multiple tumor tissues. Conversely, work from A. Minn, R. Weichselbaum, and others has shown that expression of a core set of genes (mostly ISGs) canonically regulated by STAT1 is correlated

with therapy resistance in multiple tumors types, including breast. This gene set, referred to as the IFN-related DNA damage resistance signature (IRDS), is upregulated in tumors that develop resistance to chemotherapy, endocrine therapy, radiation, and immunotherapy and can be used to predict poor prognosis.[35,40-44] Moreover, recent work from the Peter Laboratory suggests that CD95-mediated activation of IFN signaling via STAT1 promotes a cancer stemness phenotype in breast cancer cells.[45] Together, these data highlight putative differences for the role of IFN/STAT1-signaling in early (tumor initiating/immune evasion) versus late (resistance to therapy) tumor events and underscore the complexity of events that culminate in tumor formation and, ultimately, progression.

One major unanswered question in breast cancer biology is why the overwhelming majority of breast cancers (over 65%) are ER/PR positive at the time of diagnosis.[46,47] Breast cancer is a hormonally driven disease, largely driven by exposure to estrogens.[48] Estrogen, when binding to the ER, drives proliferative and survival gene programs that promote oncogenesis.[49] However, extensive animal model and clinical data suggest that both estrogen and progesterone are needed for breast tumor development.[50-52] Despite these data, the role for progesterone and PR in breast cancer remains largely unanswered.[31] Although ER and PR likely provide a proliferative advantage (affording one explanation for this preponderance of ER/PR-positive tumors), another nonmutually exclusive explanation is that these tumors have an immune-privileged phenotype, allowing for their escape from the surveilling immune system. PR-positive tumors, because of their ability to downregulate IFN signaling under liganded[10] and unliganded conditions [data presented in this study and (10)], may evade the critical first step of elimination via immune clearance. Animal experiments are currently underway in our laboratory to test this model in the context of an intact immune system.

Finally, although ligand-dependent gene regulation remains the predominant pathway through which PR regulates gene expression, a role for ligand-independent (in the absence of ligand/progestin) PR gene regulation is emerging as a potent, but not well-understood, mechanism through which PR can affect target gene regulation.[53-56] In addition to our recently published work highlighting PR ligand-dependent ISG transcriptional repression,[10] these data presented in this study suggest a novel role for unliganded PR in blocking STAT1-dependent IFN signaling. Ligand-independent effects of PR may be a significant contributor to PR-dependent gene regulation in postmenopausal women and warrant further investigation.

3.5 Supplementary Materials

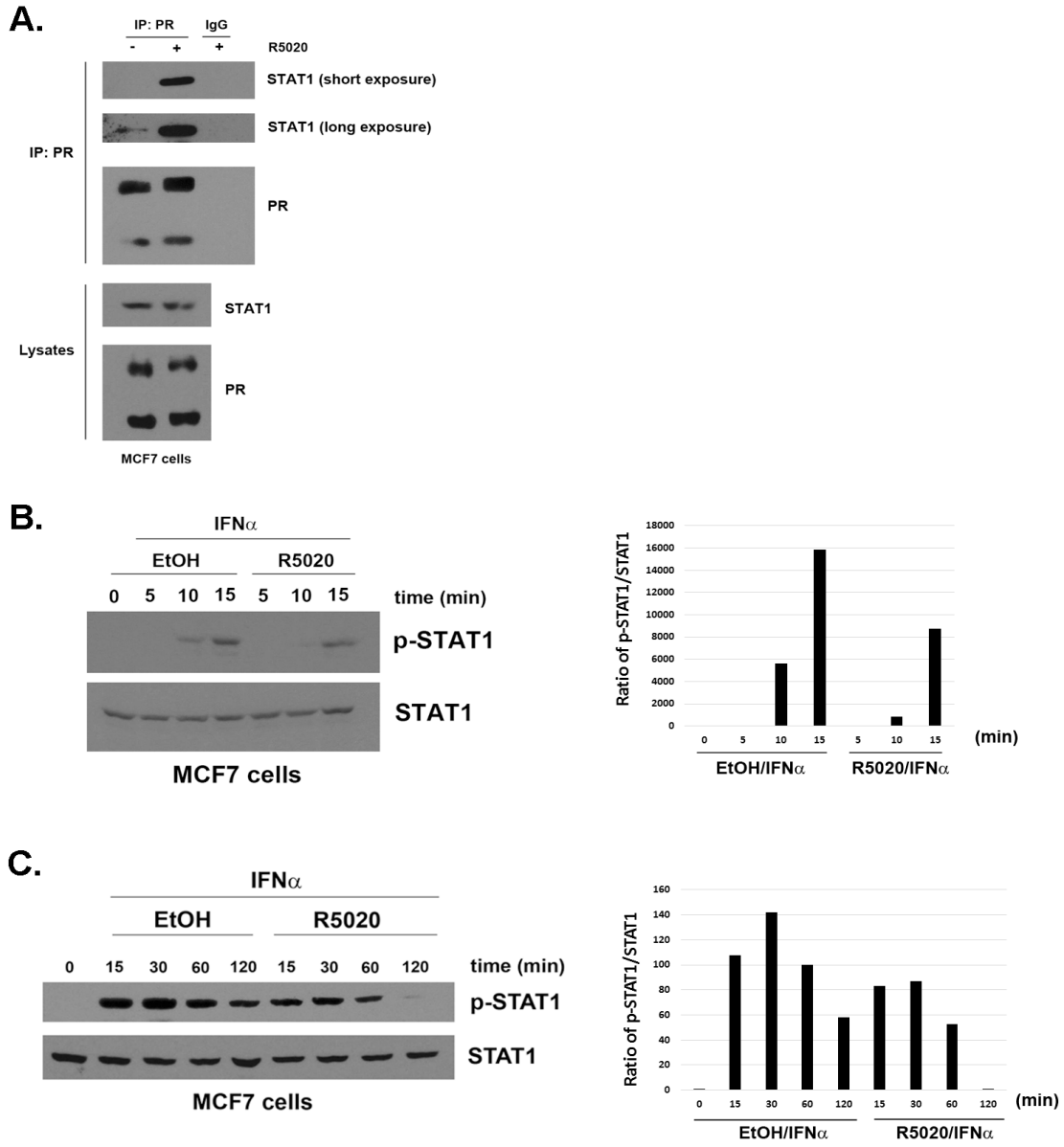


Figure 22. PR attenuates interferon-induced STAT1 signaling

(A) PR was immunoprecipitated from MCF7 cell lysates (+/- R5020) and the resulting associated protein complexes were analyzed by Western blotting. Bottom panels represent total input cell lysates. (B) and (C) MCF7 cells were treated with interferon IFNα and R5020 or vehicle (EtOH) for 0-15 min (B) or 0-120min (C). Isolated protein lysates were analyzed by Western blotting. Densitometry, as determined using Image J, is shown to the right of each blot. Ratios of pSTAT1/total STAT1 are normalized to the untreated (0 minutes) condition. These experiments were performed in triplicate, and a representative experiment is shown here.

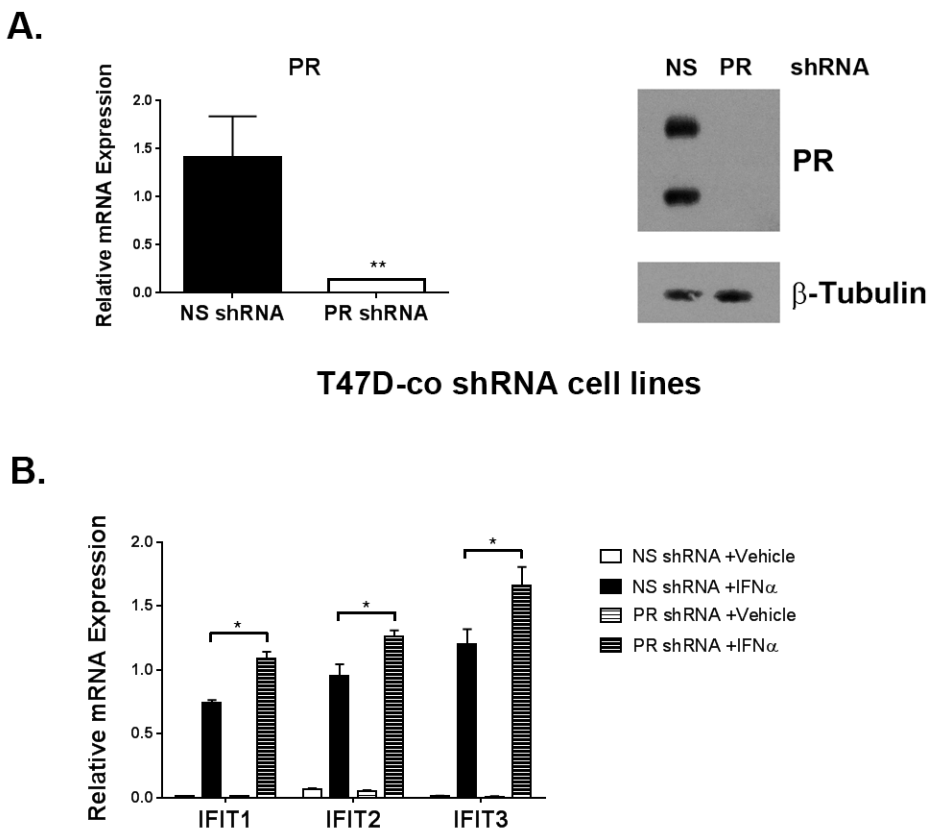


Figure 23. PR decreases interferon-induced gene expression

(A) Left: RNA was isolated from T47D-co NS and PR shRNA cell lines. Isolated RNA was analyzed for PR. Gene values were normalized to an internal control (β-actin). Error bars represent standard deviation between biological triplicates. Asterisks represent statistical significance between groups; $p < 0.01$, as determined using an unpaired Student's t-test. Right: Protein lysates isolated from T47D-co NS and PR shRNA cell lines were analyzed by Western blotting for PR expression. (B) T47D-co NS and PR shRNA cell lines were treated with 20IU/ml IFNα for 18hrs. Isolated RNA was analyzed for select genes. Gene values were normalized to an internal control (β-actin). Error bars represent standard deviation between biological triplicates. Asterisks represent statistical significance between bracketed groups; $p < 0.05$, as determined using an unpaired Student's t-test. These experiments were performed in triplicate, and a representative experiment is shown here.

3.6 Author Contribution

Katherine Walter contributed to the published manuscript in the following ways:

- Conception and design
- Acquisition of data (Figs. 20 and 21)
- Analysis and interpretation of data
- Writing, review, and/or revision of the manuscript

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Chapter 4: Progesterone receptor promotes degradation of STAT2 to inhibit the interferon response in breast cancer

4.1 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 ten years 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. 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.[10] 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.[11] Clinical trial data emerging over the last few decades have suggested a role for PR, independent of ER, in the development of breast cancer.[12,13] 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. Additionally, there is ample data showing that ER/PR-positive breast cancers, as opposed to triple negative (ER-/PR-/HER2-), are immunologically "cold," thus further reinforcing the need to identify how hormone receptor signaling mediates the immune response in tumor development.[14]

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. Because interferon

signaling is crucial in identification and destruction of nascent tumor cells, we propose that PR-mediated STAT2 degradation effectively downregulates interferon signaling and allows early transformed cells to evade immune detection and progress to clinically relevant, PR positive tumors.

4.2 Materials and methods

4.2.1 Cell lines and treatments

T47D 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 days of selection in 2.5 ug/ml Puromycin (MP Biomedicals). Target shRNA sequences are listed in **Supplementary Table 2**. Cells were treated with the following reagents where indicated: R5020 (10nM, Sigma), human rIFN-α (1000 IU/mL, IFN-α2A, SPR4594; Sigma-Aldrich), onapristone (10uM, provided through an MTA with Context Therapeutics), cycloheximide (200ug/mL, Calbiochem), and MG132 (5uM, Selleck Chemical).

4.2.2 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).

4.2.3 Co-immunoprecipitation

Co-immunoprecipitation experiment was performed as previously described.[15] In brief, 1000ug of protein from isolated protein lysates were incubated with 2mg 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 Laemmili sample buffer, then subjected to immunoblotting as described above.

4.2.4 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 2**.

4.2.5 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 2**.

4.2.6 TCGA analysis

Gene expression data for the 50-gene IRDS signature [18,19] were extracted from TCGA breast [20] 'Provisional' dataset and associated clinical metadata were accessed via the cBio portal.[21] 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.

4.2.7 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.[22]

4.3 Results

4.3.1 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, 10nM R5020, we found an increase in the formation of a PR:STAT2 complex when compared to the vehicle control (**Figure 24A**). 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.[23] To identify whether PR interacting with STAT2 impeded phosphorylation of STAT2, we treated with IFNα for 0-30min in the presence or absence of PR ligand (R5020) and found no differences in STAT2 phosphorylation with PR activation (**Figure 24B**). These data suggest that the interaction between PR and STAT2 does not affect interferon-induced STAT2 phosphorylation.

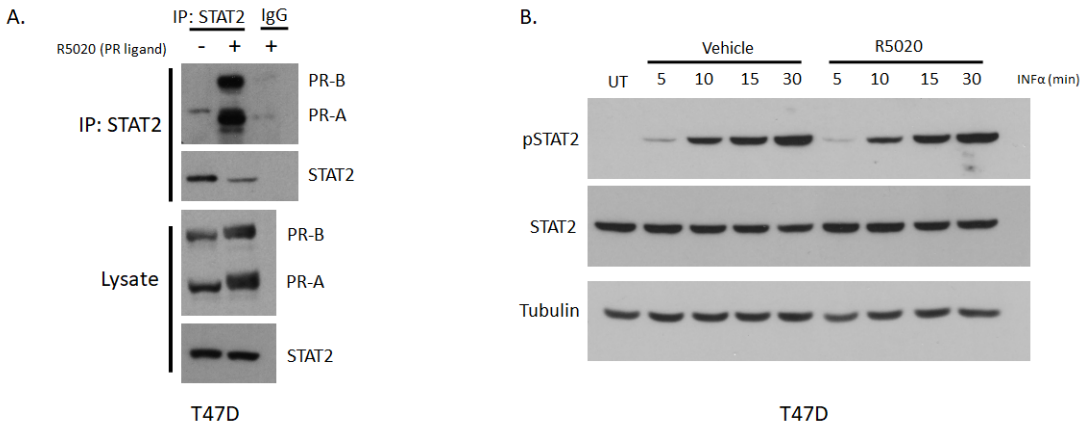

Figure 24. PR and STAT2 interact without affecting STAT2 phosphorylation

A) STAT2 was immunoprecipitated (IP) from T47D whole cell lysate treated with vehicle (EtOH) control or R5020 (10nM, 1hr) 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) for 0-30 min in the presence of vehicle (EtOH) or R5020 (10nM). Isolated protein lysate then analyzed for phosphorylated STAT2 (or total STAT2). Beta-tubulin shown as loading control. These experiments were performed in triplicate, and a representative experiment for each is shown.

4.3.2 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 production of the interferons themselves, prevention of phosphorylation of JAK/STAT proteins, promotion of JAK/STAT degradation, etc.[24] We observed that phosphorylation of STAT2 was unaffected (**Figure 24B**), 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 (0-24hr) in the presence or absence of R5020 and found that STAT2 protein was destabilized/degraded at a higher rate when PR was activated with ligand (**Figure 25A**). 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.[25] When treating with onapristone, we were able to effectively reverse the ability of PR to promote STAT2 turnover (**Figure 25B**). 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 25C**). 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 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 25D**). Taken together, these data suggest that PR interaction with STAT2 promotes ubiquitination, thereby targeting it for degradation.

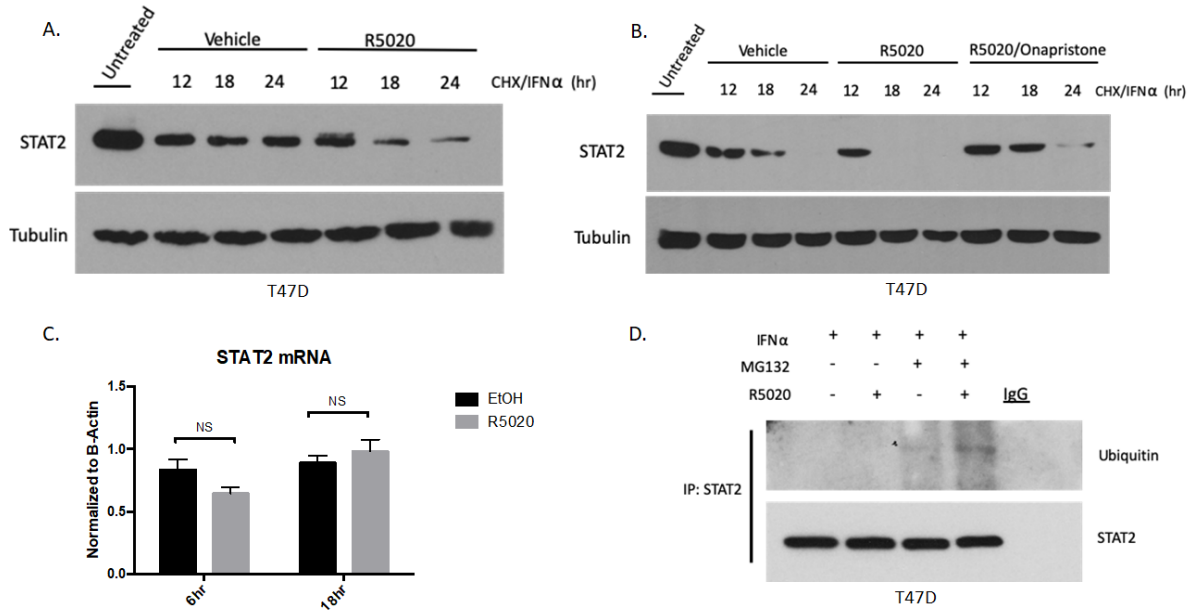

Figure 25. PR activation promotes STAT2 ubiquitination and subsequent protein degradation

A) T47D cells were treated with cycloheximide (CHX, 200ug/mL) and IFNα (1000 IU/mL) in the presence of either vehicle control or R5020 (10nM) for 12-24 hours. Isolated protein lysates were then analyzed to assess STAT2 degradation. Beta-tubulin shown as a loading control. B) T47D cells were treated with CHX (200ug/mL) and IFNα (1000 IU/mL) and either vehicle, R5020 (10nM), or a combination of R5020 and Onapristone (10uM) for 12-24 hrs. Isolated protein was then analyzed to assess STAT2 degradation. Beta-tubulin shown as a loading control. C) T47D cells treated with vehicle (EtOH) or R5020 (10nM) for 6 or 18hrs. 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 1hr then co-treated with R5020 (or vehicle) for 6hrs. Whole cell lysates were subjected to IP with STAT2-specific antibody and immunoblotted for ubiquitin. Rabbit-specific IgG used as control for IP. These experiments were performed in triplicate, and a representative experiment for each is shown.

4.3.3 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.[26,27] 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 26A**, protein in **Figure 28C**). 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 26B**); recruitment at select representative IGSs is shown. To then test the dependence upon STAT2, we used shRNA to stably knockdown STAT2 (mRNA shown in **Figure 27A**, protein in **Figure 28C**). 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 27B**). These data suggest that STAT2, not STAT1, is an 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 28A**), 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.[26,28] 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 28B**). 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 28C**). 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.

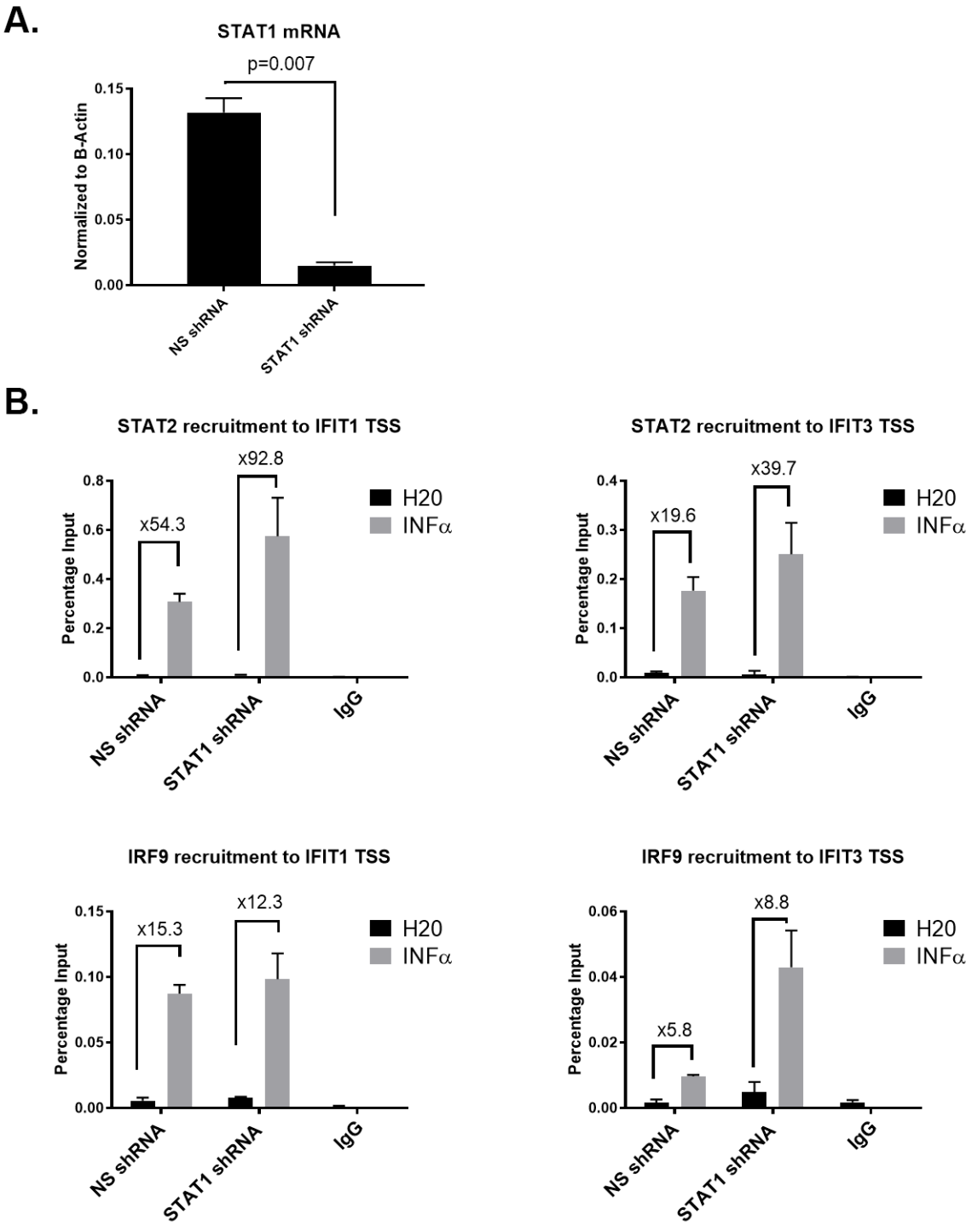

Figure 26. Interferon-induced DNA binding is maintained in the absence of STAT1

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 ($p<0.05$) determined using a Student's *t*-test. B) T47D-NS and

T47D-STAT1 shRNA cells lines were treated with IFNα (1000 IU/mL) for 4hrs 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.

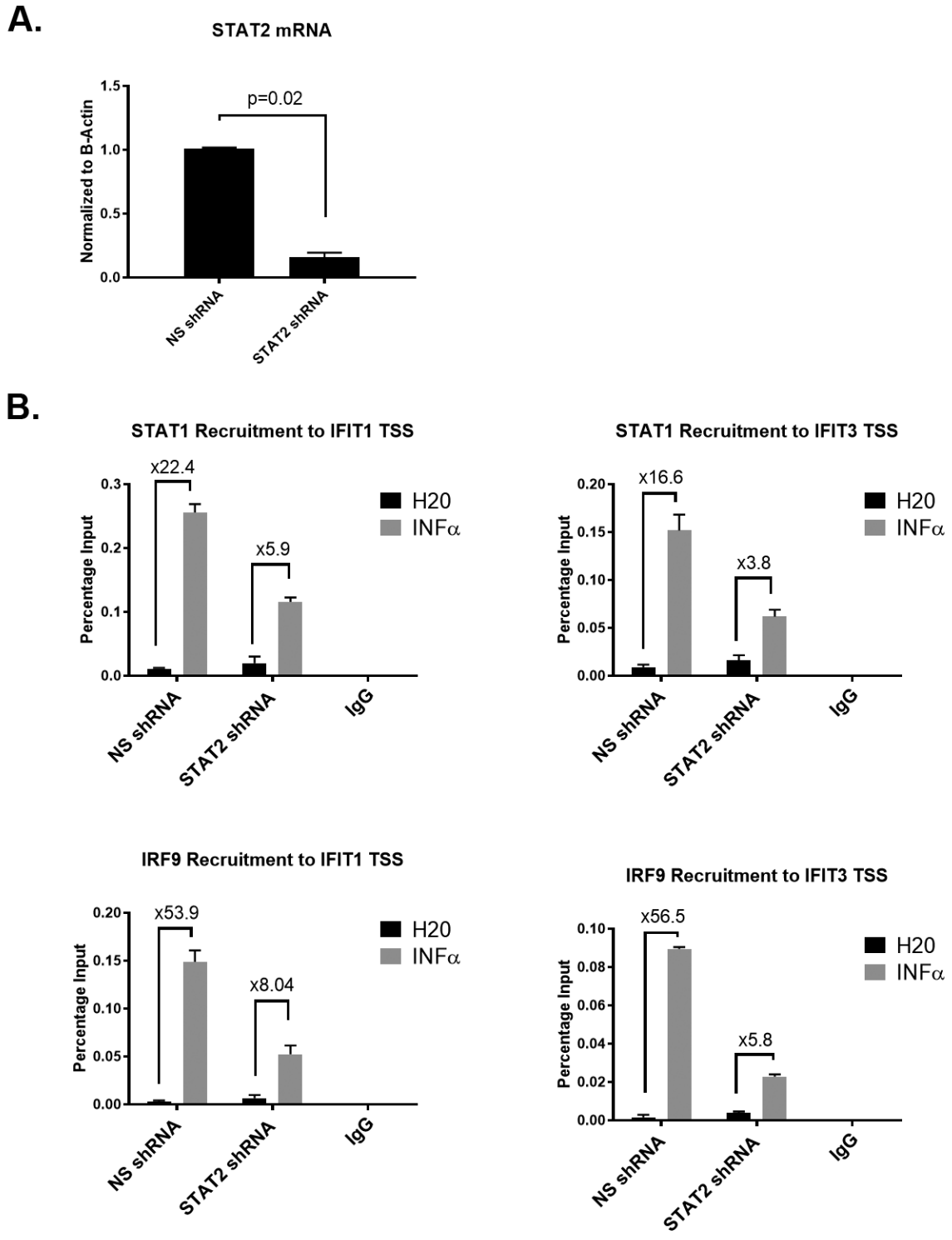

Figure 27. ISGF3 binding is severely hindered in the absence of STAT2

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 ($p<0.05$) determined using a Student's *t*-test. B) T47D-NS and T47D-STAT2 shRNA cells lines were treated with IFNα (1000 IU/mL) for 4hrs 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.

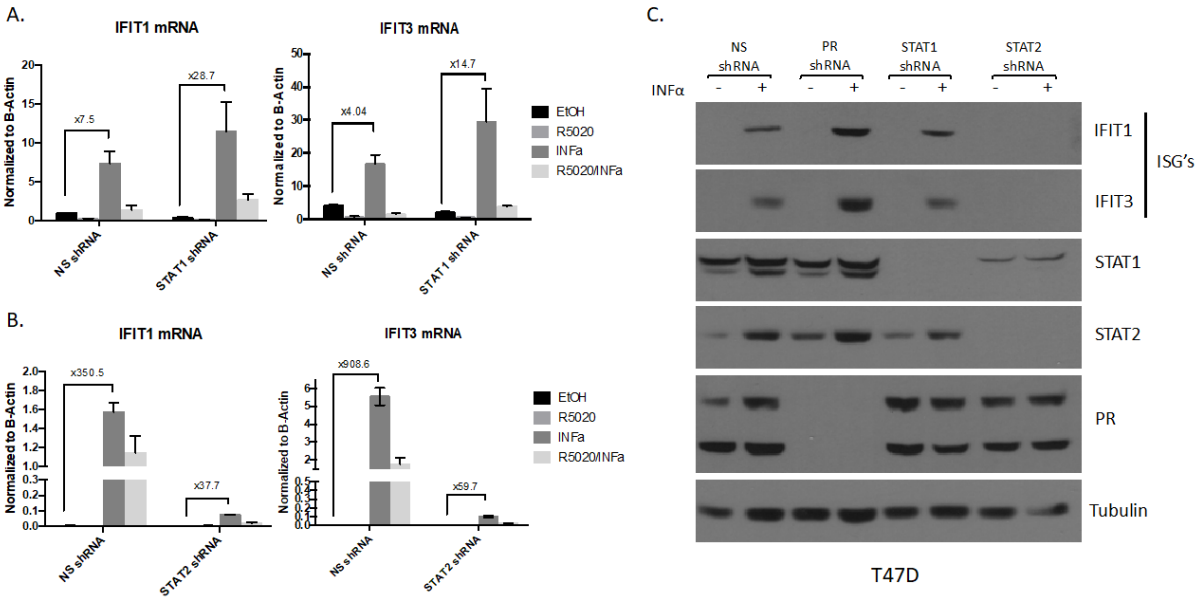

Figure 28. STAT2 is essential for efficient ISG transcription

A) T47D-NS and T47D-STAT1 shRNA cells (**Figure** 3A) were treated with vehicle, R5020 (10nM), INFα (1000IU), or a combination of both for 6hrs. 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 effect of STAT1 knockdown on interferon response. B) T47D-NS and T47D-STAT2 shRNA cells (**Figure 26C**) were treated and analyzed as in A). C). T47D cells stably expressing NS, PR, STAT1, or STAT2 shRNA were treated with IFNα (or vehicle) for 6hrs 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.

4.3.4 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[20]) and assessed differences in expression of an established interferon gene signature (that includes the ISGs shown herein[18,19]) to identify any differences between PR-positive and PR-negative breast cancers (**Figure 29A**). 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 29B**). 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.

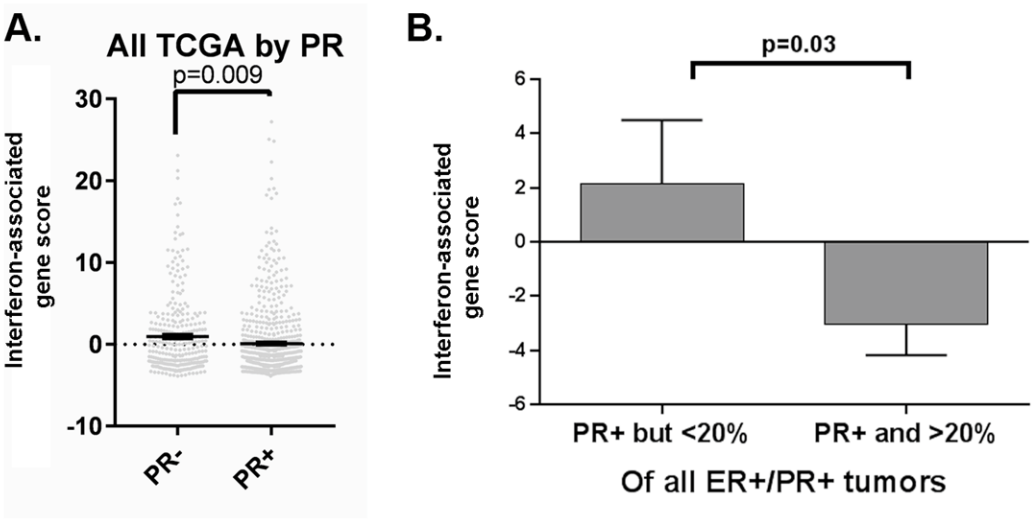

Figure 29. PR status in human tumors is correlated with decreased interferon gene signature

TCGA (Breast, provisional [20]) data analyzed for expression of interferon gene signature[18,19]. 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.

4.4 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 that in the absence of a functional STAT1 complex, compensatory STAT2-dependent signaling mechanisms are employed to maintain active interferon signaling.[17,26-30] 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.[31] 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 30**). 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).[32] While such therapies have proven to be effective in these patients, they are accompanied by undesirable side effects which can hinder compliance. Furthermore, many patients, even when compliant, will still relapse.[33,34] 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.[35] These innovative approaches to cancer treatment have proven to be effective in several tumor types but have been largely disappointing in luminal breast cancer.[36] Tumors more likely to respond to

these therapies are considered immune "hot"—higher mutational burden, cytotoxic lymphocyte infiltration, increased antigen presentation, etc.[37] Breast cancer, particularly hormone receptor-positive breast cancer, lacks immunogenicity and therefore is immune "cold."[38] 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.[39] The data shown herein suggests that PR is inducing an immunosuppressive environment through its targeting of type I interferon signaling and may therefore be a key contributor to creating a "cold," PR-positive breast tumor. It is essential to further define how PR suppresses immune signaling, as it may be a promising therapeutic target to prime tumors for immune-directed therapies.

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.[40] 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.[41] Additionally, PD-L1 expression is induced on immune cells in response to interferons.[42] 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.[43] 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]

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 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.[20] 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 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 whether targeting PR signaling could offer an advantage as a chemopreventative agent to prime immune "cold" 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*.

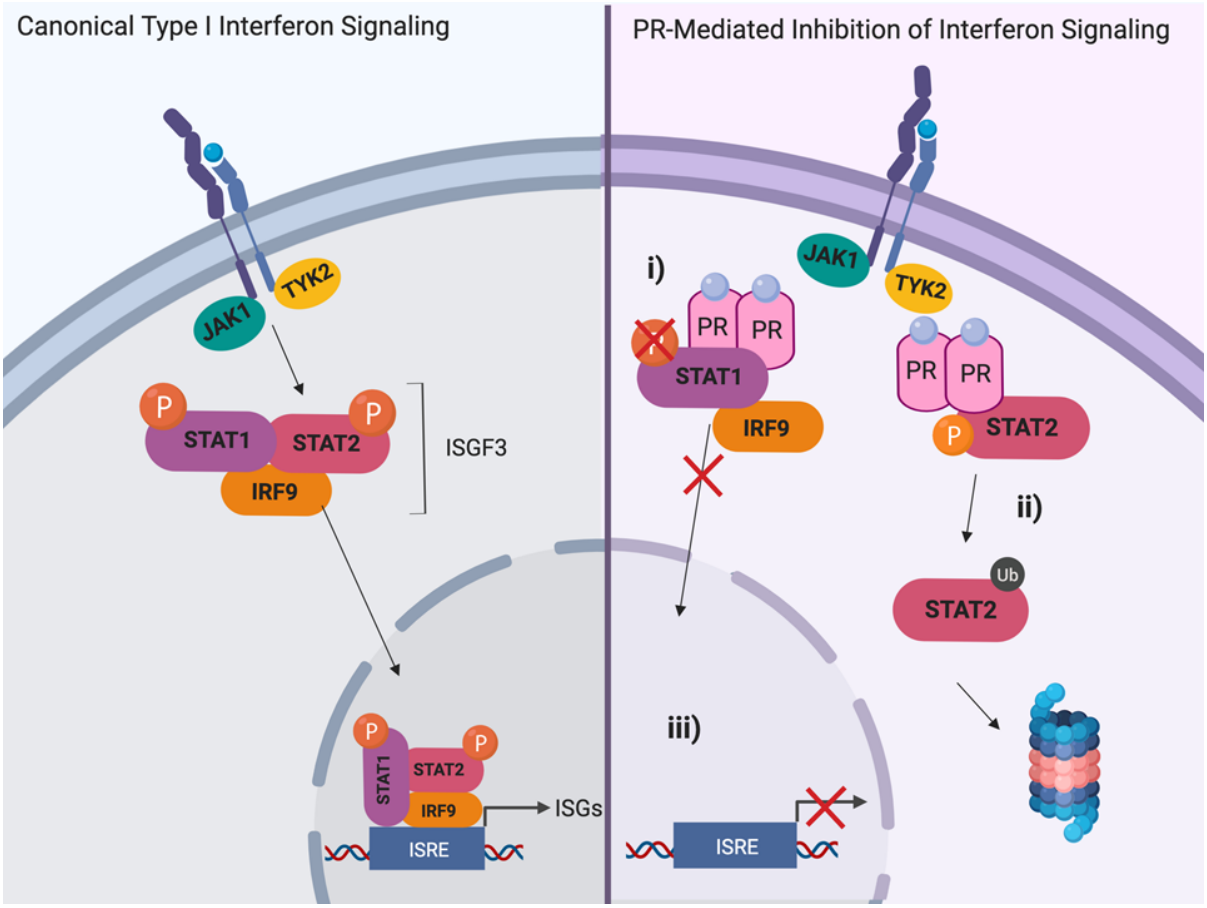

Figure 30. PR inhibits type I interferon signaling by targeting both STAT1 and STAT2

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 isn't sufficient to fully shut down interferon signaling (**Figures 26** and **28**). STAT2 compensates for loss of STAT1 functionality and PR intervenes by promoting STAT2 ubiquitination and degradation (ii) (**Figure 25**). 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).

4.5 Supplementary Materials

Table 2. Primer and shRNA sequences

qPCR IFIT1 F	GATCAGCCATATTTCATTTTGAATC
qPCR IFIT1 R	GAAAATTCTCTTCAGCTTTTCTGTG
qPCR IFIT3 F	GGAACAGCAGAGACACAGAGG
qPCR IFIT3 R	ACACTGTCTTCCTTGAATAAGTTCC
qPCR STAT2 F	GTCTTCTGCTTCCGATATAAGATCC
qPCR STAT2 R	TTTTGTCCAGTTCATTGAGAGTTTC
qPCR STAT1 F	GTATTACTCCAGGCCAAAGGAAG
qPCR STAT1 R	TTCAGACACAGAAATCAACTCAGTC
qPCR B-Actin F	AGAAAATCTGGCACCACACC
qPCR B-Actin R	GCTGGGGTGTTGAAGGTCTC
CHIP IFIT3 ISRE-F	TGTAAGTTGAGTTTCTTACTGTGCAG
CHIP IFIT3 ISRE-R	AACCCCTCAAACATCTTACAAATGC
CHIP IFIT1 ISRE-F	ACAAGACAGAATAGCCAGATCTCAG
CHIP IFIT1 ISRE-R	GAGCAAAGAAATCCTTACCTCATGG
PGR shRNA target clone# V2LHS_239356	CAGATAACTCTCATTCAGT
PGR shRNA target clone# V3LHS_355882	AGGTCAAGACATACAGTTG
PGR shRNA target clone# V3LHS_355883	ACAGCGTTTCTATCAACTT
STAT1 shRNA target clone #V2LHS_259422	CAAGCGTAATCTTCAGGAT
STAT1 shRNA target clone #V2LHS_86641	AGCTGTTACTCAAGAAGAT
STAT1 shRNA target clone #V3LHS_352184	AGGAAGTAGTTCACAAAAT
STAT2 shRNA target clone # V2LHS_70138	CCAAGTCTGTGGAACTTAA
STAT2 shRNA target clone # V3LHS_356247	AGGCTCATTGTGGTCTCTA
STAT2 shRNA target clone # V3LHS_356251	AGGATGACCCTCTGACCA

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Chapter 5: Conclusions

5.1 Summary of results

This body of work sheds light on a novel role of the progesterone receptor (PR) as a potent repressor of type I interferon signaling in ER/PR-positive breast cancer. Working with a 'ground-up' approach, we first established that PR, when activated, represses a specific subset of genes known as interferon stimulated genes (ISGs). These genes represent a diverse group of proteins produced in response to active type I interferon signaling-a pathway canonically activated in response to viral infection. We showed that PR can repress ISG transcription through inhibition of ISGF3 (STAT1/STAT2/IRF9) DNA recruitment. Importantly, we also establish that ISG repression occurs in human tumors in response to progestin treatment through human tumor explants.[1]

Our next step in interrogating this link between PR and interferon signaling inhibition was to establish precisely how PR carries out this functionality. Previously, we saw that STAT1 is unable to bind DNA as efficiently when PR was activated and we proposed that this was because PR interacted with this protein and effectively sequestered it from ISRE sites. We demonstrated this interaction between PR and STAT1 and illustrated that this interaction prevents a crucial tyrosine phosphorylation from occurring. Furthermore, PR expression in human tumors (using a tumor microarray) disrupted phosphorylated STAT1 expression, indicating that the inhibition observed in our cell lines was biologically relevant. Because STAT1 is unable to become phosphorylated in the presence of activated PR, we also demonstrated that the ISGF3 complex was unable to form, thus explaining the lack of DNA binding by these proteins, as observed previously[2].

We next demonstrated that breast cancer cells are able to adapt to the lack of functional STAT1, as persistent interferon signaling was still observed in STAT1 knockdown cells. STAT2

has the capacity to compensate for the STAT1 dysfunction, thus illustrating its own indispensability. We discovered that PR also inhibits STAT2 expression and acts as a "one-two punch" to sufficiently repress ISG transcription. Like before, we observed STAT2 interaction with PR; phosphorylation, however, was unaffected. Interrogating this interaction further, we elucidated that PR promotes ubiquitination of STAT2 and subsequent protein degradation. Taken together, these data indicate that PR intervenes at multiple points along the interferon signaling axis to inhibit a sufficient response in breast cancer cells. As interferon signaling is critical in immune-mediated elimination of nascent tumor cells, these data offer a biological mechanism by which early breast cells escape immune intervention and progress to clinically relevant, PR-positive tumors.

5.2 Significance

5.2.1 Current standard of care

As stated previously, the majority of all breast cancers are hormone receptor positive (ER/PR-positive). Because the proliferative effects of estrogen in mammary tissue are well understood, the current standard of care for patients presenting with these tumor types target the estrogen signaling axis. Selective estrogen receptor modulators (i.e. tamoxifen)/degraders (i.e. fulvestrant), and aromatase inhibitors are the current options for patients with hormonally driven tumors. These therapies, while effective, offer undesirable side effects and high rates of resistance in patients who initially respond.[3] Because of the prevalence of these tumor types in the global population, it's imperative to identify better treatment options for patients when they relapse on first and second line therapies.

5.2.2 Immunotherapy in breast cancer

Advancements in our understanding of the intricacies of the immune system's involvement in tumorigenesis has allowed for an advent in new therapies targeting some of these specific interactions[4,5]. These immune-targeting therapies include checkpoint inhibitors (anti-CTLA-4, anti-PD-1/PD-L1), CAR-T cells, vaccination, etc.[6] While effective in other tumor types, immunotherapy has been largely disappointing in breast cancer with only marginal responses in triple negative tumors (ER/PR/HER2-negative).[6]

5.2.3 Breast tumors are immune "cold"

A fundamental reason why hormone receptor positive tumors respond so poorly to immunotherapy is that most breast tumors lack the necessary therapeutic target: immune cells. Breast tumors are notoriously immune "cold," meaning that these tumors lack immune cell infiltration, cytokine signature, antigen presentation, etc.[7] Tumors lacking immunogenicity are poor candidates for immunotherapy and identifying a mechanism to effectively convert a tumor from "cold" to "hot" would open the door to other treatment options for these patients (**Figure 31)**. Our current study has shown that PR inhibits interferon signaling. As interferon signaling is critical in bridging the innate and adaptive immune systems to eliminate transformed cells, PR is effectively adding a "brake" to the ability of the host to combat tumor formation. If we are able to reverse this by say, an anti-progestin (i.e. Onapristone), we could release this "brake" and induce a more effective immune response in the tumor. Resultant increased immune infiltration into the tumor could then potentially transform the 'cold' microenvironment to 'hot' and improve patient response to immunotherapy approaches.

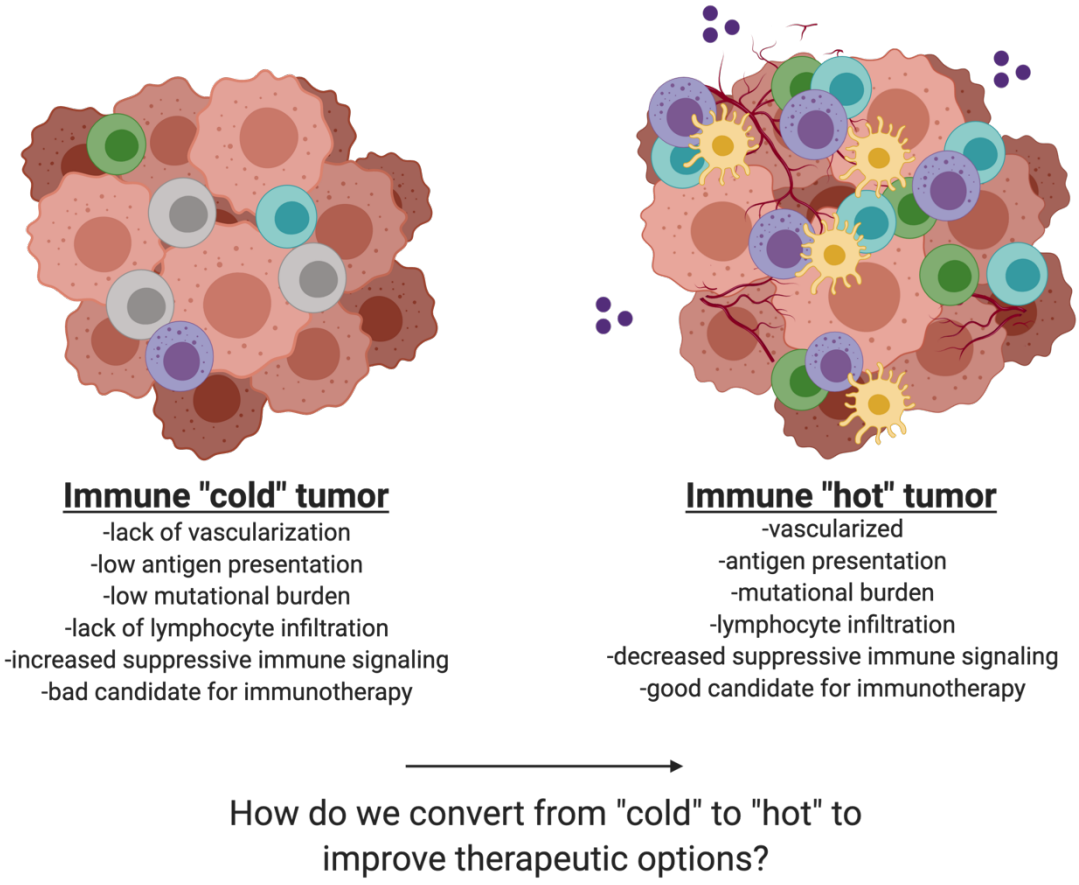

Figure 31. Breast Tumors are Immune Cold

Breast tumors lack immunogenicity and are consequently immune cold, making them poor candidates for immunotherapy. Blocking progesterone action may be a possible mechanism by which cold tumors are converted to hot tumors, thus opening up immunotherapy as a viable treatment option.

5.3 Future directions

An obvious next direction is to establish this connection with progesterone and interferon signaling *in vivo*. Current studies are underway investigating tumor development in a syngeneic mouse model where we can investigate the consequences of PR expression in the tumors themselves, as well as how treatment with exogenous progesterone (via subcutaneous time-release pellets) affects progression. We aim to identify local changes in the immune microenvironment by teasing apart different types of cells present, whether they be immune promoting (antigen presenting cells, cytotoxic T cells, etc), or immune suppressing (Tregs). We would expect that PR expression and progesterone treatment would promote a more immune suppressed environment and would allow for shorter tumor latency and ultimately a greater tumor burden.

After establishing that immune suppression does happen *in vivo* and that is PR-mediated, we next want to mitigate PR-mediated interferon repression using anti-progestins to identify whether this phenotype is reversable/druggable. To further this, it would be informative to address immunotherapy response in established tumors following anti-progestin treatment to illustrate the expected transformation from a cold tumor microenvironment to hot. These findings have the potential to revolutionize how we treat patients unresponsive to conventional endocrine therapies.

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