Prolactin Receptor-Integrin Crosstalk Mediated by SIRPα in Breast Cancer Cells

Traci Galbaugh*, Yvonne B. Feeney, and Charles V. Clevenger

Breast Cancer Program, Robert H. Lurie Comprehensive Cancer Center & Department of Pathology, Northwestern University, Chicago, IL, USA 60611

Running Title: SIRPα in PRLr-Integrin crosstalk

*To whom correspondence should be addressed

Traci Galbaugh PhD

Department of Pathology, Northwestern University
Lurie, 4-225, 303 E. Superior Street
Chicago, IL 60611
Phone: (312) 503-5755
Fax: (312) 503-0095
E-mail: t-galbaugh@northwestern.edu

Keywords: Prolactin, SIRPα, β1 integrin, collagen 1, Shp-2
Abstract:

The hormone prolactin (PRL) contributes to the pathogenesis of breast cancer in part through its activation of Jak2/Stat5, a PRLr-associated pathway dependent upon crosstalk signaling from integrins. It remains unclear, however, how this cross-talk is mediated. Following PRL stimulation, we show that a complex between the transmembrane glycoprotein signal regulatory protein-α (SIRPα) and the PRLr, β1 integrin, and Jak2 in ER+ and ER- breast cancer cells is formed. Overexpression of SIRPα in the absence of collagen 1 significantly decreased PRL-induced gene expression, phosphorylation of PRLr-associated signaling proteins, and PRL-stimulated proliferation and soft-agar colony formation. In contrast, overexpression of SIRPα, in the presence of collagen 1 increased PRL-induced gene expression, phosphorylation of Jak2, Stat5 and Erk, and PRL-stimulated cell growth. Interestingly, overexpression of a tyrosine-deficient SIRPα (SIRPα-4YF) prevented the signaling and phenotypic effects mediated by WT-SIRPα. Furthermore, overexpression of a phosphatase-defective mutant of Shp-2 or pharmacologic inhibition of Shp-2 produced effects comparable to that of SIRPα-4YF. However, the tyrosine phosphorylation of SIRPα was unaffected in the absence or presence of collagen 1. These data suggest that SIRPα modulates PRLr-associated signaling as a function of integrin occupancy predominantly through the alteration Shp-2 activity. This PRLr-SIRPα-integrin complex may therefore provide a basis for integrin-PRLr cross-talk and contribute to the biology of breast cancer.
Introduction:

Prolactin (PRL) is a pituitary hormone required for proliferation and differentiation of normal mammary epithelium and the stimulation of lactogenesis [1-3]. The canonical PRL signaling pathway begins with PRL binding, which induces PRL receptor (PRLr) activation of Jak2. Subsequently, Jak2 activates several downstream signaling proteins involved in proliferation, differentiation, survival and motility such as Stat5, Erk and Akt [1, 4].

Recently epidemiological studies have revealed that higher levels of circulating PRL in pre- and post-menopausal women increase a woman’s risk of breast cancer [5]. It has also been shown that PRL expression in tumors is higher than in normal or hyperplastic epithelium [6] and that the PRLr is expressed in 95% of breast tumors [7-9]. In addition, the overexpression of PRL or the PRLr in mice results in enhanced tumor formation [10-12]. Integrins have been shown to be critical for PRLr signaling in normal mammary epithelial cells (MECs) [13]. One report demonstrated that β1 integrin is necessary for PRL-driven MEC differentiation [14]. Furthermore, mice lacking β1 integrin have compromised Stat5 activation and milk production [15]. Despite these insights, the mechanism of β1 integrin and PRLr crosstalk in the context of breast cancer is not fully understood.

SIRPs are transmembrane glycoproteins that were first identified as novel proteins that bind Src homology 2 (SH2)-containing tyrosine phosphatases upon phosphorylation of their tyrosine residues [16]. There are two subfamilies of SIRPs, SIRPα and SIRPβ that differ only in the length of their cytoplasmic region. SIRPα contains immunoglobulin like repeats in its extra-
cellular domain as well as four tyrosine residues and a proline rich region in its cytoplasmic domain. SIRPα is ubiquitously expressed in human tissue and recent studies show that alterations of SIRPα expression may contribute to the pathogenesis of acute and chronic myeloid leukemia, hepatocellular and breast carcinoma [16-19]. Meta-analysis of publicly available expression arrays (Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization) reveal that SIRPα is significantly overexpressed in triple negative breast cancer. In addition, unpublished anti-SIRPα immunohistochemistry of primary human breast cancer reveals SIRPα is overexpressed at the invasive edge of the tumor. SIRPα binds to CD47 and has been implicated in signaling with integrins [20], growth hormone (GH) [21], insulin, and epidermal growth factor (EGF) [20]. The activation of these pathways induces tyrosine phosphorylation of SIRPα and its subsequent association with the SH2 domain-containing protein tyrosine phosphatases Shp-1 and Shp-2. Shp-2 is ubiquitously expressed in vertebrate cells and tissues and plays a critical role in development [22-24]. Typically protein tyrosine phosphatases negatively regulate cellular signaling by opposing the effects of protein tyrosine kinases. Interestingly, it has been shown that Shp-2 can promote the activation of Ras-MapK through various growth factor receptors [25]. In contrast, Shp-2 has been shown to negatively regulate Stat family proteins including Stat-1, -3, and -5 in different cell types by the dephosphorylation of Jak family kinases. In the mammary gland, Shp-2 regulates PRLr signaling by inhibiting the activity of both Jak2 and Stat5 [21, 26]. In addition, the conditional deletion of Shp-2 suppresses Stat5 activation and lobulo-alveolar outgrowth and lactation [27]. Like the PRLr, GHr is also inhibited by the actions of Shp-2. Recent studies have demonstrated that GH regulates the tyrosine phosphorylation of SIRPα and its subsequent association with...
Shp-2. Such interactions appear functionally significant, as they result in the down regulation of GHR/Jak2 signaling [21, 28].

SIRPα has also been shown to be important in integrin-mediated signaling. Integrins are critical extra-cellular matrix (ECM) receptors that have been identified as important regulators of MEC proliferation, differentiation, survival, apoptosis, and migration [29, 30]. The extra-cellular domains of integrins bind ECM proteins such as collagens, fibronectin, and laminins and activate several salient signaling networks that include Jak2/Stat5, PI-3-K, and MapK [31-33]. Integrin-mediated adhesion has been shown to promote SIRPα phosphorylation which contributes to cell migration primarily through Src family kinases and focal adhesion kinase (FAK) [29]. Thus, SIRPα may contribute to integrin-mediated cell migration.

Given the role of SIRPα and Shp-2 in GHR and integrin signaling, it was hypothesized that the SIRPα/Shp-2 complex might play a role in altering PRL/PRLr-mediated signaling in breast cancer cells. Our findings have demonstrated that PRLr, β1 integrin and SIRPα interact in both ER+ and ER- breast cancer cell lines, while SIRPα expression/function is associated with an ECM context-dependent modulation of PRLr signaling. These data reveal a novel role for SIRPα in facilitating crosstalk between the PRLr and β1 integrin and provide a basis for ECM regulation of PRLr action in breast cancer.
Materials and methods:

Cell culture: T47D (ATCC) cell lines were grown in complete media consisting of DMEM (Gibco), 10% fetal bovine serum (FBS Sigma-Aldrich), and 1% Pen Strep (P/S Gibco). Defined PRL deficient media consisted of phenol free DMEM (Gibco), .01%BSA, and 1% P/S (Gibco). MDA-MB-231 (ATCC) cell lines were grown in complete media consisting of DMEM/F12, 5% FBS, 1% P/S, 2mM Glutamine (Gibco), 1% Sodium Pyruvate (Sigma), and 2x NEAA (Gibco).

Stable cell line production: The T47D and MDA-MB-231 breast cancer cell lines were used to generate stable transfectant pools expressing either a control (GFP), WT-SIRPα (courtesy of Dr. C.Carter-Su) or a SIRPα-4YF (described in [34], courtesy of Dr. C. Carter-Su). These stable cell lines were made by inserting each plasmid into a lentiviral vector (pLenti6/V5-Dest, Invitrogen) containing a V5 tag and tested for expression by transient transfection into Hek293 cells. Hek293T cells were transfected with the lentiviral plasmids in addition to three other viral packaging plasmids with LF2000 according to manufacturer’s protocol (Invitrogen). Virus-media was harvested and used directly to infect the T47D and MDA-MB-231 breast cancer cell lines. Infected cells were grown in the presence of blastocidine (BSR, 10μg/ml) and levels of overexpressed proteins were assessed by immunoblot analysis.
Collagen coating and β1 integrin neutralization: Collagen 1 (Bovine, Cultrex and Rat Tail, Upstate) was diluted to a concentration of 3mg/ml in de-ionized H2O and added to tissue culture plates overnight at 4°C. Collagen 1 was aspirated off and wells were rinsed with de-ionized H2O and allowed to air dry. Following collagen 1 coating, an anti-β1 integrin (Chemicon) antibody was diluted to 15ug/ml in 50mM Tris pH 8.0 and added to the collagen 1 coated dishes overnight at 4°C. The next day heat-denatured 3% BSA-PBS was added to the tissue culture dishes for 30 minutes at 37°C. This was aspirated off and cells were then seeded accordingly.

Luciferase assay: T47D transfectant pools were plated in the absence or presence of collagen 1, grown in complete media and transiently transfected with 1μg of either pGL4-CISH reporter as well as DN-Shp-2 (courtesy of Dr. E. Fuch) and 10ng of pGL4-Renilla according to LF2000 manufacturers protocol (Invitrogen). Twenty-four hours after transfection the cells were arrested in defined PRL deficient media for an additional twenty-four hours. The cells were stimulated with 250ng/ml of PRL (courtesy of Dr. T. Kossiakoff) for twenty-four hours and analyzed for dual luciferase on a Victor3V (Perkin-Elmer). Cells treated with sodium stibogluconate (courtesy of Dr. E. Eklund, SSG 100ng/ml) were pre-treated for four hours prior to PRL stimulation. Results were reported as luciferase/renilla ratio (±) SEM.

Immunoprecipitation and immunoblotting: T47D, T47D transfectant pools and MDA-MB-231 cells were grown in complete media and arrested in defined PRL deficient media for twenty-four hours. They were treated with 250ng/ml of PRL for times indicated. The cells were harvested, lysed in RIPA buffer [35] and immunoprecipitated with either SIRPα (Chemicon 1μg/sample), Jak2 (Cell Signaling, 1μg/sample), β1 integrin (Chemicon, 1ug/sample) or PRLr (Zymed, 1μg/sample) antibodies as well as 40μl of a 50% solution of Protein A agarose beads.
(Invitrogen) overnight at 4°C. For immunoblots, equivalent amounts of protein were separated by SDS-PAGE and transferred to PVDF filters. The filters were blocked in 0.6% blotto for one hour. Following blocking the blots were incubated with the appropriate primary antibody overnight at 4°C and then HRP-conjugated secondary antibody for one hour at room temperature. Chemiluminescence was detected with ECL plus (Amersham) and images were collected on a CCD camera (Fuji). All blots were quantitated via scanning densitometry (Fuji, Image gauge software) and numbers reported represent fold decrease or increase as compared to the control transfectants.

**Proliferation assay:** T47D and MDA-MB-231 transfectants were plated in the absence and presence of collagen 1. Twenty-four hours after plating the transfectants were arrested in defined PRL deficient media for twenty-four hours. The cells were then treated with or without 250ng/well of PRL for seventy-two hours and ³H thymidine (0.5µCi/well) was added for four hours. The cells were lysed on a Filtermat Harvester (Perkin-Elmer) and transferred to a Filtermat (Wallac) and read on a Microbeta Luminescence counter (Perkin-Elmer).

**Soft agar colony formation:** T47D transfectant pools were suspended in 0.3% agar/regular growth media/PRL mix in the presence or absence 10% collagen 1. Once solidified an overlay of regular growth media/PRL (250ng/ml) was added and changed every two days. Cells were photographed twenty-four hours after suspension and then starting on day seven on Magnafire software (Optronics). Colony size and numbers were counted with ImageJ software (NIH) and results were analyzed by comparing number and size of colonies of control transfectant pools to the WT-SIRPα transfectants.
**Statistical Analysis:** All experiments described were performed no less than three times. Statistical analysis was performed on Graph-Pad Prism 4 (Graph-Pad Software) and results are shown as the means with error bars depicting (±) SEM. One-way ANOVA statistical tests were performed and p-values were reported.

**Results:**

**SIRPα interacts with the PRLr, β1 integrin and Jak2 in breast cancer cells**

Given literature that indicated that SIRPα could influence GHr signaling [21, 34], we sought to examine if SIRPα could interact and influence PRLr action in the context of breast cancer. To that end, the interaction of SIRPα with the PRLr, β1 integrin and Jak2 was tested (Figure 1 and 2). Co-immunoprecipitation analysis revealed endogenous interactions between the PRLr and SIRPα as well as the PRLr and β1 integrin in T47D ER+ breast cancer cells (Figure 1A) and MDA-MB-231 ER- breast cancer cells (Figure 1B). While SIRPα and the PRLr were shown to interact in T47D cells in the absence of PRL, ligand stimulation enhanced this interaction, which peaked at fifteen minutes. In contrast, co-immunoprecipitation revealed that the interaction between the PRLr and β1 integrin was PRL-dependent with their association visualized after five minutes of PRL stimulation. The interaction between the PRLr and SIRPα
in MDA-MB-231 was comparable to that seen in the T47D cells (Figure 1B). However, in these cells, unlike T47D, the data suggested that \( \beta \)1 integrin and the PRLr appear to be associated with each other in the absence of PRL and this interaction was enhanced upon PRL stimulation.

To better examine the interactions of SIRP\( \alpha \) with \( \beta \)1 integrin and Jak2, three stable T47D transfectant pools (Figure 2A, B and E) were created. These three stable transfectant pools were generated to overexpress wild type SIRP\( \alpha \) (WT-SIRP\( \alpha \)), GFP (control), or a non-phosphorylated mutant SIRP\( \alpha \) (SIRP\( \alpha \)-4YF), which is in-active in these cells therefore mimicking the control transfectants with endogenous SIRP\( \alpha \). Overexpression was assessed in WT-SIRP\( \alpha \) and SIRP\( \alpha \)-4YF transfectant pools by immunoblot analysis. Investigation in these transfectants revealed two fold higher SIRP\( \alpha \) protein expression than that of the control (Figure 2E). Co-immunoprecipitation analysis of these transfectants (Figure 2C) revealed that SIRP\( \alpha \) interacted with \( \beta \)1 integrin in the control transfectants as well as the SIRP\( \alpha \) overexpression transfectants within one minute of PRL stimulation and this interaction was maintained through sixty minutes (Figure 2A). Interestingly, the mutant form of SIRP\( \alpha \) also interacted at early time points with \( \beta \)1 integrin which suggested that the four tyrosine residues in the SIRP\( \alpha \) intra-cellular domain are not required for this interaction. Additionally, co-immunoprecipitation analysis also revealed that SIRP\( \alpha \) interacted with Jak2, independent of ligand addition in the SIRP\( \alpha \) overexpression transfectants (Figure 2B). Of importance, each co-immunoprecipitation was performed three separate times and reported as fold induction as compared to the untreated control sample (Figure 1A, 1B, 2C and 2D). Taken together these associations confirmed that SIRP\( \alpha \), the PRLr, \( \beta \)1 integrin, and Jak2 are brought into close proximity by PRL stimulation opening the possibility for functional cross-talk.
PRL-induced CISH promoter luciferase activity and protein expression is enhanced by SIRPα in a collagen 1/β1 integrin-dependent manner.

Given the requirement for integrins during PRLr signaling [14], PRL-responsive CISH reporter luciferase activity was utilized to examine the effects of SIRPα overexpression on PRL-mediated gene expression both in the presence and absence of several different ECM substrates (Table 1). Results verified that overexpression of SIRPα inhibited PRL-induced CISH promoter luciferase activity in transfectants when plated on the ECM proteins laminin and fibronectin. These ECM proteins are typically found in the basement membrane niche of the normal ductal-lobular unit. In contrast, SIRPα overexpression transfectant pools plated on collagen 1 (a structural protein of the interstitial space) showed an enhancement of PRL-induced CISH promoter luciferase activity as compared to the control or SIRPα-4YF transfectant pools (Table 1 and Figure 3B). Comparable results were obtained with two independent preparations of collagen 1 (data not shown). These data suggested that SIRPα may differentially regulate PRL-induced CISH luciferase activity depending on ECM occupancy.

In light of data in Table 1, PRL-induced CISH (Figure 3A and 3B) promoter luciferase activity was examined in transfectant pools overexpressing SIRPα plated in the presence or absence of collagen 1. These analyses revealed that the transfectant pools overexpressing SIRPα plated in the absence of collagen 1 showed a significant decrease in PRL-induced CISH promoter luciferase activity. This effect was lost in transfectants which overexpressed the control and SIRPα-4YF constructs (Figure 3A). In contrast, the transfectants overexpressing SIRPα plated in presence of collagen 1 showed a significant increase in PRL-induced CISH promoter luciferase activity (Figure 3B). Similar results were seen with the lactogenic hormone
response element (LHRE), another PRL-responsive luciferase reporter (data not shown). To confirm the results observed by luciferase reporter assay, endogenous PRL-dependent CISH protein expression was also assessed in the presence or absence of collagen 1. As noted in Figure 3C, endogenous as well as PRL-dependent CISH protein expression was inhibited in the cells overexpressing SIRPα in the absence of collagen 1. In addition, as before, cells overexpressing SIRPα plated in the presence of collagen 1, showed an increase in PRL-dependent CISH protein expression (Figure 3D). Thus, these data revealed that SIRPα overexpression differentially regulated PRL-induced CISH promoter luciferase activity and endogenous PRL-dependent CISH protein expression.

To further assess the involvement of β1 integrin on PRL-induced CISH promoter luciferase activity in the presence of collagen 1, a neutralizing anti-β1 integrin antibody was utilized. Data in Figure 4 revealed that transfectants overexpressing SIRPα plated in the presence of collagen 1 and anti-β1 integrin showed a reduction in PRL-induced CISH promoter luciferase activity as compared to the transfectants overexpressing SIRPα plated in the presence of collagen 1 only. Interestingly, the reduction resembled results observed in the cells overexpressing SIRPα plated in the absence of collagen 1. Consequently, the data revealed that SIRPα-mediated potentiation of PRL-induced CISH promoter luciferase activity is dependent on the activity of β1 integrin in the presence of collagen 1.

**Differential regulation of PRLr signaling as a function of SIRPα overexpression**

Since SIRPα overexpression modulated PRL-induced gene expression, it was reasoned that this might be due to its modulation of downstream molecules in the PRLr signaling network. Results in Figure 5 demonstrated that SIRPα overexpression inhibited the phosphorylation of
Jak2, Stat5 and Erk in the absence of collagen 1. In contrast, in the presence of collagen 1, SIRPα overexpression enhanced the phosphorylation of Jak2, Stat5, and Erk. As anticipated, the SIRPα-4YF transfectants (data not shown) showed similar results to that of the control transfectants suggesting the four tyrosine phosphorylation sites were necessary for the phosphorylation of Jak2, Stat5, and Erk. Taken together these data indicated that SIRPα overexpression regulated the phosphorylation/activation of proteins involved in cellular proliferation and survival in a collagen 1-dependent manner.

Given the documented role of Shp-2 phosphatase in the regulation of both SIRPα and Jak2 function it was also hypothesized that Shp-2 might be the signaling switch through which SIRPα mediates its differential effects in the presence or absence of collagen 1. Therefore, the PRL-induced phosphorylation of Shp-2 was also examined. In the absence of collagen 1, SIRPα overexpression resulted in a significant increase in the basal Shp-2 phosphorylation. In contrast, in the presence of collagen 1, Shp-2 phosphorylation was reduced as compared to the control transfectants. Furthermore, the tyrosine phosphorylation status of SIRPα in the absence or presence of collagen 1 was examined. Interestingly, the data demonstrated that there was no difference in the tyrosine phosphorylation of SIRPα following PRL stimulation in either the absence or presence of collagen 1. These data suggest that the phosphorylation status of Shp-2, but not SIRPα, is associated with the modulation of PRLr signaling.

In addition to signaling, the effect of SIRPα overexpression on PRL-induced cellular proliferation was measured by ³H thymidine incorporation (Figure 6). In congruence with the previous data, SIRPα overexpression significantly decreased PRL-dependent cellular proliferation in the absence of collagen 1 as compared to the control or the SIRPα-4YF
transfectants (Figure 6A). In contrast, SIRPα overexpression significantly increased cellular proliferation in the presence of collagen 1 as compared to the control or the SIRPα-4YF transfectants (Figure 6B). This effect on proliferation was not limited to only ER+ cell lines, as SIRPα overexpression in MDA-MB-231 cells also showed similar results (Figure 6C and D). This data further supports the notion that the differential regulation of PRLr signaling by SIRPα extends to the cellular level in breast cancer.

To further assess the effect of SIRPα overexpression on the biology of T47D breast cancer cells the transfectants overexpressing SIRPα were examined in soft agar. In Figure 7A, the cells were plated in a 100% soft agar/media/PRL mixture and allowed to grow for 15 days. In the absence of collagen 1 there was a significant reduction of size and number of colonies in the SIRPα overexpression transfectants as compared to the control transfectants (Figure 7C). In contrast, adding 10% collagen 1 (Figure 7B) completely reversed the outcome, as SIRPα overexpression resulted in a significant increase in colony number and size when compared to the control or SIRPα-4YF transfectants (Figure 7C). Thus, comparable to the above results, SIRPα can function as a signaling switch that can increase or decrease soft agar colony formation in a collagen 1-dependent manner.

**DN-Shp-2 or Sodium Stibogluconate (SSG) reversed the effect of SIRPα overexpression on PRL-induced CISH promoter luciferase activity in the absence or presence of collagen 1**

The above results suggested that SIRPα regulation of PRL-mediated signaling could induce the activity of the Shp-2 phosphatase (Figure 5). To test this hypothesis, two approaches were utilized to investigate the contribution of Shp-2 in the actions of SIRPα: overexpression of DN-Shp-2 or the use of a Shp-2 inhibitor SSG. As seen in Figure 8A, DN-Shp-2 co-expression
in SIRPα overexpression transfectants prevented SIRPα-mediated inhibition on PRL-induced CISH promoter luciferase activity in the absence of collagen 1. In contrast, introduction of DN-Shp-2 in SIRPα overexpression transfectants plated on collagen 1 prevented SIRPα-mediated potentiation of CISH promoter luciferase activity, restoring levels to that of the control or SIRPα-4YF transfectants (Figure 8B). As such, these results are supportive of a role for Shp-2 in the regulation of PRL-induced gene expression by SIRPα. This hypothesis was further tested through the use of a potent pharmacologic inhibitor of the phosphatase Shp-2, SSG [36]. To examine the effect of SSG, SIRPα, SIRPα-4YF or control transfectant pools were plated in the absence or presence of collagen 1 prior to pre-treatment with SSG and then stimulated with PRL. As seen in Figure 8C, SSG treatment of SIRPα overexpression transfectants reversed its effect on PRL-induced CISH promoter luciferase activity in the absence of collagen 1. In contrast, the SIRPα overexpression transfectants plated on collagen 1 showed diminished luciferase activity equivalent to the control or SIRPα-4YF transfectants in the presence of SSG (Figure 8D). Again, these data parallel the results obtained with DN-Shp-2, further confirming that SIRPα is a Shp-2-dependent signaling switch in the PRL/PRLr pathway in breast cancer cells.

Discussion:

SIRPα activation and modulation has been well documented in neurons, macrophages and myeloid cells, however, this is the first report describing its function in breast cancer cells [37, 38]. SIRPα has one known ligand, CD47, but can also be activated by various ligand engaged cell surface receptors including integrins [18, 37]. CD47, also named integrin-associated protein (IAP), is expressed in a variety of cell types in the immune system, central nervous system as well as muscle progenitor cells. One recent report has indicated that CD47 is
also expressed in a few breast cancer cell lines including MCF-7 and MDA-MB-231 [39]. This study showed that CD47 mediated apoptosis of breast cancer cells through the inhibition of protein kinase A [39]. CD47 has also been shown to regulate intestinal epithelial cell migration primarily through collagen 1-induced COX-2 expression [40]. However, the best characterized function of the CD47-SIRPα signaling complex in the immune system is to inhibit macrophage phagocytosis of red blood cells or platelets [19]. In addition, the CD47-SIRPα complex negatively regulates the immune system by suppressing both the maturation of immature dendritic cells (DCs) and the production of cytokines by mature DCs [41].

SIRPα also has been shown to negatively regulate growth hormone receptor (GHR)/Jak2 signaling in 3T3 and COS-7 cells [34]. In this report, data revealed that this negative regulation was through the ability of SIRPα to associate with the phosphatase Shp-2. In addition, these studies demonstrated that in response to GH stimulation, phosphorylation of the four tyrosine sites on the C-terminal tail of SIRPα were required for recruitment of Shp-2, while the interaction of Jak2 and SIRPα was independent of the phosphorylation status of SIRPα [21, 34]. Since the GHR is the closest PRLr homolog, we hypothesized that SIRPα may affect PRLr signaling in a similar manner. In light of these data, this study sought to examine SIRPα overexpression in the context of PRLr signaling in breast cancer cells.

The PRL/PRLr complex in mammary epithelial cells utilizes the Jak2/Stat5 as well as MapK and PI-3-K signaling cascade and this pathway in part, contributes to the proliferation, differentiation, survival and invasion of mammary epithelial cells [1, 4, 42]. Results in this report demonstrated that in the SIRPα overexpression transfectants, Jak2 is constitutively associated with SIRPα (Figure 2B), findings that are congruent with published interaction data in
COS-7 [34]. In addition, the overexpression of SIRPα in the absence of collagen 1 decreased Jak2, Stat5 and Erk phosphorylation through its inhibition of PRLr signaling (Figure 5). This resulted in down regulation of PRL-induced CISH transcription in the absence of collagen 1, as demonstrated by luciferase and western experiments (Figure 3, 4 and 5). Furthermore, overexpression of SIRPα in absence of collagen 1 inhibited anchorage dependent and independent growth as demonstrated by proliferation and soft agar colony formation experiments (Figure 6 and 7). As shown here, the inhibition of PRL-mediated signaling by SIRPα reveals that in the absence of collagen 1, SIRPα is a negative regulator of PRLr signaling. These results are consistent with results reported in the literature that SIRPα is a negative regulator of GH, EGF and PI-3-K signaling pathways in fibroblasts, neurons and macrophages [18, 34, 37].

Crosstalk between cell surface receptors leads to the modulation of signaling cascades and may alter breast cancer cell growth and progression [12]. Data presented in this study demonstrates in both ER+ and ER- breast cell lines that SIRPα, the PRLr, β1 integrin and Jak2 reside in a partially inducible complex at the cell surface (Figure 1 and 2). There is current evidence that integrins interact with other cell surface receptors to initiate and drive migration and metastasis [43]. For example, integrins can cooperate with the EGFr resulting in a chemotactic response to EGF [44]. In addition, integrins have been implicated in facilitating the epithelial to mesenchymal transformation observed in neoplastic cells [43]. Given this, and the observation that β1 integrin is required for PRLr signaling, it was hypothesized that SIRPα may function as an intermediary between the PRLr and β1 integrin [45].

Our findings revealed that SIRPα overexpression inhibited PRL-induced CISH promoter luciferase activity in the presence laminin and fibronectin (Table 1). In the presence of collagen
1, however, SIRPα overexpression enhanced PRL-induced CISH promoter luciferase activity and the inhibition of β1 integrin in the presence of collagen 1 reversed these effects (Figure 4). These novel data suggest that collagen 1 (a β1 integrin ligand) promotes a functional switch in SIRPα’s regulation of the PRLr pathway from that of a negative regulator to that of a positive regulator. Taken together, our data in Figures 1-4 and Table 1 highlight a potential role for SIRPα in mediating crosstalk between the PRLr and integrins in breast cancer cells. Such crosstalk could contribute to the aggressive behavior of breast cancer cells once they invade through the basement membrane into the interstitial space of breast tissues.

SIRPα is phosphorylated on its four cytoplasmic tyrosine residues in response to growth factors, hormones and integrins [46]. The phosphorylation of SIRPα leads to the recruitment, binding, phosphorylation and activation of Shp-2 as well as Shp-1 [21, 34]. There is some debate in the literature as to whether the phosphorylation of Shp-2 confers activity. However, recent studies suggest that the phosphorylation of Shp-2 leaves it in an open conformation allowing it to act as a phosphatase [47, 48]. Several studies have demonstrated that Shp-2 positively effects the MapK as well as the Jak2/Stat5 signaling cascade [13, 25, 49]. Conversely, Shp-2 has also been shown to negatively regulate Stat family proteins including Stat 1,-3, and -5 in different cell types by the de-phosphorylation of up-stream Jak family kinases. In the mammary gland, Shp-2 regulates PRLr signaling by inhibiting the activity of both Jak2 and Stat5 [21, 26]. Our data revealed that in the presence of collagen 1 SIRPα overexpression decreased Shp-2 phosphorylation. However, in the absence of collagen 1 there was an increase in Shp-2 phosphorylation (Figure 5). This dual regulation may be a direct result of SIRPα’s ability to alter Shp-2 activation in the presence or absence of collagen 1-bound integrins. Given that: 1)
the tyrosine residues of SIRPα are necessary for its mediation of ECM-modulated PRL-driven signaling, 2) the tyrosine phosphorylation of SIRPα did not change in the absence or presence of collagen 1 (Figure 5) and, 3) the overexpression of a DN-Shp-2 and SSG prevented SIRPα-mediated potentiation of PRLr signaling (Figure 8A), it was hypothesized that a specific tyrosine kinase or phosphatase exists within the PRLr-SIRPα-integrin complex that directly modulates Shp-2 activity as a function of integrin occupancy. Several candidates such as Jak2, Src, Fak or Shp-1 may be involved in this alteration of Shp-2. However, this is not the only possible hypothesis that could explain the regulation of Shp-2 activity. Alternatively, the sequestration of Shp-2 by SIRPα in the presence of collagen 1 and PRL may result in its functional inactivation.

Use of the Shp-2 inhibitor SSG resulted in comparable effects to DN-Shp-2. SSG is currently used in the treatment of Leishmania however, its mechanism of action has been incompletely understood [50]. A recent study demonstrated SSG as an effective inhibitor of Shp-2. In addition, this study implied that SSG may be effective in the treatment of AML by overcoming cancer cell resistance to IFN treatment [36]. In support of this notion, it was demonstrated that using SSG as a single agent in a dose-dependent manner inhibited the growth of prostate, breast, bladder and colon cancer cells in culture [36]. Our data shows that SSG blocks the effects of SIRPα overexpression on ECM-modulated, PRL-driven gene expression (Figure 8C and D). Collectively, our data suggests that SIRPα’s mechanism of action are through the phosphatase Shp-2, and that a PTP inhibitor such as SSG could be used alone or in combination with other anticancer therapies effectively in the treatment of ER+ and ER- breast cancers.
In this report, we sought to elucidate a role for SIRPα in PRLr-integrin mediated signaling. This is the first report to demonstrate that SIRPα facilitates such cross-talk in ER+ and ER- breast cancer cell lines. Importantly, data in this paper revealed that SIRPα overexpression in the presence of collagen 1 increased cellular proliferation and soft-agar colony formation (Figure 6 and 7). This regulation of PRL-induced signaling and function was associated with the phosphorylation status and activity of Shp-2. As Shp-2 can either be a negative or positive regulator of PRLr signaling, we hypothesize that SIRPα facilitates the modification of Shp-2 activity in a collagen 1/PRL-dependent manner. Our data revealed that induced alterations in Shp-2 activity or levels (by SSG or DN-Shp-2, respectively) were associated with altered cell signaling, proliferation and anchorage-independent growth, processes that contribute to tumor progression. Collectively, these data implicate an important function for SIRPα in breast cancer in its regulation of PRL-induced function and suggest a potential role for SIRPα as therapeutic target.

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Figure legends:

Figure 1: Interactions between the PRLr, SIRPα and β1 integrin in ER+ and ER- breast cancer cells: (A and B) T47D (ER+) and MDA-MB-231 (ER-) cells were treated with PRL (250ng/ml) for times indicated. Whole cell lysates were immunoprecipitated and analyzed by western blotting. All results reported were representative of three individual experiments. Each of the three experimental blots were quantitated and bar graphs represent fold increase as compared to the control transfectants (±SEM) (*p-value<.05, **p-value<.001, ***p-value<.0001).

Figure 2: Interactions between SIRPα and Jak2 as well as β1 integrin in T47D cells: (A and B) T47D transfectant pools were treated with PRL (250ng/ml) for times indicated. Whole cell lysates were immunoprecipitated and analyzed by western blotting. (E) T47D transfectant pools were harvested and analyzed by western blotting to demonstrate overexpression of SIRPα as compared to the control transfectants. Tubulin was used as a loading control. (C and D) All results reported were representative of three individual experiments. Each of the three experimental blots were quantitated and results represent fold increase as compared to the control transfectants (±SEM) (**p-value<.01, ***p-value<.001).

Table 1: Effects of extracellular matrix (ECM) on PRL-induced gene transcription in breast cancer cells overexpressing SIRPα: T47D transfectant pools were plated in the absence or presence of each ECM and transfected with the PRL-responsive luciferase reporter CISH. Cells were treated with PRL (250ng/ml) for twenty four hours and analyzed for dual luciferase. Results reported represent fold increase or decrease (±SEM) as compared to the control transfectants (**p-value<.01, ***p-value<.001).
Figure 3: PRL-induced CISH promoter luciferase activity and protein expression is enhanced by SIRPα in a collagen 1-dependent manner in T47D cells: (A and B) T47D transfectant pools were plated in the presence or absence of collagen 1 and transfected with a PRL-responsive CISH luciferase promoter. The cells were treated with PRL (250ng/ml), harvested and analyzed for dual luciferase. Results reported as luciferase/renilla ratio (three individual experiments) and represent increase or decrease in luciferase activity (±SEM) as compared to the control transfectants (**p-value<.01, ***p-value<.001). (C and D) T47D transfectant pools were plated in the presence or absence of collagen 1, stimulated with PRL (250ng/ml) for times indicated, lysed and analyzed by western blot for CISH protein expression. Tubulin was used as a loading control. (E) All results reported were representative of three individual experiments. Each of the three experimental blots were quantitated and results represent fold increase as compared to the control transfectants (±SEM).

Figure 4: PRL-induced CISH promoter luciferase activity is enhanced by SIRPα in a collagen 1/β1 integrin-dependent manner in T47D cells: T47D transfectant pools were plated in the presence or absence of collagen 1 in addition to collagen 1 plus an anti-β1 integrin antibody (+α-β1) and transfected with a PRL-responsive CISH luciferase promoter. The cells were treated with PRL (250ng/ml), harvested and analyzed for dual luciferase. Results were reported as luciferase/renilla ratio and represent increase or decrease in luciferase activity as compared to the control transfectants (**p-value<.01, ***p-value<.001). The results reported were representative of three individual experiments (±SEM).

Figure 5: SIRPα differentially regulates PRLr signaling in the absence or presence of collagen 1 in T47D cells: T47D transfectant pools plated in the presence or absence of collagen 1,
stimulated with PRL (250ng/ml) for times indicated, lysed and analyzed via western blotting for corresponding protein phosphorylation. Densitometry was performed and values are reported as fold increase (±SEM) compared to the untreated control transfectants. The results reported are representative of three individual experiments (**p-value<.01, ***p-value<.001).

**Figure 6:** SIRPα differentially regulates proliferation in the absence or presence of collagen 1: (A and B) T47D or (C and D) MDA-MB-231 transfectant pools were plated in the presence or absence of collagen 1, stimulated with PRL (250ng/ml) for seventy two hours and ³H thymidine for four hours. Results are reported as ³H thymidine incorporation (±SEM) as compared to the control transfectants (*p-value<.05, ***p-value<.001). Results are representative of three individual experiments.

**Figure 7:** SIRPα differentially regulates soft agar colony formation in a PRL and collagen 1-dependent manner in T47D cells: (A) T47D transfectants were suspended in a 0.3% Agar-media mix supplemented with PRL (250ng/ml) for 10 days. (B) T47D transfectants were suspended in a 0.3% Agar-media mix supplemented with 10% collagen 1 and PRL (250ng/ml) for 10 days. Each condition was photographed under phase contrast optics. (C) Quantification of the results of the soft-agar colony formation. Results reported as average number of colonies per field and represent differences in soft agar colony formation (±SEM) as compared to the control transfectants (**p-value<.01) and represent three individual experiments.

**Figure 8:** DN-Shp-2 or Sodium Stibogluconate (SSG) reversed the effect of SIRPα overexpression on PRL-induced CISH promoter luciferase activity in the absence or presence of collagen 1: (A and B) T47D transfectants were plated in the presence or absence of collagen 1 and transfected with a PRL-responsive CISH luciferase promoter and/or a phosphatase defective
mutant of Shp-2 (DN-Shp-2). The cells were treated with PRL (250ng/ml), harvested and analyzed for dual luciferase. (C and D) T47D transfectants were plated in the presence or absence of collagen 1 and transfected with a PRL-responsive CISH luciferase promoter. The transfectants were treated with SSG (100ng/ml) for four hours prior to PRL stimulation (250ng/ml), harvested and analyzed for dual luciferase. Results were reported as luciferase/renilla ratio (±SEM) and represent increase or decrease in luciferase activity as compared to the control transfectants (**p-value<.001). Results are representative of three individual experiments.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>WT-SIRPa</th>
<th>SIRPa-4YF</th>
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</thead>
<tbody>
<tr>
<td>No ECM</td>
<td>1.0</td>
<td>*** 0.5(±2.2)</td>
<td>0.9(±3.1)</td>
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<td>Laminin</td>
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<td>*** 0.3(±2.5)</td>
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<td>Fibronectin</td>
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<td>1.0(±2.7)</td>
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<tr>
<td>Matrigel</td>
<td>1.0</td>
<td>*** 0.5(±1.2)</td>
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<td>Collagen 1</td>
<td>1.0</td>
<td>** 2.5(±1.4)</td>
<td>0.9(±5.0)</td>
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Figure 3

A. (-) Collagen 1

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<td>CISH</td>
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<td></td>
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</tr>
<tr>
<td>α-Tubulin</td>
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B. (+) Collagen 1

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<tr>
<td>α-Tubulin</td>
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C. (-) Collagen 1

D. (+) Collagen 1

E. Fold induction

<table>
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<td></td>
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</tr>
<tr>
<td>α-Tubulin</td>
<td></td>
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</tbody>
</table>
Figure 6

T47D

A. (-) Collagen 1

B. (+) Collagen 1

MDA-MB-231

C. (-) Collagen 1

D. (+) Collagen 1

3^H Thymidine incorporation

Cont WT-SIRP 4YF Cont WT-SIRP 4YF

Cont WT-SIRP 4YF Cont WT-SIRP 4YF

Cont WT-SIRP 4YF Cont WT-SIRP 4YF

Cont WT-SIRP 4YF Cont WT-SIRP 4YF

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### Figure 7

#### A.

<table>
<thead>
<tr>
<th>Control: (-)Collagen 1 (+)PRL</th>
<th>WT-SIRP: (-)Collagen 1 (+)PRL</th>
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</thead>
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#### B.

<table>
<thead>
<tr>
<th>Control: (+)Collagen 1 (+)PRL</th>
<th>WT-SIRP: (+)Collagen 1 (+)PRL</th>
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#### C. Average number of colonies

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<th>WT-SIRPα</th>
<th>Control</th>
<th>SIRPα-4 FP</th>
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<tbody>
<tr>
<td>PRL (250ng/ml)</td>
<td>103(±2.2)</td>
<td>105(±4.3)</td>
<td>102(±5.5)</td>
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<td></td>
<td><strong>115(±2.7)</strong></td>
<td>143(±4.0)</td>
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<tr>
<td></td>
<td>125(±3.5)</td>
<td>125(±5.6)</td>
<td>116(±3.2)</td>
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<tr>
<td></td>
<td><strong>180(±3.3)</strong></td>
<td>150(±9.8)</td>
<td>144(±2.1)</td>
</tr>
</tbody>
</table>
Figure 8

A. (-) Collagen 1

B. (+) Collagen 1

C. (-) Collagen 1

D. (+) Collagen 1

Graphs showing the effect of different conditions on quantifiable parameters.
Molecular Cancer Research

Prolactin Receptor-Integrin Crosstalk Mediated by SIRPα in Breast Cancer Cells

Traci Galbaugh, Yvonne Feeney and Charles V Clevenger

Mol Cancer Res  Published OnlineFirst September 8, 2010.

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