Published in final edited form as:

Prostate. 2010 January 1; 70(1): 37–47. doi:10.1002/pros.21036.

Long Term Increased Expression of the Short Form 1b Prolactin Receptor in PC-3 Human Prostate Cancer Cells Decreases Cell Growth and Migration, and Causes Multiple Changes in Gene Expression Consistent with Reduced Invasive Capacity

Kuang-tzu Huang and Ameae M. Walker

Division of Biomedical Sciences, University of California, Riverside, Riverside, CA 92521

Abstract

Background—We have shown that treatment of human prostate cancer cells with the selective prolactin (PRL) receptor modulator, S179D PRL, inhibits growth in vitro, and the initiation and growth of xenografts in vivo. S179D PRL treatment also upregulates expression of the short form 1b (SF1b) PRL receptor, activation of which upregulates expression of the cell cycle-regulating protein, p21.

Methods—We examined the consequences of long term increased expression and activation of SF1b, at levels comparable to those resulting from treatment with S179D PRL, by creating PC-3-derived stable cell lines expressing a constitutively-active form of SF1b, Δ S2 SF1b.

Results—Increased expression of Δ S2 SF1b decreased growth and migration of the cells. This was accompanied by an increase in cell-matrix interactions, and cell-cell aggregation when cells were plated on basement membrane components. Real-time PCR evaluated the expression of genes related to invasive capacity. Of particular interest was decreased expression of the protease, urokinase-type plasminogen activator, and its receptor, uPAR, and increased expression of its inhibitors, PAI-1 and 2. Also decreased in cells with increased expression of Δ S2 SF1b was expression of basic fibroblast growth factor and vascular endothelial growth factor.

Conclusion—We conclude that at least part of the beneficial effects of S179D PRL is the result of increased expression of SF1b, and that the effects of increased expression and activation of SF1b continue to be of potential benefit in the long term.

INTRODUCTION

Evidence has accumulated over about the last decade that prolactin (PRL) is a significant growth factor for the prostate. Thus, transgenic mice over-expressing PRL developed dramatic enlargement of the prostate gland (1). In addition, knockout of PRL (2) or the PRL receptor (PRLR) (3) produced mice with smaller glands. Also, use of the dopamine agonist, bromocriptine, demonstrated that dysplasia in the dorsolateral prostate induced by prolonged treatment with testosterone and estradiol was in fact due to the effects of PRL (4). However, there is to date no correlative evidence linking circulating PRL levels to the incidence of human prostate disease (5,6). One major reason for the absence of such a correlation may be the existence of prostate autocrine PRL (7); this could produce a local elevation in PRL without significant effect on circulating levels. Using transgenic animals with prostate epithelium-specific over-expression of PRL, work from the Wennbo laboratory has indeed

been able to show the development of significant prostate hyperplasia while elevating circulating PRL by only 5–10ng/ml (8,9).

A number of additional studies in rats and on human tissue have further demonstrated that locally-produced (autocrine/paracrine) PRL affects prostate function, and that PRL is normally mainly synthesized in the epithelium (7,10). Work in our laboratory has also shown that the epithelial autocrine PRL growth loop is maintained in the human prostate cancer cell lines, LNCaP, DU145 and PC-3 (11). Furthermore, we and others have shown that blocking the autocrine growth loop with PRL receptor-specific antagonists, including $\Delta 1$ -9G129R-PRL and S179D PRL, inhibits proliferation of prostate cancer cells (11–14). S179D PRL has also been shown to inhibit both the initiation of tumors and growth of well-established tumors produced as DU145 xenografts in nude mice (11).

There are multiple isoforms of the PRL receptor (PRLR). The major isoforms in humans are the long form (LF), intermediate form (IF), and two short forms (SF1a and SF1b). These isoforms are produced by alternative splicing (15–18). They share identical amino acid sequences in their ligand-binding, extracellular domains, but differ in length in their signal-transducing, intracellular domains.

We have previously shown that inhibition of growth of prostate cancer cells with S179D PRL upregulates expression of the SF1b receptor and, through transient transfection, that this in turn upregulates the cell cycle inhibiting protein, p21, and the differentiation-promoting vitamin D receptor (VDR) (14,19). We have also recently demonstrated that a form of the SF1b receptor, lacking about half of the extracellular domain (designated Δ S2 SF1b), occurs naturally, does not bind PRL, and is constitutively active (20).

In the current study, we have taken advantage of the constitutive activity of Δ S2 SF1b to ask whether induction of long term increased expression and activation of SF1b (such as would occur with long term S179D PRL treatment) in prostate cancer cells had continuing beneficial effects. To do this, we created stable PC-3-derived cell lines expressing the constitutively active Δ S2 version. We found that PC-3 human prostate cancer cells stably expressing Δ S2 SF1b had decreased growth rates and migratory capacities.

MATERIALS AND METHODS

S179D PRL was made, purified and tested for biological activity as previously described (21).

Cell culture and transfection

PC-3 human prostate cancer cells were purchased from American Type Culture Collection. Cells were routinely cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂.

For stable transfection, PC-3 cells were seeded in 10-cm culture dishes one day before transfection. Transfection was performed when cells reached 80–90% confluency. The transfection mixture was made with 10 μ g of pcDNA3.1(+)/ Δ S2 SF1b and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in serum-free RPMI according to the manufacturer's protocol. A separate transfection for the empty vector was performed in parallel to produce control cells. After 2 days, cells were equally subcultured into ten 10-cm dishes and selected with 400 μ g/ml Geneticin (G418 sulfate, Invitrogen). Selection medium was changed every 3–4 days. After 2–3 weeks, individual colonies were isolated and cultured in 24-well plates. Cell growth was carefully observed. Expression of Δ S2 SF1b was examined by Western blotting.

Transient transfections were performed using Fugene HD transfection reagent (Roche Diagnostics, Indianapolis, MN) according to the manufacturer's protocol. Briefly, 2×10^6 cells were seeded in each 6 cm dish in regular culture medium the day before transfection. On the next day, to make the transfection mixture, 5 μ g DNA (per dish) was first incubated with 250 μ l serum-free RPMI, and 10 μ l Fugene HD reagent was then added. The mixture was incubated for 15 min and then added to the culture. Medium was changed to RPMI containing 1% FBS after 24h. Cells were harvested for analysis after another 18–24h.

Cell proliferation assay

Cell proliferation was evaluated both by direct cell counting and the MTS formazan conversion assay. For direct cell counting, 10^5 cells were seeded into triplicate wells in 6-well plates. Plating efficiency was the same for all lines. After 3 days, cells were removed from the substratum with trypsin and resuspended in 0.5 ml culture medium. Ten μ l samples of the suspension were stained with trypan blue and the cell number counted with the aid of a hemocytometer. Cells that were stained blue were excluded. The MTS assay was performed under stringent conditions as previously described (22). Briefly, cells were seeded at a density of 10^4 per well in quadruplicate in a 96-well plate. After 3 days, the culture medium in each well was changed to serum-free. MTS reagent was freshly prepared (2 mg/ml MTS, 0.92 mg/ml PMS in Dulbecco's phosphate buffered saline (DPBS)) and 20 μ l of the reagent was added to 200 μ l of medium in each well. Absorbance was recorded at 492 nm after 2h of incubation at 37°C.

Western blotting

Whole cell extracts were first prepared by solubilizing cells in cell lysis buffer (0.14 M NaCl, 20 mM Tris base, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 10 mM NaF, 1 mM Na_3VO_4 , 1% NP-40, 0.02% NaN_3 , and 0.5 mM EDTA) supplemented with 1X Complete Mini protease inhibitor cocktail (Roche Diagnostics), followed by 2 freeze-and-thaw cycles. Clear lysates were collected after centrifugation. For Western detection of Δ S2 SF1b, equal quantities of whole cell lysates (20 μ g) were run on a reducing 10% SDS gel and then proteins were transferred onto a nitrocellulose membrane. Blots were probed with mouse anti-human PRLR extracellular domain (1:1000, Invitrogen), which recognizes parts of the S1 domain. Antigen-antibody interactions were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratory, Bar Harbor, ME) and the ECL Plus chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Antibodies for additional analyses included mouse anti E-cadherin (1:2000, BD Transduction Laboratories, San Jose, CA), and goat anti-actin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Comparisons with isotype or normal serum controls in preliminary experiments demonstrated the specificity of antibody reactions.

Wound healing assay

PC-3 cells stably transfected with Δ S2 SF1b or the empty vector were seeded onto 6-well plates in duplicate, and cultured until confluent. The cell monolayer was wounded by scraping with a sterile pipette tip. Each well was then washed and the medium replaced. Wound closure was examined over time and photographed after 16h. Photographs were of three adjacent areas in the center of each well. The number of cells that migrated into the open wound areas in all three images was recorded.

Transwell migration assay

PC-3 cells stably transfected with Δ S2 SF1b or the empty vector were seeded at 10^5 cells per well into the upper region of Transwell™ chambers (8.0 μ m, 24-well, Corning Life

Sciences, Lowell, MA) in serum-free RPMI 1640. The lower chamber was filled with RPMI 1640 containing 10% FBS as a chemoattractant. Following 16h of incubation, cells were fixed with methanol for 15 min. Cells that had not migrated through the membrane were wiped out with a cotton swab. Cells on the lower surface of the membrane were stained with 1% toluidine blue and photographed. Images of each quadrant were obtained and the number of cells that had migrated to the lower surface was recorded. Assays were performed in duplicate on each occasion.

Matrigel aggregation

Once aggregation had been observed on Matrigel-coated membranes in the Transwell™ system, further experiments were conducted in normal 6-well plates. Matrigel coatings were allowed to gel for 15 min prior to plating of cells. The cells were then photographed 16h later. Four images per well were acquired and then Adobe photoshop software was used to calculate the relative pixel area occupied by each cluster of cells.

Indirect immunofluorescence staining

Immunohistochemistry was used to localize transfected ΔS2 SF1b PRLR or endogenous E-cadherin. Cells were grown to confluency on chamber slides (Nalge Nunc, Naperville, IL). Attached cells were first washed with DPBS, fixed in 4% paraformaldehyde (in DPBS) for 15 min, and then permeabilized with 0.1% Triton X-100 (in DPBS) for another 10 min. Non-specific binding was blocked by incubation in 1% BSA (in DPBS) for 1h. Cells were then incubated overnight at 4°C in mouse anti-PRLR ECD (1:100) or mouse anti E-cadherin (1:100) prepared in DPBS containing 0.1% Tween-20 (PBST). Unbound antibodies were removed by washing 3 times with PBST. Cells were subsequently incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1: 1000, Molecular Probes, Carlsbad, CA) for 1h at room temperature. After 3 washes with PBST, the slides were mounted using Prolong Antifade (Molecular Probes) and cells were visualized under the Zeiss 510 confocal microscope at × 250 magnification. Staining controls included the use of non-specific isotype-matched antibodies (not shown). All images of control vector versus ΔS2SF1b cells were obtained with the same gain settings.

Semi-quantitative and real-time RT-PCR

Total RNA was extracted using Trizol (Invitrogen). First-strand cDNA synthesis was achieved using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. For the semi-quantitative PCR, 0.5 μl first-strand cDNA was used in a 25 μl reaction with GoTaq DNA polymerase (Promega). Primer sequences are listed in Table I. Amplifications were performed as follows: initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. A final extension was carried out at 72°C for 5 min. The amplified products were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

For real-time PCR, the reaction mixture was prepared in Optical 96-well reaction plates (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems). Detection was performed on an ABI Prism 7700 (Applied Biosystems) with parameters as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Quantification of expression relative to controls was analyzed using the $2^{-\Delta\Delta CT}$ method, which can be applied when control levels are significant. PCR amplicons were examined by agarose gel electrophoresis to confirm the expected product sizes.

Statistical analyses

Analysis of statistical significance was by Student's t-test, with corrections for multiple comparisons.

RESULTS

Effect of S179D PRL on SF1b expression

We have previously demonstrated that S179D PRL treatment of several prostate cancer cell lines increases expression of the SF1b receptor at both the mRNA and protein level (14,20). Here we examined the dose-relatedness of this response in PC-3 cells and, by using traditional PCR, concurrently examined the effect on expression of the naturally-occurring Δ S2 SF1b form. This latter aspect cannot be achieved by real time PCR since the region specific to SF1b receptors is small, dictating the use of one primer common to both the SF1b and Δ S2 SF1b forms. For both receptors, the effect of a 5-day incubation in S179D PRL was greater at 100 versus 10 ng/ml (4.3 versus 0.43 nM) (figure 1A). Induction of intact SF1b protein by S179D PRL is shown in figure 1B.

Δ S2 SF1b expression

In order to evaluate the long-term effect of increased expression and activation of the SF1b receptor, we established stably transfected PC-3 lines harboring the constitutively active form. Our aim was to use the constitutively active form as a surrogate for S179D PRL-activated receptor and therefore to express the constitutively active form at a level similar to intact SF1b seen in figure 1. Expression plasmids containing the cDNA of Δ S2 SF1b were introduced into human prostate PC-3 cancer cells. After 2–3 weeks of antibiotic selection, single cell-derived colonies were isolated for analysis. Expression of the Δ S2 SF1b was demonstrated by Western blotting. Among several isolated stable clones, one with lower expression (L) and another with higher expression (H) were selected and subjected to further analyses (figure 2A). Most of the assays were performed using the L cell line since this model most closely approximated SF1b expression levels in prostate cancer cells treated with S179D PRL. A cell line stably transfected with the empty vector served as the negative control.

PC-3 cells normally express very low levels of each of the PRL receptors (14,23). Confocal microscopy after immunocytochemistry for the receptor therefore shows very low fluorescence in the vector control cells. Fluorescence is markedly increased in the Δ S2 SF1b L transfected cells. Staining is present throughout the cell as a result of translation in the rough endoplasmic reticulum, and processing and posttranslational modification in the Golgi. However, most staining is towards the cell periphery (figure 2B). H cells showed increased intracellular staining, but similar levels at the cell periphery (data not shown).

Increased expression of Δ S2 SF1b reduces cell number

Control and Δ S2 SF1b PC-3 cells were plated at the same number per well and the viable number after a 3 day incubation in normal growth medium (in the absence of added PRL) was recorded. This was achieved both by direct cell counting with trypan blue exclusion, and by a commercial dye reduction method (MTS assay). For the latter, the medium was changed to serum-free prior to the addition of reagents, as described previously (22). Results showed reduced cell number with expression of Δ S2 SF1b. For direct cell counting, there was a trend indicating dose responsiveness, but the difference in cell number was only statistically significantly different when comparing the control and H cells (figure 3A). Using the MTS indirect measure of viable cell number, there was a significant reduction even with the L cells (figure 3B). This difference in apparent degree of effect seen with the two methods could be related to the higher errors and therefore lower sensitivity of trypan

blue exclusion as a method for detecting viable cells or, since the MTS assay actually measures mitochondrial function, some other effect of expression of SF1b on mitochondrial function. Whichever is the case, there is no doubt that Δ S2 SF1b cells grew more slowly.

Δ S2 SF1b expression causes redistribution of E-cadherin

While no significant differences in morphology were observed between Δ S2 SF1b and control PC-3 cells when these cells were grown at low density, we found that when they became more confluent, Δ S2 SF1b cells were more compact and epithelioid in appearance (figure 4A). Moreover, it took 2–3 times longer (10–15 min versus 5 min.) to detach Δ S2 SF1b cells versus control cells by treatment with 0.05% trypsin-EDTA, indicating a greater production of extracellular and linking transmembrane proteins. We therefore examined expression of an adherens junction component, E-cadherin. Western blotting showed no significant differences in total protein expression (figure 4B upper), but indirect immunofluorescence staining of E-cadherin showed a different distribution of the protein between PC-3 control and Δ S2 SF1b cells (figure 4B lower): in control cells, staining was concentrated and punctate, whereas staining was more evenly spread and peripheral in Δ S2 SF1b L cells, a finding consistent with adherens junction formation. Δ S2 SF1b H cells showed very similar results (data not shown).

Increased expression of Δ S2 SF1b decreases cell migration

Increased adherence to the substratum and to other cells suggested the possibility that increased expression of Δ S2 SF1b might affect cell migration. To test this, cultures were first subjected to a “wound healing” assay. Wounds were produced in a monolayer of confluent control or Δ S2 SF1b PC-3 cells. After 16h, the extent of wound closure was observed and the number of cells that migrated into the open area was recorded. As shown in Figure 5, at the end of the assay, vector control cells migrated to fill about half the cleared area, whereas for Δ S2 SF1b cells, migration was significantly inhibited in a Δ S2 SF1b expression level-dependent manner. In this 16h assay, there was no effect on cell number, as assessed by the more sensitive MTS assay (not shown).

A second assay examined migration in a Transwell™ system: here, half as many Δ S2 SF1b L cells as control cells migrated from one side of the filter to the other (figure 6A). This is shown pictorially and after quantification. A control MTS assay was also performed in parallel with the Transwell™ assay and once again no difference in cell number was found (not shown), indicating that the different growth rate of the Δ S2 SF1b L and control cells was not a contributing factor in the 16h time frame. This assay was not performed with H cells.

When Matrigel coating of the membranes was used to similarly examine one aspect of cell invasion, this could not be accomplished since Matrigel™ caused aggregation of control PC-3 cells, which then couldn't traverse the membrane, even when the incubation period was extended to 24h. However, this assay did provide new information in that Δ S2 SF1b L cells formed larger clusters compared to the control cells. This is also shown pictorially and quantitatively, the latter as a function of the two-dimensional size of the clusters (figure 6B). This assay was not conducted with the H cells.

Effect of Δ S2 SF1b on cell migration-related genes

The expression level of matrix metalloproteases (MMP), in particular MMP-9 and MMP-2, and urokinase plasminogen activators (uPA) has been linked to increased migratory, and therefore metastatic, ability of cancer cells (24–28). We therefore examined steady state mRNA levels of MMP-9, MMP-2, uPA and its receptor (uPAR), and the tissue inhibitors of both matrix metalloproteases (TIMP-1 and TIMP-2) and plasminogen activators (PAI-1 and

PAI-2) by real-time RT-PCR. As shown in figure 7, the levels of both MMP-9 and uPA mRNA were significantly lower in Δ S2 SF1b cells, and the effect was Δ S2 SF1b expression level-dependent for uPA. MMP-2 was nearly undetectable even in the control cells (not shown). A Δ S2 SF1b expression level-dependent increase of PAI-1 mRNA was also observed. The increase in PAI-2 mRNA was greater than for PAI-1 and maximal at the lower Δ S2 SF1b expression level, although still significantly elevated with the higher expression level. To complete the picture with uPA, mRNA for uPAR was examined. Here, the response showed little effect in the Δ S2 SF1b L cells, but a decrease in the H cells. No significant change in the mRNA level of either TIMP was observed.

For two crucial growth factors implicated in increased cancer cell proliferation and migration (29–32), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), the mRNA levels were down-regulated in Δ S2 SF1b cells.

β -catenin plays different roles in the cell, dependent upon its relative expression level versus E-cadherin, as well as its distribution (33–35). With increased expression of Δ S2 SF1b, we found decreased levels of β -catenin mRNA, an effect that was dependent on the level of expression of Δ S2 SF1b.

DISCUSSION

We have previously shown that there are beneficial effects of short term treatment with S179D PRL which might suggest its use as a therapeutic for prostate cancer, especially since these effects are manifest in cell lines and xenografts representative of androgen independent stages of the disease (11). One major change that occurs in multiple cell lines (including androgen independent prostate cancer lines) in response to S179D PRL is a change in splicing of pre-mRNA for the PRLR such that there is an increase in expression of the SF1b receptor (e.g. 14,36). From previous experiments, we learned the short term effects of increased expression of SF1b, which included increased expression of the cell cycle regulatory protein, p21, and the differentiation-inducing VDR (14,19). Both effects occurred co-incidentally with a decreased growth rate (14). In the current study, our interests lay in the long term effect of increased expression and activation of the SF1b receptor. By using the constitutively active form of SF1b and eliminating the need to add ligand, we could determine the long term effects of increased signaling specifically from SF1b in cells co-expressing other forms of the PRLR. We could therefore use a relevant prostate cancer cell line rather than in an experimental model lacking PRLR which may or may not possess important signaling molecules. If we had used the intact SF1b and therefore needed ligand, we would have activated other forms of the receptor along with SF1b.

In the current study, we confirmed that S179D PRL up-regulates SF1b in PC-3 cells. Stable, increased expression of the naturally-occurring, constitutively active Δ S2 SF1b in PC-3 prostate cancer cells decreased growth when compared with vector controls. This provides evidence that the growth-inhibiting effect of S179D PRL is, at least in part, through activation of the SF1b receptor. Since the cell lines had to survive despite increased expression of Δ S2 SF1b, it is likely that the degree of effect seen in the stable cell lines is less than an initial response to increased expression of SF1b would be. However, we chose the current model because we considered stable and continued expression more appropriate than an inducible system for examination of long term responses to S179D PRL. In other words, we asked could we find evidence of a continued beneficial effect of Δ S2 SF1b once the cells had adapted in order to survive, as they would with continued drug treatment.

Comparing PC-3 cells expressing Δ S2 SF1b to control cells, we observed that the cultures were more compact, that the cell-cell junctions were more distinct, and that in general the

cells had a more epithelioid appearance. Consistent with an apparent increase in cell-cell adhesion, immunostaining for E-cadherin demonstrated a more even, peripheral location in confluent Δ S2 SF1b cells compared with vector controls. E-cadherin is an essential component of adherens junctions and is required to maintain epithelial homeostasis. Loss of epithelial polarity and cell-cell contacts is often implicated in tumorigenesis and cancer metastasis (37–39). Increased cell-cell adhesion and cell-extracellular matrix interactions increase differentiation in many cell types and decrease migratory behavior (37–39). Here we show that increased expression of the constitutively active Δ S2 SF1b not only increases cell-cell and cell-substratum interactions, but also that it inhibits cell migration towards fetal bovine serum. The lactogens in fetal bovine serum have recently been shown to interact poorly with the human PRL receptor (40) and so in all likelihood, the migratory stimulus of serum is not due to any PRL present. Nevertheless, increased expression of Δ S2 SF1b was able to counteract it.

Cancer cell migration *in vivo* also involves invasion of the extracellular matrix (ECM) through enzymatic proteolysis of ECM constituents (41,42). Two major groups of proteases, matrix metalloproteases (MMPs) and plasminogen activators (PAs) have been described as key enzymes in the degradation of ECM. Among the MMPs, MMP-2 and MMP-9 are recognized for their ability to degrade type IV collagen, a major constituent of basement membranes (24–26). PAs are serine proteases that convert plasminogen to plasmin, which is then capable of degrading ECM components, including fibronectin and laminin (27,28). The urokinase-type PA (uPA) has also been identified as a serum biomarker for prostate cancer progression (43–45). In this study, we surveyed expression of these proteases and their natural inhibitors at the mRNA level. We demonstrate that increased expression of Δ S2 SF1b decreased expression of MMP-9, but only when Δ S2 SF1b was expressed at high levels. There was also no effect on the expression of TIMPs 1 and 2, and MMP-2 was barely detectable in control and Δ S2 SF1b cells. Assuming that mRNA levels correspond to protein levels, which in all likelihood are closely related when examining differences among stable cell lines, one might therefore suggest that changes in MMP in response to increased expression of Δ S2 SF1b are likely not important components altering migration in L cells. However, reduced MMP-9 may have contributed to the further reduction in migration in the H cells. Of greater interest and potential importance are the results with uPA since there was 1) a Δ S2 SF1b expression level-dependent decrease in uPA mRNA, 2) an expression level-dependent increase in PAI-1 mRNA, 3) an increase at both Δ S2 SF1b expression levels for PAI-2 mRNA, and 4) a decrease in uPAR at high expression. These results would certainly be consistent with a decrease in invasive capacity at both levels of Δ S2 SF1b expression. Even though the increase in PAI-2 was greater in the L versus H cells (a result which may be related to a biphasic dose response peaking at lower Δ S2 SF1b levels), this was still higher than control cells and, when paired with decreased uPAR in the H cells would be consistent with similar or even greater reduction in migratory ability. Unfortunately, we were unable to quantify an actual decrease in the Transwell™ invasion assay since the addition of basement membrane components to the assay led to aggregation of control PC-3 cells, which itself inhibited the ability of these cells to migrate. Nevertheless, the increased size of the aggregates with the Δ S2 SF1b cells under these circumstances bolstered the evidence in support of increased cell-cell interactions. Thus, increased expression of Δ S2 SF1b may reduce invasion by more than one means.

The quantitative RT-PCR results also demonstrated down-regulation of β -catenin with increased expression of Δ S2 SF1b. β -catenin plays two roles in the cells: when bound to the cytoplasmic domain of E-cadherin, it creates links to the actin cytoskeleton and stabilizes adherens junctions (33,34). In addition to its structural role, it also acts as a transcription factor in pathways involved in the stimulation of cell proliferation and cancer progression

(34,35,46–50). With no change in expression of E-cadherin, decreased levels of β -catenin mRNA in the Δ S2 SF1b cells are consistent with the decreased growth rate of these cells.

The quantitative RT-PCR results also demonstrated down-regulation of the growth factors, bFGF and VEGF in cells with increased expression of Δ S2 SF1b. Both growth factors are crucial to the regulation of cell proliferation, migration, angiogenesis and tumor progression (29–32). It has also been shown that bFGF activation promotes the production of uPA (51,52), but since the down-regulation of uPA in PC-3 cells expressing Δ S2 SF1b did not parallel that of bFGF, this may not be the only route for downregulation of uPA. Increased expression of SF1b and decreased expression of both bFGF and VEGF is also a consequence of treatment with S179D PRL in human vascular endothelial cells (36,53).

The short PRL receptors have been described as dominant negatives, an activity that is presumed to be due to heterodimerization between short and long receptors and resultant inhibition of signaling through the long receptor. Indeed work from this and other labs has shown that heterodimerization of long and short human receptors does occur, both in situations where there is overexpression of the receptors and natural levels of the receptors (54–56). However, the dominant negative activity measured was confined to Stat5-mediated signaling. Previous work from this lab has shown that increased expression of SF1a and SF1b receptors in the prostate cancer cell lines, DU145 and PC-3, leads to increased activation of other signaling pathways (14). Thus, the end effect of increased expression and activation of SF1b in response to S179D PRL is likely a combination of inhibition of Stat5 signaling and the generation of alternate signals.

We conclude that the effects of increased expression and activation of SF1b continue to be of potential benefit in the long term since there is decreased cell growth, decreased cell migration and indicators of decreased invasive capacity.

Acknowledgments

This work was supported by NIH grant DK61005 and by a grant from the Prostate Cancer Foundation. KTH was supported by an individual predoctoral fellowship from the Department of Defense Breast Cancer Research Program – BC0501103.

REFERENCES

1. Wennbo H, Kindblom J, Isaksson OG, Tornell J. Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. *Endocrinology*. 1997; 138:4410–4415. [PubMed: 9322957]
2. Steger RW, Chandrashekar V, Zhao W, Bartke A, Horseman ND. Neuroendocrine and reproductive functions in male mice with targeted disruption of the prolactin gene. *Endocrinology*. 1998; 139:3691–3695. [PubMed: 9724019]
3. Robertson FG, Harris J, Naylor MJ, Oakes SR, Kindblom J, Dillner K, Wennbo H, Tornell J, Kelly PA, Green J, Ormandy CJ. Prostate development and carcinogenesis in prolactin receptor knockout mice. *Endocrinology*. 2003; 144:3196–3205. [PubMed: 12810576]
4. Lane KE, Leav I, Ziar J, Bridges RS, Rand WM, Ho SM. Suppression of testosterone and estradiol-17 β -induced dysplasia in the dorsolateral prostate of Noble rats by bromocriptine. *Carcinogenesis*. 1997; 18:1505–1510. [PubMed: 9276623]
5. Odama S, Chisholm GD, Nicol K, Habib FK. Evidence for the association between blood prolactin and androgen receptors in BPH. *J Urol*. 1985; 133:717–720. [PubMed: 2580105]
6. Saroff J, Kirdani RY, Chu TM, Wajzman Z, Murphy GP. Measurements of prolactin and androgens in patients with prostatic diseases. *Oncology*. 1980; 37:46–52. [PubMed: 6153126]

7. Nevalainen MT, Valve EM, Ingletton PM, Nurmi M, Martikainen PM, Harkonen PL. Prolactin and prolactin receptors are expressed and functioning in human prostate. *J Clin Invest.* 1997; 99:618–627. [PubMed: 9045863]
8. Kindblom J, Dillner K, Sahlin L, Robertson F, Ormandy C, Tornell J, Wennbo H. Prostate hyperplasia in a transgenic mouse with prostate-specific expression of prolactin. *Endocrinology.* 2003; 144:2269–2278. [PubMed: 12746285]
9. Dillner K, Kindblom J, Flores-Morales A, Shao R, Tornell J, Norstedt G, Wennbo H. Gene expression analysis of prostate hyperplasia in mice overexpressing the prolactin gene specifically in the prostate. *Endocrinology.* 2003; 144:4955–4966. [PubMed: 12960074]
10. Ahonen TJ, Harkonen PL, Laine J, Rui H, Martikainen PM, Nevalainen MT. Prolactin is a survival factor for androgen-deprived rat dorsal and lateral prostate epithelium in organ culture. *Endocrinology.* 1999; 140:5412–5421. [PubMed: 10537173]
11. Xu X, Kreye E, Kuo CB, Walker AM. A molecular mimic of phosphorylated prolactin markedly reduced tumor incidence and size when DU145 human prostate cancer cells were grown in nude mice. *Cancer Res.* 2001; 61:6098–6104. [PubMed: 11507059]
12. Bernichtein S, Kayser C, Dillner K, Moulin S, Kopchick JJ, Martial JA, Norstedt G, Isaksson O, Kelly PA, Goffin V. Development of pure prolactin receptor antagonists. *J Biol Chem.* 2003; 278:35988–35999. [PubMed: 12824168]
13. Dagvadorj A, Collins S, Jomain JB, Abdulghani J, Karras J, Zellweger T, Li H, Nurmi M, Alanen K, Mirtti T, Visakorpi T, Bubendorf L, Goffin V, Nevalainen MT. Autocrine prolactin promotes prostate cancer cell growth via Janus kinase-2- signal transducer and activator of transcription-5a/b signaling pathway. *Endocrinology.* 2007; 148:3089–3101. [PubMed: 17412813]
14. Wu W, Ginsburg E, Vonderhaar BK, Walker AM. S179D prolactin increases vitamin D receptor and p21 through up-regulation of short 1b prolactin receptor in human prostate cancer cells. *Cancer Res.* 2005; 65:7509–7515. [PubMed: 16103106]
15. Hu ZZ, Meng J, Dufau ML. Isolation and characterization of two novel forms of the human prolactin receptor generated by alternative splicing of a newly identified exon 11. *J Biol Chem.* 2001; 276:41086–41094. [PubMed: 11518703]
16. Kline JB, Roehrs H, Clevenger CV. Functional characterization of the intermediate isoform of the human prolactin receptor. *J Biol Chem.* 1999; 274:35461–35468. [PubMed: 10585417]
17. Boutin JM, Edery M, Shiota M, Jolicoeur C, Lesueur L, Ali S, Gould D, Djiane J, Kelly PA. Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. *Mol Endocrinol.* 1989; 3:1455–1461. [PubMed: 2558309]
18. Trott JF, Hovey RC, Koduri S, Vonderhaar BK. Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein. *J Mol Endocrinol.* 2003; 30:31–47. [PubMed: 12580759]
19. Wu W, Zanello L, Walker AM. S179D prolactin sensitizes human prostate cancer cells such that physiological concentrations of 1, 25 dihydroxy vitamin D3 result in growth inhibition and cell death. *Prostate.* 2007; 67:1498–1506. [PubMed: 17680631]
20. Tan D, Huang KT, Ueda E, Walker AM. S2 deletion variants of human PRL receptors demonstrate that extracellular domain conformation can alter conformation of the intracellular signaling domain. *Biochemistry.* 2008; 47:479–489. [PubMed: 18081308]
21. Chen TJ, Kuo CB, Tsai KF, Liu JW, Chen DY, Walker AM. Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology.* 1998; 139:609–616. [PubMed: 9449632]
22. Huang KT, Chen YH, Walker AM. Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. *Biotechniques.* 2004; 37:406, 408, 410–402. [PubMed: 15470895]
23. Peirce SK, Chen WY. Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR. *J Endocrinol.* 2001; 171:R1–R4. [PubMed: 11572805]
24. Mackay AR, Hartzler JL, Pelina MD, Thorgeirsson UP. Studies on the ability of 65-kDa and 92-kDa tumor cell gelatinases to degrade type IV collagen. *J Biol Chem.* 1990; 265:21929–21934. [PubMed: 2174891]

25. Murphy G, Cockett MI, Ward RV, Docherty AJ. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). *Biochem J.* 1991; 277:277–279. [PubMed: 1649600]
26. Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka J, Seiki M. Expression of genes encoding type IV collagen-degrading metalloproteinases and tissue inhibitors of metalloproteinases in various human tumor cells. *Oncogene.* 1992; 7:77–83. [PubMed: 1311064]
27. Dano K, Andreassen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. *Adv Cancer Res.* 1985; 44:139–266. [PubMed: 2930999]
28. Liotta LA, Goldfarb RH, Brundage R, Siegal GP, Terranova V, Garbisa S. Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res.* 1981; 41:4629–4636. [PubMed: 6458354]
29. Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. *Endocr Rev.* 1997; 18:26–45. [PubMed: 9034785]
30. Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci.* 2001; 22:201–207. [PubMed: 11282421]
31. Cross MJ, Dixelius J, Matsumoto T, Claesson-Welsh L. VEGF-receptor signal transduction. *Trends Biochem Sci.* 2003; 28:488–494. [PubMed: 13678960]
32. Delrieu I. The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. *FEBS Lett.* 2000; 468:6–10. [PubMed: 10683430]
33. Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. *J Clin Invest.* 2002; 109:987–991. [PubMed: 11956233]
34. Perez-Moreno M, Fuchs E. Catenins: keeping cells from getting their signals crossed. *Dev Cell.* 2006; 11:601–612. [PubMed: 17084354]
35. Hinck L, Nathke IS, Papkoff J, Nelson WJ. Beta-catenin: a common target for the regulation of cell adhesion by Wnt-1 and Src signaling pathways. *Trends Biochem Sci.* 1994; 19:538–542. [PubMed: 7846766]
36. Ueda EK, Lo HL, Bartolini P, Walker AM. S179D prolactin primarily uses the extrinsic pathway and mitogen-activated protein kinase signaling to induce apoptosis in human endothelial cells. *Endocrinology.* 2006; 147:4627–4637. [PubMed: 16840547]
37. Perez-Moreno M, Jamora C, Fuchs E. Sticky business: orchestrating cellular signals at adherens junctions. *Cell.* 2003; 112:535–548. [PubMed: 12600316]
38. Cowin P, Rowlands TM, Hatsell SJ. Cadherins and catenins in breast cancer. *Curr Opin Cell Biol.* 2005; 17:499–508. [PubMed: 16107313]
39. Hazan RB, Qiao R, Keren R, Badano I, Suyama K. Cadherin switch in tumor progression. *Ann N Y Acad Sci.* 2004; 1014:155–163. [PubMed: 15153430]
40. Utama FE, Tran TH, Ryder A, Lebaron MJ, Parlow AF, Rui H. Insensitivity of human prolactin receptors to non-human prolactins: Relevance for experimental modeling of prolactin receptor-expressing human cells. *Endocrinology.* 2008
41. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol.* 2001; 21:1104–1117. [PubMed: 11451738]
42. Cairns RA, Khokha R, Hill RP. Molecular mechanisms of tumor invasion and metastasis: an integrated view. *Curr Mol Med.* 2003; 3:659–671. [PubMed: 14601640]
43. Hienert G, Kirchheimer JC, Pfluger H, Binder BR. Urokinase-type plasminogen activator as a marker for the formation of distant metastases in prostatic carcinomas. *J Urol.* 1988; 140:1466–1469. [PubMed: 3193516]
44. Forbes K, Gillette K, Kelley LA, Sehgal I. Increased levels of urokinase plasminogen activator receptor in prostate cancer cells derived from repeated metastases. *World J Urol.* 2004; 22:67–71. [PubMed: 15052500]
45. Shariat SF, Roehrborn CG, McConnell JD, Park S, Alam N, Wheeler TM, Slawin KM. Association of the circulating levels of the urokinase system of plasminogen activation with the presence of

- prostate cancer and invasion, progression, and metastasis. *J Clin Oncol*. 2007; 25:349–355. [PubMed: 17264329]
46. Fodde R, Brabletz T. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. *Curr Opin Cell Biol*. 2007; 19:150–158. [PubMed: 17306971]
 47. Polakis P. The many ways of Wnt in cancer. *Curr Opin Genet Dev*. 2007; 17:45–51. [PubMed: 17208432]
 48. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A*. 1999; 96:5522–5527. [PubMed: 10318916]
 49. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*. 1999; 398(6726):422–426. [PubMed: 10201372]
 50. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. *Science*. 1998; 281:1509–1512. [PubMed: 9727977]
 51. Giuliani R, Bastaki M, Coltrini D, Presta M. Role of endothelial cell extracellular signal-regulated kinase1/2 in urokinase-type plasminogen activator upregulation and in vitro angiogenesis by fibroblast growth factor-2. *J Cell Sci*. 1999; 112:2597–2606. [PubMed: 10393815]
 52. Ribatti D, Leali D, Vacca A, Giuliani R, Gualandris A, Roncali L, Nolli ML, Presta M. In vivo angiogenic activity of urokinase: role of endogenous fibroblast growth factor-2. *J Cell Sci*. 1999; 112:4213–4221. [PubMed: 10564640]
 53. Ueda E, Ozerdem U, Chen YH, Yao M, Huang KT, Sun H, Martins-Green M, Bartolini P, Walker AM. A molecular mimic demonstrates that phosphorylated human prolactin is a potent anti-angiogenic hormone. *Endocr Relat Cancer*. 2006; 13:95–111. [PubMed: 16601282]
 54. Tan D, Johnson DA, Wu W, Zeng L, Chen YH, Chen WY, Vonderhaar BK, Walker AM. Unmodified prolactin (PRL) and S179D PRL-initiated bioluminescence resonance energy transfer between homo- and hetero-pairs of long and short human PRL receptors in living human cells. *Mol Endocrinol*. 2005; 19:1291–1303. [PubMed: 15695371]
 55. Qazi AM, Tsai-Morris CH, Dufau ML. Ligand-independent homo- and heterodimerization of human prolactin receptor variants: inhibitory action of the short forms by heterodimerization. *Mol Endocrinol*. 2006; 20:1912–1923. [PubMed: 16556730]
 56. Gadd SL, Clevenger CV. Ligand-independent dimerization of the human prolactin receptor isoforms: functional implications. *Mol Endocrinol*. 2006; 20:2734–2746. [PubMed: 16840534]

Figure 1. S179D treatment of PC-3 cells increases expression of SF1b and Δ S2 SF1b

Shown in (A) is an ethidium bromide-stained gel illustrating the amplicons specific to each form of the SF1b receptor after treatment with S179D PRL for 5 days. Shown in (B) is a Western blot detecting SF1b after immunoprecipitation. The concentration of S179D PRL is given both as ng/ml and nM. IgG designates cell extracts immunoprecipitated with a control antibody. L, basepair ladder; ECD, extracellular domain.

Figure 2. Expression of Δ S2 SF1b in stably transfected PC-3 cells

Expression was detected by Western blotting (A) or indirect immunofluorescence for the receptor (B). A cell line with lower expression (L) and one with higher expression (H) were selected for subsequent studies. The protocol for immunoprecipitation and Western blotting was the same as for figure 1. 25, is a molecular weight reference.

Figure 3. ΔS2 SF1b expression inhibits growth of PC-3 cells

Cell growth was evaluated by direct cell counting using trypan blue exclusion (A) or a colorimetric MTS assay (B). Quantification was conducted 3 days after initial plating. Data are expressed as the mean $\pm$ SD from triplicates for (A) and quadruplicates for (B). The figure is representative of 3 individual experiments. *, different from controls ($P < 0.05$); **, different from controls ($p < 0.01$).

Figure 4. Δ S2 SF1b expression in PC-3 cells changes morphology, has no effect on the expression of E-cadherin, but alters its subcellular distribution

Morphology of the L cells versus vector controls initially plated at the same density (A). Expression and localization of E-cadherin, as assessed by Western blotting and indirect immunofluorescence (B). Actin was used as the loading control for the Western blot.

Figure 5. ΔS2 SF1b inhibits migration of PC-3 cells in a wound healing assay

PC-3 cells stably transfected with ΔS2 SF1b or vector were grown until confluent. Wounds were introduced and the extent of wound closure was observed and quantified after 16h.

Data are expressed as the mean $\pm$ SD from 2 individual experiments analyzing 6 images per treatment on each occasion. *, $P < 0.05$; **, $P < 0.01$.

Figure 6. Effects of increased $\Delta S2$ SF1b expression in PC-3 cells on migration in the absence and presence of Matrigel

Migration was measured 16h after plating in the absence of Matrigel (A). The number of migrant cells per field was counted. Data are expressed as the mean $\pm$ SD from 3 individual experiments. **, different from control ($P < 0.01$). Cell aggregation occurred on Matrigel (B). The same number (10^5) of PC-3 cells, stably transfected with $\Delta S2$ SF1b or vector, were plated on Matrigel. Cells were fixed and photographed after 16h. The two-dimensional size (area) of cell aggregates was quantified. Data are expressed as the mean $\pm$ SD from 3 individual experiments. **, different from control ($P < 0.01$).

Figure 7. Effect of ΔS2 PRLR SF1b on cell migration-related genes in PC-3 cells

Real-time RT-PCR of cell migration/invasion related genes. The expression level is normalized to GAPDH. *, different from control and each other ($P < 0.05$); # different from control ($P < 0.05$).

Table 1

Primer sequences for RT-PCR

Primer name	5'-oligonucleotide sequence -3'	Product size (bp)
MMP-9 forward	5'-tgggcaaggcgctcgtggtt-3'	276
MMP-9 reverse	5'-tgggtgcagcgaggtaggatt-3'	
uPA forward	5'-ttgctcaccacaacgacatt-3'	284
uPA reverse	5'-gtcagcagcacagcattt-3'	
TIMP-1 forward	5'-ctgttctgtctgtggctgat-3'	305
TIMP-1 reverse	5'-tgcagtttccagcaatgag-3'	
TIMP-2 forward	5'-gtagtatcaggccaaagc-3'	305
TIMP-2 reverse	5'-aggctcttctctgggtggt-3'	
PAI-1 forward	5'-ctctctgcctcaccaac-3'	341
PAI-1 reverse	5'-gaggaagggtctgtccatga-3'	
PAI-2 forward	5'-gcatgttctgtgtcca-3'	364
PAI-2 reverse	5'-ccatgtccagttctccctgt-3'	
uPAR forward	5'-cacttcctgaaatgctgcaa-3'	399
uPAR reverse	5'-ggtagtggtgaggctgagat-3'	
E-cadherin forward	5'-ggctgtcatggaaggtgct-3'	337
E-cadherin reverse	5'-ttgtactgtgtgggattgaa-3'	
beta-catenin forward	5'-gcttggttcaccagtggatt-3'	305
beta-catenin reverse	5'-tcagcactctgcttgggtc-3'	
bFGF forward	5'-tgtgctaaccgttacctggct-3'	150
bFGF reverse	5'-cagtgccacataccaactg-3'	
VEGF forward	5'-gccttgctgctctacctcca-3'	401
VEGF reverse	5'-caaggccacagggatttt-3'	
GAPDH forward	5'-tgcaccaccaactgcttagc-3'	87
GAPDH reverse	5'-ggcatggactgtggtcatgag-3'	