Prolactin signaling enhances colon cancer stemness by modulating Notch signaling in a Jak2-STAT3/ERK manner

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Prolactin (PRL) is a secretory cytokine produced by various tissues. Binding to the cognate PRL receptor (PRLR), it activates intracellular signaling via janus kinase (JAK), extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription (STAT) proteins. PRL regulates diverse activities under normal and abnormal conditions, including malignancies. Previous clinical data suggest serum PRL levels are elevated in colorectal cancer (CRC) patients. In this study, we first determined the expression of PRL and PRLR in colon cancer tissue and cell lines. Higher levels of PRLR expression were observed in the cancer cells and cell lines compared with normal colonic epithelial cells. Incubation of colon cancer cells with PRL-induced JAK2, STAT3 and ERK1/2 phosphorylation and increased expression of Jagged 1, which is a Notch-1 receptor ligand. Notch signaling regulates CRC stem cell population. We observed increased accumulation of the cleaved/active form of Notch-1 receptor (Notch intracellular domain) and increased expression of Notch responsive genes HEY1, HES1 and stem marker genes DCLK1, LGR5, ALDH1 and CD44. Finally, inhibiting PRL induced JAK2-STAT3 and ERK1/2 phosphorylation using AG490 and PD98059, respectively, leads to complete abrogation of Notch signaling, suggesting a role for this pathway in regulating CRC stem cells. Together, our results demonstrate that cytokine signaling induced by PRL is active in colorectal cancers and may provide a novel target for therapeutic intervention.

Introduction

Colorectal cancer (CRC) remains one of the leading causes of cancer-related deaths in both economically developed and developing countries. It is the second leading cause of cancer deaths in both males and females in United States (1). The precancerous predisposition of colorectal epithelial polyps is no longer disputed. A plethora of malignancies. Previous clinical data suggest serum PRL levels have been shown to be complex and are influenced by various intrinsic and extrinsic factors, such as hormones (2).

Prolactin (PRL), a cytokine hormone, accumulates in the tissue microenvironment and elicits its action in an autocrine or paracrine manner to regulate diverse physiological activities that include reproduction, growth, development, metabolism and immunomodulation (3–6). Binding of PRL to the single-pass, transmembrane PRL receptor (PRLR) induces several intracellular signaling cascades that are mediated via Jak-STAT (7,8) and Jak-Ras-MAPK components (9).

PRL acts as a mitogen by promoting cell proliferation, inhibiting apoptosis and inducing chemotaxis in breast cancer cells (5,10,11). Blood PRL levels were found elevated in patients with hepatocellular carcinoma (12,13) and ovarian cancer (14). Cultured, immortalized ovarian epithelial cells and endometrial cells treated with exogenous PRL demonstrated increased proliferation and inhibition of chemotherapy-induced cell death (15). Autocrine PRL induces PRLR-mediated Jak2-STAT signaling in prostate cancer (16–19) and modulates the stem cell/basal cell population (17).

PRL and PRLR have been expressed along the gastrointestinal tract in fetal and neonatal stages during development (20). In adult rats, PRL induces active potassium-ion transport in distal colon and chloride-ion transport in proximal and transverse colon (21). IEC-6 colon crypt epithelial cells treated with PRL had increased expression of nutrient and mineral transport channel proteins, without inducing proliferation (22). Elevated serum levels of PRL have been identified in patients with colorectal malignancies (23–26). In addition, increase in PRL and PRLR expression was noted in CRC cell lines and tumor samples (27).

Cancer stem cells (CSCs), initially identified in hematological disorders as tumor-initiating cells when isolated and transplanted in NOD-SCID mice (28), are long-lived, self-renewing population of cells that initiate and sustain tumor growth and can be identified by unique set of marker proteins such as doublecortin like kinase 1 (DCLK1) (29–31), leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) (32–35), CD44 (36) and CD133 (37), which also serve as protein markers for normal colon stem cells. These cells are resistant to therapeutic interventions and cause tumor relapse and metastasis (38,39). Identifying cellular factors that regulate stem cell population are critical in understanding the process of neoplastic transformation and in developing novel therapeutics to target the CSC pool. Isolated primary mouse hippocampal cells treated with exogenous PRL showed increased number of stem cells (40). Similarly, in mouse models, inducing PRL under the control of prostate-specific probasin promoter leads to expansion in the basal cell compartment (17,41), which constitutes the stem cell population of the prostate gland. Although these data suggest that PRL can affect tissue stem cell population, its effects on CSCs have not been determined.

Notch signaling pathway is active in intestinal crypts (43) and is involved in regulating stem cell hierarchy and cell fate determination (42). Constitutive Notch activation is necessary for intestinal stem cell maintenance (44) and deregulation of the pathway has been observed in colorectal and other forms of cancer (45). There are four members in the Notch receptor family: from Notch 1 to Notch 4. Binding of specific ligands such as Jagged (JAG) 1, 2, or Delta 1, 3, 4, to the Notch receptor results in a conformational change in the receptor. Subsequent activation of the γ-secretase complex, which is composed of presenilin, nicastrin, anterior pharynx defective 1 (APH 1) and presenilin enhancer 2, cleaves the Notch receptor to release the Notch intracellular domain (NICD) (46,47). The NICD then translocates into the nucleus, interacts with co-factors recombining binding protein suppressor of hairless and mastermind-like, bind to target sequences and activate the transcription of genes such as Hes1, Hey1 and stem cell responsive genes (48) such as c-Myc, all of which can be used to assess the degree of Notch signal activation. Interestingly, extracellular signal-regulated kinase (ERK) can modulate Notch signaling by regulating the expression of its ligand JAG 1 (49).
This study is aimed at determining the role of PRL signaling in colon cancer cells. We show that presence of PRL induces Jak2-ERK1/2 mediated activation of Notch signaling, leading to an increase in spheroid formation and changes in CSC population. Furthermore, PRL signaling in these cells can be suppressed with specific inhibitors of Jak2 and ERK1/2.

Materials and methods

Cells
Colorectal cancer cell lines HT29, HCT116, SW480, SW620, DLD1 and normal intestinal epithelial fetal human colon (FHC) cells were obtained from ATCC (Manassas, VA). The cells were well characterized and used by multiple investigators. They were cultured in the recommended media supplemented with 10% fetal bovine serum (Sigma Aldrich, MO) and 1% antibiotic-antimycotics solution (Mediatech Inc, VA) at 37°C in a humidified atmosphere of 5% CO2. The cells were cultured in serum-free media overnight prior to treatment with PRL (500 ng/ml). Where indicated, cells were pretreated with 50 μM Jak2 inhibitor AG490 or 10 μM ERK1/2 inhibitor PD98059 (Selleckchem, TX).

Spheroid assay
Cells were seeded at a limiting dilution of 1500 cells/ml (total 2 ml = 3000 cells/well in a 6-well dish) in DMEM supplemented with or without PRL (0–500 ng/ml) and inhibitors AG490 or PD98059, in addition to epidemial growth factor (5 mg/ml), fibroblast growth factor (5 ng/ml), heparin (1 ng/ml) and B12 supplements (0.25x) and plated on ultra-low attachment plates (BD Biosciences, NY). An important point to note is that we reduced the amount of growth factors used in the culture conditions to prevent any growth-promoting effects by these growth factors, which can complicate the analyses, and to gain a better idea of the role of PRL in promoting spheroid formation. Specifically, we used one-fourth the dose of growth factors (epidermal growth factor, fibroblast growth factor, Heparin and B12 supplements). Treatment of the cell cultures for 24 hours in serum-free media and the media was subjected to enzyme-linked immunosorbent assay according to manufacturer’s instructions (Molecular Innovations, MI). Briefly, 100 μl of the provided standard and concentrated serum-free media collected from cells was added into wells pre-coated with PRL antibody in triplicates and allowed to bind for 30 min at which point the wells were washed and treated sequentially with primary antibody and streptavidin-horseradish peroxidase-bound secondary antibody. Colorimetric quantification after treating with substrate was done at 450nm.

Luciferase assay
Cells were plated and transfected with either 4X67 pTK-Luc (Addgene plasmid 6868 ) (51) or Hes-1A/B-Luc, a kind gift from Dr. Kimberly Foreman, Loyola University, Chicago (52), which encode firefly luciferase gene under the control of the minimal thymidine kinase promoter and four STAT3 (4X STAT3 BS) or single Hes1 (HES1 BS) binding site using Lipofectamine 2000 (Invitrogen, NY). Cells were pre-treated with PD98059 (10 μM) and/or AG490 (50 μM) for 2h prior to treatment with PRL (500 ng/ml). Renilla luciferase expressing pRL-TK plasmid (Clontech, Mountain View, CA) was used as internal control. Luciferase levels in the cell lysates were determined using Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI).

Statistical analysis
Data from at least three independent experiments were expressed as the mean ± SEM and analyzed by unpaired or paired student’s-t test using GraphPad Prism 5 (La Jolla, CA). P value ≤0.05 was considered to be statistically significant.

Results

PRLR but not PRL is upregulated in colon cancer cells
To determine whether PRL signaling occurs in colorectal cancer, we first analyzed the expression of PRL and PRLR in human colon cancer tissues and cell lines. Real-time PCR quantification using CRC patient samples indicates a significant increase in PRLR but not PRL transcript levels in the cancerous tissue compared with adjacent normal tissue (Figure 1A and B). A similar increase in PRLR mRNA levels and protein was observed in CRC cells compared with normal colonic epithelial cells (FHC) (Figure 1C and D). Moreover, no difference in expression of PRL mRNA was observed between normal colonic FHC cells and CRC cell lines (Figure 1E). Quantification of concentrated culture media from the cell lines by enzyme-linked immunosorbent assay indicated that all cells secrete PRL; however, the amount varies with time and in a cell-line-specific manner, ranging from 2 to 80 pg/ml after 24h (Figure 1F).

PRL treatment induces STAT3 and ERK1/2 phosphorylation
Upregulation of PRLR, particularly in CRC, compared with normal colonic cells, suggests a role for the pathway in the pathogenesis of colorectal cancer. Binding of PRL to PRLR is known to activate JAK2-STAT and JAK2-ERK1/2 pathways (7–9). The ERK/mitogen-activated protein kinase pathway is also known to be highly active in patients with Familial Adenomatous Polyposis (53). Cells were treated with recombinant PRL and western blot analyses were performed for Jak, STAT and ERK proteins. An increase in JAK2, STAT3 and ERK1/2 phosphorylation was observed within a minute of PRL treatment (Figure 2A and B). To validate PRL-mediated STAT3 activation, we transfected HCT116 and HT29 cells with 4X67 pTK-Luc plasmid, which encodes the firefly luciferase, under the control of a minimal promoter and four tandem STAT3 binding sites (M67 sites). This construct has been previously used to demonstrate STAT3 induced gene expression (51). PRL treatment increased luciferase activity in both cell lines in a dose- and time-dependent manner (Figure 2C). Even at 100 ng/ml of PRL, there was significant increase in luciferase activity as early as 6h. However, at 1 μg/ml dose, PRL-mediated induction in luciferase activity was observed even at 1 h. Pre-treating the cells with AG490, a pharmacological inhibitor of JAK2, prior to PRL treatment leads to a decrease in STAT3 and ERK1/2 phosphorylation (Fig 2D and E), even in the presence of PRL. However, pre-treatment with PD98059 alone led to increased STAT3 activation (Figure 2D). Moreover, cells treated with the combination of Jak2 and ERK1/2 inhibitors, AG490 and PD98059, resulted in complete

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inhibition of JAK2, ERK1/2 and STAT3 phosphorylation (Figure 2D and E). Together, the data suggest that PRL induces JAK2, ERK1/2 and STAT3 phosphorylation in colon cancer cells. Given that STAT5 phosphorylation has shown to be significantly upregulated in breast cancer cells in response to PRL (7), we also determined the level of STAT5 phosphorylation in the colon cancer cells. However, levels of total STAT5 protein were relatively low in colon cancer cells, and no significant changes in phosphorylation of the protein were observed (data not shown), suggesting that STAT3 may be a key player in PRL/PRLR signaling in colon cancer cells.

PRL induced spheroid formation and is inhibited by JAK2 and ERK inhibitors

Previous studies have demonstrated mitogenic activity for PRL in breast cancers (5). Accordingly, we determined whether PRL affects proliferation of colon cancer cells. However, PRL did not have any
Fig. 2. Prolactin induces JAK2, STAT3 and ERK1/2 phosphorylation. (A) Cells were treated with 500 ng/ml PRL and lysates were collected at regular intervals and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. PRL treatment increased phosphorylation of JAK2, STAT3 and ERK1/2, starting at 1 min post treatment and lasting for 30 min. (B) To validate STAT3 activation, a STAT3 responsive luciferase plasmid was transfected into cells and the luciferase activity analyzed after PRL treatment. A dose and time dependent increase in luciferase activity was observed following PRL treatment compared with untreated controls (*\(P < 0.05\)). (C) JAK2 and STAT3 phosphorylation is inhibited by specific inhibitors AG490 (JAK2-specific) and PD98059 (Mitogen-activated protein kinase specific) treatment. Treatment with PRL rescues the inhibition. However, PRL cannot rescue JAK2 or STAT3 phosphorylation when the combination of the two inhibitors are used. (D) Cells treated with PD98059 had decreased whereas AG490 enhanced ERK1/2 phosphorylation when treated alone. Addition of PRL did not affect ERK1/2 phosphorylation in the presence of either inhibitor.
effect on proliferation of various colon cancer cells (data not shown). Further confirmation of this was obtained when cells were subjected to cell cycle analyses by flow cytometry following propidium iodide staining. Again, there was no difference in cell cycle progression between PRL-treated cells and control cells (data not shown). These data, taken together, suggest that PRL does not affect cell growth.

We next determined whether PRL affects spheroid formation because previous studies have demonstrated that PRL stimulates neurosphere formation when hippocampal cells are treated with PRL (40). Additionally, spheroid formation remains the best available functional assay to assess the presence of CSCs in a given cancer cell pool (54,55).

We treated colon cancer cells HCT116, SW480 and HT29 with increasing concentrations of PRL (0–500 ng/ml) and allowed the spheroids to form. There was a dose-dependent increase in spheroid formation (Figure 3A), with a significant increase in both number (Figure 3B) and diameter of spheroid (Figure 3C) in all the three cell lines. We also determined the effect of inhibiting JAK-STAT and ERK1/2 signaling with the two inhibitors AG490 and PD98059. Pre-incubation with the inhibitors AG490 and PD 98059, either alone or in combination, abolished spheroid formation (Figure 3D and E). Moreover, the inhibitors affected spheroid formation in the presence of PRL. There were also less number of spheroids and the size of the spheres was smaller compared with cells treated with PRL alone. These results suggest that PRL signaling can potentially regulate colosphere formation.

**PRL induces expression of colon CSC marker genes**

Since PRL stimulated colosphere formation, a marker for stem-cell-dependent growth, we next determined whether PRL affects stem cells. Stem cells were quantified using real-time PCR and western blot of specific markers, including DCLK1 (29–31), LGR5 (32–35), CD44 (36), CD133 (37) and ALDH1A1 (38). Real time PCR analysis demonstrated that PRL treatment induced expression of DCLK1, LGR5 and ALDH1 (Figure 4A–C). Further confirmation was obtained by western blot analyses, which showed that all these proteins along with CD44 and c-Myc, an oncogene that plays a predominant role in stemness (56), are upregulated compared with untreated controls (Figure 4D). This further demonstrates that biological relevance of STAT3 activation by PRL. We also determined whether signaling through the JAK-STAT and ERK1/2 pathways affects stem cell marker expression. Pre-treatment with AG490 or PD98059 alone caused a decrease in expression of DCLK1 and LGR5, which were partially rescued upon PRL treatment (Figure 4E). These data further suggest that PRL signaling can modulate the expression of colon CSC maker protein expression.

**PRL affects CSCs and progenitor cells by inducing Notch signaling**

Notch signaling plays a significant role in stem cells and is a pathway active in colon CSCs (42,44). Phosphorylated ERK1/2 induces JAG1, a Notch receptor ligand (49). Binding of JAG1 to the Notch receptor causes a conformational change and sequential cleavage by ADAM and γ-secretase complex proteins to release the Notch intracellular domain (NICD), which translocates into the nucleus and activates the expression of target genes (57). We examined whether PRL treatment affects Notch signaling in colon cancer cells by modulating JAG1 expression. Quantitative real-time PCR analyses demonstrated increased expression of JAG1 and the Notch signaling target gene HEY1 (Figure 5A and B). Western blot analyses further confirmed the upregulation of JAG1 and HEY1 (Figure 5C). In addition, there was an increase in NICD protein levels, along with increased levels of γ-secretase complex protein anterior pharynx defective 1, presenilin 1 (PSEN1) and presenilin enhancer (Figure 5C). To further confirm that Notch signaling is activated upon PRL treatment, we transfected HCT116 and HT29 cells with a plasmid encoding the luciferase reporter gene under the control of the Hes-1 promoter. Following 500 ng/ml PRL treatment, a robust induction in luciferase activity was observed in both the cells (Figure 5D).

These results were also confirmed using the specific JAK2 and ERK inhibitors. Inhibiting either JAK2 or ERK1/2 signaling alone using AG490 or PD98059 showed decreased JAG1 expression, NICD cleavage and expression of HEY1, HES1 and PSEN1 genes (Figure 5E). This was partially rescued by PRL. Combined inhibition of both the inhibitors leads to a further reduction in JAG1 expression, Notch-1 cleavage (NICD) and expression of HEY1, HES1 and PSEN1 (Figure 5E) even in the presence of PRL, suggesting that PRL can regulate Notch signaling through either JAK2-STAT3 or JAK2-ERK1/2 pathway.

**Notch signaling is necessary to mediate PRL-induced changes**

Based on the above findings, we hypothesized that PRL induces JAK2-STAT3 and JAK2-ERK1/2 cascades that in turn activate JAG1-mediated Notch signaling. To evaluate this, we overexpressed NICD in the three colon cancer cell lines. NICD overexpression significantly induced colosphere formation, similar to that observed when cells were treated with PRL (Figure 6A). There was an increase in the number and size of the spheroids (Figure 6B and C). Furthermore, treatment with the inhibitors alone did not affect the number or size of spheroids in the presence of NICD overexpression. However, a small decrease was observed in secondary spheroids when treated with the combination of the two inhibitors (Figure 6D). Similarly, protein levels of DCLKL1, LGR5 or CD44 increased in NICD overexpressing cells, to levels comparable to PRL-treated cells (Figure 6E). The two inhibitors, either alone or in combination, did not affect the expression of stem cell markers in the NICD overexpressing cells, suggesting further that PRL-induced activation of Notch signaling is sufficient to enhance stem cell activity.

**Discussion**

PRL, a peptide hormone produced by the lactotroph cells of the anterior pituitary and other non-pituitary tissues. In lactating rats, PRL is known to modulate water and electrolyte transport in the intestine (58). It can also stimulate proliferation of the mucosal cells of the gastrointestinal tract during pregnancy (59). PRL has a mitogenic role in mammary (6), lung (60), bladder (61), prostate and ovarian (62) tumorigenesis. Elevated serum levels of PRL has been shown to be associated with increased incidence of mammary (63,64) and prostate (19) cancers. High serum levels of PRL (27) and PRLR expression (65) were also observed in CRC patients. Our findings also implicate an increase in PRLR transcript in CRC samples compared with adjacent normal tissue. Similarly, CRC cell lines had increased PRLR levels compared with normal FHC cells. In line with earlier observation (65), SW480 cells expressed relatively lower PRLR levels compared with other cells. No significant change in mRNA expression or robust secretion of PRL into the media was noted in CRC cell lines compared with FHC. These findings clearly show a preferential upregulation of PRLR in CRC cells suggesting active role for PRL–PRLR signaling in colorectal tumors.

Binding of PRL to PRL receptor (PRLR) activates JAK2/STAT and/ or RAS-RAF-ERK1/2 pathway (66,67). In fact, we observed a rapid and robust increase in STAT3 phosphorylation. We also observed an increase in STAT3 phosphorylation when treated with PD98059; a similar finding was reported earlier (68). In their studies, the authors demonstrated that treating melanoma cell line U11205 with only PD98059 induces robust phosphorylation of STAT3 and STAT5 (68). Previous studies in breast cancers have also demonstrated STAT5 activation, and not STAT3 activation, in the presence of PRL. More importantly, in breast cancers, it was determined that STAT5 and STAT3 mediate opposing effects on several key target genes such as BCL6, with STAT5 exerting a dominant role. When both STAT3 and STAT5 are activated at the same time, there is, in fact, a reduction in the proliferation of the breast cancer cells. Moreover, there was an increased sensitivity to chemotherapeutic drugs (69,70). In our studies, we observed that PRL did not affect the proliferation of CRC cells. Given our observation that STAT3 is predominantly activated in CRC following PRL treatment, suggests a differential activation of intracellular signaling modules by PRL, which may depend on the type of cancer. It would be interesting to see if overexpressing...
Fig. 3. PRL affects colosphere formation. (A) Colon cancer cells were grown in specific spheroid media in ultra-low binding plates and treated with increasing dose of PRL. After 5 days, the colonospheres were photographed and counted. (B) A dose-dependent increase in spheroid number was observed, with higher significance at 500 and 1000 ng/ml of PRL (*P < 0.05). (C) Similar increase in diameter was noted at similar doses (*P < 0.05). (D and E) Pre-treatment with AG490 and PD98059 alone or in combination leads to significant decrease in spheroid formation and number compared with PRL treatment alone (*P < 0.05).
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STAT5 in CRC and subsequent treatment with PRL would lead to an increase in chemotherapy-induced cell death, as observed in breast cancer cells.

Spheroid formation assay helps us determine the presence of cancer-initiating cells in a cancer cell population (54,55). Our results show a dose-dependent increase in spheroid formation, number and diameter in CRC cells following PRL treatment. This is in agreement with previous studies with prostate cancers, where PRL expression in mouse prostate led to increase in stem cell/basal cell population (17,41). Similarly, neurosphere formation along with the expansion of hippocampal precursor cell population (40) has been observed in a PRL-dependent manner. We also observed increased expression

Fig. 4. PRL induces stem cell marker protein expression. Colon cancer cells were treated with 500ng/ml PRL for various time points up to 12h. Total RNA and cell lysates were generated for real time PCR and western blot analyses. Real-time PCR shows increased levels of (A) DCLK1 (B) LGR5 and (C) ALDH1A1 mRNA after PRL treatment (*P < 0.05). (D) Lysates from these cells indicate significant increase in expression of DCLK1, LGR5, ALDH1A1, CD44 and c-MYC (MYC) in the presence of PRL. (E) Cells treated with either AG490 or PD98059 had decreased DCLK1 and LGR5 induction compared with PRL treatment. PRL was able to rescue this activation; however, the combination of both AG490 and PD98059 leads to complete abrogation of DCLK1 and LGR5 expression.
Fig. 5. PRL treatment activates Notch signaling. (A) Real time PCR analysis on cells treated with PRL show a time-dependent increase in expression of JAG1 (*P < 0.05). (B) Similar increase in expression of Notch target gene HEY1 was also observed in real-time PCR analysis (*P < 0.05). (C) Lysates of PRL-treated cells show increased JAG1 and HEY1 expression, NICD accumulation and induction of γ-secretase complex proteins anterior pharynx defective 1, PSEN1 and presenilin enhancer expression compared with controls. (D) Colon cancer cells, transfected with HES1 responsive luciferase plasmid, showed a PRL dependent induction of luciferase activity. (E) AG490 and PD98059 pre-treatment caused a decrease in NICD accumulation and JAG1, HEY1, HES1 and PSEN1 expression compared with PRL treatment. PRL was able to rescue this activation; however, combination of both AG490 and PD98059 leads to complete abrogation of NICD accumulation and HEY1, HES1 and PSEN1 expression to levels similar to control.
**Fig. 6.** NICD overexpression recapitulates PRL-induced changes in colosphere formation and stem cell marker protein expression. (A) Colon cancer cells were transfected with NICD overexpressing plasmid, grown in specific spheroid media in low adherent plates for 5 days in the presence of PRL and inhibitors. Neither AG490 nor PD98059 treatment alone or in combination cause any significant decrease in colosphere formation in NICD expressing cells. (B) Significant increase in spheroid number is observed in NICD expressing cells compared with untreated controls. The inhibitors did not affect the NICD overexpressing cells. (*$P < 0.005$). (C) Increased colosphere diameter was also observed in NICD overexpressing cells compared with control (*$P < 0.05$). (D) Secondary spheroids. The primary spheroids were collected, trypsinised and replated without PRL or the inhibitors. PRL primed and NICD expressing cells treated with either inhibitor maintained high colosphere formation compared with control. Combination of the inhibitors had comparable decrease in spheroid number compared with PRL-treated or NICD-expressing cells. (E) Lysates from NICD overexpressing cells either alone or treated with the inhibitors alone or in combination had increased expression of CSC markers DCLK1, LGR5 and CD44. However, expression of PSEN1 and presenilin enhancer was not affected in NICD expressing cells, compared with PRL-treated cells.
of DCLK1, LGR5, ALDH1 and CD44. However, there were differences seen in the stemness based on cell lines. HT29 cells expressed only moderately higher levels of the marker proteins compared with HCT116. This is also in line with studies on PRL effects on neural stem cells. It would be interesting to examine whether expression of stem-cell-related proteins is affected in neural stem cells and whether this expression is affected in brain tumors.

PRL also induced Notch signaling via the JAK2-ERK1/2 pathway by inducing JAG1 expression, leading to NICD accumulation along with the increase in expression of Notch target genes. This is of high significance because, clinically, increased ERK1/2 activation was noted in patients with familial adenomatous polyposis (53). Moreover, previous studies have also demonstrated that ERK1/2 can modulate Notch signaling by regulating the expression of its ligand JAG1 (49). Notch signaling is active in intestinal crypts (43) and helps regulate stem cell hierarchy and determine cell fate (42). Deregulation in this pathway can lead in colorectal cancer (45, 71). It would be interesting to determine whether PRL upregulation is essential for the tumorigenesis process.

Based on our observation, we put forward a model where the presence of PRL in the tumor microenvironment of CRC cells would activate JAK2 after binding to PRLR, which would in turn induce ERK1/2 phosphorylation. Activated ERK1/2 would induce JAG1 expression in the cells, which would translocate to the cell membrane. Binding of JAG1 extracellular domain to the single-pass transmembrane Notch-1 receptor would lead to intracellular conformational changes and cleavage by the γ-secretase complex proteins, leading to separation of the NICD from the transmembrane domain. The cleaved NICD would then translocate to the nucleus and complex with mastermind-like and recombining binding protein suppressor of hairless to activate target gene expression (Figure 7). Our findings further indicate an increase in expression of PRLR but not PRL in CRC cells. However, previous studies have demonstrated increased levels of PRL in the blood stream of patients (23, 24). This suggests that PRL expression is induced at other sites. This is also different from what has been observed in breast cancers, where the cancer tissue itself induces PRL expression (6, 17). It would be interesting to determine how and where PRL expression is induced in colon tumorigenesis. In this regard, it should be noted that PRL is believed to be a hormone whose expression is responsive to stress. PRL does increase in response to psychosocial stress, although women may have higher magnitude of increase than men, and this might be dependent on estradiol levels (62). Moreover, dietary fat was shown to induce circulating PRL under conditions of ether stress, and dietary fat can also affect tumorigenesis (63). It would be interesting to determine whether these conditions affect PRLR expression and PRL-mediated signaling—a future direction of our research program.

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