Role of Prolactin and Prolactin Receptor Signaling in Colorectal Tumorigenesis

By

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Role of Prolactin and Prolactin Receptor Signaling in Colorectal Tumorigenesis

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Abstract

Hormones are critical regulatory factors produced by the body to regulate diverse physiological activities such as energy homeostasis, growth and differentiation of a diverse array of tissues, sexual maturation and development of secondary sexual characters. Cytokines are hormones, which predominantly regulate growth, proliferation differentiation, immunomodulation and tumor progression. They act in endocrine, paracrine or autocrine manner, and elicit their action by binding to a cell surface receptor and activating diverse intracellular signaling cascades.

Prolactin (PRL) is a peptide hormone, encoded by the *PRL* gene located on chromosome 6 in humans. This gene is under the control of two independent promoters, the pituitary promoter which regulates its expression from the lactotroph cells of the anterior pituitary and an extrapituitary promoter which regulates its expression from extra-pituitary tissues such as endometrium, placenta, breast and a variety of tumors. PRL in humans binds specifically to PRL receptor (PRLR), to cause intracellular changes modulated mainly via the JAK-STAT or JAK-STAT-ERK pathways. Historically, PRL has been studied as an endocrine hormone that regulates lactation during pregnancy. However, the identification of extra-pituitary PRL and the complex clinical consequences of hyperprolactemia have prompted investigators to reevaluate its role in regulating other physiological

aspects. Studies from several groups over the last few decades have shown that PRL can regulate a spectrum of functions ranging from behavior to immune responses to tumorigenesis.

Cancer is a growth disorder which was mentioned as early as 460-370 BC, by Hippocrates who coined the term “cancer” based on its appearance post-surgery. Throughout history, cancer has been the cause of severe physical and emotional suffering and death in humans and animals alike. Cancer has an exceptional capability to take over body’s normal physiology, modulate it and uses it for its own growth and to overcome anti-cancer treatment. Over the last several decades, research efforts from several laboratories have helped gain a better understanding of the molecular mechanisms that regulate cancer initiation, development and progression. These studies have also provided new insights for identifying and developing new pharmaceutical compounds to target tumor cells.

Colorectal cancer (CRC) is the third leading cause of cancer related death in United States. Worldwide, up to 5% of all reported cancer cases are due to CRC, with 60% of them being diagnosed in industrially developed or developing countries. CRC is caused by genetic and environmental factors. Environmental factors ranging from changing dietary habits to environmental toxins are associated with the development of CRC. Germline mutations in APC, TP53 and DNA

mismatch repair genes gives rise to familial inheritable form of CRC and contribute to nearly 35% of the registered CRC cases.

This dissertation outlines the expression pattern of PRLR and the cellular mechanism(s) which are regulated or activated by PRL- PRLR signaling and the contribution of this signaling towards pathogenesis of CRC.

We have determined that CRC cells treated with recombinant human PRL show a time- and dose-dependent phosphorylation of JAK2, STAT3 and ERK1/2 proteins. Previous studies have demonstrated that breast cancer cells treated with PRL show a rapid induction of STAT5 phosphorylation. However, in our studies, we found that colon cancer cells treated with PRL show an induction of STAT3 phosphorylation. This may be in part due to low basal level of STAT5 in colon cancer cells. In addition, PRL treatment does not lead to increase in proliferation, falling in line with earlier observations, that STAT3 is not a proliferation promoting factor. Pre-incubating CRC cells with AG490 and PD98059 which are established JAK2 and ERK1/2 inhibitors prior to PRL treatment led to a complete abrogation of respective phosphorylation, suggesting that the observed activation of JAK2 and ERK1/2 is indeed induced by PRL in CRC cells.

PRL treatment induces spheroid formation, a hall mark of cancer stem cells and does so, by activating Notch signaling. The Notch signaling pathway is critical in

maintaining cancer stem cell populations both *in vitro* and *in vivo*. PRL activates Notch signaling by inducing the expression of Jagged 1(JAG1), a Notch receptor ligand. Binding of JAG1 to Notch receptor induces conformation changes in the receptor, leading to its cleavage and translocation of the cleaved intracellular domain (NICD) into the nucleus where it activates expression of Notch responsive genes. PRL treatment induces a time dependent increase in Notch cleavage. In addition, an increase in expression of Hes1 and Hey1, established Notch target genes, clearly implicate PRL treatment in activation of Notch signaling in colon cancer cells. In addition, the treatment induced expression of established colon cancer stem cell marker proteins such as LGR5, DCLK1 and CD44 suggests that PRL contributes towards modulating colon cancer stem cell population. Pretreating CRC cells with AG490 and PD98059, leads to loss of Notch activation and decreased expression of cancer stem cell marker proteins, again implicating the role of PRL in modulating the Notch signaling, thereby playing a critical role in regulating colon cancer stem cell population.

One of the critical aspects associated with human PRL signaling is its receptor specificity. Human PRL can bind only to PRLR, which is a specific PRL receptor. Our findings indicate that PRL can modulate critical aspects associated with colorectal cancer. We were interested in examining the expression pattern of PRLR in colorectal cancer patients with an aim to develop novel diagnostic tools or

therapeutically target PRL signaling. Our findings clearly indicate that PRLR is expressed in normal tissues throughout the GI tract, with predominant expression in the large intestine. Additionally, PRLR expression is significantly increased in colorectal cancer biopsy samples compared to adjacent normal samples. This suggests that PRL signaling can play a critical role in colorectal cancer tumorigenesis. A couple of factors may contribute to the increase in expression of a gene: first being an increase in copy number of the gene and second being an enhanced transcription of the gene. In order to examine the existence of chromosomal variation, we analyzed TCGA data sets pertaining to expression and copy number data. Data analysis suggested the possibility of an increase in copy number of not only PRLR but also of a couple other genes located in the vicinity in some patients. Among the other patients, some had an increase in expression without any change in copy number. To identify the reason for increased expression of PRLR in these patients, we evaluated the possibility of tumor specific transcription factor binding and subsequent increase in PRLR transcription. We analyzed a 2 Kb upstream region of the PRLR promoter and identified binding sites for SREBP-1, a transcription enhancer that regulates expression of enzymes necessary for lipid metabolism and energy homeostasis. SREBP-1 is highly expressed in colorectal tumors. Studies using ChIP and RT-

PCR analysis indicate that SREBP-1 is actively recruited to the PRLR enhancer region and can potentially be involved in regulating its expression.

Collectively, this dissertation provides novel insights into the role of PRL in colorectal tumorigenesis. It also implicates the critical role of PRLR signaling in colorectal cancer and suggests that PRLR can be exploited as a diagnostic marker.

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Finally I thank God for all of my life's blessings.

Dedication

पितासि लोकस्य चराचरस्य
त्वमस्य पूज्यश्च गुरुर्गरीयान् ।
न त्वत्समोऽस्त्यभ्यधिकः कुतोऽन्यो
लोकत्रयेऽप्यप्रतिमप्रभाव ॥ ४३ ॥

(Bhagvadgita Chapter 11: 43)

I dedicate this dissertation to the preceptor of the universe. I thank him for giving me this opportunity to understand a small portion of his marvelous and mysterious creation.

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List of Abbreviations

Abbreviation	Full name
15- PGDH	15- prostaglandin dehydrogenase
ADAM-TACE	ADAM Metallopeptidase Domain 17/Tumor necrosis factor-α-converting enzyme
AGXT2	Alanine-glyoxylate aminotransferase 2
ALDH1	Aldehyde dehydrogenase 1
APC	Adenomatose polyposis coli
APH-1	Anterior pharynx defective-1
bHLH	Basic helix-loop-helix
BMI-1	Polycomb complex protein BMI-1
CAE	Carcino embryonic antigen
CaP	Prostate cancer
CBC	Crypt based columnar cells
CD44	Cluster of division-44
cDNA	Complimentary DNA

CK-1	Casein kinase-1
CNV	Copy number variation
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
DCLK1	Double cortin like kinase 1
E2	Esotrgen
ECD	Notch Extracellular Domain
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked Immunosorbent assay
ER	Estrogen receptor
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
Fz	Frizzled
GH	Growth receptor

GHR	Growth hormone receptor
GSK-3β	Glycogen synthase kinase 3β
IBD	Inflammatory bowel disease
IRS2	Insulin receptor substrate 2
ISC	Intestinal stem cells
JAG1	Jagged 1
JAK	Janus Kinase
KGFR	Keratinocyte growth factor
LBW	Liver to body weight
LGR5	Leucin-rich repeat containing G-protein coupled receptor
LRP	LDL receptor protein
MAML	Mastermind like protein
MAPK	Mitogen Activated Protein Kinase
Min	Multiple intestinal neoplasia
MMR	Mismatch repair

mRNA	Messenger ribonucleic acid
Msi-1	Musashi-1
NECD	Notch extracellular domain
NICD	Notch Intracellular Domain
NICD	Notch intracellular domain
NSAID	Non-steroidal anti-inflammatory drugs
P4	Progesterone
PDAC	Pancreatic ductal adenocarcinoma
PEN-2	Presenilin enhancer-2
PGE2	Prostaglandin E2
PH	Partial hepatectomy
PI3K	Phosphoinositol 3 kinase
PKC	Protein kinase C
PL	Placental lactogen
PR	Progesterone receptor

PRL	Prolactin
PRLR	Prolactin receptor
PSEN-1	Presenilin 1
PSENEN	Presenilin enhancer
RBPJ	Recombining binding protein suppressor of hairless
RT-PCR	Real time polymerase chain reaction
SCID	Severe combined immuno-deficient mice
SOCS3	Suppressor of cytokine signaling 3
SRE-1	SREPB-1 response element
SREBP	Steroid response element binding protein
STAM	Signal transducing adapter molecules
STAT	Signal transducers and activators of transcription
TA	Transient amplifying cells
TF	Transcription Factor/s
TGCA	The cancer genome atlas

TGFBR2	Transforming growth factor beta receptor 2
TK	Thymidine kinase
VEGF	Vascular endothelial growth factor

Chapter 1

Background and introduction

1.1 Cancer Statistics

The earliest mention of cancer can be traced to Hippocrates in 460- 370 BC. He coined the term “cancer” based on the appearance of the tissue mass post-surgery. Throughout the world, cancer is responsible for the severe physical and emotional suffering and death in both humans and animals. It has been proposed that most tumors are clonal in origin, which implicates arising from a single cell. During growth, this clonal cell acquires genetic variation and mutations which confer collective growth advantage to these cells leading to full-fledged cancer [1].

Cancer constitutes a rising health issue in developed and developing countries. It is a major public health problem in the United States accounting for one in four deaths. Prostate cancer alone accounts for 28% of registered cancer related incidents in men. Breast, lung and bronchus, and colorectal cancers in women account for 51% of cancer cases. According to predictions based on clinical statistics, prostate cancer, lung and bronchial cancer, and colorectal cancers (CRC) will account for about 50% of all newly diagnosed cancers. It is estimated that between 500,000 to 600,000 Americans will be affected by cancer in 2014 [2].

1.2 Intestine and its cellular architecture

1.2.1 Anatomy of the intestine

The intestinal tract is a tube like organ lined with three concentrically arranged tissue layers. The outer smooth muscle layer is heavily innervated with nerve terminals which regulate the rhythmic peristaltic movements of the intestine. The inner luminal surface consists of a single-cell layer simple epithelium and an acellular mucosa (mucosal layer) responsible for nutrient absorption and stool compaction. The connective tissue called stroma fills the space between these two layers and contains blood and lymph vessels, nerve fibers, and immune cells [3]. The intestinal tract can be anatomically divided into two segments: the small intestine and the large intestine or colon. Small intestine forms the absorptive surface and has numerous finger-like protrusions called “villi” pointing into the lumen and invaginations called “crypts of Lieberkühn” which are embedded into the submucosa. These villi and crypts increase the absorptive surface of the intestine. The colon lacks villi but retains the deeply embedded crypts [4]. Prenatally and at birth, intestinal epithelial proliferation is limited to small pockets along the length of the intestine. A few weeks postnatally, clear and distinguishable villi and crypts begin to appear, spreading across the intestine. As

development progresses, the number of crypts continuously increases to accommodate the growth of the organ by dividing through a process called “crypt fission” [5].

1.2.2 Cell types of the intestine

The epithelial lining of the intestine has an average turnover time of 7-8 days and is routinely replaced by new cells. Four distinct cell types constitute the intestinal epithelium: the absorptive enterocytes, enteroendocrine cells, mucus secreting goblet cells and Paneth cells [3] (Fig 1.1). Enterocytes constitute the absorptive cells of the intestine and are more abundant in the small intestine. Each enterocyte possesses numerous villi like projections that increase the absorptive surface. These cells secrete digestive enzymes and help in absorption of digested nutrients from the lumen. Enterocytes of the colon are completely devoid of the villi structures. Goblet cells secrete mucus and play a role in stool compaction. Their population is high in the colon compared to the small intestine. Enteroendocrine cells secrete hormones like serotonin, substance P and secretin. Paneth cells, which reside at the bottom of each crypt, secrete antimicrobial agents such as defensins and lysozyme. These help in regulating and controlling intestinal microbial population. In addition, crypts of both the small intestine and colon harbor a distinct stem cell population. These stem cells are located at the base of the crypt

and can regenerate the cell types lining the intestine [6]. The relative abundance of each cell type differs depending on the context of the segment; small intestine has a relative abundance of absorptive enterocytes, while the population of mucous secreting goblet cells increases in the colon [4].

1.3 Stem Cells

Stem cells are pluripotent cells that can differentiate into cells of diverse tissue types. They were first isolated and identified by Martin Evans in mouse blastocysts [7]. Human stem cells were identified and isolated by James Thompson from human blastocysts [8]. Later, stem cells were identified in several tissue types and are thought to be responsible for routine tissue regeneration. Hematopoietic stem cells were the first to be identified [9]. Stem cells of the intestine are located at the base of the crypt and have the potential to regenerate all of the cell types of the crypt [10].

1.3.1 Intestinal stem cells (ISC)

Stem cells of the crypt as described earlier are present at the base of the crypt and are critical in regenerating various cells of the intestinal epithelium [6], however, there are a few of these cells in the intestine. For more than a century, crypt stem cells were thought to be localized in a “proliferative zone” below the Paneth cells. Label retaining experiments by Potten *et* al., clearly demonstrated that cells located

at the 4th position (+4 position) from the base of the crypt constitutes the stem cell population [11] (Fig 1.1). These cells divide asymmetrically to produce a +4 cell and a transient amplifying cell (TA) [11]. ^{3}H-thymidine and BrdU incorporation studies including immunochemical labeling using Ki67 have shown that TA cells constitute the rapidly dividing cells of the intestine, with a cycling time of 12- 14 hours and are capable of differentiating into all four cell types of the of the crypt-villi axis. Both +4 cells and TA cells are pluripotent and can generate all of the intestinal cell types of the crypt-villus axis [10]. Radiation studies identified that +4 cells are generally resistant to low dose radiation but not high doses, a feature that protects the stem cells from genetic damage. In this model, TA cells help reestablish the stem cell population by falling into the +4 position to dedifferentiate into stem cells [12]. A second school of thought proposed by Leblong, Cheng and Bjerknes, believes that Crypt Base Columnar (CBC) cells, located at the base of the crypt, interspersed between Paneth cells, are small and undifferentiated, and constitute the stem cell population [13, 14] (Fig 1.1).

Irrespective of their origin (+4 cells or CBC), ISC's are critical in maintaining normal GI architecture and dysregulations in the ISC population can lead to neoplastic growth. Various factors, intrinsic or extrinsic, can contribute to this dysregulation and eventually lead to development of GI cancers.

1.3.2 Cancer stem cells (CSC)

CSCs were initially identified in human leukemia as a distinct group of cells capable of proliferation and self-renewal [15]. Singh and colleagues later identified in disintegrated brain tumors that CSC also express normal neural stem cell surface markers and were able to form neurospheres when cultured in adhesion free conditions. The number of these cells varied with tumor grade with high numbers in more aggressive tumors compared to benign forms [16]. Later research identified cancer stem cell population in diverse cancer types including breast [17], melanoma [18], ovarian [19], prostate [20], and colorectal cancer [21]. It is now well established that cancer stem cells are resistant to therapeutic interventions, responsible for tumor recurrence after a successful therapeutic intervention and ensure continued growth of the tumor [22]. Current therapeutic development research is directed towards targeting the CSC population. In spite of the awareness of the existence of CSC, an equal number of investigators argue against the existence of a distinct CSC population.

1.4 Signaling pathways and markers of ISC and CSC

ISC are thought to be located in a niche that regulates the balance between stem cell renewal and tissue regeneration. A number of signaling pathways are active and regulate the establishment of this niche and proliferation of ISC.

Figure 1.1: Structure of a colonic crypt

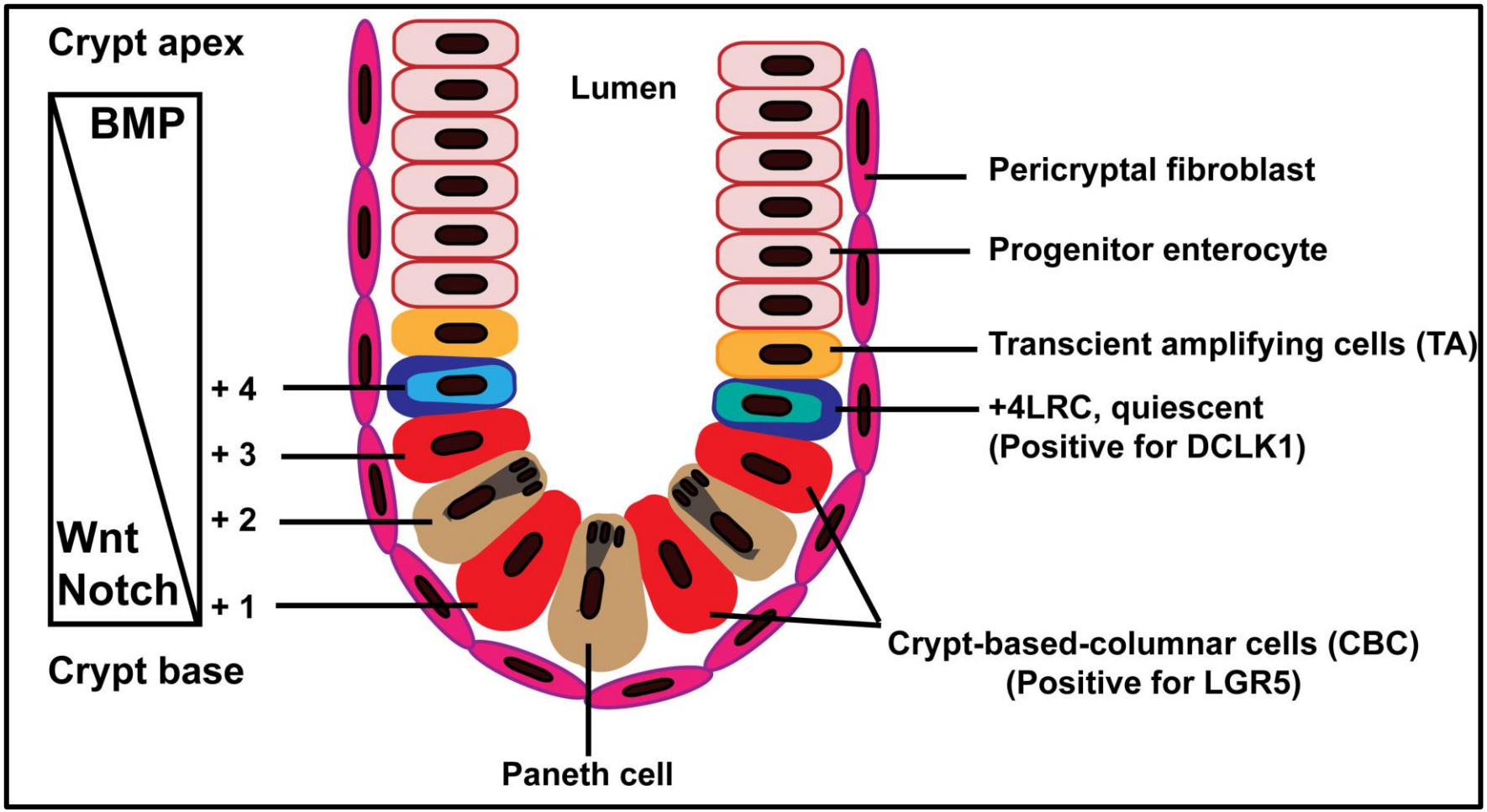


Figure 1.1: Structure of a colonic crypt: Colon consists of deeply embedded crypts, which are lined by columnar epithelial cells called enterocytes. Enterocytes can be categorized as crypt based columnar cells (CBC) and +4 DCLK1 and LGR5 positive cells, which make up the stem cells and transient stem cell population, mucin secreting Paneth cells, hormone secreting enteroendocrine cells and goblet cells. Moving along the crypt axis, the Wnt signaling pathway, critical for maintaining stem cell population, is active at the base and decreases in activity as we move up the axis. Whereas, Notch signaling, active in transient amplifying cells, increases in activity as we move up the crypt axis.

Genetic studies have provided evidence that the Wnt/ β-catenin signaling is important for ISC maintenance and self-renewal. Notch signaling pathways regulate ISC fate and differentiation, while BMP, secreted by the mesenchymal cells surrounding the crypt, down regulates both Wnt/ β-catenin and Notch signaling and induces differentiation [23] (Fig 1.1).

1.4.1 Signaling pathways

1.4.1.1 Notch signaling pathway

Notch signaling is a pathway that is active in intestinal stem cells and contributes significantly to stem cell proliferation and differentiation (Fig 1.2). It is involved in regulating stem cell hierarchy and determining cell fate [24] and is active in the intestinal crypts [25]. The Notch family of receptors consists of four transmembrane proteins designated as Notch 1- 4. Each Notch receptor consists of an extracellular domain (NECD) and intracellular domains (NICD). These domains are translated as a single protein; however, this peptide undergoes S1 cleavage and is later bound by disulfide linkages and held across the membrane. Notch receptor ligands (Jagged 1, 2 or Delta 1, 3, 4) are single pass transmembrane proteins that are localized on adjacent cell membranes. Binding of the ligand with the receptor leads to a conformational change in the Notch receptor leading to S2 cleavage. This cleaves off the Notch extracellular domain (NECD) and activates the γ-

Figure 1.2: Notch Signaling

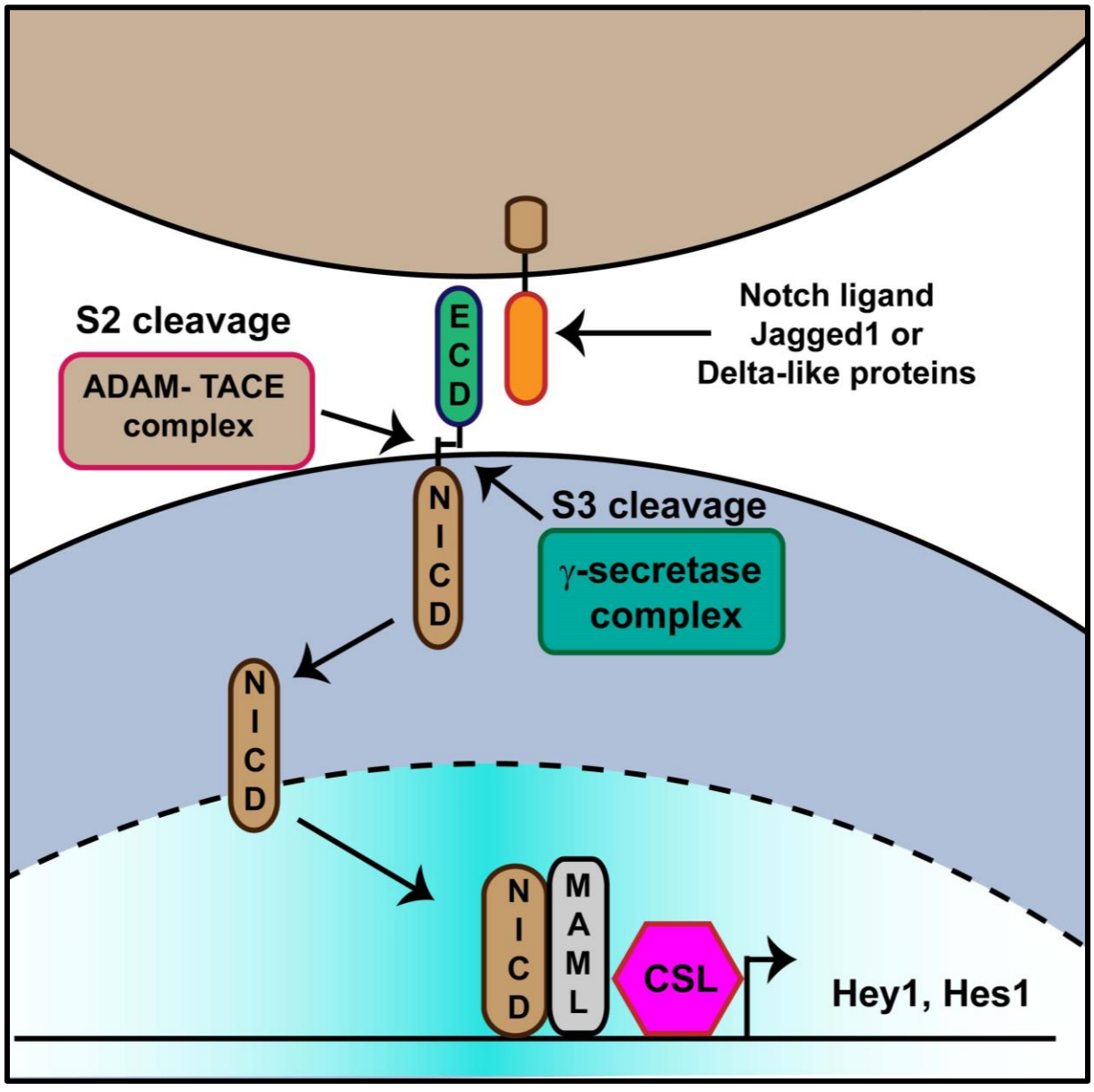


Figure 1.2: Notch Signaling: Notch signaling is necessary for maintain the crypt transient stem cell pool. Notch receptor and ligands are both transmembrane proteins. Interaction of the ligand to the notch extracellular domain (ECD), leads to a conformational change in the receptor, opening up cleavage sites initially to be acted upon by protease tumor necrosis factor α-converting enzyme (ADAM-TACE) (S2 cleavage) and later by the proteins of the γ-secretase complex (S3 cleavage), leading to release of the notch intracellular domain (NICD). NICD translocates into the nucleus to form a transcription initiating complex with Master Mind (MAML) and CBF1/ suppressor of hairless-1 (CSL) proteins and activates transcription of Notch responsive genes.

secretase complex consisting of Presenilin, Nicastrin, Anterior Pharynx Defective 1 (APH 1) and Presenilin enhancer 2 (PEN-2) proteins, which leads to the last S3 cleavage, resulting in the release of the Notch intracellular domain (NICD) [26, 27]. Cleaved NICD would translocate into the nucleus and bind to transcription enhancer proteins Mastermind and RBPJ, leading to the activation of Notch target genes. Hes-1 and Hey1 have long serves as a powerful target of Notch activation [28], and can be used to assess the degree of Notch signal activation. Constitutive Notch activation is necessary for intestinal stem cell maintenance [29] and deregulation of this pathway have been observed in CRC and other forms of cancer [30, 31].

1.4.1.2 Wnt signaling pathway

Canonical Wnt signaling is active at the crypt base and is critical in maintaining the colonic stem cell population. Wnt genes were initially identified in *Drosophila melanogaster* as factors that regulate the segmental behavior of larval development [32], and were later identified as the gene that is activated in virally induced breast tumors [33]. It is thought that perycryptic myofibroblasts produce the Wnt ligand that can bind to its cognate receptors located on the adjacent crypt base cells. Wnt receptor consist of a seven transmembrane domain containing Frizzled (Fz) proteins, that would bind to the Wnt ligand in the presence of LDL receptor protein

(LRP), also a single pass transmembrane protein to activate intracellular signaling. β-catenin is one of the critical intracellular proteins, which mediates canonical Wnt signaling. Binding of β-catenin to the TCF-LEF enhancer complex localized in the promoter regions of the Wnt responsive genes, regulates the expression of these genes. β-catenin has a very low turnover and in the absence of Wnt ligands, it forms a complex with Adenomatous polyposis coli (APC), Glycogen synthase kinase 3 β (GSK 3β), Axin and Casein Kinase-1 (CK-1). Phosphorylation of β-catenin mediated by CK1 and GSK 3β, directs it to ubiquitin-mediated degradation. Binding of Wnt ligand to the heterodimeric Fz and LRP receptor leads to CK1 mediated phosphorylation LRP receptor. Phosphorylated LRP acts as binding site for the Auxin causing a conformational change in the protein leading to the release of APC and β-catenin, which can eventually translocate into the nucleus and activate gene expression [34, 35]. Mutations in components of this pathway have been implicated in inheritable CRC, as described below.

1.4.1.3 Hedgehog signaling

The *hedgehog* (*hh*) gene was identified from genetic screens aimed at evaluating genes involved in body segmentation in *Drosophila melanogaster* [32]. Orthologous signaling pathway was later identified in higher vertebrates including humans. In humans and rodents, hedgehog signaling is involved in embryogenesis, tissue homeostasis, tissue repair and tumorigenesis by modulating stem cells [36].

Three Hh family ligands have been identified in humans: Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH). These ligands undergo auto processing and lipid modifications to generate mature proteins [37]. In the absence of the ligands, Patched family receptors (PTCH1 and 2) will interact and inhibit Smoothened (SMO) signal transducer protein. Sequestration of SMO leads to formation of GLI degradation complex. This complex is composed of casein kinase Iα (CKIα), glycogen synthase kinase- 3β (GSK-3β) and protein kinase A (PKA) and would bind to GLI proteins and phosphorylate them leading to their ubiquitin mediated degradation [38]. Binding of hedgehog ligand to the Patched receptor leads to release of SMO, which is later activated by STK36 (Serin/ threonine kinase), preventing formation of the GLI degradation complex, leading to stabilization and nuclear translocation of GLI proteins. Hedgehog signaling activates and leads to GLI-dependent transcriptional activation of target genes like Cyclin D2, FoxL1 and Jagged 2 [39].

1.4.1.4 Hippo signaling pathway

The Hippo signaling pathway was discovered in *Drosophila melanogaster* and regulates organ size across species [40]. Deregulation in this pathway is associated with disease and cancer in various tissues. Hippo pathway modulates tissue/ tumor size by directly regulating stem cell proliferation and maintenance [41]. Canonical

Hippo signaling pathway is mediated mainly by MST1/2 and LATS1/2 serine kinases that inhibit the YAP and TAZ transcriptional cofactors by phosphorylating them on Ser127 and Ser89, respectively. YAP and TAZ activate TEAD and other transcription factors to regulate gene expression. YAP and TAZ can also regulate a plethora of other activities including tumorigenesis. YAP expression is typically restricted to the crypt compartment. Expression of constitutively active YAP (YAP-S127A) protein in the intestine leads to expansion of undifferentiated cells in the crypt. A similar phenotype is observed when YAP protein is activated in the skin. Along similar lines, conditional deletion of MST1/2 lead to an intestinal phenotype similar to that of the YAP overexpressing model, with an increase in stem cell population. Canonical Hippo components SAV1 and MST1/2 actively restrict nuclear translocation of YAP in the ISCs, thus regulate their proliferation under normal conditions. Under pathological conditions such as CRC, active Hippo signaling together with Wnt and Notch signaling contribute to tumor progression by modulating ISC population [40].

1.4.2 ISC markers

1.4.2.1 Doublecortin like kinase 1 (DCLK1)

DCLK1, earlier known as Doublecortin and Calmodulin Kinase Like 1 (DCAMKL1) is a microtubule-associated protein which is highly expressed in

developing brain [42]. Work by Giannakis and colleagues using gene expression microarray analysis of small intestinal crypt cells identified DCLK1 as a potential stem cell marker [43]. Co-labeling studies with BrdU and DCLK1 demonstrated that DCLK1 cells have low BrdU retention and are generally located at the base of the crypt. Based on co-staining, Gagliardi and colleagues concluded that DCLK1 co-localizes with LGR5 (another colon crypt stem cell marker, described below) at the base of the crypt and with Chromogranin-A (CgA), a marker for enteroendocrine cells, throughout the crypt, further suggesting that DCLK1 can mark a specific subset of crypt stem cells specifically in the colon [44]. Work by May and colleagues demonstrated that DCLK1 is an epithelial cell surface protein and that even though coexpressed with LGR5, DCLK1 is expressed and retained more in the quiescent crypt stem cells while LGR5 is expressed more in actively dividing stem cell population [45]. Most recently, work done by Nakanishi using the $Apc^{Min/+}$: $Dclk1^{CreERT2/+}$ mouse model, showed that in a normal colon, DCLK1 may be expressed in crypt stem cells but is specifically expressed in tumor stem cells and potentially contributes towards maintaining CSC phenotype [46].

1.4.2.2 Leucin-rich-repeat-containing G-protein-coupled receptor 5 (LGR5)

LGR5, earlier known as an orphan G-protein coupled receptor GPR49, was identified as a stem cell marker protein identified in intestinal *Wnt* target gene

panel by Baker and colleagues [47]. In addition using the *Lgr5*$^{Cre/+}$: *Rosa2* $^{lacZ/+}$ mouse model, they showed that these cells are located at the base of the crypt, interspersed with Paneth cells and that they can give rise to all the other intestinal cell types over the course of time [47, 48]. Lineage tracing studies by Schepers and colleagues using a *Lgr5*$^{EGFP\text{-}Ires\text{-}CreERT2}$: *Apc*$^{fl/fl}$ mouse model, suggested that, LGR5+ cells constitute 5- 10% cells of the adenomas generated in this model. They also demonstrated that these LGR5+ cells are capable of forming adenomas when used in a xenograft model [49]. Recently, LGR5 expression was also detected in specific cells of the embryonic metanephric mesenchyme cells, a group of cells that give rise to adult kidneys. Knock down of LGR5 expression in these cells led to improper kidney development [50].

1.4.2.3 Other putative stem cell markers

Musashi-1 (Msi-1) is an RNA binding protein identified in neural stem cells of *Drosophila melanogaster* [51]. It is involved in down regulating Notch signaling by translational regulation of its target gene Hes-1, that is required for differentiation of stem cells into secretory lineage. Msi-1 marks cells located at the base of the crypt are interspersed among Paneth cells [52]. Mice lacking Msi-1 expressing cells, however, do not have defective crypt and intestinal development [53].

Polycomb complex protein BMI-1 (BMI-1), also known as Polycomb group RING finger protein 4 (PCGF4) or RING finger protein 51 (RNF51), plays a critical role in maintenance of chromatin silencing [54] and is highly expressed and necessary for self-renewal of neuronal, hematopoietic, and leukemic cells. Lineage tracing using *Bmi-1*$^{Cre/+}$mouse model, demonstrated that Bmi-1 expression is found in crypt based cells and colocalizes with LGR5+ cells, indicating that Bmi-1 cells also mark crypt stem cells [55].

1.5 Colorectal cancer

Colorectal cancer (CRC) is the third leading cause of cancer related death in United States. Worldwide, up to 5% of all reported cancer cases are due to CRC, with 60% of them being diagnosed in industrially developed or developing countries. CRC is caused by genetic and environmental factors. Environmental factors ranging from changing dietary habits to environmental toxins are associated CRC. In addition, genetic factors such as germline mutations in APC, TP53, and DNA mismatch repair genes also contribute to CRC pathogenesis and demographically constitute nearly 35% of the registered CRC cases [56].

1.5.1 Genetic mutations in colorectal tumorigenesis

Colorectal tumorigenesis is a multistep process and provides an excellent model of study to elucidate the sequential molecular events that lead to cancer initiation and

Figure 1.3: Vogelstein Model

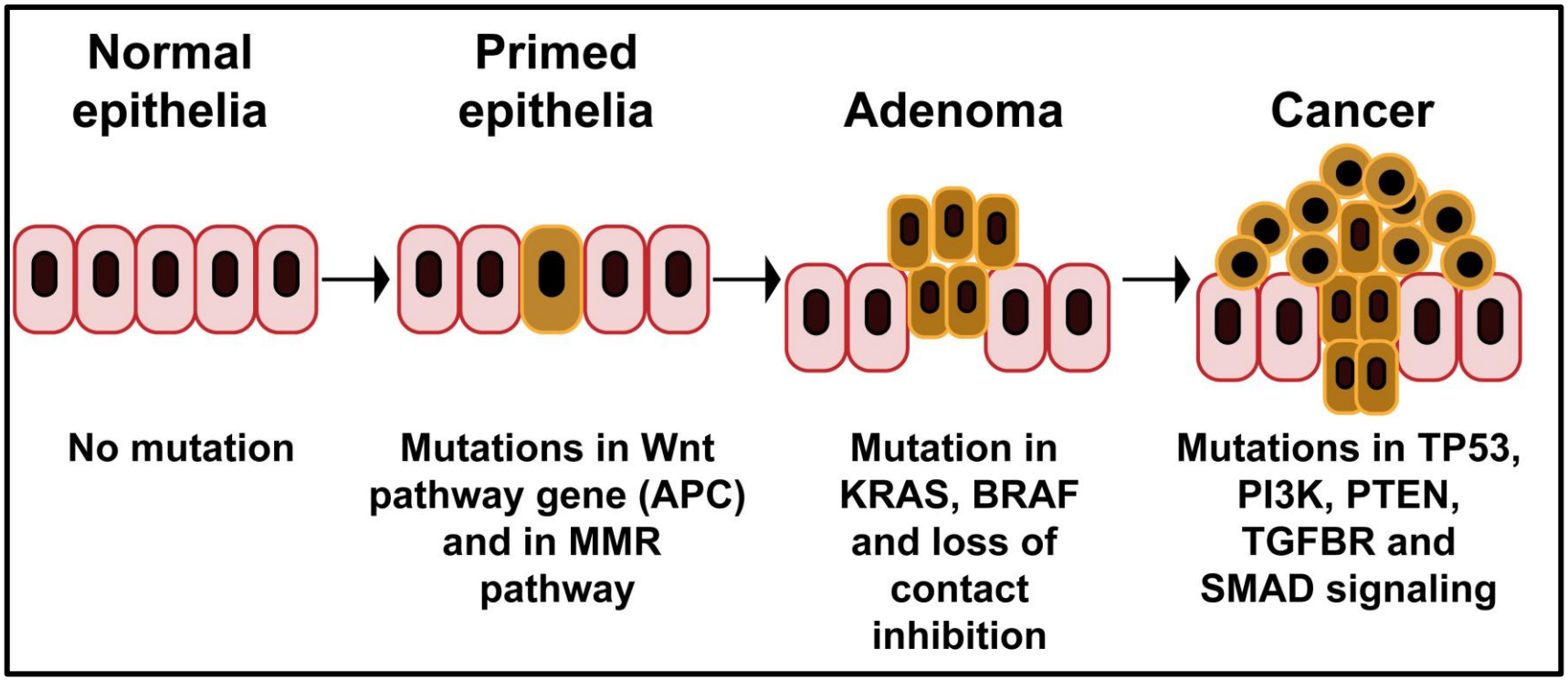


Figure 1.3: Vogelstein Model: According to this model, mutations in the APC gene (a critical component of the Wnt pathway) and mis- match repair (MMR) pathways are some of the initial events in neoplastic transformation. Following this, dysregulation in KRAS-BRAF (Ras/Raf) signaling pathways leads to an increase in cell proliferation and adherence free cell growth. Further mutations in TP53 gene and activation of PI3K, PTEN, and SMAD pathways would lead to a complete neoplastic transformation and development of full-fledged adenoma.

progression [57]. The occurrence of CRC is preceded by a sequence of mutation events in genes whose normal function would be to maintain chromosomal integrity and regulate proliferation. According to "Vogelstein Model" (Fig 1.4), colorectal tumors arise as a result of the mutational events, which cause inactivation of tumor suppressor genes and simultaneously cause oncogenic activation. Mutation in at least four to five tumor suppressor genes is required for initiation and formation of a malignant tumor. Fewer mutational events lead to the formation of a benign tumor. Most of these mutational events often occur in a sequential order in CRC. It is the accumulation of these mutations that determines the biological properties of the tumor [58]. These mutations account for most of the chromosomal instability associated with colorectal tumors and lead to loss/ mutation of the wild type copy of APC, P53, and SMAD family member 4 (SMAD4) of tumor suppressor genes whose normal function is to oppose malignant phenotype [59] (Fig 1).

1.5.1.1 Adenomatous polyposis coli (APC) gene mutation

A variety of signaling pathways play an active role in establishing CRC but clearly the Wnt signaling pathway stands out as the prime pathway. As discussed earlier (Section 1.3.1 and Fig 1.2), Wnt signaling is critical in establishing a normal crypt axis and ensures survival of crypt stem cells. Most of the initial mutational events

which lead to colorectal tumors, both in the hereditary form or the spontaneous form, occur in genes that code for protein involved in this pathway. A direct involvement of the Wnt pathway in colorectal tumors was identified in patients with familial adenomatous polyposis (FAP) which a form of inheritable CRC.

APC is a tumor suppressor gene that is mutated in most FAP patients. Apart from serving as a carrier and playing a critical role in regulating cytoplasmic verses nuclear level of β-catenin, it also translocates into the nucleus either independently or in complex with β-catenin [60]. Recent evidence suggest that APC can regulate expression of Wnt target genes by recruiting H3K4 histone demethylase and α-catenin onto the regulatory regions of these genes [61, 62]. Mutations in APC are mostly truncations leading to complete lack of the β-catenin/ axin binding domain. This results in increased nuclear translocation of β-catenin and subsequent activation of Wnt responsive genes. Multiple intestinal neoplasia (Min) is a mutant allele of the murine *Apc* locus developed using ethylnitrosurea and has high penetrance [63]. Like humans with FAP, $Apc^{Min/+}$ mice show extensive predisposition to spontaneous intestinal tumors and have been used as models to study intestinal tumorigenesis [64, 65]. Taken together, these observations suggest that mutations in *APC* gene serve as the starting point for colorectal cancer development.

1.5.1.2 Tumor protein p53 (TP53) mutations

Mutations in the *TP53* gene are the second key genetic event in development of CRC. *TP53* encodes for p53 a tumor suppressor protein which mediates cell-cycle arrest, cell death checkpoint, and activates multiple cellular stresses. Most of the colorectal adenomas have either a missense mutation or deletion of entire 17p chromosomal locus containing the TP53 gene leading to inactivation of one or both the alleles of *TP53* gene. *TP53* mutations cause transition of large adenomas to invasive carcinomas [59].

1.5.1.3 Transforming Growth Factor Beta Receptor Type 2 (TGFBR2) mutations

Somatic mutations in the *TGFBR2* are noted in a majority of patients with advanced colorectal cancer [66]. The *TGFBR2* gene is subject to frameshift mutations which occur primarily due to lack of mismatch-repair mechanism in advanced CRC. In addition, mutations in the downstream components of the TGF-β pathway, such as mutations in *SMAD* genes, lead to high-grade carcinomas [67].

1.5.1.4 Mutations in RAS- BRAF and PI3K pathway genes

Oncogenic mutations in the *RAS* gene occur in a majority of CRC. These mutations enhance the GTPase activity of the protein rendering it constitutively

active. Activated RAS in turn activates the RAF protein by phosphorylation and together they activate (via phosphorylate) proteins of the mitogen-activated protein kinase (MAPK) signaling pathway to stimulate growth and proliferation [68]. Similarly, independent mutations in BRAF alone can activate the serine–threonine kinase activity and activate the MAPK signaling cascade.

BRAF mutations are most commonly detected in small polyps compared to RAS mutations, which are more common in hyperplastic polyps, serrated adenomas, and proximal colon cancers. Patients with numerous and large hyperplastic lesions, a condition called hyperplastic polyposis syndrome, which carry activated RAS-RAF mutations, have an increased risk of CRC [69].

Activating somatic mutations in PI3K-CA, which encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), can be detected in advanced cases of CRC. The loss of PTEN, an inhibitor of PI3K signaling, is a less common genetic alteration and can substitute and augment the effects of PI3K mutations. In addition, amplification of the insulin receptor substrate 2 (IRS2), an upstream activator of PI3K signaling, along with coamplification of the downstream mediators of PI3K signaling component like AKT and PAK4, are also detected in advanced colorectal tumors [70, 71].

Figure 1.4: Involvement of growth factors in CRC

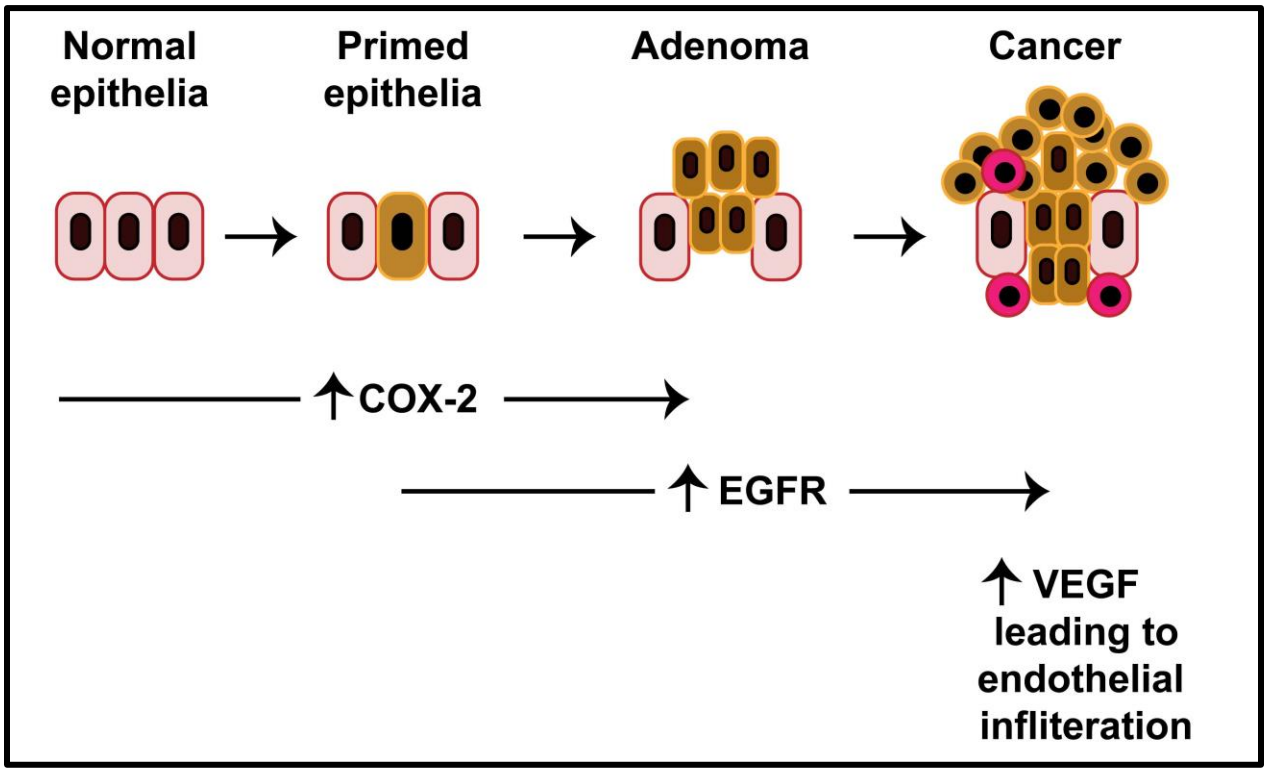


Figure 1.4: Involvement of growth factors in CRC: Activation of various growth signaling pathways plays a critical role in establishment of colorectal cancer. COX-2, an inflammation related protein, is upregulated in the initial stages of CRC. Subsequent activation of EGFR and TGFBR signaling leads to the establishment of tumor. Progressive activation of VEGF signaling induces formation of new blood vessels, ensuring tumor survival and aiding metastasis.

1.6 Growth factor pathway activation in colorectal cancer

Numerous growth factor pathways are activated in CRC. These factors directly or indirectly influence surrounding tissue environment to help in the growth of the tumor and tumor progression (Fig 1.5).

1.6.1 Prostaglandin E2

Activation of prostaglandin signaling is an early and critical step in the development of an adenoma. Abnormal activation of COX-2 due to inflammation or mitogen signaling, mediates the synthesis of prostaglandin E2 (PGE2), an agent strongly associated with CRC. 15-prostaglandin dehydrogenase (15-PGDH) is a rate-limiting enzyme which catalyzes the degradation of prostaglandin E2. In the early stages of CRC, the β-catenin/ T-cell factor-1 (TCF-1) complex binds to and inhibits the expression of 15-PGDH leading to complete loss of the enzymatic activity and increased accumulation PGE2 and COX-2 in the majority of colorectal adenomas and cancers. Clinical trials using non-steroidal anti-inflammatory drugs (NSAID), which inhibit COX-2 activity not only prevented development of new adenomas but also induced regression of established adenomas [72].

1.6.2 Epidermal Growth Factor Receptor

Epidermal growth factor (EGF) is a soluble protein that has trophic effects on intestinal cells. Clinically, in a subset of colorectal cancer cases, active EGF signaling through the EGF receptor (EGFR) has been shown to play a critical role. EGF- EGFR signaling activates the MAPK and PI3K signaling cascades. Recent clinical data has shown tumor promoting mutations of this pathway. Activating mutations in KRAS, BRAF and the p110 subunit of PI3K have been shown in advanced colorectal cancer [73].

1.6.3 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is an angiogenic factor that is responsible for production of new blood vessels, and is produced in states of injury and during the growth of normal tissue. Clinically angiogenic signaling pathways play a critical role in the growth of colorectal cancer even conferring metastatic advantage to the tumor. Anti-VEGF therapy using bevacizumab (an anti-VEGF antibody) increased the overall survival of colorectal cancer patients [74].

Inferring from the above results and clinically speaking, colorectal cancer is a result of multifactorial interactions that ultimately manifest into a disease. However, the challenge is to determine the factor(s) and understand how these

factors initiate the development of the tumor, drive its progression, and determine its responsiveness to therapeutic agents.

1.7 Role of endocrine hormones in colorectal cancer

Endocrine hormones are synthesized and secreted by specialized cell types in the body, released into the blood and are involved in regulating growth, development, and differentiation of various tissues in the body. They also play an active role in regulating various physiological functions such as growth, metabolism and reproduction by modulating specific signaling pathways. Any disruption either in the synthesis and production of these hormones or their downstream signaling cascade would lead to a disease state. Endocrine hormones particularly PRL, play a relevant role in breast, lung, hepatic and prostate cancer. Accumulating evidence indicates PRL's active involvement in colorectal cancer**.**

1.8 Prolactin (PRL)

PRL is a peptide hormone produced and secreted by the lactotrophs of the anterior pituitary was initially identified in 1928 by Stricker and Grueter [75]. Later experiments by Oscar Riddle in 1933, using the pigeon crop-sac assay, clearly elucidated for the first time that this pituitary extract can stimulate milk production and named it "Prolactin" [76]. The existence of a distinguishable human homologue of PRL was a highly debated issue prior to 1970. Histochemical studies

by independent labs identified PRL secreting cells in the pituitary during pregnancy. This has provided compelling evidence for the first time for the existence of PRL, thus establishing its identity, independent from growth hormone [77]. Serum levels of PRL are high in pregnant and lactating women [78] and neonates [79].

In humans, the *PRL* gene is located on chromosome 6 [80] spanning a region of 10 Kb, and it consists of 5 exons coding for a peptide of 227 residues (of which 28 residues make up the signal peptide) [81]. PRL gene and protein share considerably homology with growth hormone (GH) and placental lactogen (PL) and are grouped into the same protein family. It is believed that all these genes arise from the same ancestral gene through gene duplication and mutation events [82, 83]. Several PRL variants have been identified which arise due to posttranslational modification and considerably expand the functionality of the hormone [84].

Contributions by Horseman and Ormandy using animal models including mice deficient in PRL [85] confirmed that PRL was the hormone responsible for lactation. Synthesis and secretion is PRL is a tightly regulated process which is multifactorial involving both negative (dopamine) and positive regulators (Estrogen, cAMP, Insulin, Thyroid Releasing Hormone).

Figure 1.5: Prolactin gene and transcripts

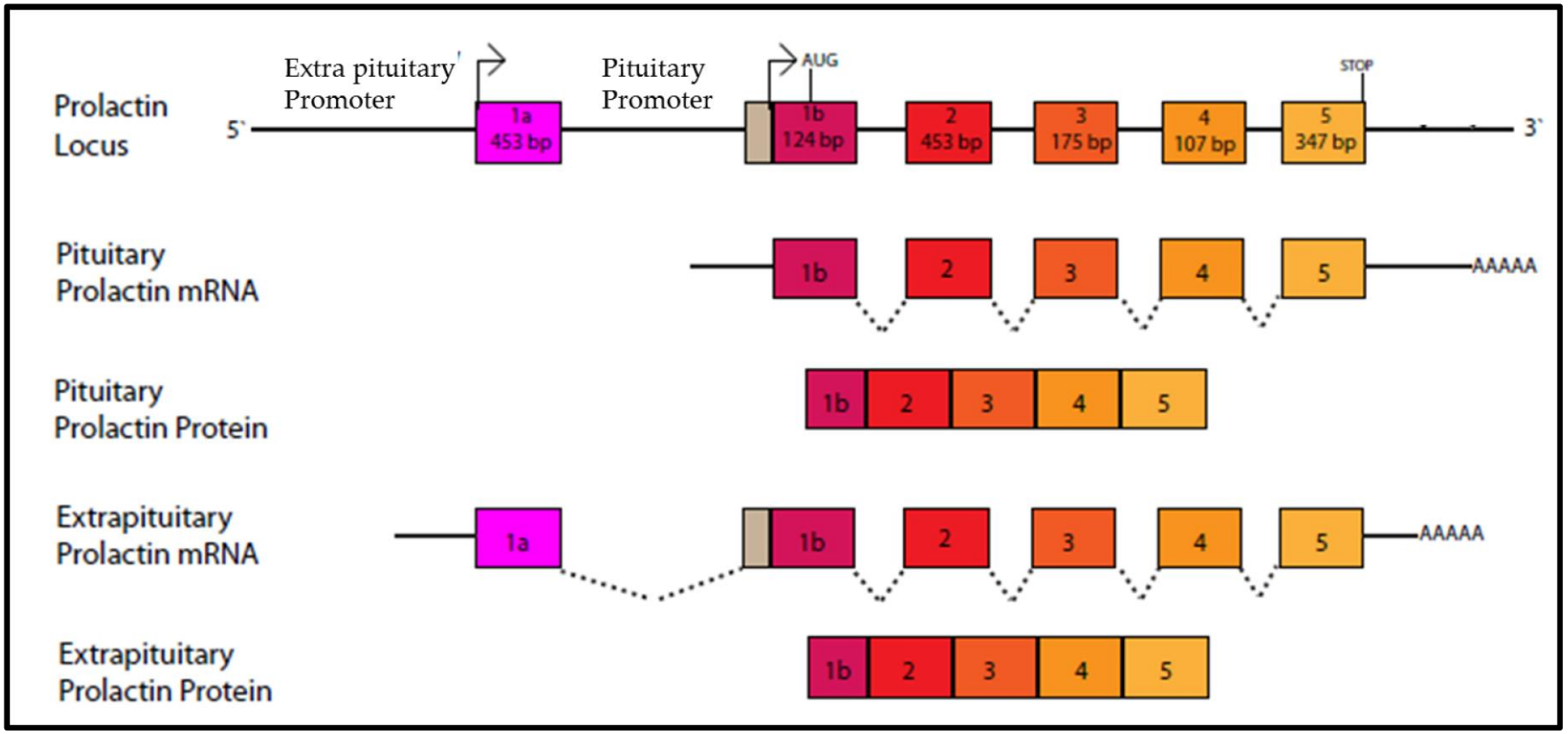


Figure 1.5: Prolactin gene and transcripts: PRL gene is located on chromosome 6, and codes for two distinct transcripts from two different and independent promoters. The pituitary transcript is shorter than the extrapituitary transcript, which has an additional exon ‘1a’. However, irrespective of the promoter used or the length of the transcript, they both produce the same 23 kDa active PRL protein.

Neuroendocrine regulation at the hypothalamic level is regulated by dopamine, and contributes to the daily variation in serum PRL levels and the stress induced increase in serum PRL [86, 87]. Secretion of pituitary PRL is regulated by a short-loop feedback regulation. First, synthesis and secretion of pituitary PRL is controlled by dopamine, a catecholamine neurotransmitter. Dopamine is produced in the hypothalamus and acts through the D2 subclass of dopamine receptors on the lactotroph cells of the anterior pituitary to negatively regulate PRL expression and secretion [88]. Second, after reaching a threshold serum concentration, active PRL signaling through PRL receptor (PRLR) increases dopamine production leading to inhibition of PRL [89]. Supportive evidence for the negative feedback effect of PRL comes from work by Binart and colleagues who observed increased serum PRL levels in *Prlr* knockout (*Prlr*$^{-/-}$)mouse models [90, 91]. Several factors such as neurotransmitters, neuropeptides and other hormones have also been implicated in regulating PRL production [92]. Ectopic production of PRL has also been detected in mammary [93], lung [94], bladder [95], uterine decidua [96, 97], prostate and ovary tumors [98]. PRL or PRLR transcripts and protein were found to be overexpressed in malignant tissue as compared to normal tissue and this increase is localized mainly to the epithelial cells. This suggests that epithelial cells are the main ectopic source of the peptide hormone [99].

PRL expression is regulated by two distinct promoter elements; a pituitary promoter located upstream of the transcription start site is necessary and sufficient for transcription of PRL in response to pituitary signals [100]. A second promoter regulates extrapituitary expression of PRL and was initially described as directing PRL expression in lymphoid and decidual cells [101, 102]. The transcript lengths differ depending on the promoter used and the tissue type, but encode identical 23 kDa (23k PRL), mature peptide. This represents the predominant form synthesized and secreted by the pituitary and extra-pituitary tissues (Fig 1.5). In addition, a novel N-Terminal 16 kDa (16K PRL) fragment of PRL has been described which inhibits angiogenesis by affecting endothelial cell proliferation [103, 104].

1.9 Prolactin and its role in normal development and pathologies

Since its initial identification, the involvement of PRL in regulating normal development of various tissues and its role in various growth abnormalities, particularly cancer has been a subject of active research. We now know from published work over the last decade that PRL and the subsequent signaling induced by it regulate not only normal development of various tissues, but also have a supportive role in development of various pathologies.

1.9.1 Role of PRL in brain, stress, fertility and pregnancy

Apart from circulating PRL being transported to the brain, work done by Fuxe and colleagues using PRL specific probes and antibodies showed the existence of PRL like transcripts and protein in brain sections of hypophysectomized rat brains suggesting that brain produces PRL [105-107]. Similarly, estradiol (E2)-treated hypophysectomised rats or direct injection of E2 into the pons-medulla region of the brain caused increased PRL expression compared to untreated controls, further supporting pituitary independent expression of PRL in brain [108]. PRL is an anxiolytic agent capable of inducing a dose-dependent suppression of anxiety behavior and acute stress response [109, 110]. Injecting PRL antisense oligos into the pituitary portal system prevented anxiety and acute stress response supporting the role of PRL as an anti-stress factor [110]. Corticotrophins are released in response to acute stress. Chronic PRL treatment blocked stress-induced increases in corticotrophin releasing hormone in para-ventricular nucleus thus reducing neuronal activation in response to stress [109].

Pituitary production of PRL increases during pregnancy and lactation. It is necessary for the proper establishment and maintenance of pregnancy. Elevated levels of PRL are maintained by the suckling stimulus in the post parturition period [111, 112]. Along similar lines, increased immuno-reactive PRLR was observed in

brain sections of rats having active and ample sucking stimulus compared to control non-pregnant rats, demonstrating increased sensitivity of brain and associated neurons to PRL during pregnancy [113]. PRL causes pregnancy induced increases in food intake by inducing leptin (a hormone responsible for satiety) resistance [114], an adaptation necessary to meet the energy demands of the developing fetus and/ or lactation [115]. The anxiolytic effect of PRL suppresses stress responses induced by glucocorticoids during late pregnancy, lactation, and pregnancy induced hyperthermia an adaption responsible for decreasing fetal growth and abnormalities [116, 117]. In addition, PRL-induced transient suppression of fertility as adaptation to balance the nutritional need and energy expense necessary for lactating females and birth spacing [118]. Finally, PRL-induced hypothalamic neurogenesis during pregnancy is important in establishing maternal recognition of the offspring, contributing to enhancement of maternal behavior [119].

1.9.2 Role of PRL in normal breast development and breast cancer

PRL is the principal lactogenic hormone secreted by the lactotroph cells of the anterior pituitary and is critical in inducing and establishing lactation, milk production and milk macronutrient content. The concentration of circulating prolactin increases during pregnancy so that by the end of gestation, levels are 10

to 20 times over normal non pregnant levels. Acting both as a mitogenic and a differentiating agent, PRL profoundly influences the normal development and differentiation of the mammary gland. Data obtained using PRL and/or PRLR knockout mice show that PRL regulates branching of ductal epithelia during puberty and controls lobulo-alveolar development and lactogenesis during pregnancy [120]. Elevated levels of progesterone, observed during pregnancy prevent pre-parturition PRL induction of milk secretion. Following fetal delivery, clearance of progesterone and estrogen releases the inhibitory influence on PRL, ensuring copious milk secretion [120]. Experiments on bovine models indicate that inhibiting PRL delays mammary gland development and differentiation.

In differentiated mammary cells of a nursing female, PRL stimulates synthesis and secretion of milk protein β-casein, a part from stimulating the synthesis of alpha-lactalbumin (the regulatory protein of the lactose synthetase enzyme system) and increasing lipoprotein lipase activity. Post parturition, PRL ensures continued milk production. PRL is also secreted into milk at levels nearly close to the circulating concentration, however the physiological significance of this PRL in the infant is unknown [121].

Evidence supporting the mitogenic role of PRL on mammary epithelia comes from studies where PRL was knocked down either in mammary epithelium, stroma, or

both. These mice demonstrated normal mammary development until parturition. However, a significant decrease in mammary epithelial cell proliferation was observed postpartum, as demonstrated by decreased bromodeoxyuridine (BrdU) incorporation in the PRL knockouts compared to wild type controls, suggesting the involvement of autocrine/paracrine PRL in mammary epithelial cell proliferation [122].

A large number of publications in the early 1970's indicated a strong association between excess PRL in the serum with increased risk of developing breast cancer. The role of PRL in mammary cancer was suggested several decades ago, based on initial observations in rodent models of breast cancer [123]. Welsch and colleagues showed that mammary PRL contributes significantly to the pathogenesis and progression of neoplastic mammary tumors in mouse models [124]. Isograft of pituitary under the renal capsule in rodent models leads to elevated serum PRL levels. Multiple incidences of spontaneous mammary tumors were noted in these model suggesting active production of mammary PRL and its involvement in mammary tumorigenesis [125]. Oakes and colleagues identified a decrease in neoplastic lesions in PRLR knockout mouse [126].

To demonstrate the autocrine/ paracrine function of PRL in human breast cancer, PRL responsive estrogen receptor (ER) negative breast adenocarcinoma cells

T47Dco and ER+ve MCF7 cells were treated *in vitro* with monoclonal anti-human PRL antibodies (mAb 631 and mAb 390). This resulted in 86 and 68% inhibition of cell growth in T47Dco cells and 20 and 71% reduction in the MCF7 cells respectively as compared to untreated cells implicating the mitogenic effect of local PRL in human breast cancer. Additionally, PRL responsive, Nb2 rat lymphoma cells, cultured in conditioned medium collected from PRL antibody treated T47Dco had decreased growth, as compared to cells cultured in media from untreated T47Dco cells, further supporting active secretion and an autocrine action of PRL in these cells [93]. Together, these data suggest that human breast tissue, both normal and malignant, is a source of extrapituitary PRL. Not only PRL but transcript and protein levels of PRLR were also increased in a vast majority of breast cancer biopsies independent of estrogen and progesterone receptor status [127, 128].

1.9.3 Role of PRL in normal prostate development and prostate cancer

The prostate gland is a hormone-dependent organ. Androgens and PRL plays an important role in growth, development, and differentiation of the prostate. Prostate cancer (CaP) currently represents the second most common cause of cancer death and is the most frequently diagnosed cancer in men [2]. Worldwide 8-103 cases per every 100,000 individuals are diagnosed with CaP with a mortality rate of 2-32

per 100,000 individuals. In the United States, CaP represents the most frequent tumors representing 25% of all new reported male cancer cases occupying the second most common cause of male mortality [2].

In 1955, Grayhack identified that prolactin was necessary for complete prostate formation and development, using rat models where PRL secretion was inhibited in embryonic stages, leading to abnormal development prostate [129]. Similarly, chronic hyperprolactinemia rodent models showed a significant prostate enlargement [130]. *In vitro* PRL treatment caused an increase in survival and decreased apoptosis in PC3 prostate cancer cells [131]. Transgenic mice expressing PRL in a prostate specific probasin induced model had an expansion/ increase of the basal/stem cell compartment in the prostates [132]. Additionally, homozygous PRLR knockout mice had a 30- 40% reduction in occurrence of prostrate tumors compared to wild type mice when challenged with tumor inducing virus, such as SV40T [133]. Clinically, elevated preoperative levels of serum PRL were also noted in hypernephroma patients and the levels dramatically decreased after surgical removal of the tumor [95]. These observations clearly implicate the critical role of PRL not only in prostate development, but also in prostate cancers.

1.9.4 Role of PRL in normal liver development and hepatological cancer

PRL is a potent liver mitogen and circulating levels of PRL increase during physiological and pathological liver growth. Clinically, a significant increase in serum PRL levels was noted in a cohort of patients with hepatic cirrhosis (27-30 ng/ml in males and 38-42 ng/ml in females) compared to control subjects. Among them, patients with suspected encephalopathy had significantly higher serum PRL than others which was significantly correlated with mortality [134]. Intravenous PRL injection into mice with chemically induced hepatic cancer, for six weeks, led to hepatomegaly, large tumor like foci along with an increase in hepatic DNA synthesis and an increase in liver-to-body weight ratio (LBW), suggesting a mitogenic role of PRL in hepatic tumors [135]. An additional, PRL treatment for 23 weeks lead to further increase in the number of tumor foci [135]. In rodents, partial hepatectomy (PH) led to an elevation in serum PRL levels as early as 5-15 min post operation. Protein kinase C (PKC) signaling is a critical pathway that regulates hepatic cell proliferation. A simultaneous increase in nuclear PKC activity with increased serum PRL in PH rats suggests active PRL-PKC signaling leading to hepatic proliferation post hepatectomy [135]. At a molecular level, PRL administration after PH causes an increase in several transcription factors involved in hepatic cell proliferation such as AP-1, c-Jun and STAT3 along with liver-specific differentiation and maintenance of energy metabolism such as CEBPα,

HNF-1, HNF-4 and HNF-3 [136]. Further, isolated hepatocytes from lactating rat treated with exogenous PRL showed an increase in Src- tyrosine kinase activity along with an increase in expression of c-fos, c-jun, and c-src genes, which promote proliferation and cell division implicating the critical role of PRL in promoting liver growth and regeneration [137].

1.9.5 Role of PRL in gynecological cancer

Serum levels of PRL are elevated in women with ovarian and endometrial tumors to variable levels and serves as a strong diagnostic biomarker in these tumors [138]. Additionally, an increase in expression of PRLR was observed in tumor biopsies from patients with ovarian and endometrial cancer compared to healthy tissues. This increase in PRLR may be responsible for the PRL induced increase in proliferation, cell survival in ovarian and endometrial cancer. *In vitro* ovarian cancer cells treated with exogenous PRL had a rapid activation of Ras-signaling; a critical event which initiates ovarian and endometrial cancer development [139], implicating the role of PRL in regulating at least some gynecological cancers.

1.9.6 Prolactin and its role in normal intestine

PRL stimulates proliferation of the mucosal cells of the gastrointestinal tract in the lactating rat model [140] suggesting a pregnancy induced role of PRL in regulating growth and proliferation in the gastrointestinal tract. Rodent models

where pituitary explants were transplanted into the renal capsule showed cellular and mucosal hyperplasia in both jejunum and ileum [141]. Pregnancy-induced PRL also increased intestinal specific vitamin D and calcium absorption [142]. PRL induces expression of bicarbonate transporter necessary for water and electrolyte transport suggesting that PRL is helpful maintaining intestinal ion homeostasis [143] [144].

1.9.7 Implication of prolactin in colorectal tumors

Bhatavdekar and colleagues reported high serum concentration of PRL in preoperative colorectal cancer patients [145, 146]. Ilan and colleagues also observed that in 53% of the patients with colorectal malignancy, there was elevated levels of PRL that decreased after surgical removal of the tumor [147]. Conflicting data by Baert and colleagues suggested hyperprolactinemia to be a secondary effect and that it occurs more common in rectal cancer and do not support the hypothesis of ectopic PRL production by colorectal tumors [148]. Similar observations were reported by Carlson and colleagues, suggesting that the increase in circulating PRL may be a consequence of renal, endocrine and psychiatric disorders, medications and/ or a premenopausal situation in patients with colorectal neoplasms [149].

Studies by Jan-Michel Otte and colleagues with colon cancer cell lines showed that exogenous PRL treatment increased H^3-thymidine incorporation suggesting a mitogenic role of PRL in colorectal cancers [99]. Soroush and colleagues observed that 76% of patients with colorectal malignancies had an increase in circulating PRL levels and suggested that PRL can be a better prognostic marker than carcinoembryonic antigen (CEA), an established colorectal tumor marker, a finding further supported by Bhatavdekar [145, 150, 151]. However, a further large scale studies on an extended patient population comprising both preoperative and postoperative patients is necessary to validate the use of PRL as a valid marker for colorectal cancer.

1.9.8 Role of prolactin in stem cells

Autocrine/ paracrine PRL has been implicated in promoting proliferation and growth of stem cell populations in high grade prostate cancer via the activation of Jak/STAT pathway [132, 152]. Inhibiting Jak/STAT activation using prostate specific PRL knock down and using competitive PRLR-antagonist, Route and colleagues, reported prevention of the expansion of prostate stem cell populations [153].

PRL has also been indicated to play a critical role in the expansion of the neural stem cell population during severe brain injury and pregnancy [154]. Isolated

neuronal cells treated with recombinant PRL showed an increase in neurosphere formation *in vitro* by a significant proportion compared to untreated cells. Direct infusion of PRL into adult dentate gyrus induces an expansion of precursor cell population [155].

Isolated ovarian cancer cells, treated with exogenous PRL showed an expansion of clonogenic population and an increase in adherence free growth indicating that PRL-induced expansion of primordial/ stem cell population [139]. These findings clearly demonstrate that PRL can regulate organ/ tissue specific stem cell population. However, the intracellular signaling modules activated in the presence of PRL which can regulate growth and expansions of stem cell population are not well characterized.

Figure 1.6: Prolactin receptor gene structure

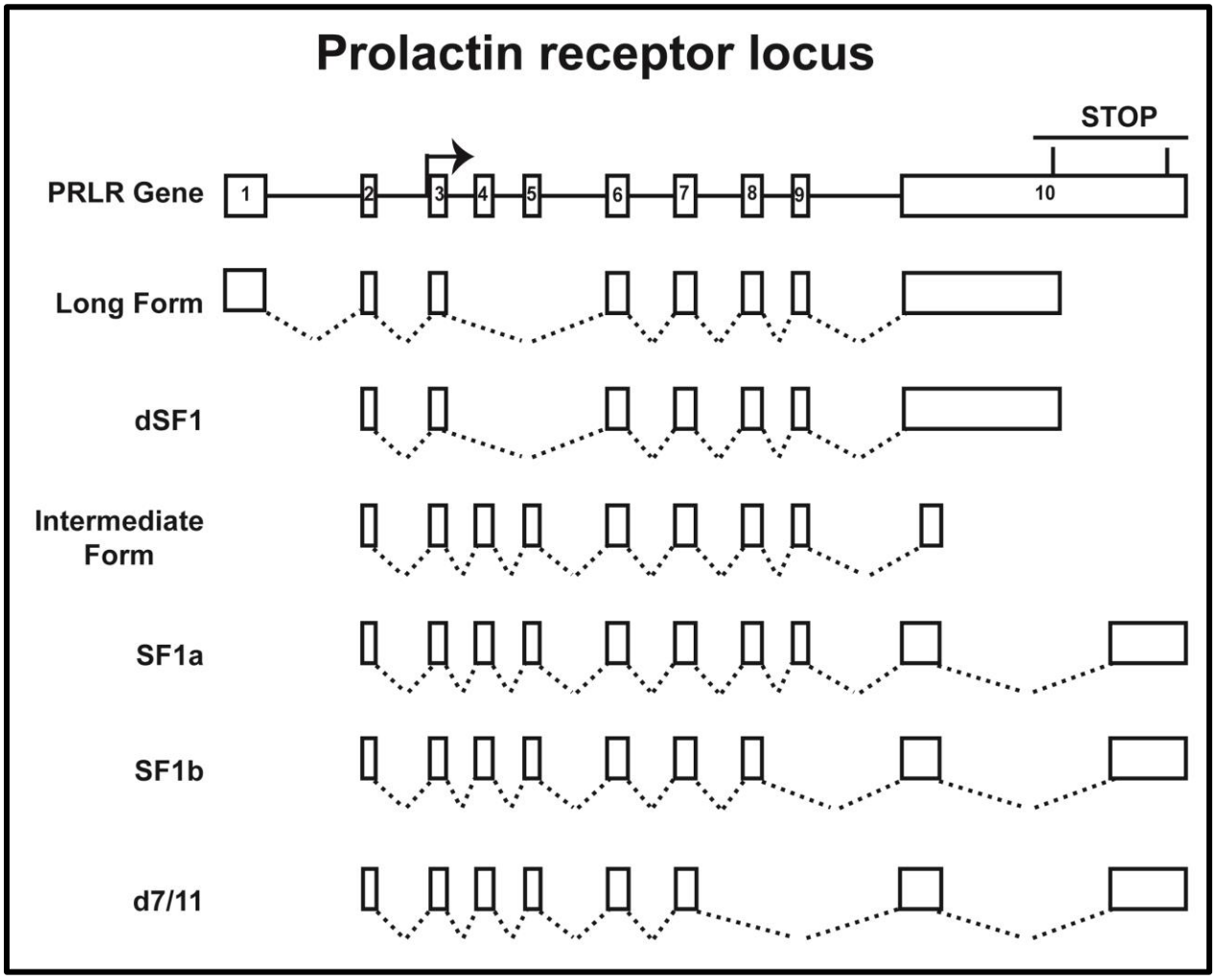


Figure 1.6: Prolactin receptor gene structure: Located on chromosome 5 at p13 locus, prolactin receptor gene codes for a single transcript driven from same promoter region. However, alternate transcription leads to the production of multiple variants as depicted in the figure. These have deletion of various exons and have different stop sites.

Figure 1.7: Prolactin receptor isoforms

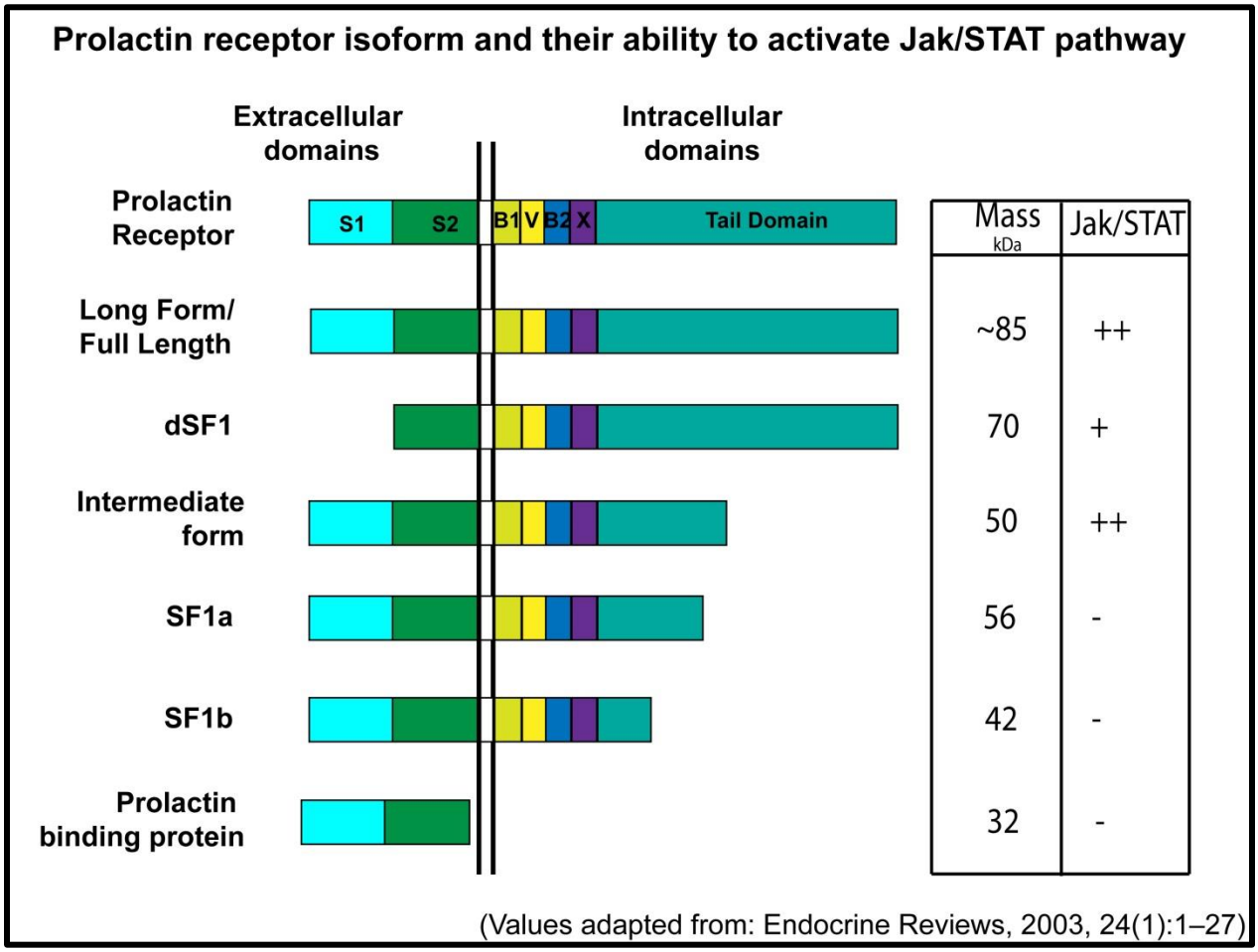


Figure 1.7: Prolactin receptor isoforms: Prolactin receptor (PRLR) is a single pass transmembrane protein, consisting of extracellular, transmembrane, and intracellular domains. The extracellular domain consists of ligand binding S1 and S2 domain with S1 having a higher affinity for the ligand compared to S2 domain. The intracellular domain consists of B1, V, B2, X-box and Tail domain. B1 and B2 interact with Jak proteins that can phosphorylate various domains on the tail domain. This phosphorylated tail domain now acts as docking sites for STAT proteins, which are responsible for intracellular transduction of the signals.

1.10 Prolactin receptor

PRL signals through PRLR, a high-affinity membrane bound, class I cytokine receptor family protein [156]. Located on chromosome 5p13, the PRLR gene consists of 10 exons with a single promoter region (Fig 1.6) [128]. Multiple PRLR isoforms generated by alternate splicing have been identified in both normal and malignant tissues (Fig 1.7) [157, 158]. The long form (LF), also called the full length (FL) isoform of PRLR, represents the classical type I transmembrane protein [159, 160]. The extracellular region is made of 24 amino acids and is composed two subdomains, the ligand binding S1 domain and a receptor dimerization S2 domain, which also has some residual ligand binding activity. The intracellular domain contains five juxtamembrane motifs called Box 1, Variable Box (V-Box), Box 2, Extended Box 2 (X-Box) and a tail domain, which are conserved across the cytokine receptor superfamily. The function of each of these motifs is poorly understood; however, it is suggested that the Box 1 motif is involved in interaction and activation of Janus kinase 2 (Jak2) after ligand stimulation [161-163]. The C-terminal tyrosine residue interacts with STAT5 [164] and SH2-containing protein tyrosine phosphatase (SHP-2) [165]. Considerable differences exist in the C terminal region of PRLR between humans and rodents, in terms of the number and location of the tyrosine residues and the surrounding amino acid residues. Further, posttranslational modifications of PRLR and the need

of other accessory proteins to modify PRLR upon receptor dimerization have not been well elucidated in rodents.

The intermediate form of PRLR has a deletion of all coding sequence C terminal to the X-Box and has been implicated to mediate cell survival but not cell proliferation in transfected cells [166]. ΔS1 has a complete deletion of S1 domain in the extracellular region and hence has a decreased affinity to PRL [167]. In comparison, the long form of PRLR has a relatively higher affinity for PLR compared to other isoforms. PRL binding protein (PRLbp) is a freely circulating form of PRLR which has been recently identified in human serum, however, its real presence and function are being actively investigated [168]. *In vitro*, it can bind to circulating PRL and prevent its interaction with PRLR thereby suppressing PRL signaling. Stoichiometrically, PRLbp can potentially sequester up to 35% of circulating PRL, indirectly reducing the amount or PRL available for active signaling [168]. PRLR is expressed in all organs and tissues at varying levels. The expression of various isoforms is thought to vary with hormonal status, estrous cycle, pregnancy, and lactation [156].

1.10.1 PRLR and its implication in pathologies and cancer

Evaluating the expression of PRLR in diverse tissues has shown that most of the tissues expressed both the long and the short form of the receptor in a tissue

specific manner. At a tissue level, muscle, thymus and kidney expressed both forms in equal amounts. Ovary, uterus and cerebral cortex, expressed the long form, only in the estrous phase, the liver expressed predominantly the short form [169].

Clinically, more than 50% percent of patients with breast cancer showed a significant increase in expression of PRLR [170]. At the transcript level there is a difference in expression pattern. Grade-I breast tumors predominantly express the LF while the grade-II tumors express higher SF and low levels of LF and grade-III tumors express similar amounts of LF and SF [171].

Multiple fibroadenomas (MFA) are a form of spontaneous benign breast tumors, which occurs most frequently in young women. In a cohort of 74 MFA patients and 170 control subjects, heterozygous activating mutations leading to substitution of isoleucine-146 with leucine (Ile(146)-->Leu) was noted in some of the patients. This mutation resulted in a constitutively active form of PRLR, leading to increased STAT5 signaling and rapid cell proliferation [172]. *In vivo*, stable overexpression of PRLR in breast cancer cell lines caused an increase in PRL signaling leading to increased cell proliferation and tumor formation in xenograft models. Knocking down of PRLR led to decreased proliferation and an overall reduction of tumor mass [173, 174]. Given the widespread expression of PRLR and the corresponding PRL-PRLR signaling in breast cancer, several

pharmacological interventions have been developed aimed at targeting PRLR to treat breast cancer [175]. LFA102 is a humanized neutralizing monoclonal antibody directed against the extracellular domain of PRLR. Nb2-11 rat lymphocyte cells, which respond to PRL by increasing proliferation, however, when treated with LFA102 they showed a significant decrease in cell division and xenograft formation [176]. Clinically, LFA102 is being recommended to patients with breast cancer, where it antagonizes PRL signaling leading to reduction of tumor load [176].

1.10.2 PRLR and its implication in gastrointestinal cancer

The widespread expression of PRLR mRNA along the GI tract and associated lymphatic tissue, suggests an active role for PRL in regulating GI immune functions [177] and CRC. PRLR transcripts were detected in isolated gastric glands, gastric cell fractions, and intestinal mucosa lineages from human, rabbit, as well as fetal and adult rat [177]. Human gastric mucosal adenomas, gastrointestinal cancer cell lines, and intraepithelial lymphocytes also expressed high levels of PRLR.

1.11 Prolactin Signaling

Binding of PRL to PRLR triggers an intracellular signaling cascade involving the Jak/ STAT pathway, Jak-Ras-Raf-MAPK pathway and/ or the Src tyrosine kinase pathway proteins [156, 178]. Site directed mutagenesis studies led to the

identification of specific features in the intracellular domain that can be linked to activation of specific signaling cascade via the recruitment of STAT proteins, insulin receptor substrates (IRS) and other adaptor proteins to the receptor dimer with the presence or absence of these features, dictating the activation of a specific signaling pathways [165, 179]. PRLR isoforms can also heterodimerize, which is important, as PRL target cells usually express more than one PRLR isoform further increasing the complexity of signaling associated with PRL.

PRLR deficient mice have served as a useful model to study the effects of PRL in a detailed manner. Mice having germ line deletion of *Prlr* were produced by homologous recombination by Ormandy and colleagues [91]. A detailed analysis of these mice was done by Kelly and colleagues who identified that circulating PRL levels in these mice were elevated by 30 -100 fold, suggesting a direct feedback regulation of PRL expression. Homozygous mutant ($Prlr^{-/-}$) females are completely sterile with defects in blastocyst implantation and breast development apart from other reproductive tract abnormalities. In hemizygous condition ($Prlr^{+/-}$) females exhibited severe, breast development and nursing defects occurred in their first pregnancy but recovered in subsequent pregnancies. Homozygous null females also exhibited a higher degree of maternal anxiety when challenged with foster pups and suffered a general decrease in bone development and density [180].

1.12 JAK-STAT signaling

Darnell and colleagues identified that interferon-α and γ treatment leads to activation of *Drosophila melanogaster melanogaster* homologue of Jak proteins in human cells. Activated Jak in turn lead to the activation of STAT proteins [181, 182]. This pathway is a pleotropic cascade responsible for transducing multiple signals that regulate development, immunity, and homeostasis from flies to humans. In higher mammals, such as humans, the Jak-STAT pathway is the principle pathway regulating cytokine and growth factor responses that stimulate cell proliferation, differentiation, migration and apoptosis, events that are critical for hematopoiesis, immune development, mammary gland development, lactation, adipogenesis and sexual maturation. Activation mutations in proteins of this pathway lead to neoplastic transformation of these tissues. Similarly, dominant negative mutations inactivate of these proteins, lead to several early developmental defects and embryonic lethality [183, 184] .

Mechanistically (Fig 1.8), receptors that activate Jak-STAT signaling are normally single pass transmembrane receptors for cytokines and growth factors. Binding of these ligands to the extracellular domain leads to receptor activation and subsequent dimerization. Dimerized receptors serve as docking sites for the Jak family of proteins in a region designated as Box1 and 2 domains. In humans, the Jak family has four members: Jak1, Jak2, Jak3 and Tyk2. Receptor bound Jak

transphosphorylates multiple other sites on the Jak and the receptor itself. These phosphorylated sites on the receptors act as binding sites for STAT proteins [185, 186].

STAT proteins are latent transcriptional factors/initiators that reside in the cytoplasm until activated through phosphorylation. Receptor bound STAT proteins are phosphorylated by receptor bound Jak proteins at the evolutionary conserved C-terminal domain at the tyrosine residue leading to its activation. Activated STAT proteins release the receptor and dimerize in the cytoplasm via the conserved SH2 domain and subsequently translocate into the nucleus, aided by the importin-Ran proteins. Once inside the nucleus, STAT proteins bind to the conserved sequencesin the enhancer regions of STAT responsive genes. Jak mediated STAT activation is sometimes aided by adapter proteins such as STAM (signal transducing adapter molecules). STAM1 and 2A are predominant forms in humans, which interact with protein phosphatase. These proteins can interact with either Jak or STAT proteins and inactivate them through dephosphorylation [187]. Apart from binding to STAT proteins, Jak phosphorylated residues on the receptor also serve as docking sites for the SH2-domain containing adapter protein complex consisting of GRB2, SHP-2 and SHC proteins. This complex of proteins lead to subsequent downstream activation of mitogen activated kinase proteins (MAPK).

Figure 1.8: Jak- STAT signaling

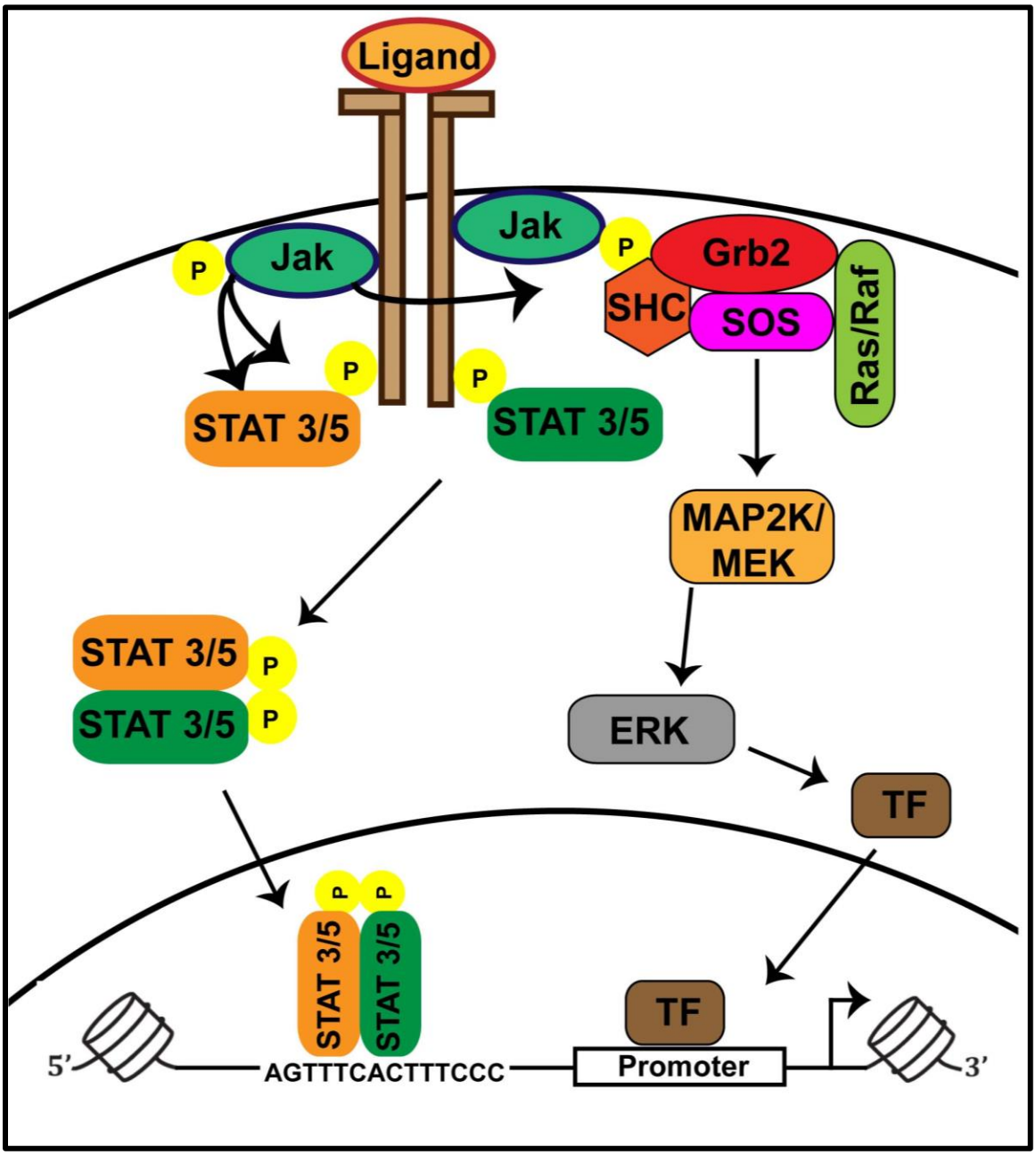


Figure 1.8: JAK- STAT-ERK signaling: This signaling pathway is predominantly activated by cytokines and growth factors. Binding of the ligand to receptors leads to receptor dimerization. JAK proteins bind to the dimerised receptor and get auto phosphorylated and also phosphorylate several tyrosine residues on the receptor which act as binding sites for STAT family of proteins. These STAT proteins are later phosphorylated by JAK leading to their activation, dissociation from the receptor, and dimerization in the cytoplasm. Dimerized STAT proteins translocate into the nucleus and bind to respective enhancer elements. Additionally, Jak proteins can activate ERK signaling mediated by the Grb2-Ras/Raf complex.

1.12.1 Role of JAK-STAT signaling in pathologies

Cytokine signaling plays a critical role in maintaining appropriate immune responses and also in several autoimmune diseases. The cytokine activated Jak-STAT signaling cascade is hyperactivated in autoimmune diseases [188]. Renal cancer cell lines treated with cytokines had increased activation of STAT3 leading to increased cell proliferation and decrease apoptosis [189]. AG490 mediated Jak inhibition causes complete abrogation of STAT activation implicating Jak-STAT signaling has a pivotal role in renal cancer progression [189].

Pancreatic ductal adenocarcinoma is an aggressive, highly metastatic form of pancreatic cancer with hyper active Jak2/ STAT3 signaling pathways that regulate both initiation and progression of the disease. Therapeutically, inhibiting both these pathways using the JAK2 inhibitor, in cell lines and animal model decreases growth, cell proliferation, migration, invasion and increases apoptosis compared to untreated cells, implicating the critical role of Jak/STAT signaling in pancreatic cancer [190].

Bonni and colleagues demonstrated the critical role of Jak-STAT in glial differentiation [191]. They showed that Jak-STAT signaling is active in embryonic cortical precursor cells and initiated differentiation of these cells into astrocytes, a phenomenon augmented by cytokines [192]. Clinically, STAT3 was not active in normal brain however, increased levels of STAT3 phosphorylation along with

increased expression of STAT3 responsive genes that promote cell cycle progression and decrease apoptosis, was observed in brain tumor cell lines. In addition, multiple other pathways also seem to converge on STAT3 in brain tumors. Further support for this comes from that fact that inhibiting STAT3 alone, genetically or pharmacologically leads to a decrease in tumor phenotype, implicating the central role of STAT3 in brain tumor pathologies [193].

1.12.2 Significance of JAK-STAT signaling in colorectal cancer

Corvinus and colleagues demonstrated that Jak/STAT signaling apart from Wnt signaling plays a significant role in colorectal cancer. Dedifferentiated cancer cells and infiltrated lymphocytes of CRC patient samples showed a constitutively active STAT3 compared to uninvolved colon epithelium. Isolated CRC cells from these patients eventually lost their STAT3 activity. However subsequent implantation of these cells into flanks of nude mice led to reactivation of STAT3. Blockade of STAT3 activation slowed down the development of CRC derived xenograft tumors [194, 195]. Xiong and colleagues showed that either inhibiting JAK activity using AG490 and STAT3 expression using specific siRNA, decreased expression of genes responsible for cell growth, survival, invasion, and migration such as Bcl-2, p21, p27, E-cadherin, VEGF, and MMPs. They also showed that Jak2 is subjected to proteasome-mediated proteolysis and dephosphorylation. An increased level of nuclear phopho-STAT3 immunostaining was identified in adenomas and

adenocarcinomas while phospho-Jak2 immunoreactivity was found in the cytoplasm suggesting a positive correlation between Jak-STAT and CRC [196]. Adenoviral mediated overexpression of the suppressor of cytokine signaling (SOCS3) gene (an inhibitor of the JAK/STAT3 pathway) suppressed CRC cell growth and induced apoptosis both *in vitro* and *in vivo*, suggesting the critical role of JAK-STAT3 pathway in CRC. This action efficiently inhibited the activation of this pathway and decreased expression of downstream tumor promoting factors [197]. Clinically, genetic variations in JAK1, JAK2, TYK2, SOCS1, SOCS2, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 were predominant in patients with colorectal cancer. Among them JAK2, SOCS2, STAT1, STAT3, STAT5A, STAT5B, and STAT6 were predominant in patients with colon cancer while STAT3, STAT4, STAT6, and TYK2 were associated with patients with rectal cancer further implicating that JAK-STAT polymorphism and signaling have a critical role in CRC, and may be potential therapeutic targets [195, 198].

Chapter 2:

Prolactin signaling enhances colon cancer stemness by modulating Notch signaling via JAK2-STAT3/ERK pathway

This part of the dissertation has been accepted for publication:

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

Neradugomma NK, Subramaniam D, Tawfik OW, Goffin V, Kumar TR, Jensen RA, Anant S.

Carcinogenesis, 2014 Apr; 35(4):795-806.

2.1 Abstract

Prolactin (PRL) is a secretory cytokine produced by various tissues. Binding of PRL to the cognate prolactin receptor (PRLR) activates intracellular signaling via Jak, ERK and STAT proteins. PRL regulates diverse activities under normal and abnormal conditions including malignancies. Previous clinical data suggest that serum PRL levels are elevated in colorectal cancer patients. In this study, the expression of PRL and PRLR in colon cancer tissue and cell lines was assessed. Higher levels of PRLR expression in the cancers and cell lines were observed compared to normal colonic epithelial cells. Incubation of colon cancer cells with PRL induced JAK2, STAT3, and ERK1/2 phosphorylation and increased expression of Jagged 1 (JAG1), a Notch-1 receptor ligand. The cleaved/ active form of Notch-1 receptor (NICD), increased expression of Notch responsive genes HEY1, HES1 and stem cell marker genes DCLK1, LGR5, ALDH1 and CD44 were all increases in CRC cell lines. Finally, pharmacologically inhibiting the PRL induced JAK2-STAT3 and JAK2-ERK1/2 signaling abrogated Notch activation an important component of CRC stem cell. Together, the results demonstrate that cytokine signaling induced by PRL is active in colorectal cancers and may provide a new target for therapeutic targeting.

2.2 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 [2]. The precancerous predisposition of colorectal epithelial polyps is no longer disputed. There is a plethora of morphologic and molecular studies that carefully analyzed the progression of a non-cancerous polyp, into invasive cancerous lesions. These process are complex and influenced by various intrinsic and extrinsic factors such as hormones [199].

Prolactin (PRL), a cytokine hormone, accumulates in the tissue microenvironment and elicits its action in an autocrine and/ or paracrine manners to regulate diverse physiological activities that include reproduction, growth, development, metabolism, and immunomodulation [93, 120, 200, 201]. Binding of PRL to the single pass, transmembrane Prolactin receptor (PRLR) induces several intracellular signaling cascades mediated via JAK-STAT [153, 202] and JAK-RAS-MAPK components [203].

PRL acts as a mitogen promoting cell proliferation, inhibiting apoptosis, and inducing chemoattraction in breast cancer cells [120, 204, 205]. Blood levels of PRL were found elevated in patients with hepatocellular carcinoma [206, 207] and ovarian cancer [138]. Cultured immortalized ovarian epithelial cells and

endometrial cells treated with exogenous PRL demonstrated increased proliferation and inhibition of chemotherapy induced cell death [139]. Autocrine PRL induces PRLR-mediated Jak2-STAT signaling in prostate cancer [130, 131, 152, 208] and modulates the stem cell/basal cell population [152].

PRL and PRLR are expressed all along the GI tract in fetal and neonatal stages during development [177]. In adult rats, PRL induces active potassium ion transport in distal colon and chloride ion transport in proximal and transverse colon [209]. IEC-6 colon crypt epithelial cells treated with PRL had increased expression of nutrient and mineral transport channel proteins without inducing proliferation [210]. Elevated serum levels of PRL have been identified in patients with colorectal malignancies [145-147, 150]. In addition, increases in PRL and PRLR expression were noted in CRC cell lines and tumor samples [99].

Cancer stem cells (CSC) initially identified in hematological disorders as tumor initiating cells when isolated and transplanted in NOD-SCID mice [15], 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 DCLK1 [44-46], LGR5 [47, 48, 50, 211], CD44 [212] and CD133 [23], which also mark normal colon stem cells. These cells are resistant to therapeutic interventions and cause tumor relapse and metastasis [213, 214]. Identifying cellular factors that regulate the stem cell population are critical in understanding the process of neoplastic

transformation and for development of novel therapeutics to target the cancer stem cell pool. Isolated primary mouse hippocampal cells treated with exogenous PRL showed increased number of stem cells [155]. Similarly, in mouse models, inducing PRL under the control of prostate specific probasin promoter led to expansion in the basal cell compartment [132, 152], which constitutes the stem cell population of the prostate gland. Although these data suggest that PRL can affect the tissue stem cell population, its effects on cancer stem cells have not been determined.

The Notch signaling pathway is active in intestinal stem cells [215]. It is involved in regulating stem cell hierarchy and determining cell fate [24] and is active in the intestinal crypts [25]. Constitutive Notch activation is necessary for intestinal stem cell maintenance [29] and dysregulation of the pathway has been observed in colorectal and other forms of cancer [30]. There are four members in the Notch receptor family Notch 1 to Notch 4. Binding of specific ligands like Jagged 1, 2 or Delta like 1, 3, 4 to the Notch receptors results in a conformational change in the receptor. Subsequent activation of the γ-secretase complex, composed of Presenilin, Nicastrin, Anterior Pharynx Defective 1 (APH 1) and Presenilin enhancer 2 (PEN-2) cleaves the Notch receptor to release the intracellular domain (NICD) [26, 27]. The NICD then translocates into the nucleus, interacts with co-factors CSL and MAML, bind to target sequences and activate the transcription of

genes such as Hes1, Hey1 and stem cell responsive genes [28] such as c-Myc, all of which can be used to assess the degree of Notch signal activation. Interestingly, ERK can modulate Notch signaling by regulating the expression of its ligand, Jagged 1 [216].

The current study is aimed at determining the effect of PRL on signaling in colon cancer cells. We show that PRL induces JAK2-ERK1/2 mediated activation of Notch signaling, leading to an increase in spheroid formation and changes in cancer stem cell population. Furthermore, PRL signaling in these cells can be suppressed with specific inhibitors of JAK2 and ERK1/2.

2.3 Materials and Methods

2.3.1 Cells

Colon cancer cell lines HT29, HCT116, SW480, SW620, DLD1 and normal intestinal epithelial FHC cells were obtained from ATCC (Manassas, VA). The cells are well characterized and have been 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% CO_2. The cells were cultured in serum free media overnight prior to treatment with PRL (500 ng/ml). Where indicated, cells were pre-treated with 50 μM Jak2 inhibitor AG490 or 10 μM ERK1/2 inhibitor PD98059 (Selleckchem, TX).

2.3.2 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 EGF (5 ng/ml), FGF (5 ng/ml), heparin (1 μg/ml) and B12 supplements (0.25X) and plated on ultra-low attachment plates (BD Biosciences, NY). An important point to note is that the amount of growth factors used in the culture conditions were reduced to prevent any growth promoting effects by these growth factors which can complicate the analyses in order to gain a better idea of the role of prolactin in promoting spheroid

formation. Specifically, 1/4th the dose of growth factors (EGF, FGF) was used along with, Heparin and B12 supplements recommended by earlier studies [217]. The limiting dilution of cells was also determined based on this concentration of aggregates that lasts for 8 days. Colosphere formation was assessed after 4 to 6 days and the number and size of colospheres was determined using Celigo (Cyntellect, CA).

2.3.3 RT- PCR analysis

Colon cancer cDNA panel with matched adjacent tissue controls was obtained from Origene (Rockville, MD). Total RNA from cell lines was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. 2 μg RNA was used to synthesize complimentary DNA using Superscript II reverse transcriptase and random hexanucleotide primers (Invitrogen, CA). Individual gene expression was quantified using SYBR green reagent (Molecular Probes, OR) and specific primers with GAPDH as internal standard. Primers for the PCR include 5`-GCATATTGCGATCCTGGAAT-3` and 5`-CGTTTGGTTTGCTCCTCAAT-3` for PRL, 5`-GGAGCTGGCTGTGGAAGTAA-3` and 5`-CTCCCACTCAGCTGCTTTCT-3`for PRLR, 5`-GTGCGGTATATTTCCTCCAA-3` and 5`-GTTCCCGTGAAGCCTTTGT-3` for JAG1, 5`-CCTCTCTTCCCTCCGGACT-3` and 5`-GGTCAGTCACTTAATACAGCTCTCTCT-3` for HES1, 5`-

Figure 2.1: Expression of Prolactin and its cognate receptor in colorectal cancers

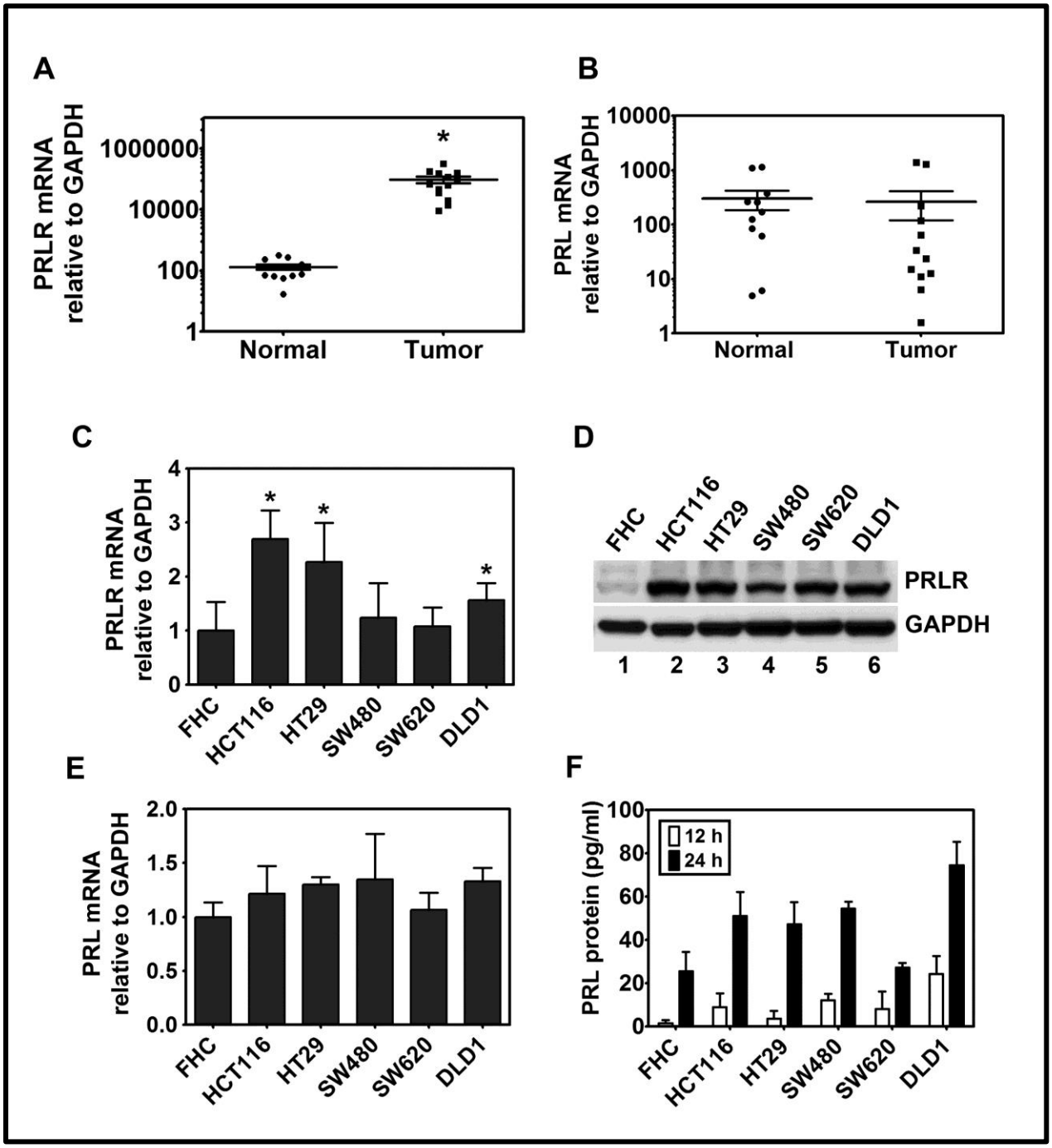


Figure 2.1: Expression of Prolactin and its cognate receptor in colorectal cancers:

(A) Real time PCR to evaluate expression of PRLR in colon tumors. Increased expression of PRLR is noted in tumor samples compared to adjacent controls. **(B)** Real time PCR for PRL colon tumors. Data suggest no change in expression of PRL between tumor samples and adjacent controls. **(C)** Real time PCR for PRLR in colon cancer cell lines compared to normal (FHC cells). Increased PRLR expression was observed in all colorectal cancer cell lines. **(D)** Western blot analysis. There were higher levels of PRLR in colorectal cancer cells when compared to FHC cells. **(E)** Real time PCR for PRL in normal and cancer cell lines. PRL mRNA was present in all cell lines. **(F)** ELISA bases quantification of PRL.

GCTGGTACCCAGTGCTTTTGAG-3` and 5`-TGCAGGATCTCGGCTTTTTCT-3` for HEY1, 5`-GGTGAACGTCAAGACCACCT-3` and 5`-GTCCTGAAGGCACATCACCT-3` for DCLK1, 5`-AACAGTCCTGTGACTCAACTCAAG-3` and 5`-TTAGAGACATGGGACAAATGCCAC-3` for LGR5, 5`-TGTTAGCTGATGCCGACTTG-3` and 5`-TTCTTAGCCCGCTCAACACT-3` for ALDH1, 5`-CAGCCTCAAGATCATCAGCA-3` and 5`-GTCTTCTGGGTGGCAGTGAT-3` for GAPDH.

2.3.4 Western Blot Analysis and Enzyme-Linked Immunosorbent Assay

Protein samples were prepared in RIPA buffer (Thermo Scientific, IL). Following quantification using BCA Kit (Thermo Scientific, IL), the lysates were subjected to poly acrylamide gene electrophoresis and transferred onto PVDF membrane (EMD Millipore, MA). Antibodies for PRLR (ab87992), DCLK1 (ab37994) and LGR5 (ab119012) were obtained from Abcam (Cambridge, MA), Jagged1 (sc6011), Hes1 (sc25392), Hey1 (sc28746) and ACTB (sc1616) were obtained from Santa Cruz Biotech Inc (Dallas, TX), Nicastrin (A00883), Presenilin1 (A00881), APH1 (A00884) and Presenilin enhancer protein (PEN2) (A00882) were obtained from Millipore (Billerica, MA) and Jak2 (3230S), pJak2 (3776S), STAT3 (4904S), pSTAT3 (9131S), Erk1/2 (p42/44) (9102S), pErk1/2 (p-p42/44) (9101S), cMyc

Figure 2.2: Prolactin induces Jak2, STAT3 and ERK1/2 phosphorylation

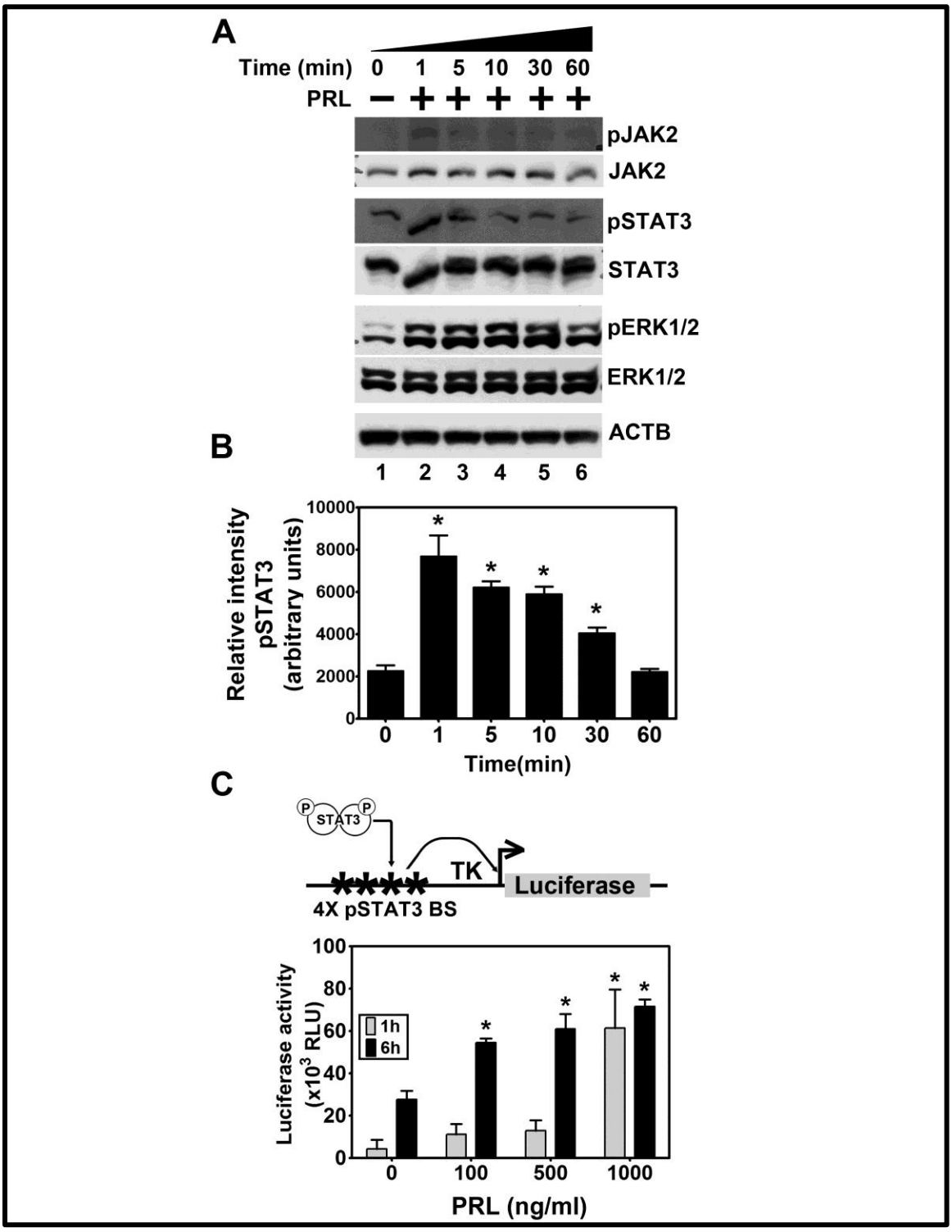


Figure 2.2: Prolactin induces Jak2, STAT3 and ERK1/2 phosphorylation: (A, B) HCT116 cells were treated with 500ng/ml PRL, and lysates were collected at regular intervals and subjected to SDS-PAGE. PRL treatment increased phosphorylation of Jak2, STAT3 and ERK1/2, starting at 1 min post treatment and lasting for 30 min. **(C)** STAT3 responsive luciferase plasmid was transfected into cells and the luciferase activity analyzed after PRL treatment. Dose- and time-dependent increases in luciferase activity were observed following PRL treatment as compared to untreated controls (*$p<0.05$).

(5605S), NICD (4380S), CD44 (3570S) and GAPDH (2118S) were obtained from Cell Signaling (Boston. MA). Specific proteins were detected using chemiluminescence (GE Healthcare, NJ). To determine PRL levels, cells were cultured in serum free media and the media was subjected to ELISA 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 minutes at which point the wells were washed and treated sequentially with primary antibody and streptavidin-HRP bound secondary antibody. Colorimetric quantification after treating with substrate was done at 450 nm.

2.3.5 Luciferase Assay

Cells were plated and transfected with either 4XM67 pTK-Luc (Addgene plasmid 8688) [218] or Hes-1A/B-Luc, a kind gift of Dr. Kimberly Foreman, Loyola University, Chicago [219], which encode firefly luciferase gene under the control of the minimal thymidine kinase (TK) promoter and four STAT3 (4X STAT3 BS) or a 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 2 h prior to treating 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).

Figure 2.3: Prolactin induces Jak2, STAT3 and ERK1/2 phosphorylation

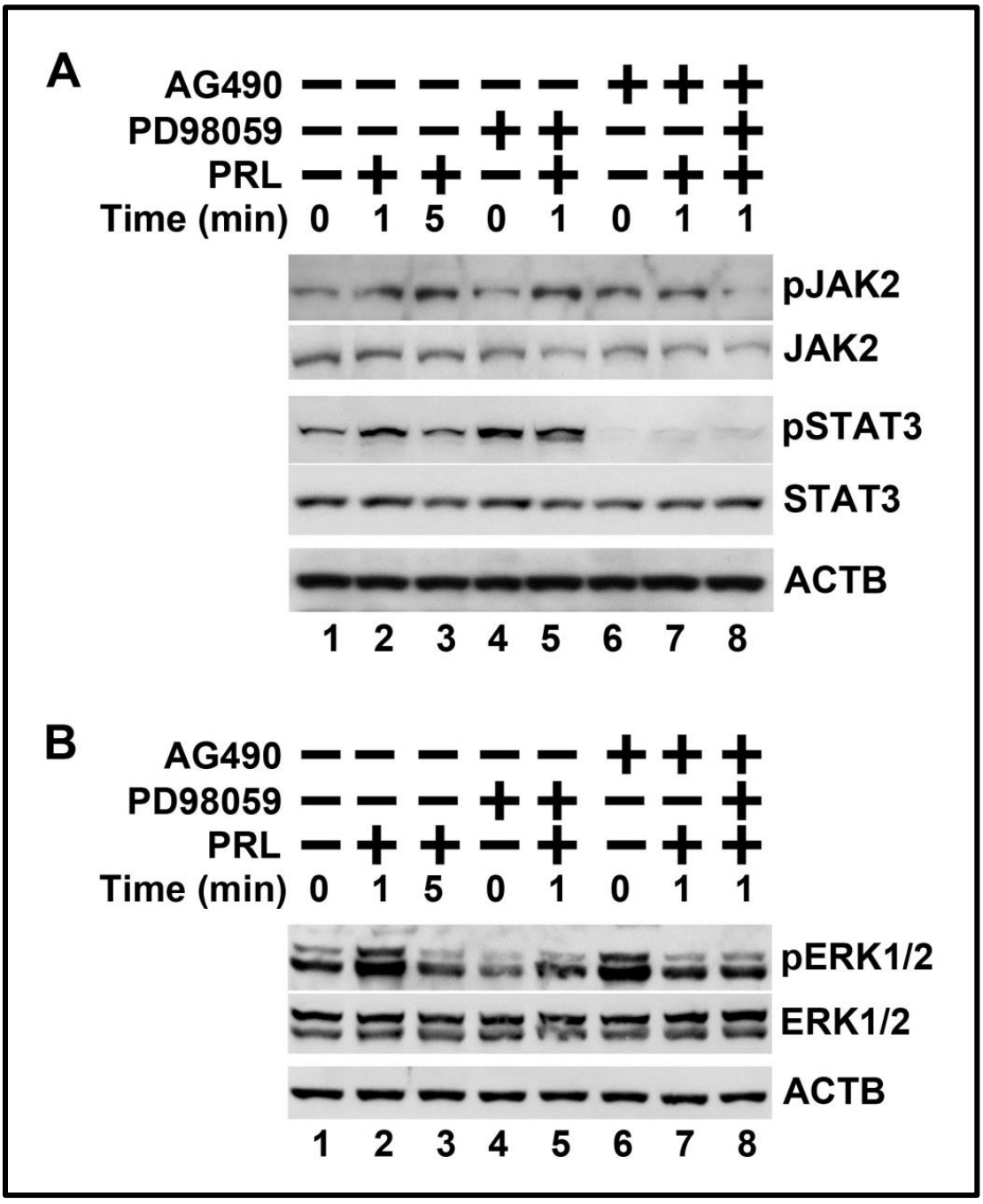


Figure 2.3: Prolactin induces Jak2, STAT3 and ERK1/2 phosphorylation: (A) AG490 or PD98059 treatment alone inhibited Jak2 and STAT3 activation which was rescued by PRL treatment. However, the combination of both inhibitors completely abrogated Jak2 and STAT3 activation which could not be rescued by PRL treatment. **(B)** Cells treated with PD98059 had decreased ERK activation while AG490 enhanced ERK1/2 phosphorylation when treated alone. However, the combination of AG490 and PD98059, significantly inhibited ERK activation even in presence of PRL.

2.3.6 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). $P \leq 0.05$ was considered to be statically significant.

Figure 2.4: STAT5 is not induced by PRL

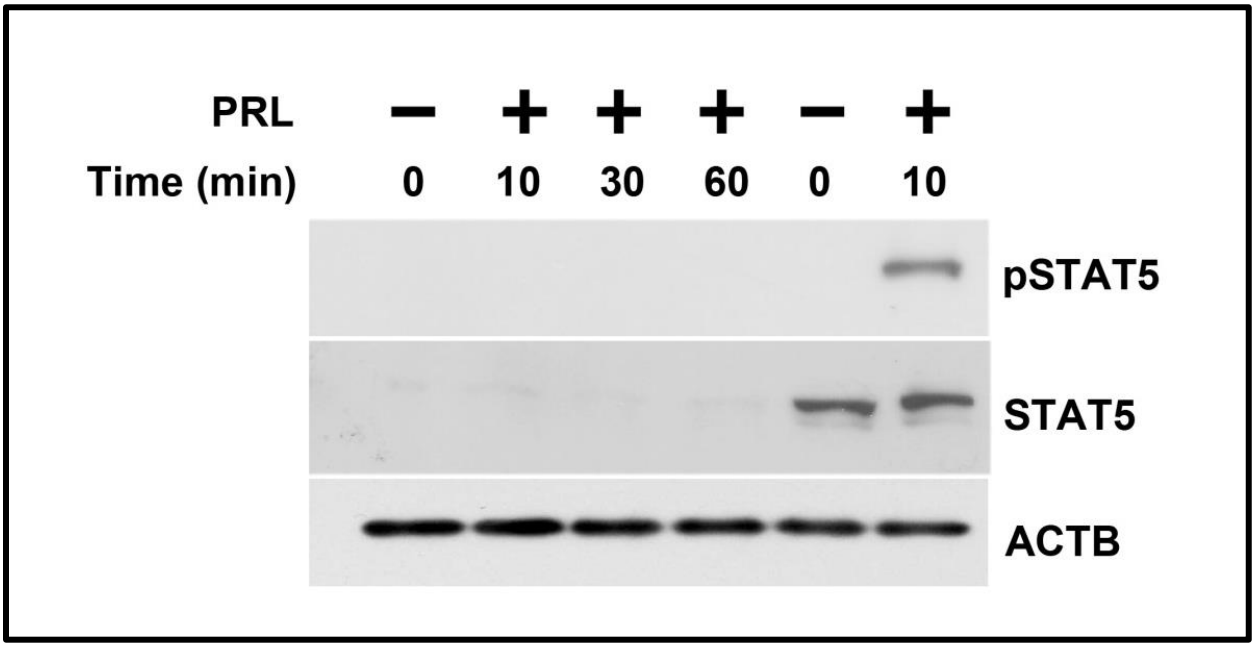


Figure 2.4: STAT5 is not induced by PRL: HCT116 colorectal cancer cell line has low basal level of STAT5. In these cells, PRL (500 ng/ml) treatment did not induce an activation of STAT5. T47D breast cancer cell line has higher basal STAT5 and in these cells PRL treatment led to a significant STAT5 phosphorylation as early as 10 minutes post treatment.

2.4 Results

2.4.1 PRLR but not PRL is upregulated in colon cancer cells

To determine whether prolactin 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 colorectal cancer patient samples indicated a significant increase in *PRLR* but not *PRL* transcript levels in the cancerous tissue when compared to adjacent normal tissue (Fig 2.1 A, B). A similar increase in *PRLR* mRNA levels and protein was observed in CRC cells compared to normal colonic epithelial cells (FHC) (Fig 2.1 C, D). Moreover, no difference in expression of *PRL* mRNA was observed between normal colonic FHC cells and CRC cell lines (Fig 2.1 E). Quantification of concentrated culture media from the cell lines by ELISA indicatd that all cells secrete PRL; however, the amount varies with time and in a cell line specific manner ranging from 2- 80 pg/ml after 24 h (Fig 2.1 F).

2.4.2 PRL treatment induces STAT3 and ERK1/2 phosphorylation

Upregulation of PRLR particularly in CRC compared to normal colonic cells suggests a role for the pathway in the pathogenesis of colorectal cancer. Binding of PRL to PRLR is known to activate the JAK/STAT and JAK/ERK pathways [153, 202, 203]. The ERK- MAP kinase pathway is also known to be highly active in patients with Familial Adenomatous Polyposis [220]. HCT116 cells were treated

with recombinant PRL and western blot analyses were performed for JAK, STAT and ERK proteins. There was an increase in Jak2, STAT3 and ERK1/2 phosphorylation within a minute of PRL treatment (Fig 2.2 A,B). To validate PRL-mediated STAT3 activation, we transfected HCT116 and HT29 cells with 4XM67 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 [218]. PRL treatment increased luciferase activity in both cell lines in a dose- and time- dependent manner (Fig 2.2 C). Even at 100 ng/ml of PRL, there was significant induction in luciferase activity observed even at 1h. Pre-treating the cells with AG490, a pharmacological inhibitor of JAK2, prior to PRL treatment, led to a decrease in STAT3 and ERK1/2 phosphorylation (Fig 2.3 A,B) even in the presence of PRL. However, pretreatment with PD98059 alone led to increased STAT3 activation (Fig 2.3 A). Moreover, cells treated with the combination of JAK2 and ERK1/2 inhibitors, AG490 and PD98059, resulted in complete inhibition of JAK2, ERK1/2 and STAT3 phosphorylation (Fig 2.3 A, B). Together, the data suggest that PRL induces JAK2, ERK1/2 and STAT3 phosphorylation in colon cancer cells. Given that STAT5 phosphorylation has been 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.

Figure 2.5: Prolactin affects colosphere formation

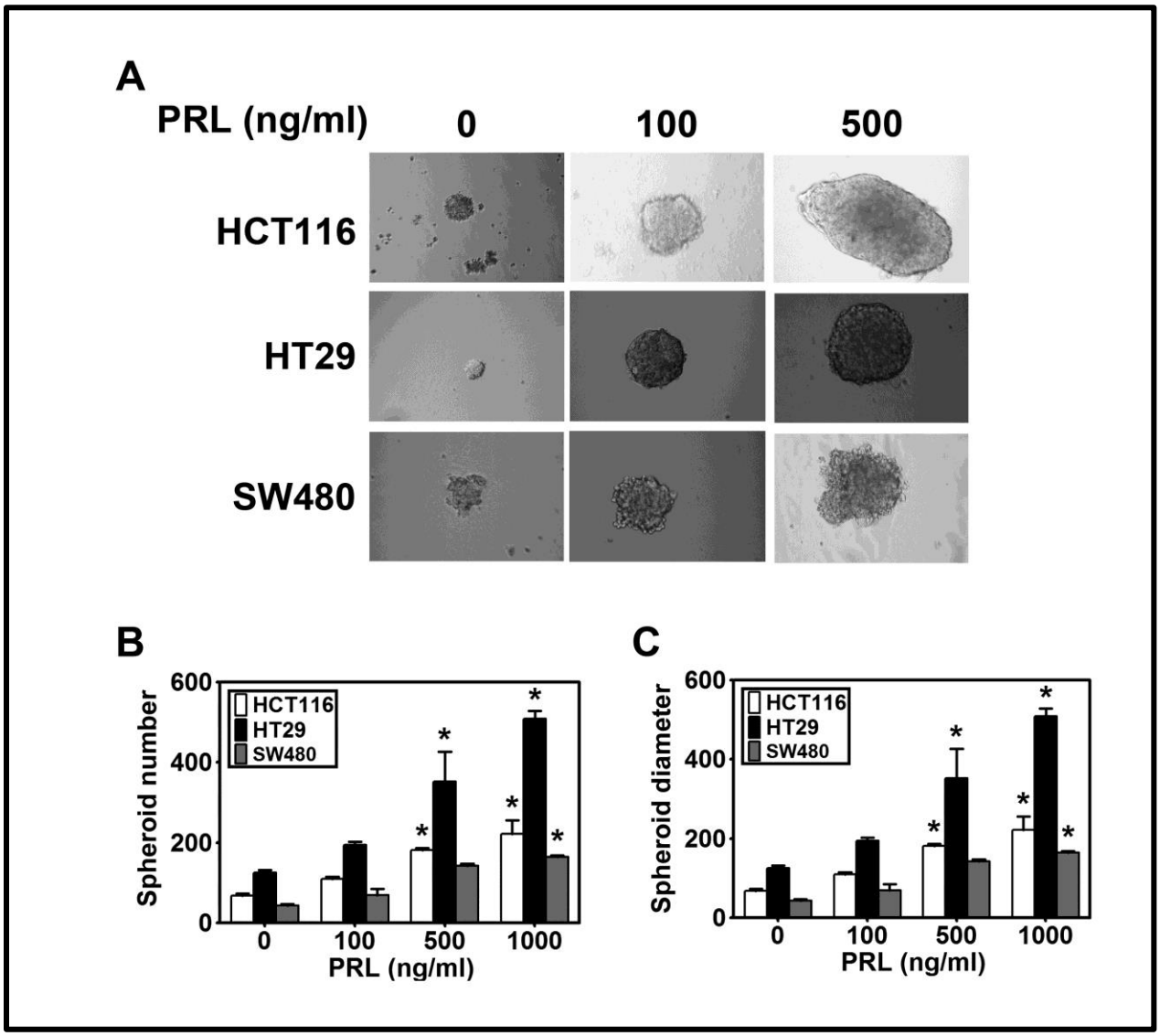


Figure 2.5: Prolactin affects colosphere formation: (A) Colon cancer cells were grown in specific spheroid media in ultra-low binding plates and treated with increasing doses of PRL. After 5 d, the colospheres were photographed and counted. **(B)** A dose-dependent increase in spheroid number was observed with statistical significance at 500 and 1000 ng/ml of PRL (*$p \leq 0.05$). **(C)** Similar increases in diameter were noted at similar doses (*$p \leq 0.05$).

However, levels of total STAT5 protein were relatively low in colon cancer cells, and no significant changes in phosphorylation of the protein were observed (Fig 2.4), suggesting that STAT3 may be a key player in PRL:PRLR signaling in colon cancer cells.

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

Previous studies have demonstrated mitogenic activity for PRL in breast cancers [120]. Accordingly, we determined whether PRL affects proliferation of colon cancer cells. PRL did not have any effect on proliferation of various colon cancer cells (Chapter 4, Fig 4.1). 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 and control cells (data not shown). These data, taken together suggest that PRL does not affect proliferation of CRC cells *in vitro*.

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 [155]. Additionally, spheroid formation remains the best available functional assay to assess the presence of cancer stem

Figure 2.6: Prolactin affects colosphere formation

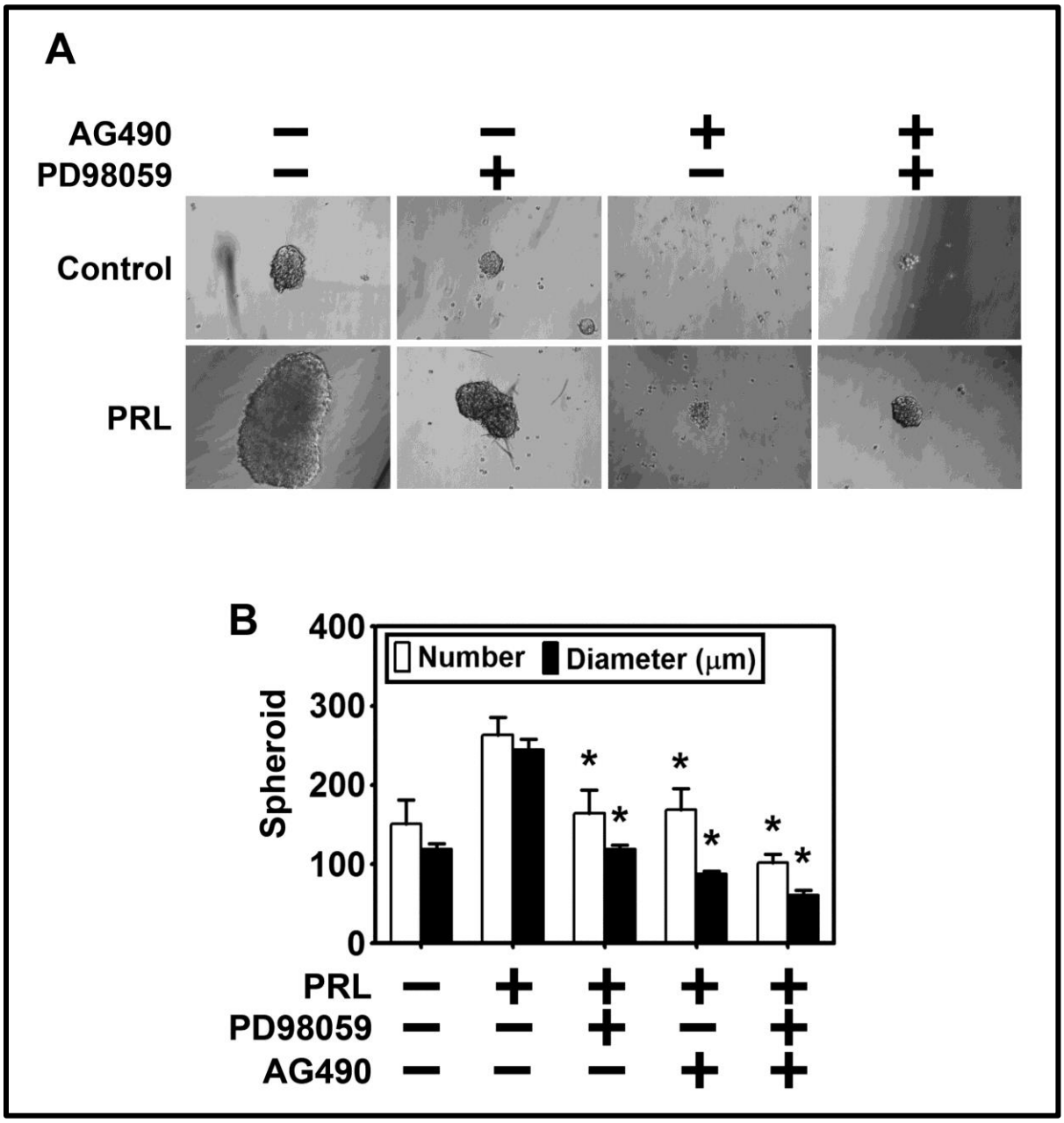


Figure 2.6: Prolactin affects colosphere formation: (A and B) Pre-treatment with AG490 and PD98059 alone or in combination led to a significant decrease in spheroid formation and number as compared to PRL treatment alone (*$p \leq 0.05$).

cells in a given cancer cell pool [16, 221]. We treated colon cancer cells HCT116, SW480 and HT29 with increasing concentrations of PRL (0-500 ng/ml) and allowed the colospheres to form over a period of 6-8 days. There was a dose dependent increase in colosphere formation (Fig 2.5 A), with a significant increase in both number (Fig 2.5 B) and diameter of spheroid (Fig 2.5 C) 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 colospheres formation (Fig 2.5 D,E). Moreover, the inhibitors affected colosphere formation in the presence of PRL. There were also fewer numbers of colospheres and the size of the spheres was significantly smaller, when compared to cells treated with PRL alone. These results suggest that PRL signaling can potentially regulate colosphere formation.

2.4.4 PRL induces expression of colon cancer stem cell marker genes

Since PRL stimulated colosphere formation, a marker for stem cell-dependent growth, we next determined whether PRL affects stem cell related gene. Stem cell gene were quantified using Real-Time PCR and western blot of specific markers including DCLK1 [44-46], LGR5 [47, 48, 50, 211], CD44 [212], CD133 [23] and ALDH1A1 [38]. Real-time PCR analysis demonstrated that PRL treatment induced expression of DCLK1, LGR5

Figure 2.6: Prolactin induces stem cell marker protein expression

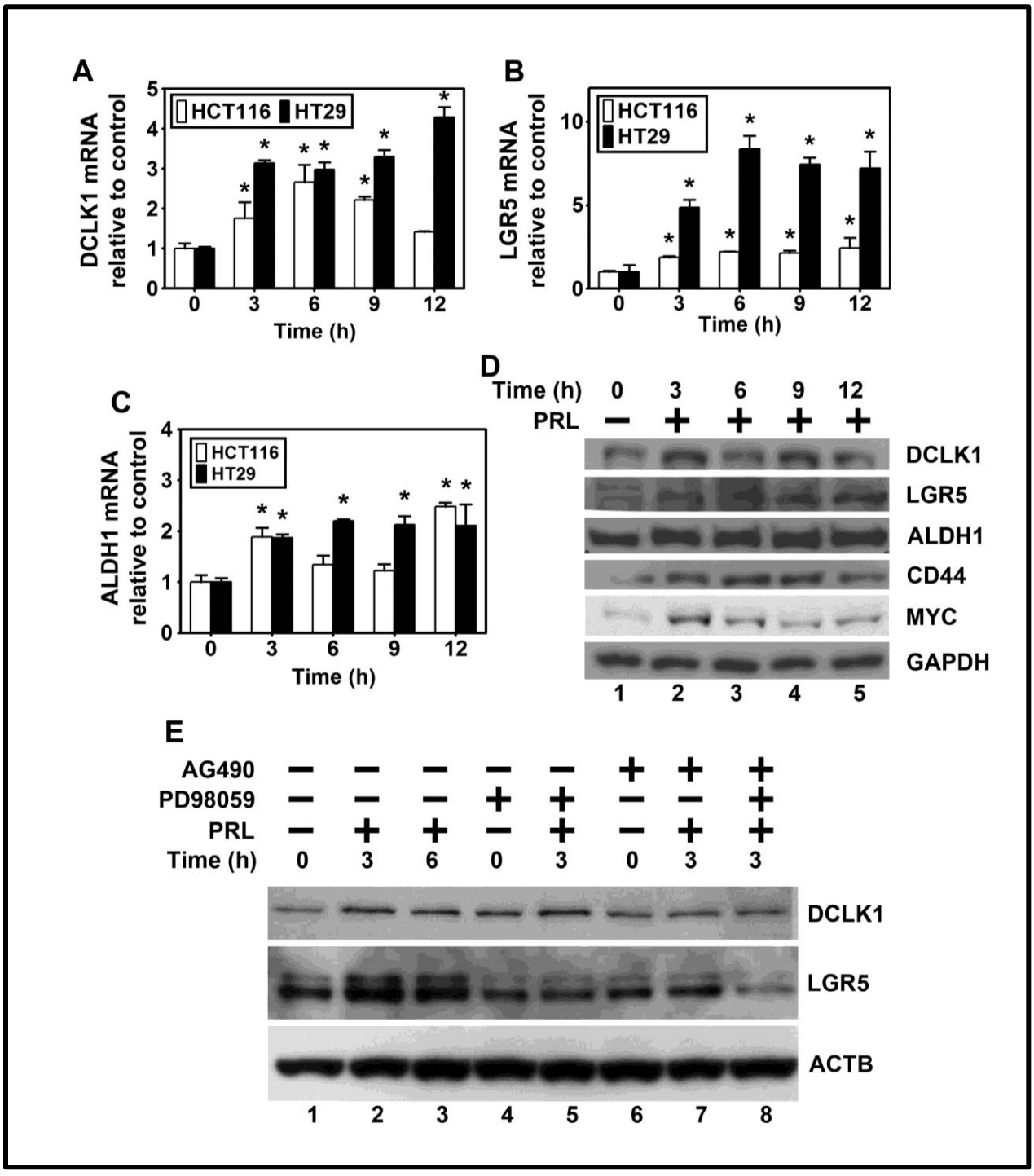


Figure 2.6: Prolactin induces stem cell marker protein expression: CRC cells were treated with PRL (500 ng/ml) showed increased levels of **(A)** DCLK1 **(B)** LGR5 and **(C)** ALDH1A1 mRNA and **(D)** protein levels. **(E)** Treatment with either AG490 or PD98059 alone decreased DCLK1 and LGR5 protein levels (lane 4, 6) compared to PRL treatment samples (lane 2, 3). PRL was able to rescue this inhibition (lane 5, 7); however, the combination of both AG490 and PD98059 led to complete abrogation of DCLK1 and LGR5 expression even in presence of PRL (lane 8).

and ALDH1 expression (Fig 2.6 A-C). Further confirmation was obtained by western blot analyses which showed that all three proteins along with CD44 and c-Myc, an oncogene that plays a predominant role in stemness [222], are upregulated compared to untreated controls (Fig 2.6 D). This further demonstrates the 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 was partially rescued upon PRL treatment (Fig 2.6 E). These data further suggest that PRL signaling can modulate the expression of colon cancer stem cell maker protein expression.

2.4.5 PRL affects cancer stem and progenitor cells by inducing Notch signaling

Notch signaling plays a significant role in stem cells, and is a pathway active in colon cancer stem cells [24, 29]. Phosphorylated ERK1/2 induces Jagged 1 (JAG1), a Notch receptor ligand [216]. 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

expression of target genes [215]. We determined 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 (Fig 2.7 A,B). Western blot analyses further confirmed the upregulation of JAG1 and HEY1 (Fig 2.7 C). In addition, there was an increase in NICD protein levels, along with increased levels of γ-secretase complex proteins anterior pharynx defective 1 (APH1), Presenilin 1 (PSEN1) and Presenilin enhancer (PSENEN) (Fig 2.7 C). 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 cell lines (Fig 2.7 D). These results were also confirmed using the specific JAK 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, HES1and PSEN1 proteins (Fig 2.7 E). This was partially rescued by PRL. Combined inhibition of both the inhibitors lead to a further reduction in JAG1 expression, Notch-1 cleavage (NICD) and expression of HEY1, HES1 and PSEN1 (Fig 2.7 E) even in presence of PRL, suggesting that PRL can regulate Notch signaling through either JAK2-STAT3 or JAK2-ERK1/2 pathways.

Figure 2.7: Prolactin treatment activates Notch signaling

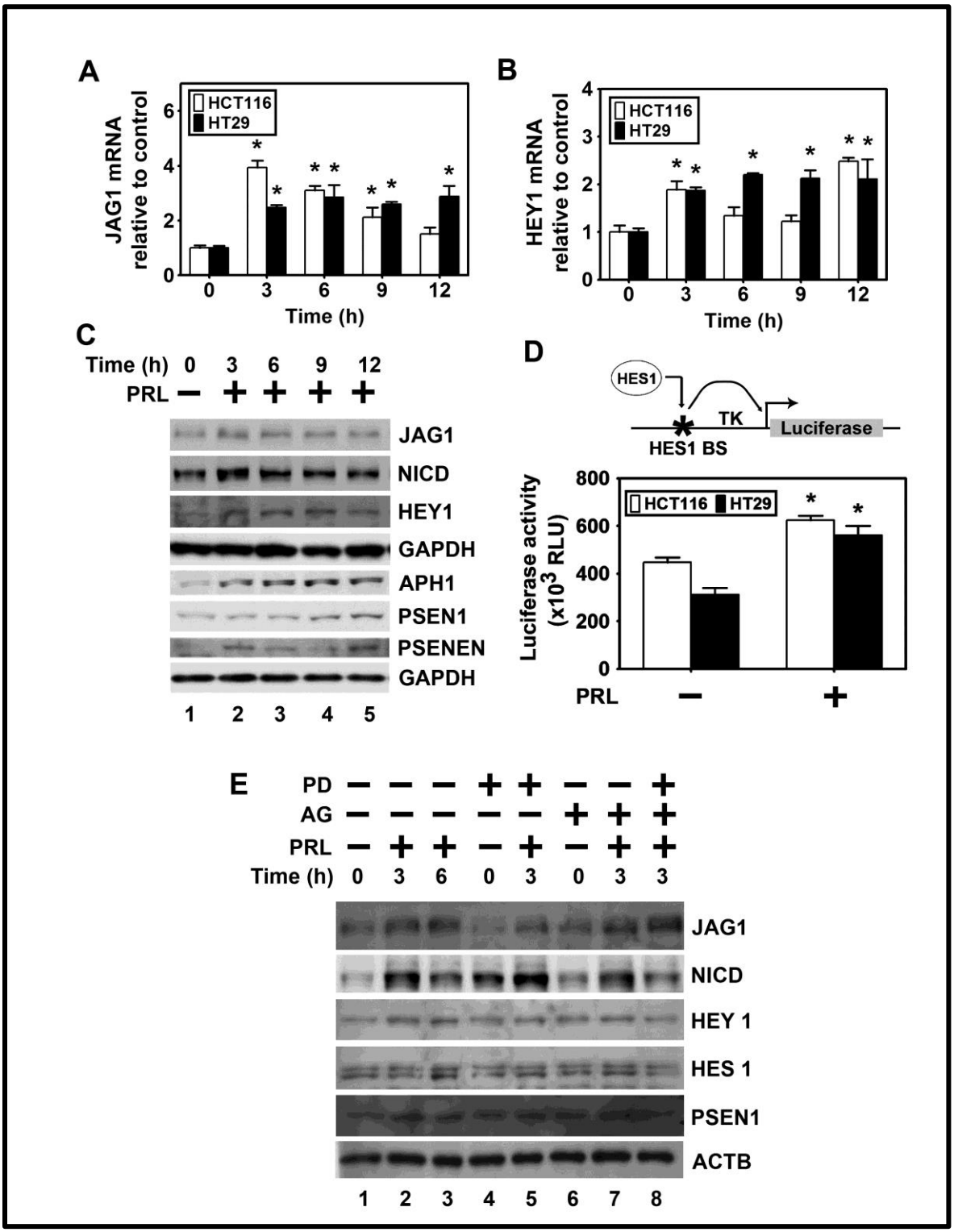


Figure 2.7: Prolactin treatment activates Notch signaling: (A) Real-Time PCR analysis of cells treated with PRL show a time dependent increase in expression of JAG1 (*$p<0.05$). **(B)** Similar increase in expression of Notch target gene HEY1 was also observed in Real-Time PCR analysis (*$p<0.05$). **(C)** Lysates of PRL treated cells showed increased JAG1 and HEY1 expression, NICD accumulation and induction of γ-secretase complex proteins APH1, PSEN1 and PSENEN expression as compared to 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 (lanes 4-7) caused a decrease in NICD accumulation and JAG1, HEY1, HES1 and PSEN1 expression compared to PRL treatment. PRL was able to rescue this activation; however, the combination of both AG490 and PD98059 (lane 8) led to complete abrogation of NICD accumulation and HEY1, HES1 and PSEN1 expression to levels similar to control.

2.4.6 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 (Fig 2.8 A). There was an increase in the number and size of the spheroids (Fig 2.8 B, 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 (Fig 2.8 D). Similarly, protein levels of DCLK1, LGR5 or CD44 increased in NICD overexpressing cells to levels comparable to PRL treated cells (Fig 2.8 E). The two inhibitors either alone or in combination did not affect expression of stem cells markers in the NICD overexpressing cells further suggesting that PRL-induced activation of Notch signaling is sufficient to enhance stem cell activity.

Figure 2.8: NICD overexpression recapitulates loss of PRL signaling

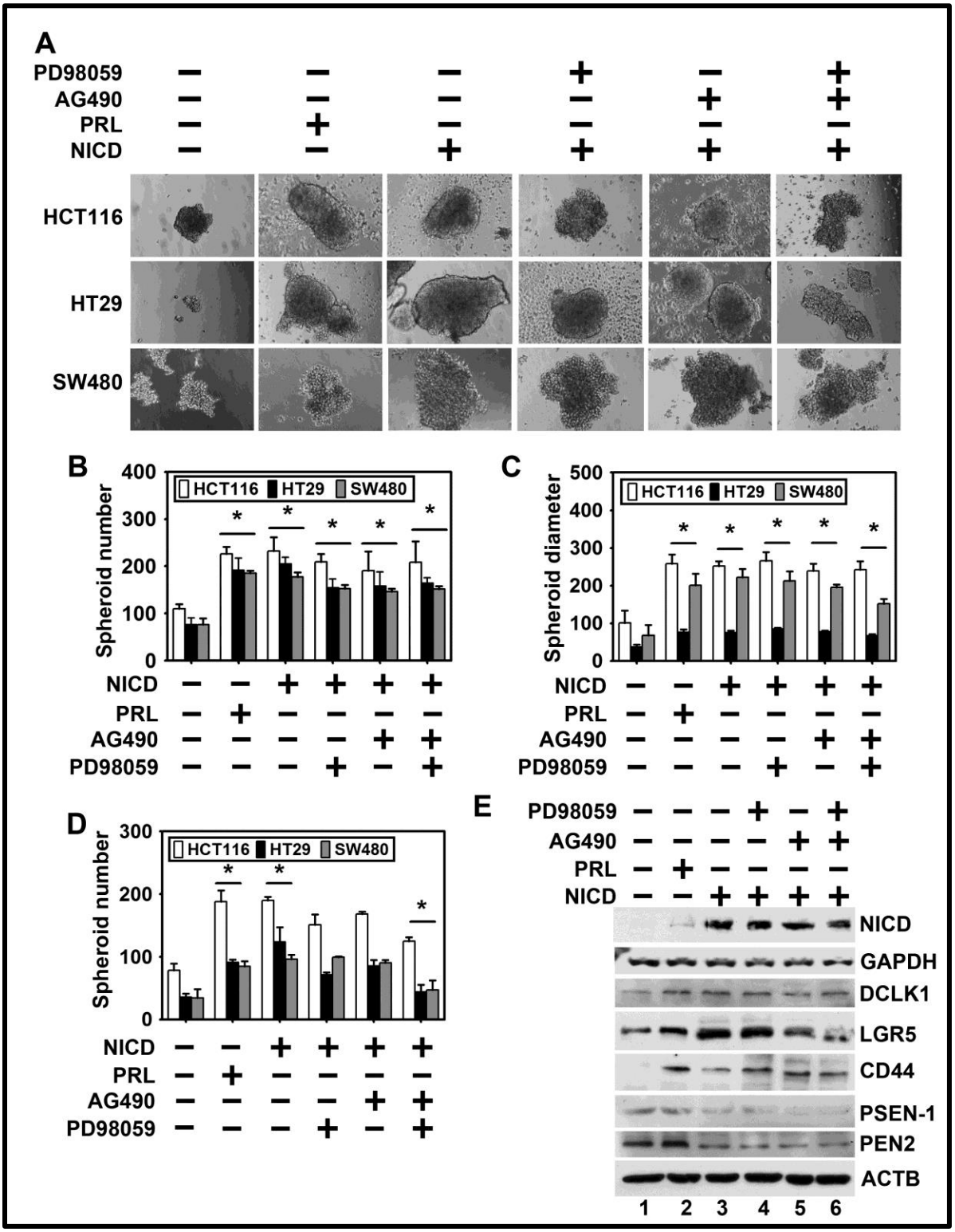


Figure 2.8: NICD overexpression recapitulates PRL-induced changes in colosphere formation and stem cell marker protein expression: (A) Colon cancer cells were transfected with NICD overexpressing plasmid, grown in specific spheroid media in low adherent plates for 5 d in the presence of PRL and inhibitors. Neither AG490 nor PD98059 treatment alone or in combination caused any significant decrease in colosphere formation in NICD expressing cells. **(B)** Significant increase in spheroid number was observed in NICD expressing cells compared to untreated controls. The inhibitors did not affect the NICD overexpressing cells (*$p \leq 0.005$). **(C)** Increased colosphere diameter was also observed in NICD overexpressing cells compared to control (*$p \leq 0.05$). **(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 to control. Combination of the inhibitors had comparable decrease in spheroid number compared to 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 cancer stem cell markers DCLK1, LGR5 and CD44. However, expression of PSEN1 and PEN2 was not affected in NICD expressing cells as compared to PRL-treated cells.

2.5 Discussion

Our findings implicate an increase in *PRLR* transcript in CRC samples compared to adjacent normal tissue. Similarly, CRC cell lines had increased *PRLR* levels compared to normal FHC cells. In line with earlier observations [223], SW480 cells expressed relatively lower PRLR levels compared to other cell lines. No significant change in mRNA expression or robust secretion of PRL into the media was noted in CRC cell lines compared to FHC. These findings clearly show a preferential upregulation of PRLR in CRC cells suggesting a role for PRL-PRLR signaling in colorectal tumors.

Binding of PRL to prolactin receptor (PRLR) activates JAK/STAT and/or RAS-RAF-ERK1/2 pathway [156, 178]. 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 reported earlier [224]. In their studies, the authors demonstrated that treating the melanoma cell line LU1205 with only PD98059 induces robust phosphorylation of STAT3 and STAT5 [224]. 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 and increased sensitivity to chemotherapeutic drugs [225, 226]. In our studies, we observed that PRL did not affect the proliferation of CRC cells. The fact that only STAT3 is activated in CRC cells suggests differential activity for PRL that depends on the cancer type. It might be interesting to overexpress STAT5 in CRCs and determine whether PRL treatment would mimic the reduction in proliferation as observed in breast cancer.

The spheroid formation assay helps us determine the presence of cancer initiating cells in a cancer cell population [16, 221]. 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 [132, 152]. Similarly, neurosphere formation along with expansion of hippocampal precursor cell population [155] has been observed in a PRL dependent manner. We also observed increased expression 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 when compared to HCT116. This is also in line with studies of PRL effects on neural stem cells [155]. It would be interesting to determine 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 an 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 [220]. Moreover, previous studies have also demonstrated that ERK1/2 can modulate Notch signaling by regulating the expression of its ligand JAG1 [216]. Notch signaling is active in intestinal crypts [25] and helps regulate stem cell hierarchy and to determine cell fate [24]. Dysregulation in this pathway can lead in colorectal cancer [30, 31]. It would be interesting to determine whether PRL upregulation is essential for tumorigenesis.

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 (MAML) and CSL to

induce respective gene expression (Fig 2.9). However, it is important to note that PRL expression itself is not upregulated in the cancer tissues, but rather only PRLR. However, previous studies have demonstrated increased levels of PRL in the blood stream of patients with CRC [145, 146]. 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 [93, 152]. 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 either stress, and dietary fat can also affect tumorigenesis [227].

Figure 2.9: Proposed model of PRL signaling in CRC

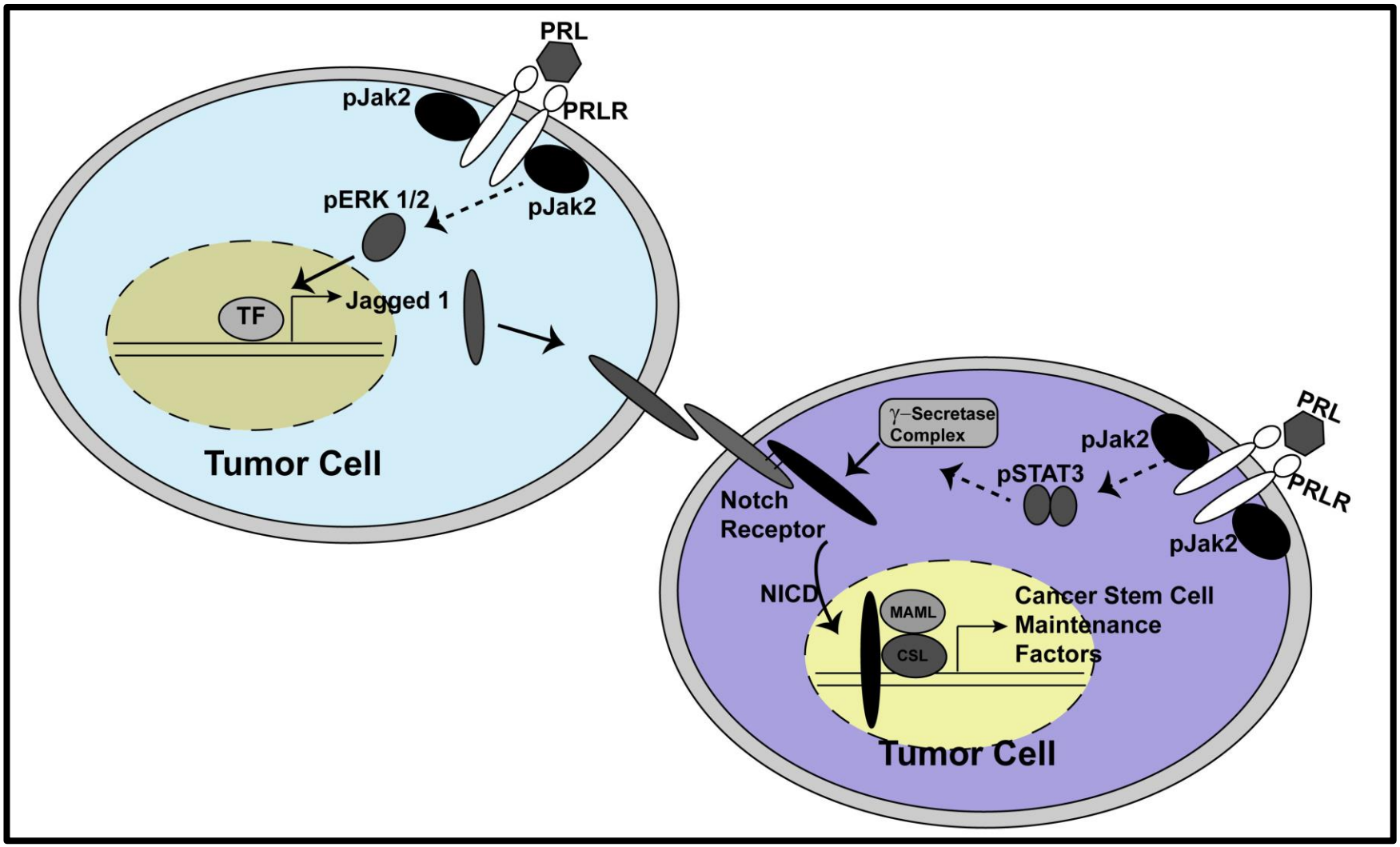


Figure 2.9: Proposed model of PRL signaling in CRC: Prolactin present in the tumor microenvironment would bind to PRLR and induce Jak2-ERK1/2 phosphorylation. The activated ERK1/2 induces expression of Jagged 1 (JAG1), a Notch-1 ligand. JAG1 would translocate to the cell membrane and bind to the transmembrane Notch receptor in the neighboring cell. This binding would induce a conformational change in the receptor leading to sequential cleavages by various enzymes including the γ-secretase complex in the neighboring cell. This results in the release of the Notch intracellular domain (NICD) that then translocates into the nucleus, complexes with Mastermind-like (MAML) and CBF1/ suppressor of hairless-1 (CSL) to activate target gene expression.

Chapter 3:

Heterogeneity in expression of Prolactin receptor in colorectal cancer

3.1 Abstract

Prolactin receptor (PRLR) is a single pass transmembrane protein that activates proliferative and growth responses upon interacting with its ligand Prolactin (PRL). Overexpression of PRLR was reported in several cancers with higher incidences particularly in breast cancer. Our study illustrates a significant increase in expression of PRLR in colorectal cancer patient samples and cell lines compared to adjacent normal tissue or cells. In addition, we observed a stage specific increase in expression of PRLR in patient samples with adenomas and carcinomas having high expression while it is low in polyps and inflammation. *In silico* analysis for expression and copy number variation on data retrieved form The Cancer Genome Atlas (TCGA) data base pertaining to colorectal cancer patients suggest both an increase in expression and copy number of genes in the 5q13 chromosomal locus. This locus harbors the *PRLR* gene along with other genes which may play a critical role in colorectal cancer. Sterol response element binding protein 1 (SREBP1) is a transcription enhancer and is upregulated in colorectal cancer. It has binding regions in the PRLR promoter regions and can regulate its expression in colorectal cancer cells. Taken together, our observations indicate that there is a stage specific upregulation of PRLR in colorectal cancer and that this upregulation may be a

contribution of locus amplification and/ or increase in gene expression due to enhanced transcription.

3.2 Introduction

Cytokines present in the tumor microenvironment play a critical role in tumor progression and metastasis. Upregulation of several cytokines and their receptors have been noted in several cancer types. Cancer cells respond to host-derived cytokines by increasing cell proliferation, attenuating apoptosis, and increasing the invasion and metastasis phenotype. A more detailed understanding of cytokine-tumor cell interactions provides new opportunities for improving cancer immunotherapy [228, 229]. Cytokines interact with specific receptors which are transmembrane proteins. This interaction leads to dimerization of the receptors. These dimerised receptors can activate several intracellular signaling proteins/ cascades that promote cell growth or death.

Several clinical observations and experimental studies indicated an increase in cytokine receptor expression in breast, prostate, ovarian, and endometrial cancers. Analysis on a cohort of seventeen breast cancer tissues and adjacent normal tissue revealed that growth hormone receptor (GHR) and progesterone receptor (PR), showed a significant increase, suggesting that growth hormone (GH) and progesterone (P4) may be acting together to promote breast cancer development [230]. However, tumor grade (MIB-1 index) and GHR have an inverse correlation

indicating that GHR and PR may serve as early prognostic marker [230]. A similar increase in expression of GHR, EGFR, keratinocyte growth factor receptor (KGFR), in addition to receptors for several colony stimulating factors, was observed in inflammatory bowel disease (IBD) [231].

DNA copy number variations (CNVs) constitute an important component of genetic variation. Since their advent, they have been identified in a variety of conditions implicating their role in susceptibility to a wide spectrum of pathologies. In addition, somatic CNVs can be used to identify regions of the genome involved in these disease phenotypes [232]. CNVs occurring in a specific gene or affecting the whole chromosomes have been identified to cause various pathologies and developmental abnormalities as well as act as a source for genetic diversification, adaptation and evolution [232]. CNVs occurring in many genes simultaneously can be very detrimental. Compared to higher organisms, CNVs are beneficial, increasing survival under selective pressure and augmenting drug resistance in microorganisms [233]. Until recently, the significance of CNVs in cancer was underappreciated. However, *in vitro* and clinical studies have characterized genomic instability while chromosomal structural dynamism was observed in a plethora of cancer types, leading to the conclusion that these genetic variations form an important part of cancer pathogenesis [232].

Human epidermoid carcinoma cells A431 have high levels of EGFR. The *EGFR* gene is located on short arm of chromosome 7 and cytological studies have identified that this locus is duplicated/ amplified in epidermoid carcinomas. This correlates with increased EGFR expression [234]. Similarly, chromosomal band analysis on human pancreatic cancer cell lines T3M4, PANC-1, COLO 357, and UACC-462, suggested structural alterations of chromosome 7p in T3M4, PANC-1, and COLO 357 cells, while UACC-462 showed multiple copies of chromosome 7 [235, 236]. Amplification of 20q12-13 chromosomal region encompassing genes coding for *AIB1, PTPN1, MYBL2, BTAK* and *ZNF217* is common in breast cancer and in ovarian tumors. Clinical studies done on 24 sporadic, 3 familial and 4 hereditary ovarian carcinomas and in 8 ovarian cancer cell lines showed amplification of at least one of the five regions of 20q12-13.2 chromosomal locus in 54% of sporadic and 100% of hereditary tumors. *AIB1* and *PTPN1* genes were preferentially amplified in sporadic ovarian tumors while *BTAK, MYBL2* and *ZNF217* gene locus were preferentially amplified in sporadic breast tumors. Such high frequency of gene amplification at 20q12-q13.2 suggests that the genes present in this region may play a central role in the pathogenesis of ovarian and breast cancers [237]. CNV of 1q21-23, 2p12-16, 8q24, 9q34, 12q12-14, 13q32, 16p12, 18q21-22, and 22q12 locus identified in large B-cell lymphomas (DLBL) demonstrates the significance of genes located in this region in lymphoma-genesis

[238]. Oral squamous cell carcinomas often possess a triploid karyotype which is associated with disease severity and drug resistance [239]. As regards GI-related cancer, amplification of *ERBB2* gene was identified in MKN-7 gastric cancer cell line [240]. Apart from amplification, deletion of a locus or the complete chromosome can also confer selective advantage. For example, haploinsuffecieny of *BRCA1* in breast cancer leads to increase in GHR and PR leading to an increase in cell survival [241].

In addition to the a forth mentioned genetic changes which can contribute to the increase in gene expression, an increase in binding of transcription enhancing factors to the enhancer regions of the *PRLR* gene can also lead to increase in PRLR expression at a transcription level.

Aberrant increase in lipogenesis is a metabolic feature of proliferating tumor cells. Normal cells, acquire their fatty acid stock from the circulation: however, in tumor cells there is an active *de novo* synthesis of lipids. Sterol regulatory element-binding proteins (SREBP), also referred to as sterol regulatory element-binding transcription factor (SREBF) encoded by the *SREBF* genes in humans, is a member of the basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor family of proteins [242], and is a master regulator of lipogenic gene expression. SREBP-1a, SREBP-1c, and SREBP-2 are the three isoforms of SREBP proteins. SREBP-1a and SREBP-1c are transcribed from the same *SREBP-1* gene, but from two

distinct promoters, while SREBP-2 is encoded by *SREBP-2* gene as a single mRNA. The relative levels of SREBP-1a and -1c mRNA vary depending on the tissue type. SREBP-1c is the predominant isoform in adult liver and adipocytes, while, SREBP-1a is predominant in spleen and in cancer cells.

SREBP-1a interacts with co-activators such as p300/CREB-binding protein and Sp1 to regulate transcription. SREPB proteins bind to a highly conserved sequence called sterol regulatory element-1 (SRE1) in the promoter upstream regions of target genes. SREBP-1a and 1c are potent transcription activators of genes that mediate synthesis of cholesterol, fatty acids, and triglycerides, while SREBP-2 preferentially activates cholesterol synthesis genes [243]. SREBP-1 mRNA is translated as a precursor which attaches to the nuclear membrane and endoplasmic reticulum. Following activation and cleavage, the mature protein translocates to the nucleus and activates transcription by binding to the SRE1. Sterols inhibit the cleavage of the precursor, and the mature nuclear form is rapidly catabolized, thereby reducing transcription [244, 245].

Small molecule mediated inhibition of fatty acid synthesis in the HCT116 CRC cell line resulted in a dramatic increase in SREBP-1 protein levels [246]. Similarly SW480 and SW620 CRC cells treated with oridonin, a potent antiproliferative

diterpenoid agent, isolated from *Rabdosia rubescens*, caused a dramatic decrease in SREBP-1 levels resulting from decreased fatty acid synthesis [247].

Earlier, we have demonstrated that PRL treatment activates the stem cell population in CRC. In humans PRL specifically binds specifically to PRLR to activate intracellular signaling events [248]. We have shown that an increase in expression of PRLR was observed in CRC patient samples and cell lines compared to adjacent normal tissue or normal intestinal epithelial cell line. However, the reason for this upregulation is not yet known. This work is directed towards identifying the expression pattern of PRLR and the molecular basis for its upregulation in CRC.

3.3 Materials and Methods

3.3.1 Cell lines

Well characterized colon cancer cell lines used in the study were obtained from ATCC (Manassas, VA). These cells were cultured in DMEM 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% CO_2.

3.3.2 RT-PCR Analysis

Total cDNA from various sites along the normal human gastrointestinal tract was obtained from Clontech (Clontech, CA). Colon cancer cDNA panel with matched adjacent tissue controls was obtained from Origene (Rockville, MD). Trizol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from cell lines following manufacturer's instructions. 2 μg RNA was used for cDNA synthesis using Superscript II reverse transcriptase and random hexanucleotide primers (Invitrogen, CA). PRLR expression was quantified using SYBR green reagent (Molecular Probes, OR) and specific primers with GAPDH as internal standard. Primers for the PCR include 5`-GGAGCTGGCTGTGGAAGTAA-3` and 5`-CTCCCACTCAGCTGCTTTCT-3`for PRLR, 5`-CAGCCTCAAGATCATCAGCA-3` and 5`-GTCTTCTGGGTGGCAGTGAT-3` for GAPDH, 5`-CTGCTGTCCACAAAAGCAAA -3` and 5`-GGTCAGTGTGTCCTCCACCT-3` for SREBP-1, 5`-

GCTACCTGTCCGGCTACATC-3` and 5`-CGATGCCCATAATGTTGTTG-3` for NR2F1/ COUP-TF1, 5`-CCAGGCACCATGCTAGGTAT-3` and 5`-GTAAGAACAGACCCAGCCACTT-3` for GFI1.

3.3.3 Western Blot Analysis

Protein samples were extracted using RIPA buffer (Thermo Scientific, IL) supplemented with protease and phosphatase inhibitors. 10-30 μg of protein was subjected to poly acrylamide gene electrophoresis and transferred onto PVDF membrane (EMD Millipore, MA) following quantification using BCA Kit (Thermo Scientific, IL). Antibodies for PRLR (sc-377098), SREBP-1 (sc-365513) and ACTB (sc-1616) were obtained from Santa Cruz Biotech Inc. (Dallas, TX). Proteins were detected using chemiluminescence (GE Healthcare, NJ).

3.3.4 Histology

Tissue microarray slides containing colorectal cancer, adjacent normal, liver and lymph node metastatic and normal samples (CO702) and colon adenocarcinoma, mucinous, papillary, squamous cell carcinoma of different grades and normal colon tissue (CO802) were obtained from Biomax (Rockville, MD). The tissue sections were fixed in 1% formaldehyde, following de-waxing at 60^{o} C. Following sequential hydration and blocking, the sections were incubated overnight with PRLR antibody. Staining was developed using the Histostain kit (Invitrogen, CA) following manufacturers protocol. Staining was scored using Clarient Automated

Cellular Imaging System (ACIS) (San Juan Capistrano, CA) as previously described [249] and the scores obtained were used for further analysis.

3.3.5 ChIP Assay

ChIP assay was performed based on protocol from Dr. Jeffery Rosen's lab (https://www.bcm.edu/rosenlab/index.cfm?PMID=13002) with little modification. Briefly, HCT116 and DLD1 cells were plated at a confluence of 2 $X10^6$ in 100mm dishes and were grown to 90% confluence, at which point the cells were cross-linked by adding 4% formaldehyde (Cat # 433284, Sigma, St. Louis, MO) directly into the media to a final concentration of 1%. Fixing was stopped by adding glycine to a final concentration of 125 mM. The cells were washed in ice cold PBS and cells were scraped and collected. Cell were lysed using a mild buffer (5mm PIPES in KOH (pH 8.0), 85mM KCl and 0.5% NP-40, supplemented with protease and phosphatase inhibitor) and dounced (Type B) repeatedly to ensure thorough cytoplasmic lysis as seen under a microscope. The nucleus was pelleted by centrifuging (5000 rpm for 5 min) and suspended in nuclear lysis buffer (50 mM Tris (pH 8.1), 10 mM EDTA and 1% SDS supplemented with protease/ phosphatase inhibitors) and sonicated to obtain DNA fragments of 0.3- 1 kb size. The chromatin was diluted 1:5 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1 mM EDTA, 16.7 mM Tris (pH 8.1), 167 mM NaCl supplemented with protease inhibitors) and pre-cleared using salmon sperm DNA treated protein A

agarose slurry. A part of the pre-cleared lysate was saved to be used as input and the remainder was mixed with 5-8 μg of SPREBP-1 antibody and following an overnight incubation, the antibody/DNA complex was precipitated using salmon sperm DNA/ protein A agarose slurry. Reverse crosslinking and DNA extraction was followed by semi-quantative PCR using primers specific to upstream region of PRLR gene: 5`- GAACTTCAAGAGGAGGAAGT-3` and 5`-CCTACAACTTCTACATCTTCTT-3` which produce a 415bp fragment and 5`-CCTGCATTAGAAGCTCTGCAA-3` and 5`-CTTCCCTCTCAGTGCCTTAA-3` that produce a 114 bp fragment.

3.3.6 Analysis of TCGA Data Sets

The Z-score values for gene expression and the log2 ratios for copy number variations for each of the five genes PRLR, AGXT2, RAD1 and DNAJC21 were obtained for 156 patients from TCGA portal (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) with the collaboration of Dr. Andrew Godwin. Samples with a tumor/normal expression level of more than or equal to 0.5 were considered up-regulated. Samples showing a tumor/normal log2 values of >0.3 for CNV were considered to possess a copy number gain [250]. Correlation and regression analysis was used to analyze the data.

3.3.7 Statistical Analysis

GraphPad Prism 5 (La Jolla, CA) was used to perform all the descriptive statistics

including mean, standard deviation and student's t-test. Data from at least three independent experiments were expressed as the mean ± SEM. *P* value ≤ 0.05 were considered significant.

3.4 Results

3.4.1 Expression of PRLR along the gastrointestinal tract

We first determined the expression of PRLR along the GI tract to get a better idea about the expression pattern. Specific primers were designed to detect the full length and dSF1 isoforms (together called long form) or the SF1a, SF1b and the d7/11 isoforms (short form) (Fig 3.1A and B). Real-Time PCR data suggests that both PRLR long and short forms are differentially expressed along the length of the GI tract with the long form being the predominant isoform. Ileocecum, colon and liver have higher long form expression compared to other sites, with colon having the highest expression (Fig 3.2 A). While the expression of long form is predominant in the intestinal portion of the GI tract, short form expression is spread throughout, with detectable expression all the way from stomach to rectum, and the liver (Fig 3.2 B).

3.4.2 Expression of PRLR in CRC and normal tissues

Next, we evaluated the expression of PRLR in CRC and adjacent normal human tissues using the same primer pairs. We noted that there was a three-fold increase in expression of the long form and a four - fold increase in short form (Fig 3.3 A and B) in the tumor samples compared to adjacent normal tissue. However, there was a considerable variation in the expression of short form ranging from one to five fold increase (Fig 3.3 B). On the other hand, the expression pattern for the

Figure 3.1: PRLR transcript grouping and primer binding sites

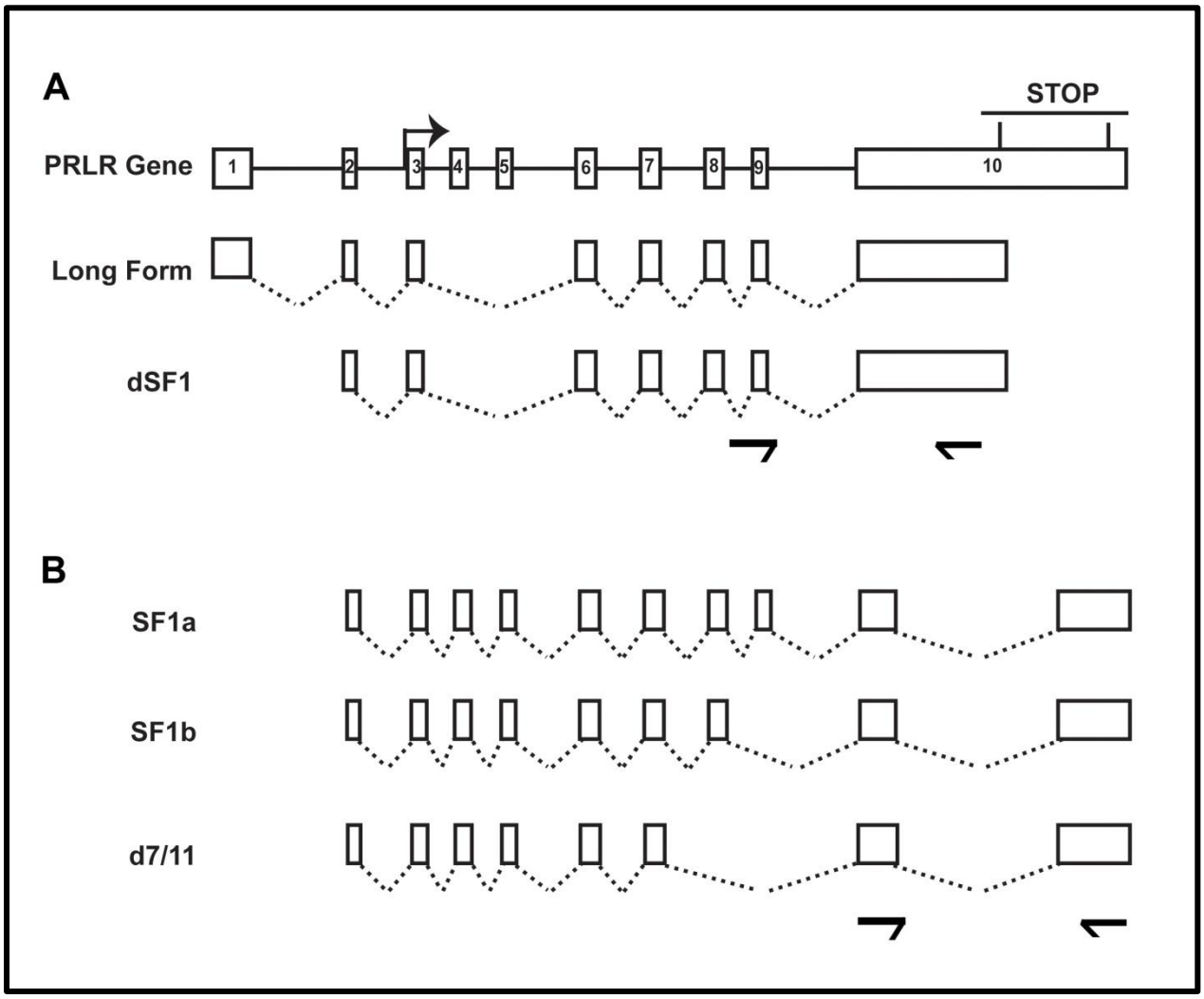


Figure 3.1: PRLR transcript grouping and primer binding sites: For the ease of desigiing primers, PRLR transcripts were catagorized into two groups. **(A)** The long form including the full length and the dSF1 isoforms and the **(B)** short form including the SF1a, SF1b and the d7/11 isoforms. For the long form, primers were designed spanning exon 9-10, while that for the short form were designed spanning only exon 10, but the binding site for the reverse primer is very unique for the short form.

Figure 3.2: Expression of PRLR along the GI tract

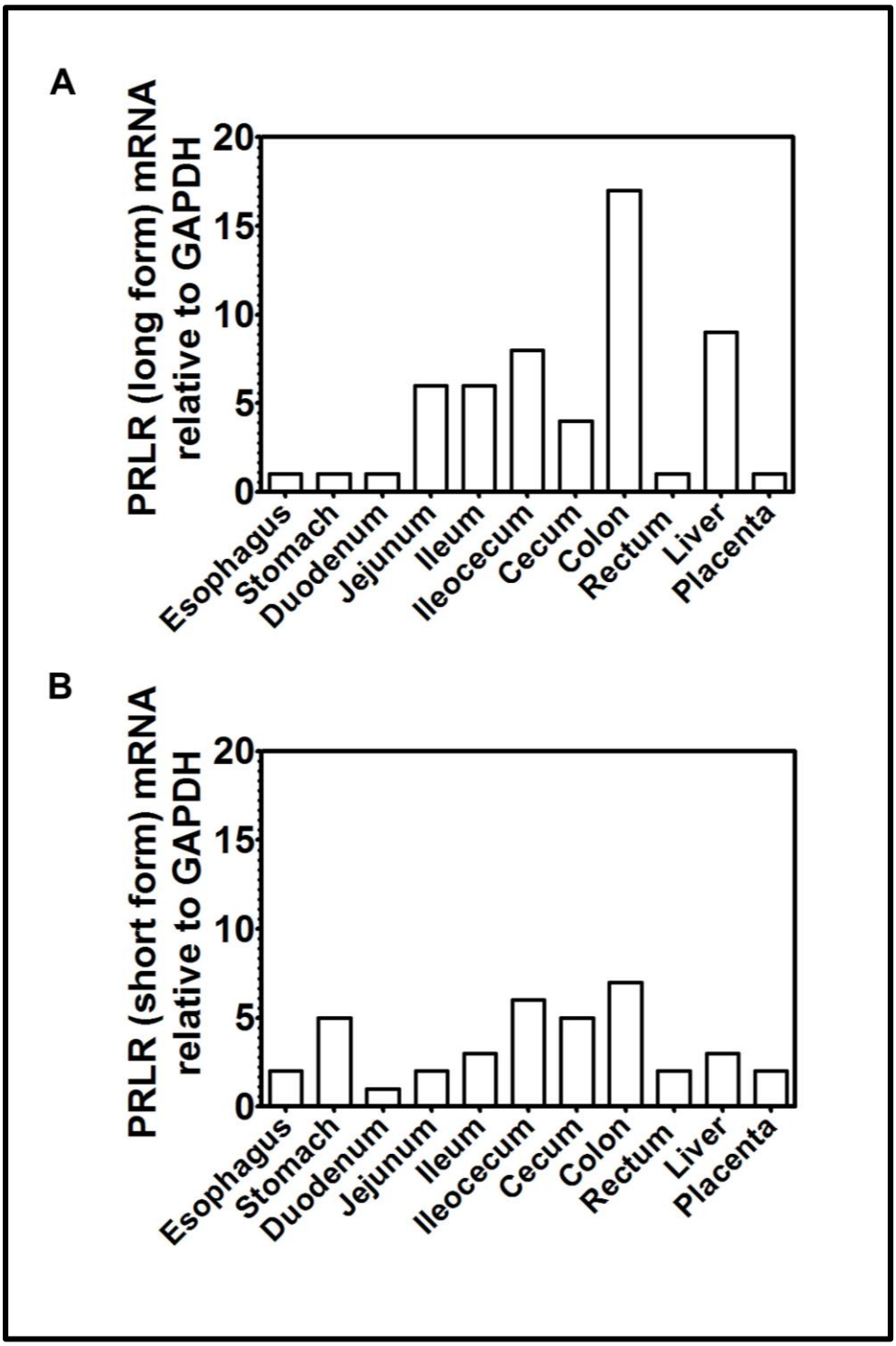


Figure 3.2: Expression of PRLR along the GI tract: Evaluation of PRLR across the GI tract indicate that both the long **(A)** and the short **(B)** form are expressed at varying levels across the GI tract with the long form predominantly in the intestine and short form more generally expressed. Expression of long form is more prominent in the colon as compared to other intestinal sites. Placental tissue was used as experimental controls.

Figure 3.3: Expression of PRLR in patient samples from CRC and adjacent normal tissue

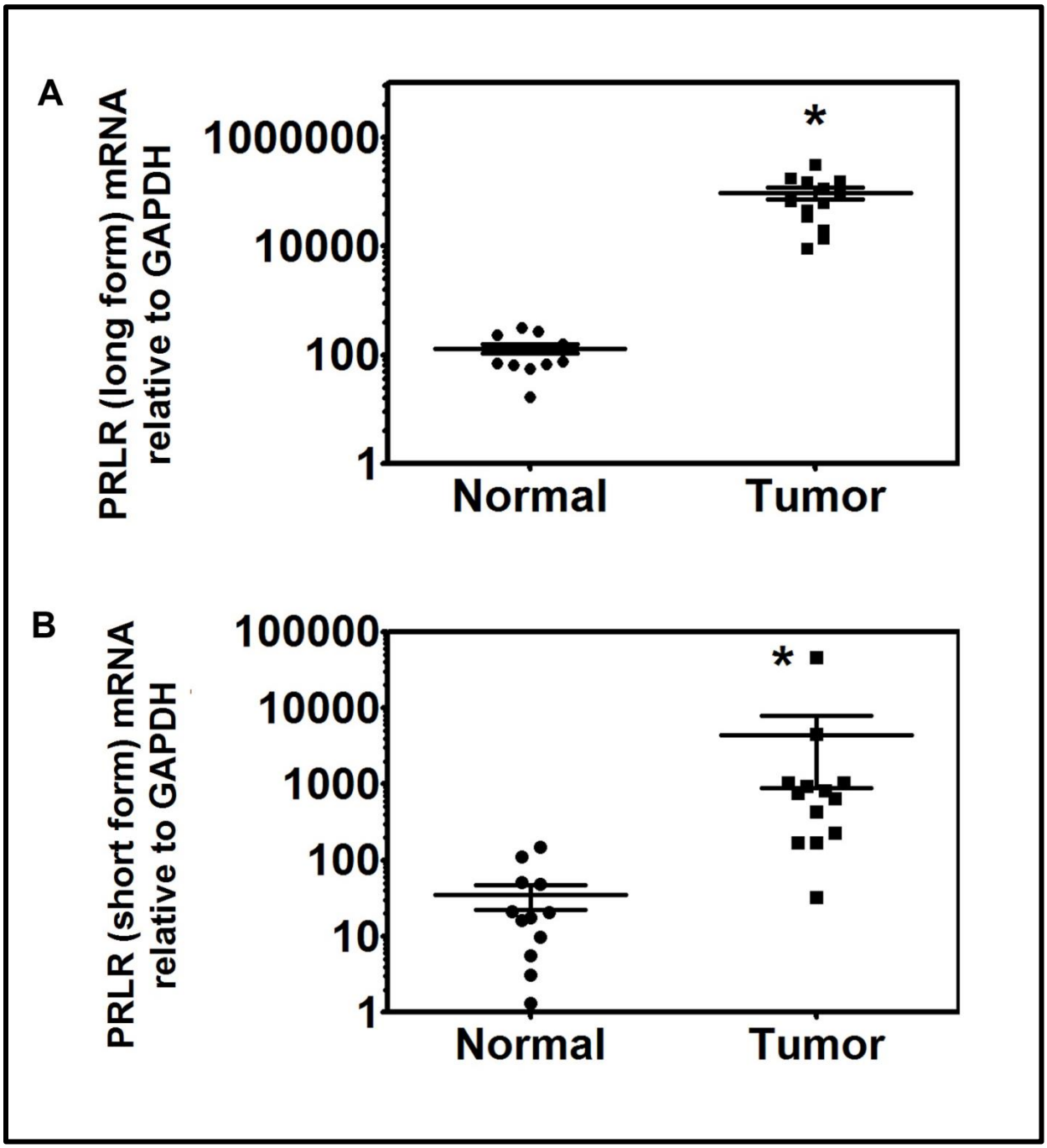


Figure 3.3: Expression of PRLR in patient samples from CRC and adjacent normal tissue: In general, a significant overall increase in expression of both the isoforms of PRLR is observed in CRC tumor samples compared to adjacent normal tissue samples. **(A)** While the expression of the long form is more compact and significant, **(B)** short form expression is more variable among both the normal and maligant samples.

long form was tighter with most of tumor samples showing a significant increase in expression as compared to adjacent normal samples (Fig 3.3 A).

3.4.3 Histological validation of PRLR expression in CRC and normal tissues

We next used immunohistological studies to further validate the increase in PRLR expression. CRC samples and normal tissue sections were stained with PRLR antibody which detects the C-terminal region of PRLR receptor and scored for intensity. Detectable levels of PRLR was noted along the crypt length in normal colon (Fig 3.4 A, B), predominantly in the membrane of the epithelial cells lining the crypt and a few stromal cells. However, in adenoma samples, the crypt structure is completely lost accompanied with a significant and distorted expression of PRLR (Fig 3.4 C, D). Further more in some cells, nucelar staining for PRLR was also observed. Histoscore analysis indicated a significant increase in PRLR expression in adenomas and adenocarcinomas (Fig 3.5 A). In addition, this increase occurs in a stage specific manner with decresed expression noted in inflammation and polyps while increased expression was observed in adenomas and adenocarcinomas (Fig 3.5 B).

3.4.4 Analysis of PRLR expression in metastatic sites

Further histological analysis of samples derived from distant metastatic sites such as lymph nodes and liver indicates that the increase in PRLR expression observed in adenomas and adenocarcinomas is maintained in these metastatic foci localized

to these regions. Lymph nodes have a lower level and diffuse expression of PRLR (Fig 3.6 A, B), however, metastatic foci have significantly higher levels of PRLR compared to surrounding tissue (Fig 3.6 C, D). Normal liver samples (Fig 3.7 A, B) have a significant higher PRLR expression compared to metastatic foci (Fig 3.7 C, D). Histoscore analysis also indicates the same with higher PRLR expression in metastatic foci of the lymph nodes as compared to liver site (Fig 3.8 A).

3.4.5 TCGA based evaluation of changes in PRLR expression

Changes in the expression of a particular gene can occur due to a number of reasons, including chromosomal anomalies. Numerical variations, referred to as copy number variations (CNV), constitute one of the frequently observed chromosomal variations in most cancers [251]. The Cancer Genome Atlas (TCGA), data sets containing expression and CNV data pertaining to PRLR and four other genes flanking the PRLR gene *DNAJC21*, *RAL14*, *RAD1* and Alanine-glyoxylate aminotransferase 2 (*AGXT2*), were analyzed to determine whether the increase in expression was due to underlying genetic variation. We noted a significant increase in expression *of PRLR, DNAJC21, RAD1* and *RAL14* in 35-40% of patient samples (Fig 3.9 A, B, C and D). *AGXT2*, on the other hand, did not show any significant change in expression (Fig 3.9 E). The remaining patient population had a decrease in expression of PRLR; however, the decrease is not statistically significant.

Figure 3.4: Histological analysis of PRLR expression in CRC and adjacent normal tissue

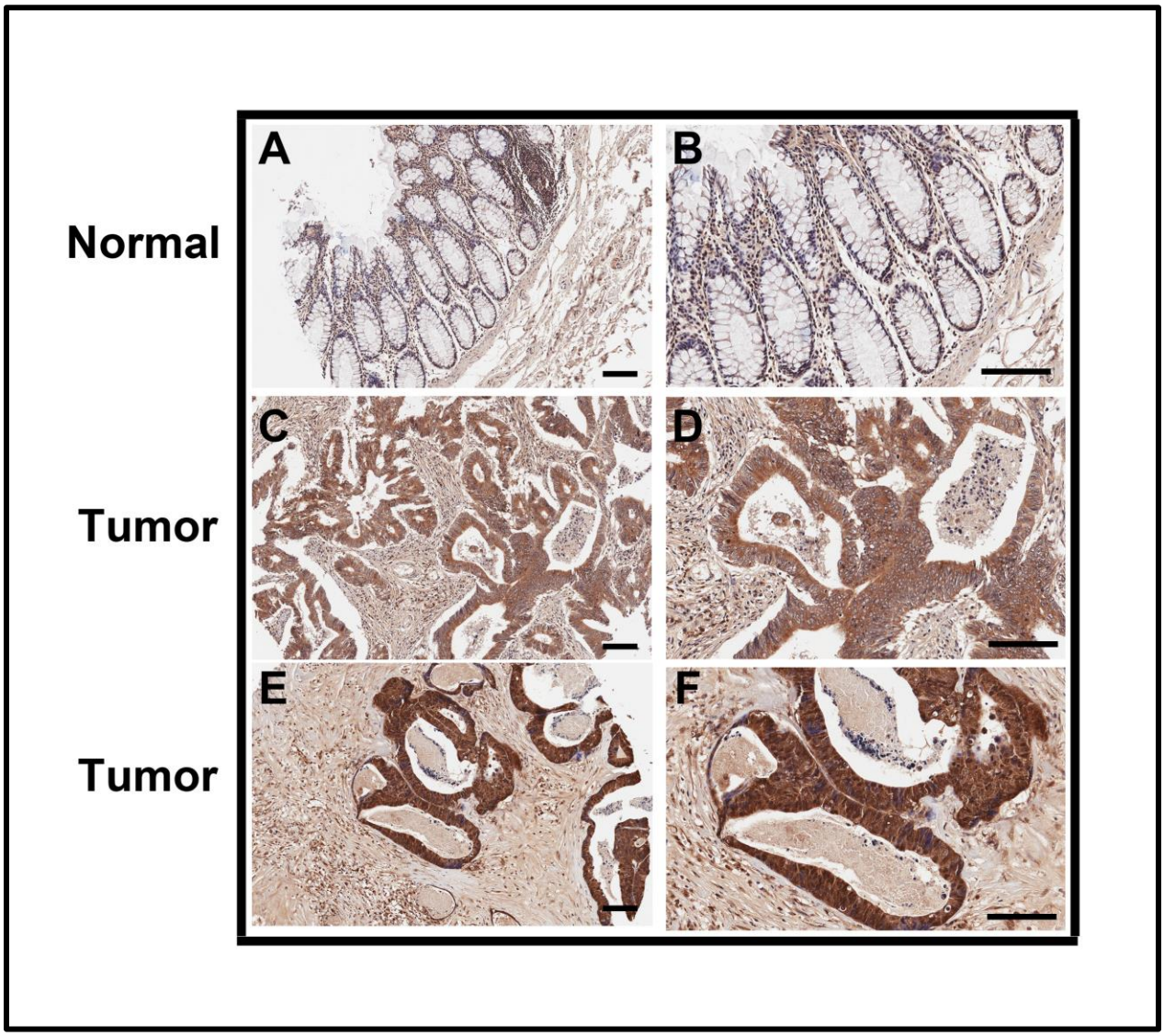


Figure 3.4: Histological analysis of PRLR expression in CRC and adjacent normal tissue: (A, B) PRLR is expressed at very low levels and is primarily localized to the basolateral membrane surface. **(C- F)** In colorectal tumor samples, the crypt structure is completely distorted accompanied with an overall increase in PRLR expression in all the cells of the tumor. A few cells also show nuclear staining.

Figure 3.5: Histoscore analysis of PRLR expression in normal and CRC tissue samples

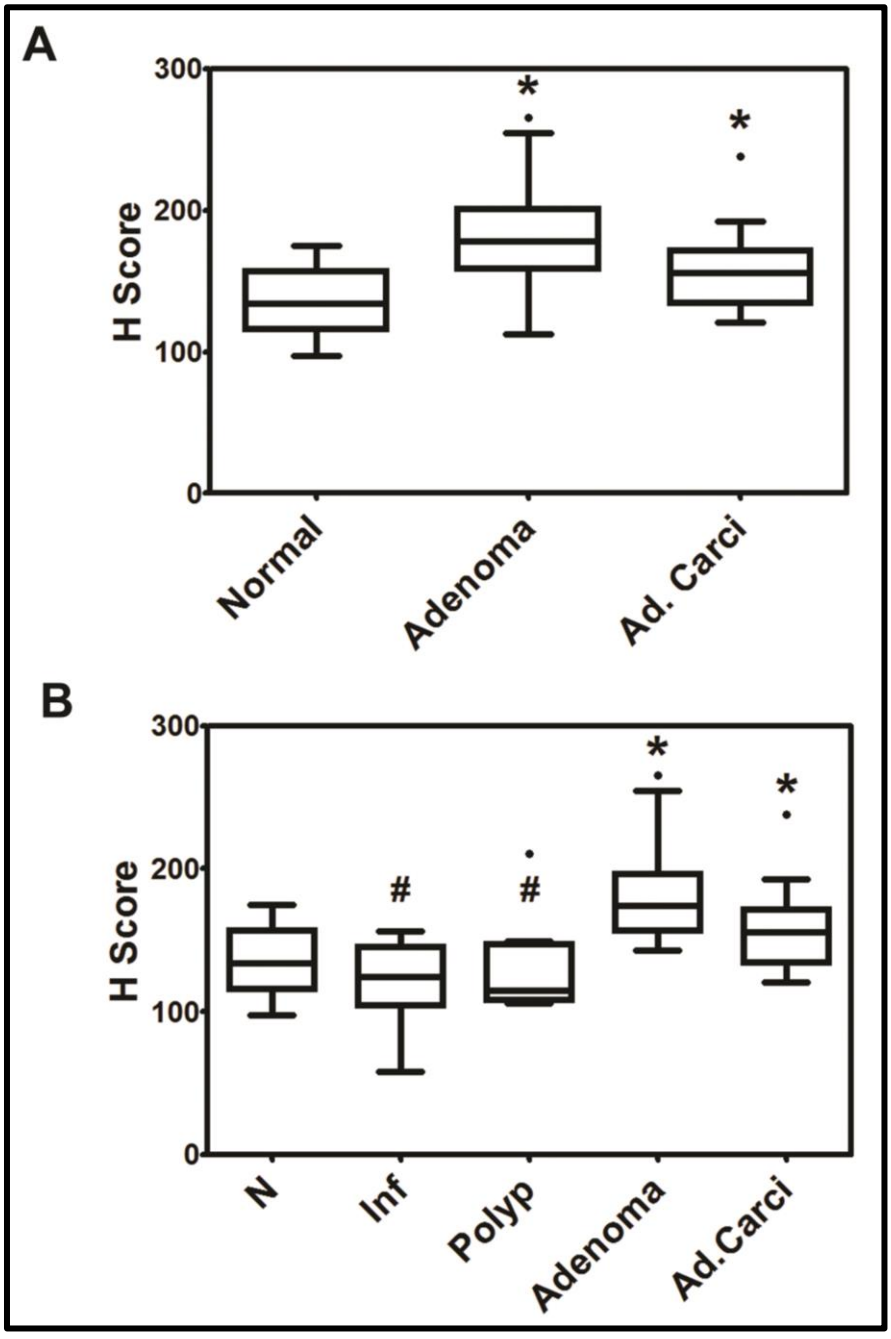


Figure 3.5: Histoscore analysis of PRLR expression in normal and CRC tissue samples: (A) In comparison to normal colon sections, increased expression of PRLR is observed in adenomas and adenocarcinomas. **(B)** However, during the initial stages of CRC progression, that is during inflammation and polyps, a decraese in PRLR expression is observed.

Figure 3.6: Histological analysis of PRLR expression in lymph node metastatis site

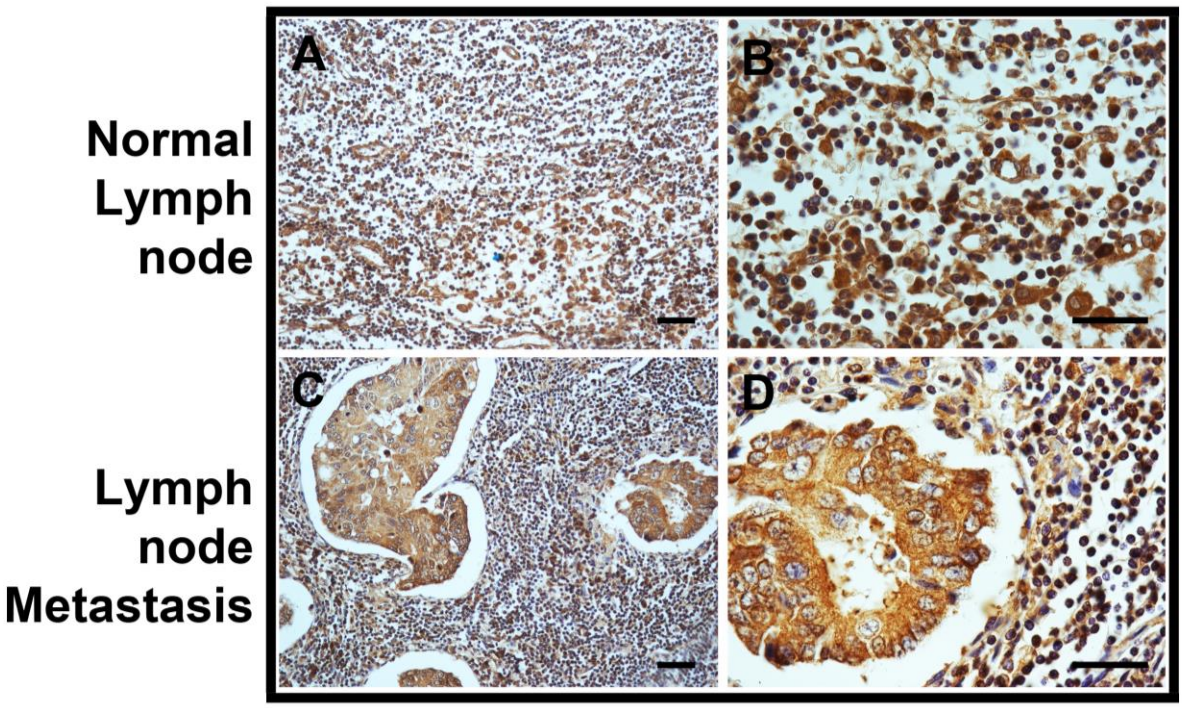


Figure 3.6: Histological analysis of PRLR expression in lymph node metastatis site: (A, B) Normal lymph exhibits diffused PRLR staining. **C,D)** metastatic foci in the lymph node expressed higher levels of PRLR compared to surrounding lymph nodes tissue. Metastasis also led to complete loss of tissue integrity.

Figure 3.7: Histological analysis of PRLR expression in liver metastatis site

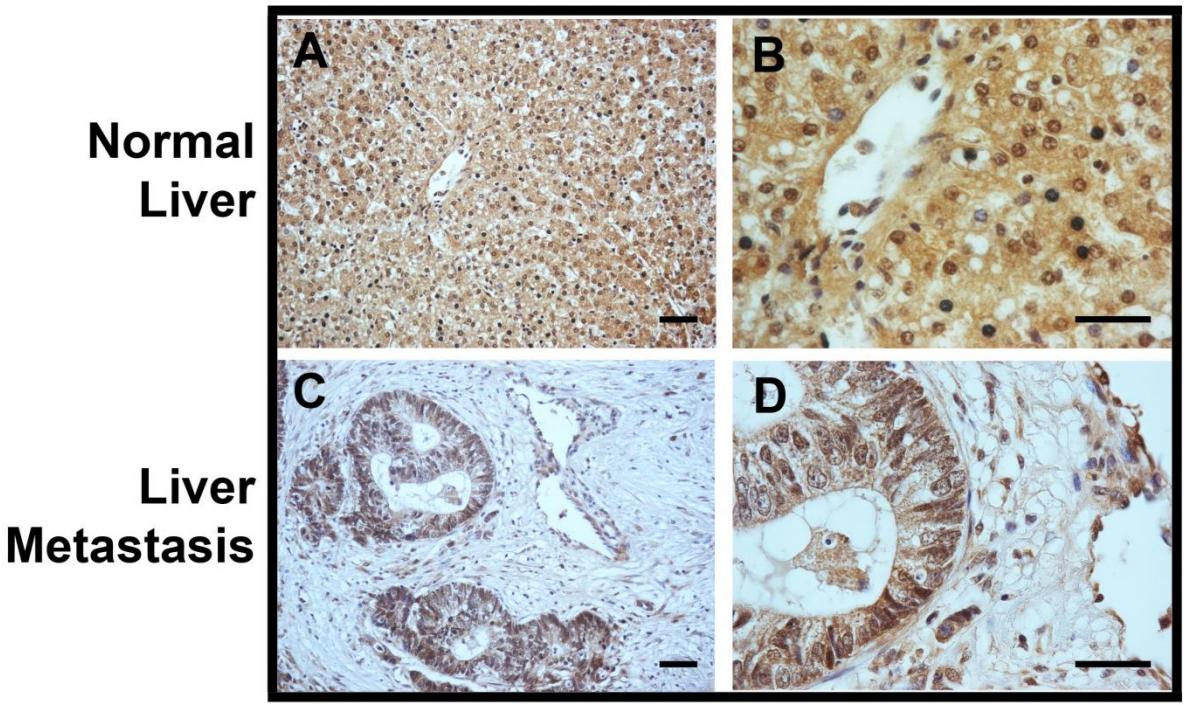


Figure 3.7: Histological analysis of PRLR expression in liver metastatis site: (A) PRLR is expressed at high levels in a diffuse manner with deep staining in the nuclei of some cells. **(B)** Metastatic foci have higher PRLR expression.

Figure 3.8: Histoscore analysis of PRLR expression in metastatic sites

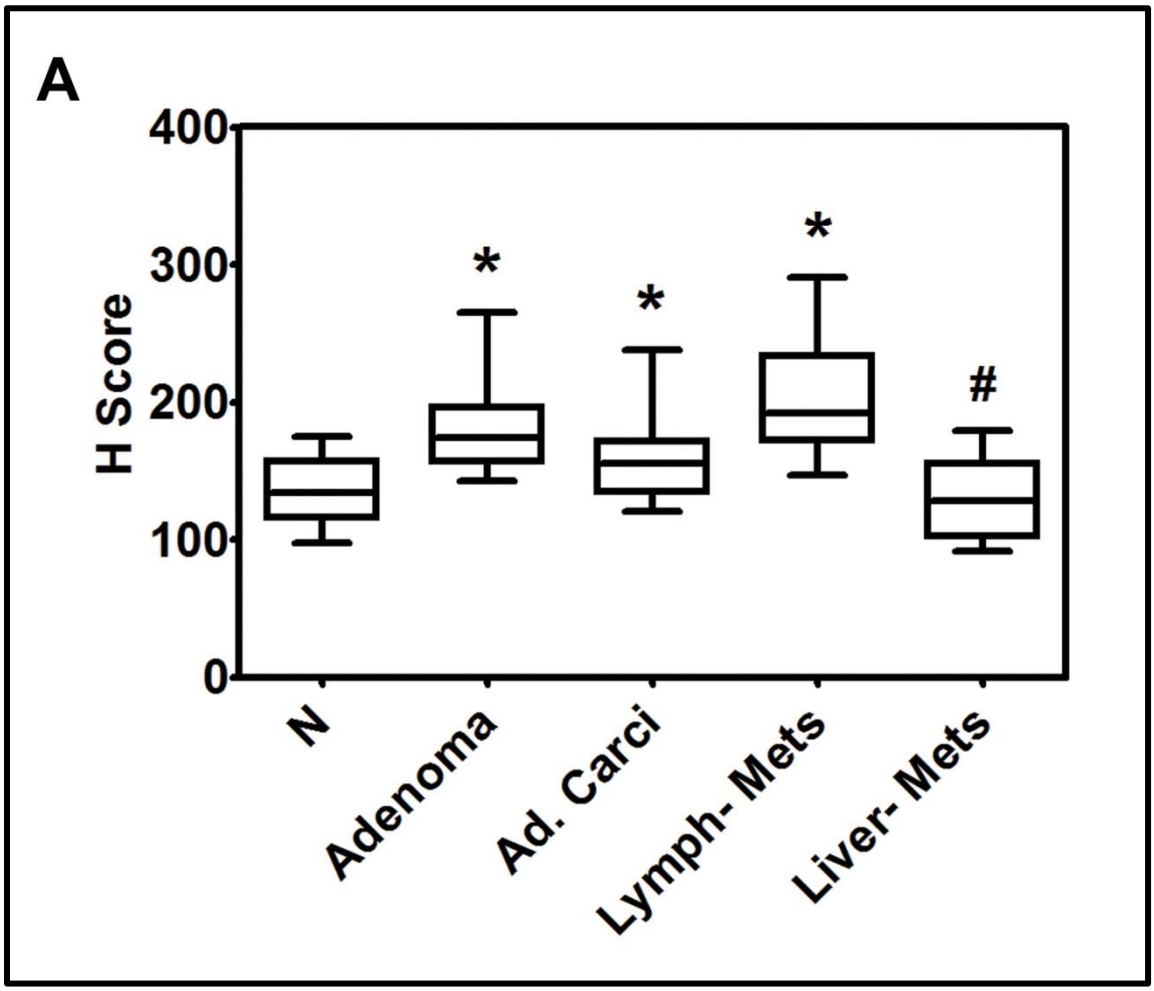


Figure 3.8: Histoscore analysis of PRLR expression in metastatic sites: (A) Compared to normal intestinal tissue, PRLR expression is increased in adenomas and adenocarcinomas and increases further in lymph node metastatic sites. However, in the liver metastatic foci PRLR expression is decreased.

Figure 3.9: Evaluating changes in expression of genes located in the PRLR locus

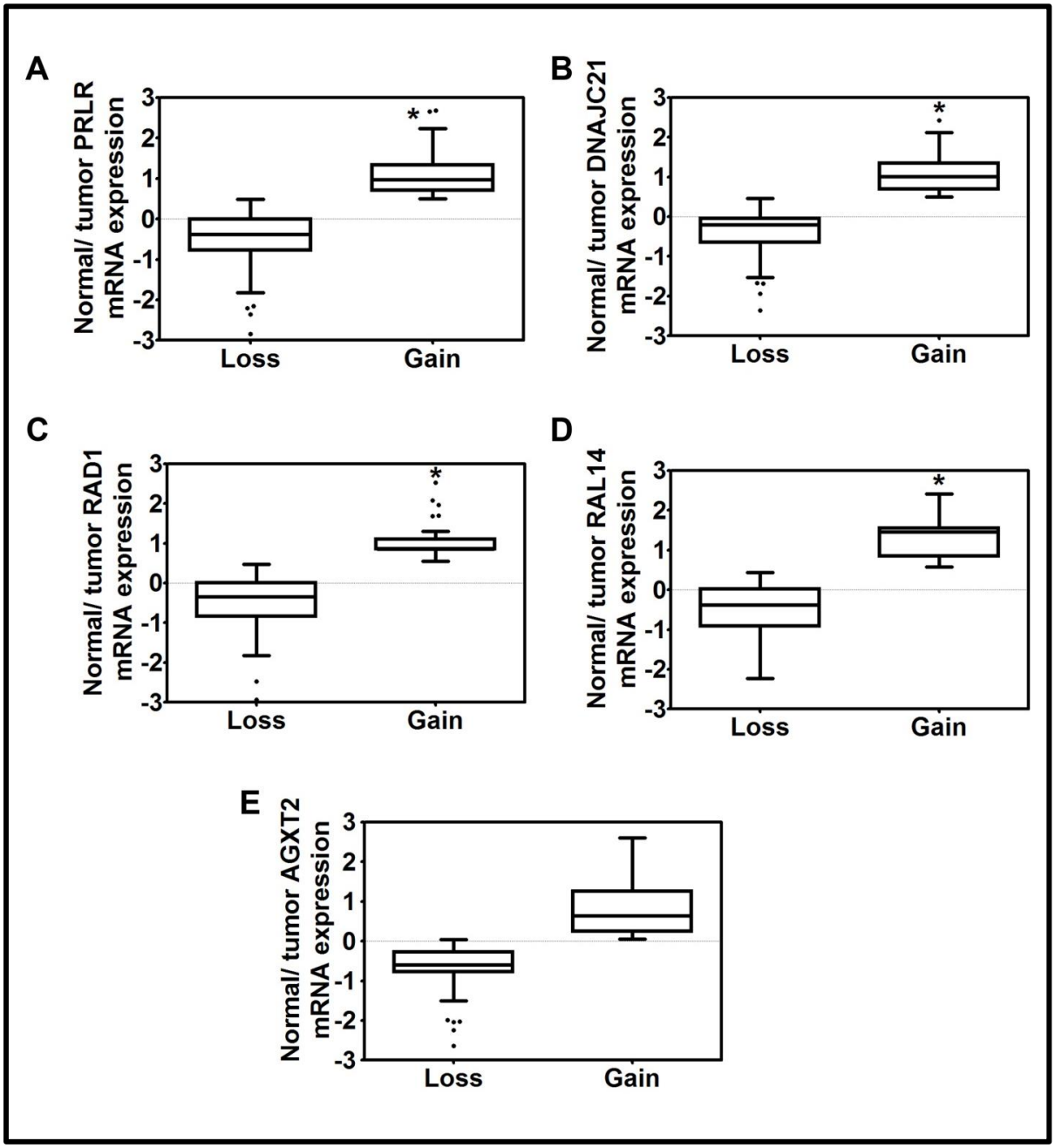


Figure 3.9: Evaluating changes in expression of genes located in the PRLR locus: Expression data sets pertaining to genes *DNAJC21, RAD1, RAL14, AGXT2* which are in close vicinity of *PRLR* gene for 156 patients was obtained from TCGA data base. Data indicates that there is a significant increase in expression of **(A)** *PRLR,* **(B)** *DNAJC21,* **(C)** *RAD1*and **(D)** *RAl14* in 30-40% of patient population, while expression of (**E)** *AGXT2* seems to increase, but is not stastically significant. *= $p<0.05$.

Figure 3.10: Evaluating changes in the copy number of genes located in the PRLR locus

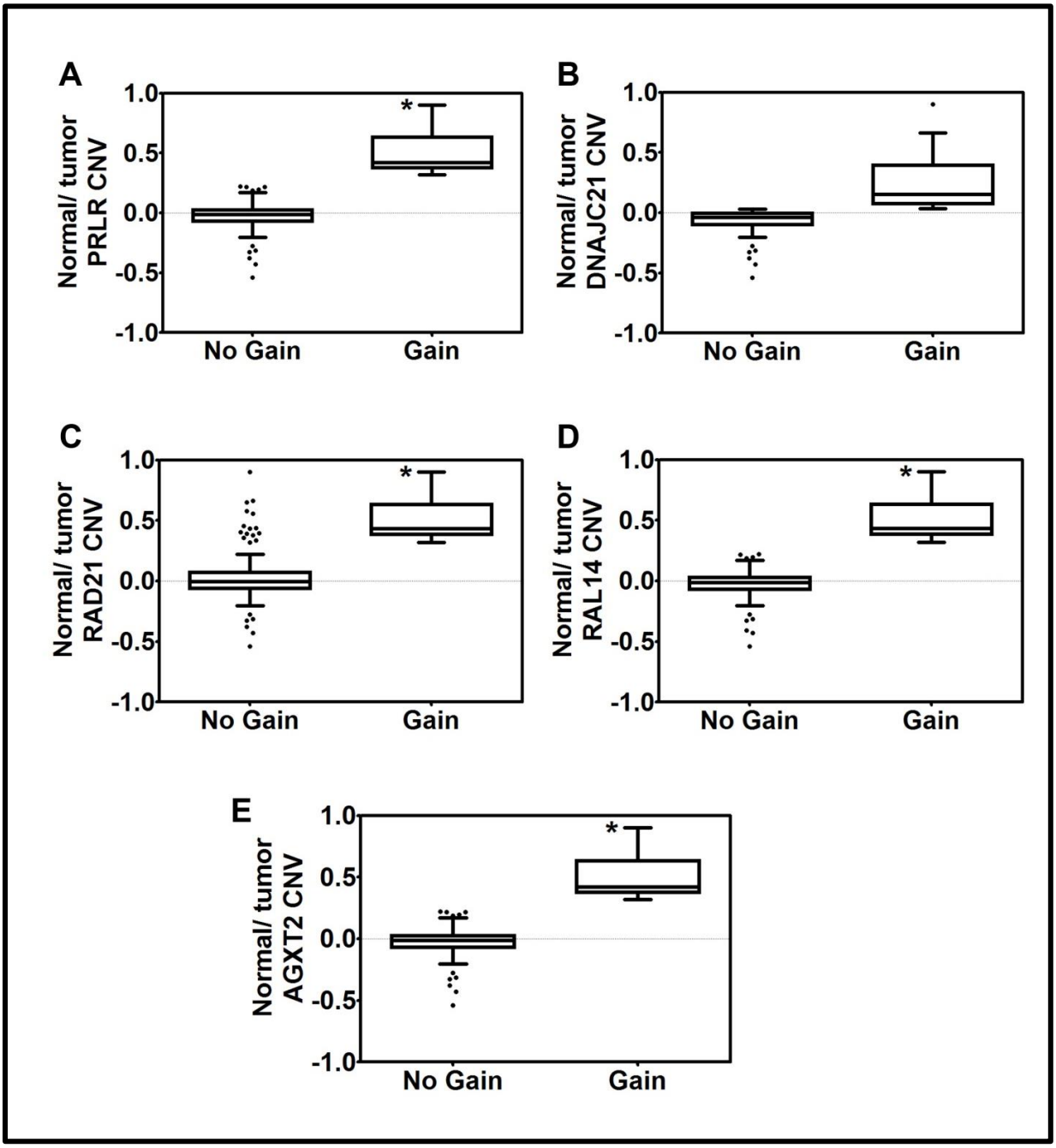


Figure 3.10: Evaluating changes in the copy number of genes located in the PRLR locus: Patient data sets pertaining to copy number analysis for *DNAJC21, RAD1, RAL14, AGXT2* genes which are in close vicinity of PRLR gene for 156 patients was obtained from TCGA data base. Data indicates that there was a significant increase in copy number of **(A)** *PRLR*, **(C)** *RAD1*, **(D)** *RAl14* and **(E)** *AGXT2* while the copy numer of **(B)** *DNAJC21* does not change significantly (*= $p \leq 0.05$).

In addition to the expression data, we also evaluated for any changes in the copy number as an increase in copy number has also been identified as a contributory factor for increase in gene expression [252]. TCGA analysis demonstrated that there is a significant increase in mean copy number of *PRLR, RAD21, RAL14* and *AGXT2* (Fig 3.10, A, C, D, E) with no significant change in DNAJC21 (Fig 3.10 B). Next we performed a correlation and regression analysis on normalized data to identify existence of correlation between the increase in expression and the CNV of the genes. Data indicates a strong correlation in the increase in expression of *PRLR, DNAJC21, RAD1* and *RAL14* (Fig 3.11 A, B, C, D) with an increase in copy number of these genes indicated by a positive correlation, while in the case of *AGXT2*, this correlation is lost (Fig 3.11 E).

Figure 3.11: Evaluating changes in the copy number of genes located in the PRLR locus

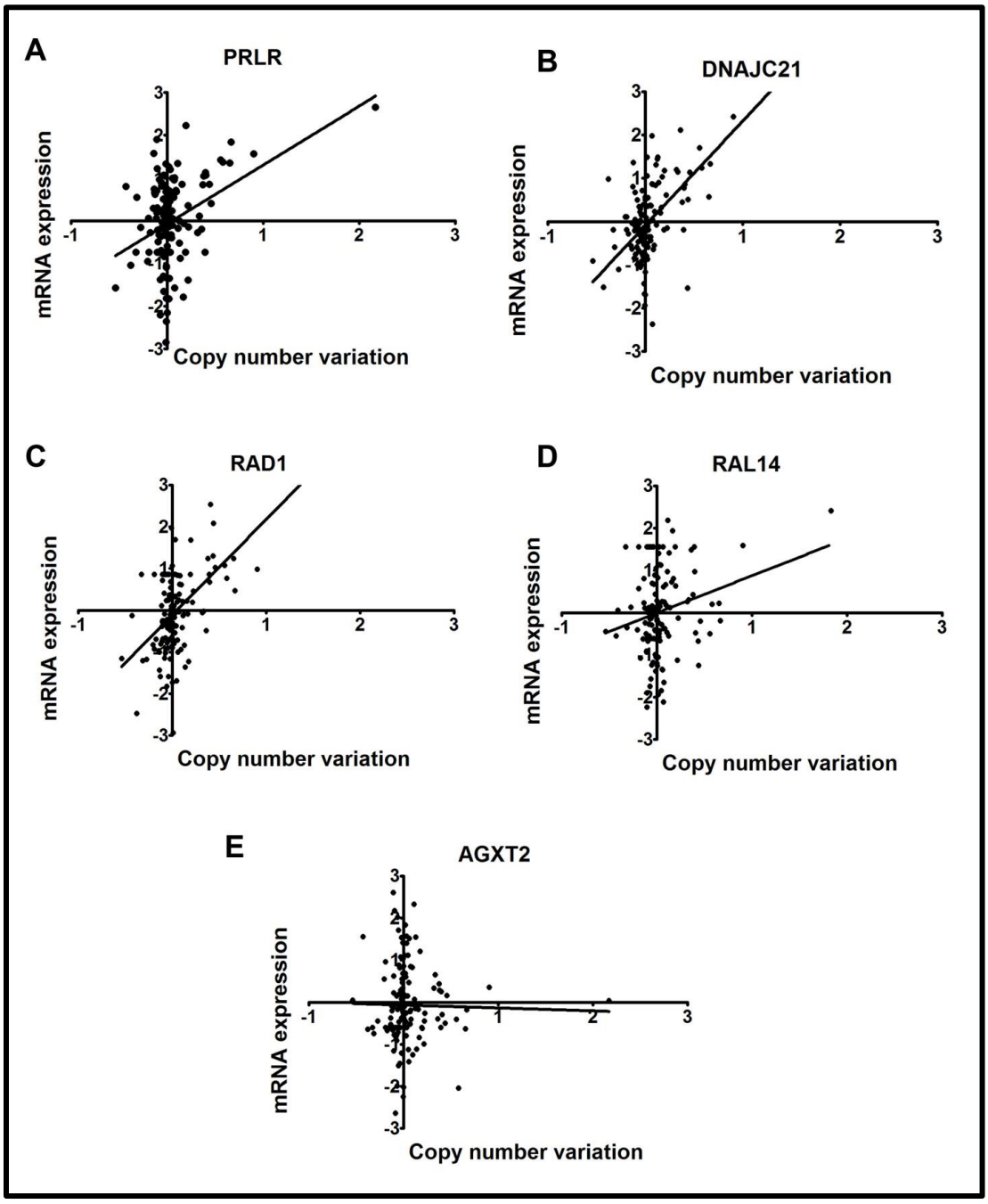


Figure 3.11: Evaluating changes in the copy number of genes located in the PRLR locus: Correlation analysis to identify if there is a relationship between the observed changes in expression and copy number. It may be speculated that the increase in expression observed in **(A)** *PRLR*, **(B)** *DNAJC21*, **(C)** *RAD1*and **(D)** *RAl14* may be due to an increase in copy number of the genes in some patients. Such positive correlation did not exist in the case of **(E)** *AGXT2*.

3.4.6 Intrinsic transcription factors which regulate PRLR expression

In the above study, some patients show an increase in PRLR gene expression but no change in copy number, suggesting a different mechanism. To identify the cause for increase in expression of PRLR noted in these samples, we investigated the intrinsic transcription enhancer factors that can potentially regulate PRLR expression. We analyzed the 2.5 Kb promoter upstream region for PRLR transcription start site for several transcription enhancers (Table 2.1). Of the multiple factors, we identified only three, SREBP1, NR2F1 and GFI1, that had single binding sites. Real-time PCR analysis to identify expression levels of these factors in colorectal cancer cells shows that HCT116 cells have a significant increase in expression of SREBP-1 compared to other cells types (Fig 3.12 A) and is predominantly localized to the nucleus (Fig 3.12 B). Based on this, we directed our further studies to identify if SREBP-1, which can regulate expression of metabolic genes [247], is also involved in regulating PRLR expression in CRC. To this end, chromatin-immuno precipitation followed by semi-quantative real-time PCR was performed using two different primer pairs which flank the SREBP-1 binding sites of PRLR enhancer region (Fig 3.12 C). There was an increase in SREBP-1 occupancy in HCT116 cells, compared to isotype control (Fig 3.12 D). Collectively, this data suggests that expression of PRLR in CRC patients can be regulated individually or in combination by in CNV or an increase in transcription.

Figure 3.12: Evaluating SREBP-1 expression and its occupancy of the PRLR enhancer elements.

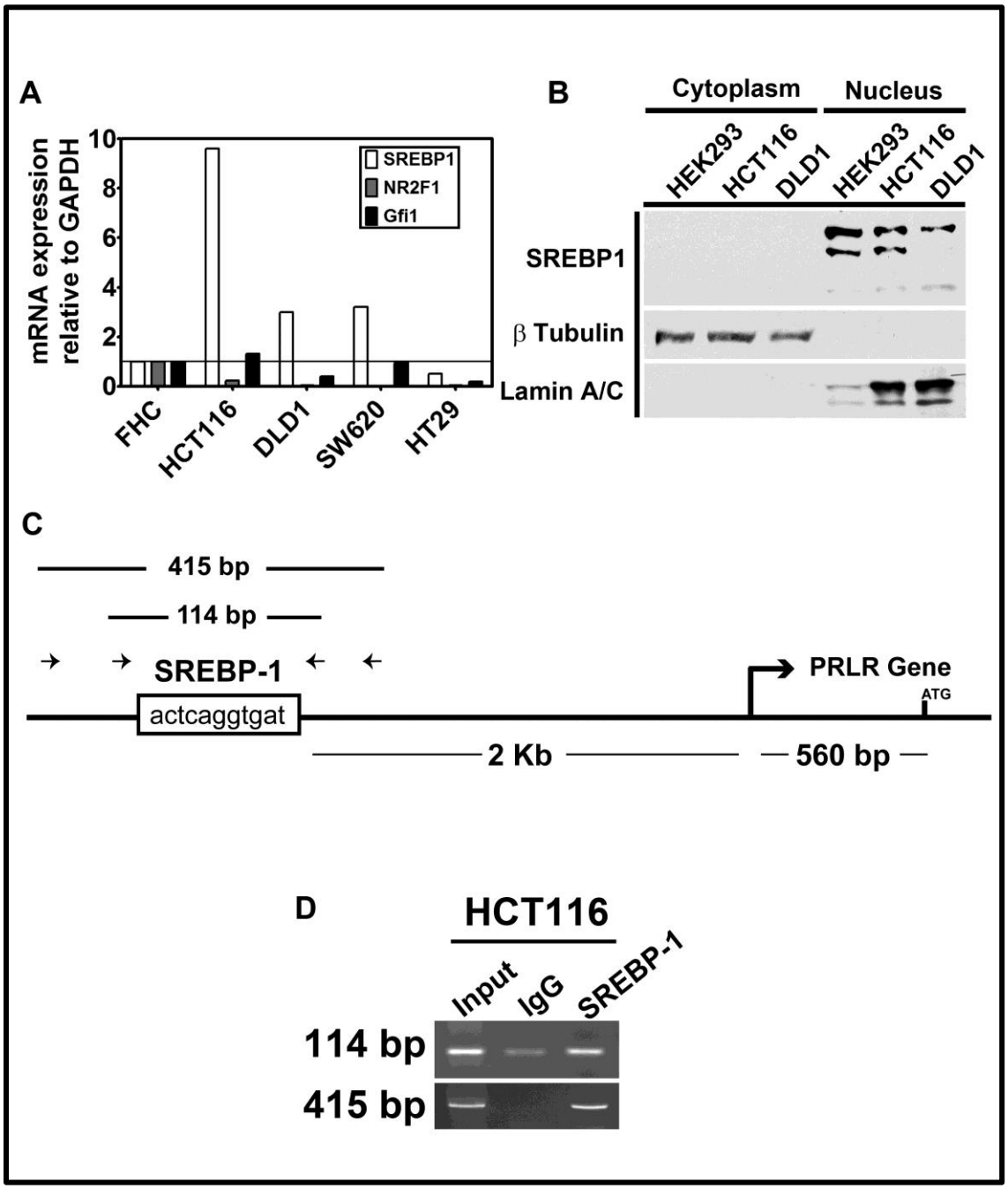


Figure 3.12: Evaluating SREBP-1 expression and its occupancy of the PRLR enhancer elements: (A) Evaluating expression of three transcription enhancer proteins which have a single binding site in the PRLR promoter region. Data indicates that SREBP-1 was upregulated in HCT116, DLD1 and SW620. **(B)** Western blot indicates that SREBP-1 accumulated in the nucleus. **(C)** Depiction of SREBP-1 binding site in the PRLR enhancer region, two primers of 114 and 415 bp were designed to evaluate binding. **(D)** ChIP data indicating active recruitment of SREBP-1 onto the PRLR promoter region.

3.5 Discussion

PRLR is a single pass non-tyrosine kinase receptor belonging to the class 1 cytokine receptor family. Localized to chromosome 5p13-14, it contains 10 exons and codes for a single transcript, which is then alternately spliced to generate diverse functional isoforms. Binding of PRL leads to dimerization of the receptor and activation of intracellular signaling mediated predominantly by JAK-STAT or JAK-ERK pathways. Several tissues have been identified to express PRLR where it plays a critical role in tumorigenesis. Our earlier studies have shown that CRC cells treated with recombinant PRL show activation of Jak2- STAT3 signaling, clearly demonstrating the presence of functional PRLR in CRC cells [253]. In humans, PRL is only known to interact with PRLR. This intrigued us to evaluate the levels and pattern of PRLR expression in patient population and to identify if there is existence of any heterogeneity in the expression pattern with an intention to exploit it as a diagnostic tool.

For the ease of desigining primers, we catagorized, PRLR isoforms into two major groups. The full length and the dSF1 forms were grouped into long form and the SF1a, SF1b and d7/11 isoforms were grouped as short form (Chapter 1, Fig 1.7). We evaluated the expression of PRLR in various regions of the GI tract and identified that the long form is predominantly expressed in the intestinal region. The long form was expressed more in the colon compared to short form, which is

expressed at varying level across the GI tract (Fig 3.2 A, B). Next we evaluated PRLR expression in CRC patient samples, and observed an increase in the expression of both the long and the short form of PRLR transcripts in a cohort of patient samples. However, the levels of long form of receptor is higher than that of the short form (Fig 3.3 A, B). Similar findings were reported earlier [99, 223]; however, our study provides in depth analysis and demonstrates an isoform dependent variation in expression of PRLR. The increase in long form of PRLR compared to short forms clearly indicates that PRL signaling potentially plays a significant role in CRC tumorigenesis.

Next, we evaluated PRLR expression in tissue sections pertaining to tumor and adjacent normal samples. PRLR is expressed both in normal (Fig 3.4 A,B) and malignant sections (Fig 3.4 C,D). It is expressed in all the cells lining the crypt and predominantly on the basolateral membrane surface. However, in malignant samples the crypt structure is completely lost with a distorted expression pattern of PRLR, a finding further supported by histoscore analysis (Fig 3.5 A). PRLR expression is decreased in the initial stages of CRC (such as the inflammatory stages) as compared to adenoma and adenocarcinoma (Fig 3.5 B). These data clearly indicate that PRLR expression changes in a stage specific manner with higher expression observed in malignant tissue. This increase in PRLR expression is retained in metastatic sites of lymph nodes (Fig 2.6 B) and liver (Fig 3.7 B),

where invasion leads to loss of native tissue structure and formation of metastatic foci.

We next analyzed gene expression and CNV data sets derived from TCGA datasets to identify the underlying reason for the observed increase in PRLR levels in colorectal cancer patient samples. Our data suggest that there is increase in expression of *PRLR,* including *DNAJC21, RAD1, RAL14* and *AGXT2* genes located in the same locus. These genes have also been shown to play a critical role in tumorigenesis of various cancer types. In addition, *PRLR, RAL14, RAD1* and *DNAJC21* genes also exhibited an increase in gene copy number (Fig 3.8- 3.10). There exists a correlation between the increase in expression and increase in copy number of *PRLR*, *RAL14* and *RAD1* genes. However, *DNAJC21* did not follow that relationship, there was a increase in expression but no change in CNV. *AGXT2* on the other hand shows only an increase in copy number but not expression. This may have been due to improper design of probe sets used to quantify changes in expression and CNV or changes in promoter region of this gene.

We next evaluated whether, there are any intrinsic factors that can regulate expression of PRLR. For this, we analyzed the 2.0 kb region upstream of the transcriptional start site for PRLR gene. Consences sequences for binding of several transcription enhancer factors was found in this region (Table 3.1). However, among the factors which have unique binding sites, only SREPB-1

seemd to be expressed at high levels compared to other factors. SREBP-1 plays a critical role in lipid biosynthesis and energy homeostasis in CRC. Our findings, based on ChIP analysis followed by real-time PCR, clearly indicate that SREBP-1 can potentially bind to and regulate the expression of PRLR.

Collectively, our findings indicate increase in expression of PRLR in colorectal tumor samples compared to normal samples. We also demonstrate that PRLR is expressed in normal colon crypt and is predominantly localized to the membrane. The study also suggests that the increase in expression may be a combined contribution of increase in copy number of PRLR gene and an increase in transcription, facilitated by increased binding of enhancer factor to the promoter proximal region of the PRLR gene. Further studies directed towards cytological evaluation to validate this increase in copy number and including a wider population may provide a clearer view of the expression pattern of PRLR in CRC and may provide new directions in evaluating PRLR based early diagnostic tools for detection of CRC.

Table 3.1: List of transcription factors or enhancers that have potential binding sites in the 2 Kb region upstream of the PRLR promoter region.

Transcription factor/ enhancer	**Number of sites**
CDX2	70
AML1a	16
SRY	51
GATA-X	3
MZF1	8
CP2	4
GATA2	12
GATA1	28
LYF1	3
DeltaE	6
NKX2	5
SREBP1	1
USF	8

TST1	4
S8	5
OCT1	11
EVI7	6
AP1	9
P300	1
C/EBPB	5
c-ETS	9
SP1	1
IK2	5
AP4	1
C-REL	4
COUPT	1
HNF3b	11
XFD1	1
N-MYC	2
CDPCR	2
GFI1	1
HFH2	10

Chapter 4

Discussion and future direction

4.1 Discussion

PRL was identified in the early 1920's [75, 76] and since then it has been shown to affect a diverse array of physiological activities. Released in a circadian manner [254] from the pituitary, PRL regulates stress related responses [110]. In humans PRL regulates reproduction [255] and milk production and secretion [118] and cancer [123]. Even though at a slow rate, new attributes are continuously being connected or advocated as being caused by PRL, such as its role in regulating the normal neuronal stem cell population [155], including our finding that it regulates cancer stem cells [253] in colorectal cancers.

The first evidence of the role of PRL in GI tract comes from the work of Muller and Dowling [141] who induced hyperprolactinemia by injecting perphenazine, an antipsychotic drug that increased PRL secretion (as a side effect) [256] and found that this led to an increase in mucosal hyperplasia. Nagano and colleagues later identified that PRLR is expressed in the intestine [177], additional studies have shown that intestinal PRL can regulate calcium homeostasis and ion transport [142, 209]. Clinically high serum levels of PRL was observed in patients with colorectal cancer [147]. Surgical removal of the tumor led to normalization of serum PRL levels, based on these observations; the authors propose the use of serum PRL as a

better prognostic marker for early detection of CRC. On similar lines, an increase in PRLR expression was observed in CRC tumors [223]. These findings implicate that expression of both PRL and PRLR is upregulated in CRC patients. However, the biological significance of this upregulation and the intracellular signaling pathways which are activated or regulated by PRL via PRLR in CRC remain elusive. Similarly, the expression pattern of PRLR in normal GI tract and how the expression varies in CRC has not been well characterized. This thesis is aimed at providing a clear understanding of the signaling induced by PRL and to identifying the expression pattern of PRLR in normal GI tract and in CRC, in order to develop better diagnostic/ prognostic tools and identify therapeutic agents to inhibit PRL-PRLR signaling.

4.2 Intracellular pathways activated by PRL in CRC

The first part of this dissertation deals with understanding the intracellular signaling pathways that are activated by PRL in CRC. Based on *in vitro* studies, using established colorectal cancer cell lines, we first showed that PRLR is expressed at varied levels in these cells implicating that they are PRL responsive. Second, culturing these CRC cells in presence of recombinant PRL showed an increase in spheroid formation, an assay used to quantify the presence of stem cells. In addition, we showed that PRL induces, JAK2, STAT3 and ERK

phosphorylation. JAK, STAT and ERK family of proteins are established “signal transducers” which are responsible for transducing an extracellular event to the nucleus either directly or through other accessory proteins to induce changes in gene expression in response to extracellular cues [257].

PRL activates JAK2- STAT5 signaling pathways in breast cancer [258] and prostate cancer [153]. However in CRC, we showed that PRL activates JAK2-STAT3-ERK pathway compared to other cancer types. Several publications including our own (Chapter 2, Fig 2.4) have demonstrated that the basal level of STAT5 in the CRC cells lines used in this study significantly low. This implicates that PRL can differentially activate either STAT5 or STAT3 depending on the cell type and abundance of STAT proteins. The differential activation also implicates different downstream effect, such as STAT5 promotes proliferation, but STAT3 does not [259]. This may be the reason that we did not observe a change in proliferation in our study (Fig 4.1). However, we observe an increase in cancer stem cell population (Chapter 2, Fig 2.5).

Notch signaling, as elaborated in Chapter 1, is critical in regulating colorectal cancer stem cell population. The fact that PRL can regulate CRC stem cell population, by inducing Notch signaling, suggests that it can activate additional pathways that help coordinate its action on stem cells. Goh and colleagues

demonstrated that phosphorylated ERK can induce JAG1, a Notch receptor ligand [216]. We have demonstrated that PRL treatment induces JAG1 expression which in turn activates Notch signaling pathway. These results indicate that PRL can activate not only JAK-STAT-ERK pathways, but also accessory pathways that can regulate critical events in colorectal tumorigenesis.

4.3 Expression pattern of PRLR in colorectal cancer

Next, we looked at levels of PRLR, which is critical in regulating PRL induced signaling in CRC. In a patient population based study, we observed an increase in PRLR expression in CRC tissue compared to adjacent normal tissue samples. Similarly, histological analysis shows an increased PRLR staining in CRC biopsy samples compared to adjacent normal tissue (Chapter 3, Fig 3.3- 3.5). Similar changes in expression pattern of PRLR was earlier reported in breast cancer [175] where tumor specific increases in PRLR expression were observed. However a similar and dramatic change in PRLR expression is also seen in normal breast tissue under varied physiological condition limiting its application as a prognostic tool in breast tumors. In comparison, an increase in PRLR distinctly in CRC tissue compared to adjacent normal, may implicate its diagnostic relevance.

Some of the histological sections and *in vitro* studies show nuclear localization of PRLR particularly in CRC cells treated with PRL (Fig 4.2). Similar nuclear

Fig 4.1: Presence of PRL does not induce proliferation neither does it induce cell death.

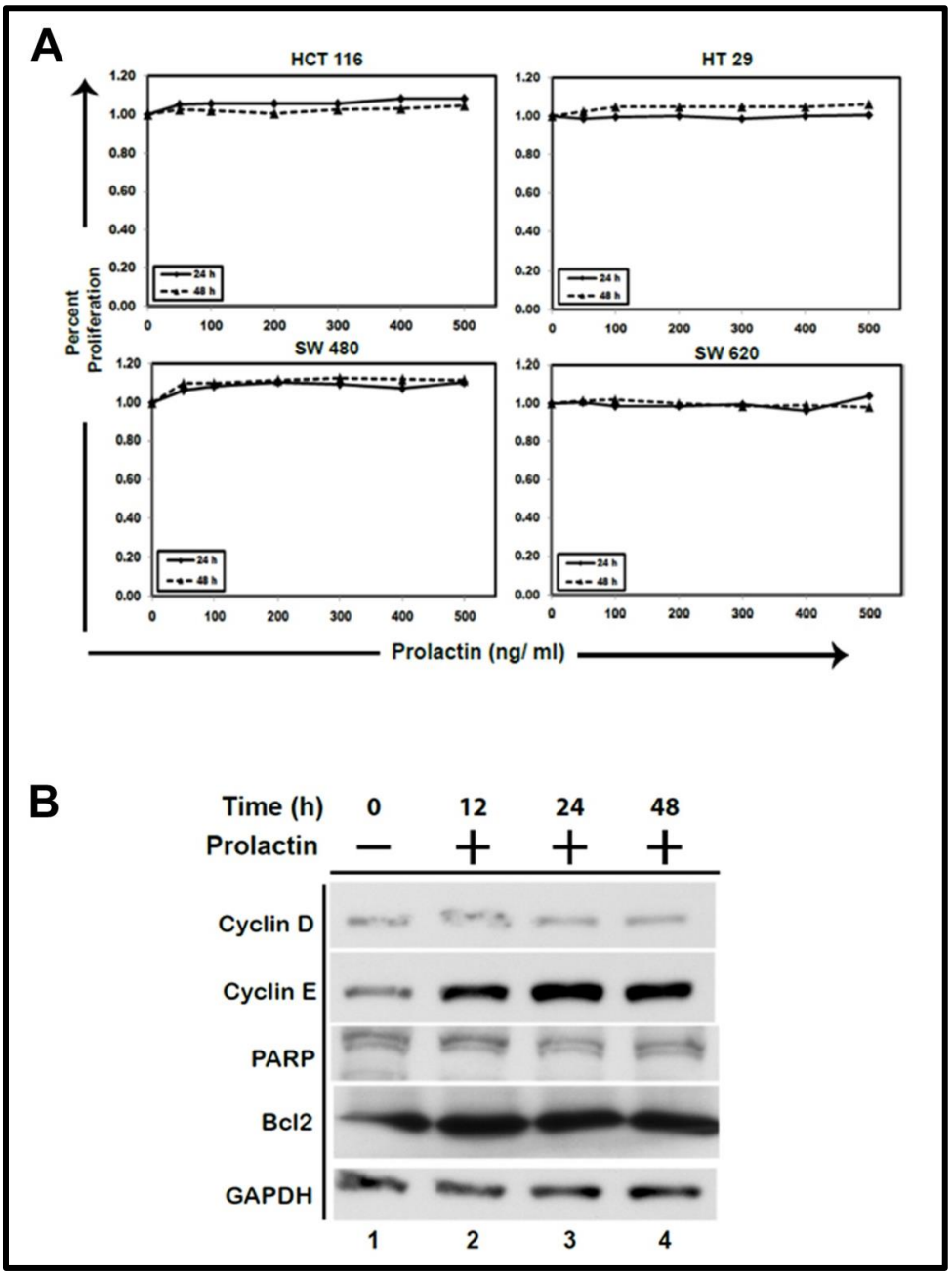
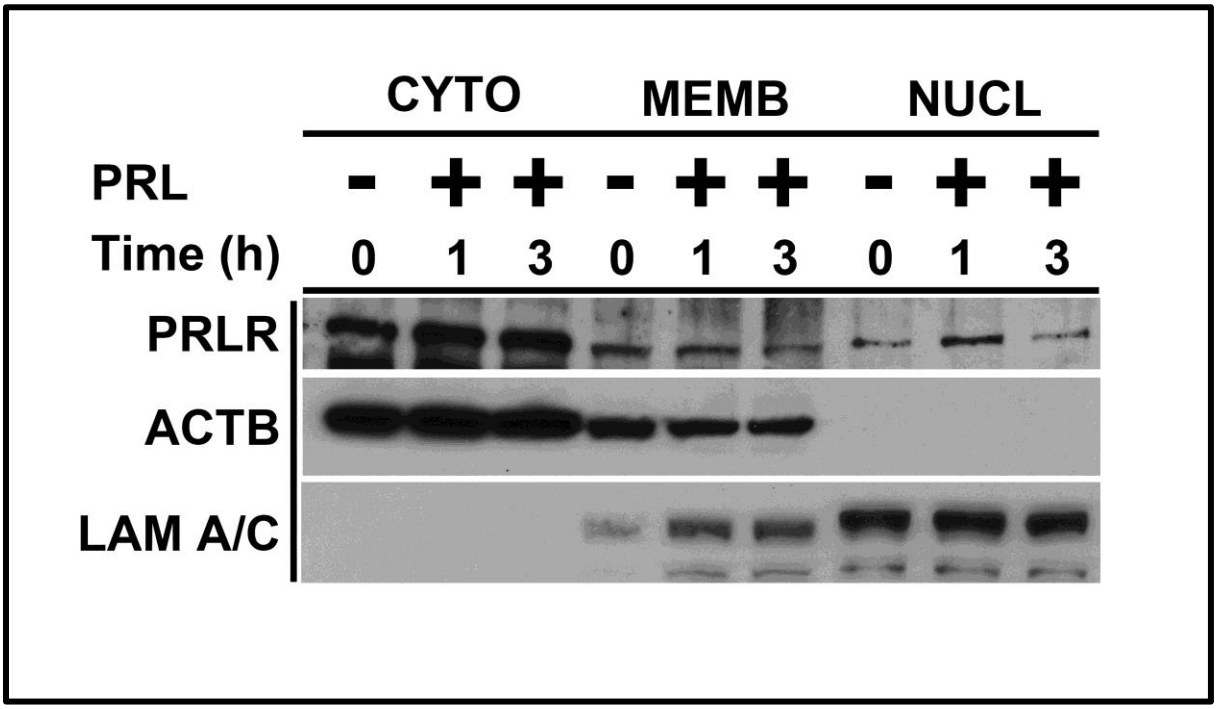


Fig 4.1: Presence of PRL does not induce proliferation neither does it induce cell death proteins: (A) HCT116, HT29, SW480 and SW620 colorectal cancer cells treated with exogenous PRL for 24 and 48 hours do not show any increase in proliferation. **(B)** An increase in Cyclin E levels, clearly implicate active PRL signaling, however an absence in increase in Cyclin D, a critical protein to promote S-G1 transition, suggest that PRL treatment may not induce proliferation. Similarly, an absence of PARP cleavage and an increase in levels of anti-apoptic protein BCL2, suggest that prolactin may prevent cell death in CRC cell lines.

4.2: Nuclear localization of PRLR upon PRL treatment in CRC


CYTO
MEMB
NUCL
PRL
- + + - + + - + +
Time (h)
0 1 3 0 1 3 0 1 3
PRLR
ACTB
LAM A/C

4.2: Nuclear localization of PRLR upon PRL treatment in CRC: CRC cells treated with exogenous PRL (500 ng/ml) led to nuclear translocation of PRLR as early as a 1h post treatment as compared to untreated sample. At 3h, there was a decrease in nuclear and membrane localization of PRLR but the cytoplasmic levels remain unchanged.

localization of PRLR was observed in breast cancer tissue [260] where it can bind to HMGN2 to induce expression of STAT5 responsive genes. Given the fact that, PRL signaling in CRC leads specifically to STAT3 activation and that STAT3 and STAT5 activate different sets of genes, it will be interesting to identify the proteins that can interact with PRLR in CRC and the genes that are regulated by this complex.

4.4 Future Directions

4.4.1 Analyzing PRLR locus amplification

In silico data analyzed and presented earlier (Chapter 3, Fig 3.9- 3.11) clearly indicate the possibility of occurrence of locus amplification. Several early reports indicate similar amplification in genes in other cancers that have been exploited as diagnostic tools [261, 262]. These chromosomal amplifications can be identified using PCR [263] or through chromosomal staining. Current focus in the lab is directed towards identifying means to analyze 5p13-14 locus amplification in colorectal cancer. Data from this analysis would help us develop novel diagnostic tool for early detection of CRC.

4.4.2 Identifying novel small molecular inhibitors to target JAK-STAT signaling

Data presented in Chapter 2 indicates that PRL actives JAK-STAT pathway in CRC cell lines. In addition, the dose and time dependent increase in STAT3-luciferase activity further supports the observation. The lab intends to use this plasmid to scan for novel STAT3 inhibitors, from the existing small molecule library available at the NCI and KU repository.

4.4.3 Evaluating SREBP-1 binding to the PRLR enhancer elements

Data presented in Chapter 3, clearly demonstrates that SREBP-1 can bind to the enhancer region of the PRLR gene. SREBP-1 binds to a specific sequence known as Sterol Response Element- 1 (SRE-1) in the enhancer region. Current studies in the lab are focused on cloning wild type and mutated SRE element upstream of a luciferase promoter. The luciferase activity from this plasmid would be used as a read out for the active binding of SREBP-1 to the PRLR promoter regions.

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